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PRISCILA MARCELINO DOS SANTOS SILVA

**DESENVOLVIMENTO DE BIOENSAIOS UTILIZANDO CRAMOLL 1,4 COMO
ALTERNATIVA PARA O DIAGNÓSTICO DO CÂNCER DE PRÓSTATA**

Recife

2019

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Tese apresentada ao Programa de Pós-graduação em Ciências Biológicas do Centro de Biociências da Universidade Federal de Pernambuco como parte dos requisitos parciais para obtenção do título de doutor em Ciências Biológicas.

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Orientador: Prof^a. Dr^a. Maria Tereza dos Santos Correia

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Aos meus pais Aluizio e Josefa, à minha irmã Abigail e ao meu esposo Allames, por estarem ao meu lado, pelo afeto, incentivo e confiança em todos os momentos,

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“A curiosidade constante reacende todos os dias o interesse pela vida.” (MARX, 1987).

RESUMO

O câncer de próstata destaca-se por sua elevada incidência e mortalidade, e seu diagnóstico ainda representa um desafio na medicina. Alterações nos perfis de glicosilação têm sido identificadas como potenciais marcadores da doença. Lectinas têm sido utilizadas para investigar e detectar essas alterações, integradas a diferentes métodos bioanalíticos e ao uso de nanomateriais. Neste trabalho, foram desenvolvidos bioensaios em microplacas e biossensores baseados em eletrodos sólidos e impressos empregando a lectina Cramoll 1,4, para analisar seu potencial na detecção de alterações nos perfis de glicosilação em soros de pacientes com câncer de próstata. Os ensaios foram padronizados com a glicoproteína fetuína, para a qual a lectina apresenta elevada afinidade. Foi desenvolvido um biossensor eletroquímico utilizando eletrodo sólido de carbono vítreo modificado com o polímero poli-L-lisina, nanotubos de carbono e Cramoll 1,4 para caracterização eletroquímica dos perfis de glicosilação em soros de pacientes com hiperplasia benigna prostática e câncer de próstata. Um estudo eletroquímico também foi efetuado em eletrodo impresso de carbono com nanotubos de carbono, para melhor analisar o fenômeno de interação de Cramoll 1,4 com fetuína. Um ensaio colorimétrico em microplaca utilizando Cramoll 1,4 conjugada à peroxidase e um ensaio fluorescente em microplaca com a lectina conjugada à nanocristais fluorescentes (*quantum dots*) também foram desenvolvidos. O biossensor de Cramoll 1,4 mostrou sinais de corrente significativamente diferentes para as amostras de hiperplasia e câncer de próstata após incubação com os soros, com um aumento do sinal correlacionado ao grau de agressividade do tumor. A detecção linear de fetuína por Cramoll 1,4 foi possível tanto no eletrodo sólido como no eletrodo impresso, revelando-se uma nova estratégia para avaliar a interação Cramoll 1,4 – fetuína, e futura aplicação na avaliação de perfis de glicosilação em amostras de soros. Os resultados parciais dos ensaios colorimétricos revelaram alterações dos perfis de glicosilação detectáveis em soros de câncer de próstata em relação à hiperplasia, a partir da observação de um aumento na reatividade de Cramoll 1,4 aos soros, correlacionado ao grau de agressividade do tumor. O ensaio colorimétrico tipo “sanduíche” para analisar os padrões de glicosilação do marcador de câncer de próstata, PSA, composto por anticorpo de captura e Cramoll 1,4 foi preliminarmente padronizado. O conjugado fluorescente de Cramoll 1,4 – *Quantum dots* mostrou-se ativo para marcação de *Candida albicans* e ligação à manana. Os métodos baseados em Cramoll 1,4 foram úteis na detecção da glicoproteína fetuína e mostraram potencial na detecção de perfis de glicosilação associados ao câncer de próstata, sendo alternativas úteis na investigação de alterações de glicosilação em diferentes fluidos biológicos, e até mesmo em células, relacionadas ao câncer de próstata e a outras doenças.

Palavras-chave: Câncer de próstata. Cramoll 1,4. Nanotubos de carbono. *Quantum dots*.

ABSTRACT

Prostate cancer stands out because of its high incidence and mortality, and its diagnosis still poses a challenge in medicine. Changes in glycosylation profiles have been identified as potential markers of the disease. Lectins have been used to investigate and detect these changes, integrated with different bioanalytical methods and the use of nanomaterials. In this work, bioassays were developed in microplates and biosensors based on solid and printed electrodes using the lectin Cramoll 1,4, to analyze its potential in the detection of alterations in the profiles of glycosylation in sera of patients with prostate cancer. The assays were standardized with the fetuin glycoprotein, for which the lectin exhibits high affinity. An electrochemical biosensor was developed using a solid carbon glass electrode modified with poly-L-lysine, carbon nanotubes and Cramoll 1,4 for the electrochemical characterization of glycosylation profiles in sera from patients with benign prostatic hyperplasia and prostate cancer. An electrochemical study was also carried out on a carbon electrode modified with carbon nanotubes, to better analyze the interaction phenomena of Cramoll 1,4 with fetuin. A colorimetric assay on microplate using Cramoll 1,4 conjugated to peroxidase and a fluorescent assay on microplate with lectin conjugated to fluorescent nanocrystals (quantum dots) were also developed. The Cramoll 1,4 biosensor showed significantly different current signals for the prostate cancer and hyperplasia samples after incubation with the sera, with an increase in the signal correlated to the degree of tumor aggressiveness. The linear detection of fetuin by Cramoll 1,4 was possible both on the solid electrode and on the printed electrode, revealing a new strategy to evaluate the interaction Cramoll 1,4-fetuin, and future application in the evaluation of glycosylation profiles in sera samples. Partial results from the colorimetric assays revealed changes detectable in glycosylation profiles from prostate cancer sera when compared to hyperplasia, from the observation of an increase in the reactivity of Cramoll 1,4 to the sera, correlated with the degree of tumor aggressiveness. The "sandwich" colorimetric assay for analyzing the glycosylation patterns of prostate cancer marker, PSA, composed of capture antibody and Cramoll 1,4 was preliminarily standardized. The fluorescence conjugate of Cramoll 1,4-Quantum dots was active for labeling *Candida albicans* and binding to mannan. The methods based on Cramoll 1,4 were useful in the detection of fetuin glycoprotein and showed potential in the detection of glycosylation profiles associated with prostate cancer, being useful alternatives in the investigation of glycosylation alterations in different biological fluids, and even in cells, related to prostate cancer and other diseases.

Keywords: Carbon nanotubes. Cramoll 1,4. Prostate cancer. Quantum dots.

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LISTA DE ABREVIATURAS E SIGLAS

ALL	<i>Aleuria aurantia lectin</i>
Ac	Anticorpo
Ag	Antígeno
AMA	Ácido mercaptoacético
AMS	Ácido mercaptosuccínico
ANOVA	<i>Analysis of variance</i>
AQT	Antiquimotripsina
ATCC	<i>American Type Culture Collection</i>
BC	Banda de condução
BPH	<i>Benign prostatic hyperplasia</i>
BPSA	<i>Benign PSA</i>
BSA	<i>Bovine serum albumin</i>
BV	Banda de valência
CA-125	<i>Cancer antigen 125</i>
CaP	Câncer de próstata
CdS	Sulfeto de cádmio
CdSe	Seleneto de cádmio
CdTe	Telureto de cádmio
CEA	<i>Carcynoembryonic antigen</i>
CETENE	Centro de Tecnologias Estratégicas do Nordeste
CIS	Cisteína
CISTM	Cisteamina
CNT	<i>Carbon nanotube</i>
ConA	Concanavalina A
cPSA	<i>Complexed PSA</i>
Cramoll	<i>Cratylia mollis lectin</i>
CV	<i>Cyclyc voltammetry</i>
DAPI	<i>4',6-diamidino-2-phenylindole</i>
DNA	<i>Deoxyribonucleic acid</i>
DSL	<i>Datura stramonium lectin</i>

ECV	Eletrodo de carbon vítreo
EDC	<i>1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide</i>
Eg	Energia do <i>band gap</i>
ELISA	<i>Enzyme linked immunosorbent assay</i>
ELLA	<i>Enzyme linked lectin assay</i>
E-PHA	<i>Phaseolus vulgaris erythro agglutinin</i>
EQM	Eletrodos quimicamente modificados
et al.	e outro
EUA	Estados Unidos da América
FDA	<i>Food and Drug Administration</i>
FITC	<i>Fluorescein isothiocyanate</i>
fPSA	<i>Free PSA</i>
FR	Fluorescência Relativa
FT-IR	<i>Fourier - Transform Infrared Spectroscopy</i>
Gal	Galactose
GalNAc	N-acetil-galactosamina
HBP	Hiperplasia benigna da próstata
hK2	<i>human Kallikrein 2</i>
hK3	<i>human Kallikrein 3</i>
hK4	<i>human Kallikrein 4</i>
INCA	Instituto Nacional do Câncer
IUPAC	<i>International Union of Pure and Applied Chemistry</i>
iPSA	<i>Intact PSA</i>
LCA	<i>Lens culinaris agglutinin</i>
LD	Limite de detecção
Ln III	Lantanídeos III
LOD	<i>Limit of detection</i>
MAL I e II	<i>Maackia amurensis lectin I and II</i>
MedMan	Metil- α -D-manopirranose
MEV	Microscopia eletrônica de varredura
MUC5b	Mucina 5b

MWNT	<i>Multi-walled carbon nanotube</i>
Neu5Ac	Ácido N-acetilneuramínico
NTC	Nanotubo(s) de carbono
NTCPM	Nanotubos de carbono de paredes múltiplas
NTCPS	Nanotubos de carbono de parede simples
OMS	Organização Mundial da Saúde
p	Coeficiente de correlação de Pearson
PBS	<i>Phosphate buffered saline</i> (Tampão fosfato salino)
PCa	<i>Prostate cancer</i>
PLL	Poli-L-lisina – <i>Poly-L-lysine</i>
PNA	<i>Peanut agglutinin</i>
PSA	<i>Specific prostatic antigen</i>
PSALT	Relação PSA livre/total
QDs	<i>Quantum dots</i>
r	Coeficiente de correlação linear
SAM	<i>Self-assembled monolayer</i>
SBU	Sociedade Brasileira de Urologia
SNA	<i>Sambucus nigra agglutinin</i>
SPE	<i>Screen-printed electrode</i>
STn	<i>Sialyl tumor-associated antigen</i>
Sulfo-NHS	<i>Sulfo-N-hydroxysulfosuccinimide</i>
SWV	<i>Square wave voltammetry</i>
Tn	<i>Tumor-associated antigen</i>
TRITC	<i>Tetramethylrhodamine-isothiocyanate</i>
UEA-I	<i>Ulex europaeus agglutinin I</i>
UFPE	Universidade Federal de Pernambuco
VC	Voltametria cíclica
WGA	<i>Wheat-germ agglutinin</i>

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1 INTRODUÇÃO

Nos dias atuais, milhares de pessoas no mundo são diagnosticadas com diversos tipos de câncer. Câncer é um grupo de doenças caracterizadas pelo crescimento e multiplicação de células anormais, e se não controladas, podem facilmente levar à morte. Dentre os tipos de câncer que acometem a população masculina mundial, o câncer de próstata (CaP) é o segundo mais incidente (INCA, 2017).

O rastreamento do CaP inicia-se com a realização do toque retal e do teste do PSA (do inglês *prostatic specific antigen*), sendo o diagnóstico confirmado através da biópsia. O PSA, considerado o marcador tumoral da próstata, é normalmente é secretado no sangue em concentrações muito baixas ($0 \leq 4 \text{ ng mL}^{-1}$), podendo atingir concentrações elevadas em indivíduos com CaP. O uso do teste do PSA isolado no diagnóstico do CaP é controverso, pois doenças benignas da próstata, como hiperplasia benigna prostática (HBP) também podem cursar com níveis séricos elevados de PSA, bem como o registro da incidência significativa de CaP com níveis reduzidos (UMBEHR et al., 2015; GRONBERG et al., 2015; ARMSTRONG et al., 2017), dificultando o diagnóstico do CaP.

Sabe-se que alterações na glicosilação de proteínas circulantes e em superfícies celulares são frequentemente observadas no surgimento e progressão do câncer, e podem ser utilizados como biomarcadores do câncer (ZHANG; WUHRER; HOLST, 2018). O PSA e outras glicoproteínas oriundas de tecido prostático, soro e fluido seminal de indivíduos com tumores prostáticos tem revelado alterações significativas no padrão de glicosilação que permitem distinguir entre HBP e CaP (KAMMEIJER et al., 2018). Lectinas são proteínas de origem natural e não imune que possuem sítios de ligação a carboidratos, com as quais interagem de modo específico e reversível. Assim, têm sido amplamente empregadas no desenvolvimento de bioensaios para investigar alterações de glicosilação relacionados a doenças, podendo auxiliar na detecção precoce do câncer (TOTTEN et al., 2018). Recentemente, os estudos de análise de padrões de glicosilação com lectinas têm buscado utilizar tecnologias simplificadas, de fácil manuseio, baixo custo, rápidas, e que ao mesmo tempo sejam sensíveis e específicas.

O emprego de biossensores vem ganhando espaço na área diagnóstica, pois são dispositivos de fácil manuseio capazes de detectar e quantificar um determinado analito com rapidez, especificidade e sensibilidade eficientes (GIRIGOSWAMI; AKHTAR, 2019). Lectinas podem ser imobilizadas às superfícies sensoras e atuar como biorreceptores de afinidade para detecção de carboidratos e glicoconjugados em amostras biológicas. Neste contexto, os biossensores eletroquímicos destacam-se pela diversidade de materiais e designs de eletrodos, facilidade e rapidez nas mensurações, além da possibilidade de modificação dos eletrodos com polímeros iônicos e nanomateriais, os quais podem melhorar as propriedades analíticas dos biossensores (HE et al., 2015; KURALAY; DUKAR; BAYRAMLI, 2018).

Métodos baseados na tecnologia de imunoensaio são valiosos devido à simplicidade e versatilidade que fornecem para detecção de um analito. O ensaio de imunoabsorção ligado à enzima (ELISA – do inglês *Enzyme-linked Immunosorbent Assay*) e o ensaio de lectina ligado à enzima (ELLA - do inglês *Enzyme-linked Lectin Assay*) têm sido desenvolvidos para estudos de glicosilação. São tipicamente realizados em microplacas de 96 poços, requerendo etapas sucessivas de incubação e lavagem; a detecção do sinal é obtida com o uso de substratos colorimétricos, por absorbância. Outro método empregado é o ensaio fluorescente em microplaca baseado em lectina, que utiliza lectinas conjugadas a marcadores fluorescentes para detecção.

Os nanomateriais são conhecidos por apresentarem propriedades diferenciadas, sendo amplamente empregados no desenvolvimento de bioensaios e biodispositivos. Os nanotubos de carbono (NTC) apresentam uma nanoestrutura que favorece a transferência de elétrons nos processos eletroquímicos, o aumento da área de superfície eletródica, possibilidade de funcionalização pela adição de grupamentos químicos, e imobilização de biomoléculas, sendo amplamente utilizados na preparação de biossensores (SILVA et al, 2015; GHRERA; PANDEY; MALHOTRA, 2018). Nanocristais inorgânicos semicondutores conhecidos como pontos quânticos (*quantum dots*), tem se destacado como sondas fluorescentes mais fotoestáveis que os fluoróforos orgânicos, úteis em métodos diagnósticos e processos terapêuticos (ZHENG et al., 2014; CABRAL FILHO et al., 2016).

Nesta tese, descreve-se o desenvolvimento de um biossensor eletroquímico baseado na lectina de sementes de *Cratylia mollis*, Cramoll 1,4, para detectar e

distinguir CaP com diferentes escores de Gleason e HBP. Também foi desenvolvido um biossensor de Cramoll 1,4 utilizando eletrodo impresso. Foram preparados ensaios tipo sanduíche lectina-anticorpo, ELLA e ensaio fluorescente em microplaca utilizando Cramoll 1,4 conjugada à peroxidase ou a pontos quânticos como nanossondas fluorescentes para detectar esses diferentes padrões de glicosilação em amostras de soro humano de CaP e HBP. Essas metodologias poderão ser uma alternativa complementar para auxiliar na detecção precoce do CaP.

1.1 OBJETIVOS

1.1.1 Objetivo geral

Desenvolver bioensaios colorimétricos e fluorescentes em microplacas e biossensores baseados em eletrodos sólidos e impressos empregando Cramoll 1,4 livre e conjugada à peroxidase e a QDs de CdTe para detectar a glicoproteína fetuína, e posteriormente distinguir os níveis de glicosilação em soros de pacientes com HBP e CaP (graus de Gleason 5, 6, 7 e 9).

1.1.2 Objetivos específicos

- Purificar Cramoll 1,4 a partir de sementes de *Cratylia mollis*;
- Modificar a superfície de eletrodo sólido de carbono vítreo (ECV) com PLL e NTC-COOH, e depois caracterizar as superfícies modificadas por técnicas eletroquímicas, FT-IR e MEV;
- Imobilizar a lectina Cramoll 1,4 nas superfícies sensoras;
- Otimizar os parâmetros cinéticos e experimentais, tais como velocidade de varredura, concentração de PLL e NTC-COOH, estudos de estabilidade, concentração da lectina e tempo de imobilização;
- Avaliar a resposta analítica do sensor a diferentes concentrações de fetuína e estabelecer uma curva analítica;
- Realizar estudos de seletividade e verificar a reprodutibilidade e estabilidade das medidas eletroquímicas do biossensor;
- Analisar os níveis de glicosilação em glicoproteínas dos soros de HBP e CaP e distingui-las entre si.
- Imobilizar Cramoll 1,4 na superfície sensora de eletrodos impressos de tinta de carbono contendo NTC;
- Avaliar a resposta analítica do eletrodo impresso de Cramoll 1,4 frente a diferentes concentrações de fetuína;
- Conjugar covalentemente Cramoll 1,4 à peroxidase;
- Confirmar a atividade biológica do conjugado Cramoll 1,4 - peroxidase através de ensaio colorimétrico em microplaca e ensaio de inibição de carboidratos;

- Definir as etapas e condições analíticas do ensaio colorimétrico em microplaca utilizando Cramoll 1,4 – peroxidase;
- Utilizar o ensaio colorimétrico em microplaca baseado em Cramoll 1,4 – peroxidase para detectar e discriminar o perfil de glicosilação nos soros do pacientes de HBP e CaP;
- Realizar estudos de linearidade e reprodutibilidade das medidas obtidas nos soros;
- Conjugar por adsorção Cramoll 1,4 aos QDs de CdTe;
- Analisar a eficiência da conjugação através de ensaio fluorescente em microplaca com revestimento de manana, ensaio de inibição de carboidratos e marcação de *C. albicans* visualizada por microscopia de fluorescência;
- Definir as etapas e condições analíticas do ensaio fluorescente em microplaca utilizando Cramoll 1,4 – QDs CdTe;
- Efetuar através de pesquisa bibliográfica exaustiva revisões que destaquem o potencial dos glicobiomarcadores no diagnóstico precoce de doenças como o CaP, bem como o desenvolvimento de bioensaios baseados em lectina, direcionados para a detecção e análise desses biomarcadores.

2 REFERENCIAL TEÓRICO

2.1 CÂNCER: CONSIDERAÇÕES GERAIS

O câncer é considerado uma doença crônica em que células anormais se dividem incontrolavelmente com potencial de invadir e espalhar-se pelos tecidos e órgãos do corpo, sendo uma das principais causas de morte no mundo, excedida apenas por doenças cardiovasculares no mundo desenvolvido. De acordo com estimativas mundiais do projeto Globocan 2012, da Agência Internacional para Pesquisa em Câncer (Iarc, do inglês *International Agency for Research on Cancer*), da Organização Mundial da Saúde (OMS), houve 14,1 milhões de casos novos de câncer e um total de 8,2 milhões de mortes por câncer, em todo o mundo, em 2012 (OMS, 2013). Entre os tipos de câncer, os mais incidentes no mundo foram pulmão, mama, intestino e próstata, sendo o de pulmão, próstata, intestino, estômago e fígado mais frequente em homens, enquanto nas mulheres os mais incidentes foram mama, intestino, pulmão, colo do útero e estômago, de acordo com informações do projeto Globocan (INCA, 2017).

No Brasil, o número de casos novos de câncer tem aumentado a cada ano. A estimativa para o biênio 2018/ 2019 indica aproximadamente 600 mil casos novos de câncer para cada ano, incluindo os casos de câncer de pele não melanoma, reforçando a magnitude do problema do câncer no país. O câncer de pele do tipo não melanoma é o mais incidente na população brasileira, além dos demais tipos mais frequentes, sendo próstata, pulmão, intestino, estômago e cavidade oral em homens; e mama, intestino, colo de útero, pulmão e tireóide em mulheres, como pode ser observado na Figura 1 (INCA, 2017).

A segunda parte do século XX caracteriza-se por um considerável progresso na melhoria da saúde e sobrevivência em todo o mundo. A expectativa de vida ao nascer para a população mundial aumentou de 48 anos em 1950-1955 a 68 anos em 2005-2010 (NAÇÕES UNIDAS, 2012), como resultado da transição demográfica observada nos países desenvolvidos e em países em desenvolvimento como o Brasil. Tal transição reflete na transição epidemiológica, caracterizada por quedas na

incidência de doenças infecciosas e transmissíveis, seguida por aumentos subsequentes na incidência e mortalidade por doenças não transmissíveis de caráter crônico, como o câncer, cujo número de novos casos e mortalidade tem aumentado consideravelmente. Na tentativa de controlar a incidência do câncer, a OMS tem desenvolvido estratégias de prevenção primária, que objetivam minimizar a incidência do câncer através do controle da exposição a fatores de risco e ações de rastreamento para detecção precoce (OMS, 2013). Também são propostas ações de apoio à terapêutica, aos cuidados paliativos e às ações clínicas para o seguimento dos doentes tratados. Tais estratégias podem reduzir a incidência e a mortalidade do câncer em diferentes proporções para alguns tipos de câncer mais comuns (FRANCESCHI; WILD, 2013; BYERS et al., 2016).

Figura 1. Distribuição proporcional dos dez tipos de câncer mais incidentes estimados para 2018 por sexo, exceto pele não melanoma *.

Localização Primária	Casos	%	Homens	Mulheres	Localização Primária	Casos	%
Próstata	68.220	31,7%			Mama Feminina	59.700	29,5%
Traqueia, Brônquio e Pulmão	18.740	8,7%			Cólon e Reto	18.980	9,4%
Cólon e Reto	17.380	8,1%			Colo do Útero	16.370	8,1%
Estômago	13.540	6,3%			Traqueia, Brônquio e Pulmão	12.530	6,2%
Cavidade Oral	11.200	5,2%			Glândula Tireoide	8.040	4,0%
Esôfago	8.240	3,8%			Estômago	7.750	3,8%
Bexiga	6.690	3,1%			Corpo do Útero	6.600	3,3%
Laringe	6.390	3,0%			Ovário	6.150	3,0%
Leucemias	5.940	2,8%			Sistema Nervoso Central	5.510	2,7%
Sistema Nervoso Central	5.810	2,7%			Leucemias	4.860	2,4%

Fonte: INCA, 2017. * Números arredondados para múltiplos de 10.

O câncer tem sido alvo de muitas pesquisas científicas visando o desenvolvimento de novas ferramentas para o diagnóstico precoce e eficiente com melhoramento nas chances de cura, sobrevivência e qualidade de vida. A investigação de biomoléculas e alterações moleculares relacionadas ao desenvolvimento do câncer é fundamental na busca de novos biomarcadores e métodos alternativos que possam complementar os exames preventivos e auxiliar na detecção precoce da doença.

2.2 CÂNCER DE PRÓSTATA

A última estimativa mundial apontou o câncer de próstata (CaP) como o segundo mais frequente em homens, com cerca de 1,1 milhão de casos novos no ano de 2012. CaP é o mais frequentemente diagnosticado em homens ao lado do câncer de pele, sendo a 2º causa principal de morte por câncer em homens, ficando atrás apenas de câncer de pulmão (SIEGEL et al, 2012; INCA, 2017; Sociedade Americana do Câncer, 2018). As taxas de incidência de CaP permanecem maiores nas regiões de maior renda do mundo, incluindo América do Norte, oeste e norte da Europa, e Austrália/ Nova Zelândia, ao passo que as taxas de mortalidade por CaP tendem a ser mais elevadas em ambientes de baixa renda média, incluindo partes da América do Sul, Caribe e África Subsaariana. As maiores taxas de incidência observada nos países desenvolvidos são, em parte, devido ao aumento das notificações da doença baseadas no rastreamento populacional pelo teste do PSA, mais difundido nesses países, bem como a alta longevidade da população (TORRE et al., 2016). Para o Brasil, estimam-se 68.220 mil casos novos de CaP no biênio 2018/ 2019, sendo o câncer mais incidente em todas as regiões do país, excetuando casos de câncer de pele não melanoma (INCA, 2017). As taxas de incidência do CaP no Brasil têm crescido ao longo dos anos devido ao aumento da expectativa de vida, a melhoria na qualidade dos sistemas de informação do país e evolução dos métodos diagnósticos, refletindo na maior adesão da população aos exames de rastreamento (INCA, 2017).

Mais do que qualquer outro tipo, o CaP é considerado um câncer da terceira idade, ocorrendo principalmente em homens idosos. A idade é um fator de risco cuja relação com o CaP já está bem estabelecida. Cerca de 62% dos casos diagnosticados no mundo ocorrem em homens com 65 anos ou mais, sendo esperado um aumento de cerca de 60 % devido ao aumento da expectativa de vida mundial (INCA, 2015). Outro fator de risco considerado é a etnia. O CaP é aproximadamente duas vezes mais frequente em homens negros que em homens de outras raças (70% maior em afrodescendentes que em brancos) e são duas vezes mais propensos a morrer de CaP que homens brancos (REBBECK et al,

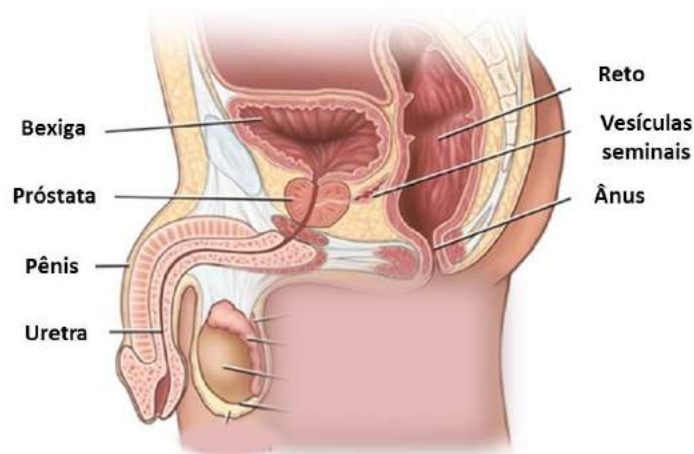
2013). Estudos genéticos sugerem que a predisposição familiar pode ser responsável por 5-10% dos casos. Ter um pai ou irmão com CaP, principalmente antes dos 60 anos, aumenta para mais que duas vezes a possibilidade do homem desenvolver a doença. O risco é maior para homens com vários parentes afetados, particularmente se seus parentes eram jovens quando o câncer foi encontrado (FITZGERALD et al, 2009; Sociedade Americana do Câncer, 2016; WU; GU, 2016). Outros fatores de risco são investigados quanto à relação com a doença, como mutações genéticas, dieta, obesidade, tabagismo, inflamações na próstata, infecções sexualmente transmissíveis e vasectomia (LEITZMANN; ROHRMANN, 2012; WU; GU, 2016).

A próstata é uma glândula exócrina muito pequena em forma de maçã, faz parte do sistema reprodutor masculino e se localiza na parte baixa do abdômen, abaixo da bexiga e à frente do reto (Figura 2). A próstata produz um fluido que nutre e transporta o sêmen, um líquido espesso que contém os espermatozoides, e é composto basicamente por fosfatase ácida, ácido cítrico, fibrinolizina, antígeno específico da próstata, enzimas proteolíticas e zinco. A uretra, que é o tubo que transporta urina e sêmen para fora do corpo através do pênis, atravessa o centro da próstata. A próstata tem o tamanho aproximado de uma castanha, podendo apresentar-se muito maior em homens idosos, devido a um crescimento benigno da glândula que ocorre na maioria dos homens a partir da fase adulta (WILSON, 2014). As doenças que mais afetam a próstata são prostatites e hiperplasia benigna prostática, consideradas não cancerosas, e o câncer de próstata (AGHAJANYAN & ALLEN, 2016).

A maioria dos cânceres de próstata tem origem nas células glandulares prostáticas, sendo chamados de adenocarcinoma. Alguns cânceres de próstata podem crescer e espalhar-se rapidamente para outros órgãos, mas a maioria cresce lentamente. Estudos de autópsia mostram que muitos homens idosos e alguns homens jovens que morreram de outras doenças tinham CaP e nunca os afetaram durante suas vidas (INCA, 2011; Sociedade Americana do Câncer, 2012). Em seus estágios iniciais, o CaP usualmente não causa sintomas, fato que dificulta a detecção precoce da doença, principalmente em indivíduos que não fazem os exames de rastreamento. Na doença mais avançada, o crescimento do tumor frequentemente pressiona e obstrui a uretra, causando os sintomas característicos:

fluxo urinário fraco ou interrompido; incapacidade de urinar ou dificuldade para controlar o fluxo de urina; necessidade frequente de urinar, especialmente à noite; presença de sangue na urina e dor ou ardência ao urinar (DEBRUYNE et al., 2016). Encontrando-se em estado avançado, o câncer de próstata pode se disseminar pelo corpo, vindo a atingir outros órgãos e, principalmente os ossos, podendo atingir a coluna vertebral, quadril, costelas, fêmures, entre outras áreas. Uma dor na coluna vertebral num indivíduo na idade de risco ou fratura espontânea do fêmur sem qualquer trauma pode ser provocada por uma disseminação do tumor (MENG et al., 2016).

Figura 2. Localização da próstata no sistema reprodutor masculino.



Fonte: <http://www.labluxor.com/exames-prostata> (adaptado).

A hiperplasia benigna prostática (HBP) é outra condição patológica muito comumente associada ao envelhecimento, bastante prevalente em homens a partir dos 40 anos, atingindo mais da metade da população masculina na sétima década de vida e a quase totalidade na oitava década (VUICHOUD; LOUGHLIN, 2015). HBP é uma condição clínica caracterizada pelo aumento benigno da próstata, resultante da hiperplasia progressiva das células glandulares prostáticas, levando a formação de nódulos e consequente aumento volumétrico, podendo interferir no fluxo normal de urina causada pela compressão da uretra prostática e pelo relaxamento inadequado do colo vesical (MARRA et al., 2016). Evidências sugerem que os

hormônios andrógenos iniciam a hiperplasia do tecido prostático, levando a obstrução do colo vesical ou uretra prostática devido à hipertrofia dos lobos, dificultando o fluxo da urina e causando o esvaziamento incompleto da bexiga com retenção urinária, vindo a ocorrer uma dilatação gradual dos ureteres e rins. A avaliação do paciente com HBP é realizada através da anamnese com aplicação do escore de sintomas prostáticos, exame físico com toque retal, avaliação laboratorial (PSA sérico, exame de urina e função renal), além dos exames de imagem e urodinâmica (VUICHOUUD; LOUGHLIN, 2015).

2.2.1 Diagnóstico do Câncer de próstata

A Sociedade Americana do Câncer e a Sociedade Brasileira de Urologia (SBU) postulam o diagnóstico do CaP através do rastreamento populacional pelo toque retal em conjunto com o teste do PSA, com periodicidade anual, em homens com idade igual ou superior a 50 anos e a partir dos 45 anos em homens pertencentes a grupos de risco (afro-americanos ou com um parente diagnosticado com CaP antes dos 65 anos), e ainda a partir dos 40 anos em homens com vários parentes já diagnosticados com CaP em idade precoce (SBU, 2015; Sociedade Americana do Câncer, 2018).

O PSA, também conhecido como hK3, é considerado o marcador tumoral da próstata, sendo rotineiramente quantificado em amostras séricas através do teste do PSA para rastreamento do CaP. Essa glicoproteína é sintetizada no epitélio ductal e acinal da glândula prostática e secretada no interior do lúmen prostático, passando a compor o fluido seminal. A maior parte do PSA produzido está presente no fluido seminal em concentrações relativamente altas (0,5 a 5 mg mL⁻¹) e apenas uma pequena fração é liberada para a circulação sanguínea, onde a concentração varia de 0 a 4 ng mL⁻¹ (ROOBOL et al., 2010). Em indivíduos saudáveis, os níveis de PSA sérico são comumente $\leq 0,1$ ng mL⁻¹, sendo considerado normal até 4 ng mL⁻¹. Porém, qualquer alteração na arquitetura da glândula prostática permite que maiores concentrações de PSA entrem na circulação. Valores acima de 10 ng mL⁻¹ são fortes indícios de câncer; entre 4 e 10 ng mL⁻¹ são classificados dentro da categoria de zona “cinzenta” de diagnóstico, não permitindo uma diferenciação clara entre CaP e

outras patologias, fazendo com que a biópsia seja indicada em níveis de PSA a partir de 4 ng mL^{-1} (ROOBOL et al., 2012).

Embora o PSA tenha revolucionado os testes oncológicos desde a sua primeira aprovação pela FDA em 1986, a sua especificidade clínica para detecção do CaP é questionável. Outras doenças da próstata, como HBP e prostatites também causam aumento do PSA na circulação, ou ainda casos de CaP que ocorrem quando os níveis de PSA são considerados normais (POMPEU et al., 2015). Estudos têm relatado uma incidência de até 27% de CaP quando os níveis de PSA são $\leq 0,5$ a 4 ng mL^{-1} e 25% dos casos de CaP ocorrem com PSA entre 4 e 10 ng mL^{-1} (HEIDENREICH et al., 2011; ROOBOL et al., 2012; POMPEU et al., 2015). Esses fatores reduzem a sensibilidade e especificidade do PSA como teste diagnóstico para o CaP. Considerando um ponto de corte em $4,0 \text{ ng mL}^{-1}$, apresenta sensibilidade estimada de 71% e especificidade de 46% para o CaP (NARDI et al., 2015). Estudos que estimaram seu valor preditivo positivo apontam para valores em torno de 28%, o que significa que cerca de 72% dos pacientes com dosagem do PSA alterada são submetidos a biópsias desnecessárias (ROOBOL et al., 2010; NARDI, et al., 2015).

Quando o PSA total está entre 4 e 10 ng mL^{-1} é recomendado considerar a relação PSA livre/ total (% PSALT), que está inversamente proporcional ao volume prostático e tumoral e ao escore de Gleason. A relação $< 25 \%$ é sugestivo de CaP, e $> 25\%$ é sugestivo de HBP (NARDI et al., 2015). Estudos indicam que a expressão do PSA não é tecido específica. O antígeno já foi detectado por imunohistoquímica no endométrio, glândulas sudoríparas, periuretrais e anais masculinos, tumores de mama, adenocarcinoma de pulmão, entre outros tumores (CHEN et al., 2008). Considerando esta afirmação e os demais fatores citados, é importante associar o teste do PSA a outros exames, como exame digital retal, para o câncer ser detectado.

O toque retal tem sido recomendado devido ao aumento de possibilidade diagnóstica, sobretudo em pacientes com PSA dentro da normalidade. A presença de alteração observada pelo toque retal é um forte indicativo para biópsia. Estudos mostram que até 1/5 dos pacientes tem alteração apenas no toque retal, e entre esses, 1/3 tem CaP, mesmo com PSA normal (NARDI et al., 2015). As estimativas de sensibilidade variam entre 48% e 59% e a especificidade de 89 - 92%. O valor

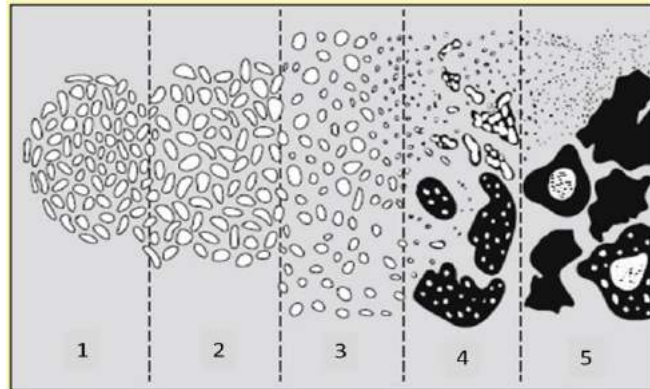
preditivo positivo é estimado entre 28% e 40%. Apesar da recomendação da realização do exame em cada consulta, a aderência na prática clínica é cerca de 20%, considerada baixa (NARDI et al., 2013; 2015).

Havendo anormalidades nas avaliações no toque retal ou dosagem do PSA igual ou superior a 4 ng mL^{-1} , ou ambos, é indicada a realização do exame histopatológico do tecido prostático obtido através de biópsia (NARDI et al., 2015). A ultrassonografia transretal é o método preferido para orientar a biópsia da próstata, na qual se obtém amostras de tecidos para análise histopatológica e definição do diagnóstico de CaP. Além de orientar o local da biópsia, a ultrassonografia transretal também é utilizada para acompanhar alterações no volume prostático, que é fator preditivo de detecção precoce de CaP, bem como avaliar a extensão local da doença (HOU et al., 2015; OCHIAI, 2017).

Uma vez detectado o tumor, o relatório anátomo patológico deve fornecer a graduação histológica do sistema Gleason, desenvolvido por Donald Gleason e colaboradores entre 1966 e 1974 (GLEASON; MELLINGER, 1974), que informa sobre o grau de agressividade do tumor, além de auxiliar na definição do melhor tratamento (HEINDERECH et al., 2014). Na graduação histológica, as células do câncer são comparadas às células prostáticas normais; quanto mais diferentes das células normais forem as células cancerosas, mais agressivo será o tumor e mais rápida será sua disseminação. A escala de graduação do sistema de Gleason baseia-se em padrões histológicos que variam de 1 a 5 (Figura 3), com o grau 1 sendo a forma menos agressiva e o grau 5 representando o tumor anaplásico.

Como pode ser observado na figura 3, o grau 1 é caracterizado por apresentar células uniformes, pequenas e com formação de glândulas regulares, bem agrupadas e com bordos bem definidos. No grau 2, já pode-se perceber variação na tamanho e forma das células, agrupamento mais frouxo e bordos irregulares. As células no grau 3 variam ainda mais em tamanho e forma, com formação de glândulas muito pequenas, individualizadas e espalhadas pelo estroma. No grau 4, muitas células estão constituindo massas amorfas ou formando glândulas irregulares, invadindo tecidos adjacentes. Quando classificado no grau 5, as células estão soltas ou se agrupando em massas amorfas, que invadem órgãos e tecidos vizinhos.

Figura 3. Sistema de classificação de Gleason para CaP baseado em padrões histológicos de 1 a 5, de acordo com o nível de organização celular e formação de glândulas na próstata.



Fonte: EPSTEIN et al, 2010.

Para obtenção do escore total da classificação de Gleason, que varia de 2 a 10, gradua-se de 1 a 5 as duas áreas mais frequentes do tumor e soma-se os resultados. Quanto mais baixo a pontuação, menos agressivo é o tumor e melhor será o prognóstico do paciente, e vice-versa. O primeiro número atribuído na soma é o grau mais comum no tumor. Por exemplo, a pontuação $3 + 4 = 7$ significa que a maior parte do tumor apresenta grau 3, e o grau 4 correspondem às partes menos frequentes do tumor, resultando em um escore total de 7. Escores de 2 a 4 significam que o tumor provavelmente crescerá lentamente. Os escores intermediários (5 a 7) indicam que o câncer poderá evoluir lento ou rapidamente, a depender de uma série de fatores. Os escores de 8 a 10 indicam que o câncer crescerá rapidamente, com elevada chance de disseminação para outros órgãos.

Porém, a evolução do diagnóstico histológico e tratamento do CaP levou à revisões do sistema de Gleason, as quais resultaram em modificações desse sistema. A classificação utilizada atualmente não mais atribui a pontuação 2 – 5, e alguns padrões anteriormente classificados como 6 agora são classificados como 7, sendo os tumores classificados dentro de um grupo de três níveis: 6, 7 e 8 - 10. No nível 6, o câncer é considerado de baixo grau e tendem a ser menos agressivos, ou seja, tendem a crescer e se disseminar lentamente. Cânceres com pontuação 8 – 10 são considerados de alto grau e tendem a ser agressivos, e com pontuação 7 são considerados de grau intermediário.

Apesar das mudanças, o sistema de Gleason utilizado apresenta algumas deficiências, como a classificação final não reconhecer que os tumores $3 + 4 = 7$ e $4 + 3 = 7$ apresentam prognósticos muito diferentes. Outra deficiência é o fato de considerar a menor pontuação igual a 6, embora a escala inicie na pontuação 2, levando à suposição de que o câncer é mais grave e despertando expectativas e medo do diagnóstico pelos pacientes (EPSTEIN et al., 2016). Em 2013, um novo sistema de classificação de Gleason foi proposto na tentativa de minimizar essas deficiências, consistindo em cinco grupos separados em graus de 1 a 5: grupo de grau 1 (Grau de Gleason < 6), grupo de grau 2 (Grau de Gleason $3 + 4 = 7$), grupos de grau 3 (Grau de Gleason $4 + 3 = 7$), grupo de grau 4 (Grau de Gleason 8); e grupo de grau 5 (Graus de Gleason 9 - 10) (EPSTEIN et al., 2016). Dessa forma, pode-se obter uma classificação e tratamentos mais adequados, principalmente de CaP de baixo grau.

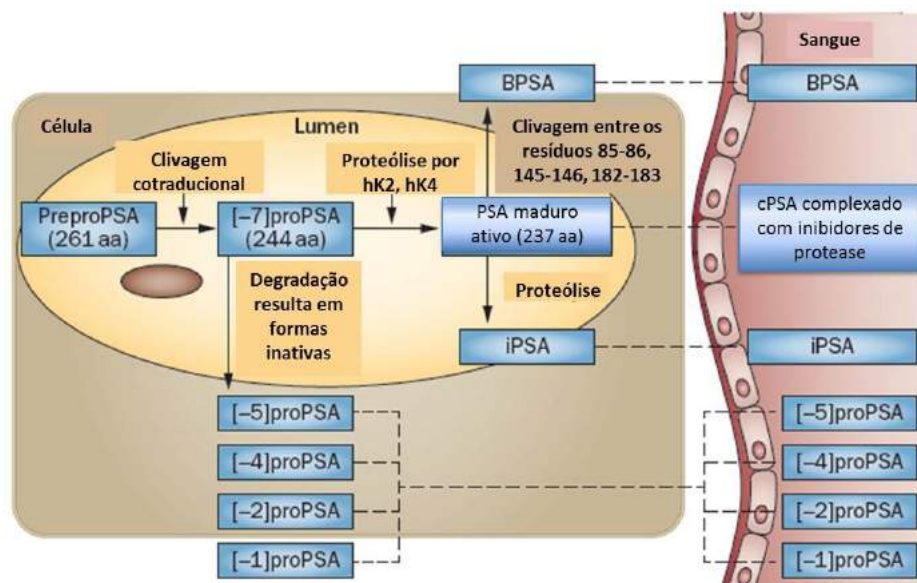
Apesar de permitir o diagnóstico definitivo do CaP, estudos mostram que em até um terço dos casos, o câncer não é detectado na biópsia inicial (VOURGANTI et al., 2012). Quando a primeira biópsia é negativa, é recomendada a realização de uma segunda biópsia, que quando negativa também, reduz a possibilidade de desenvolvimento e diagnóstico do CaP (NARDI et al., 2013).

2.3 BIOQUÍMICA DO PSA

PSA é uma serino-protease regulada por andrógeno pertencente a família das calicreínas, codificadas por um aglomerado de genes localizados dentro de uma região de 300 kb no cromossomo 19q13.4 humano (CLEMENTS et al., 2001). O PSA é sintetizado no epitélio ductal e acinal da próstata e inicialmente, é traduzido como um pre-propolipeptídio com uma sequência líder de 17 aminoácidos, que é cotraducionalmente clivada e removida na passagem através da via secretória, produzindo um precursor enzimaticamente inativo denominado [-7]proPSA. Este precursor contém um pro-peptídeo líder de sete aminoácidos além de 237 aminoácidos do PSA maduro. Uma vez secretado dentro dos ductos prostáticos, o pró-peptídeo líder é removido por outras duas calicreínas da próstata, hK2 e hK4, que cliva 7 aminoácidos na porção N-terminal do proPSA, que torna-se rapidamente ativado para PSA, uma protease ativa de 33 kDa, consistindo de cinco pontes

dissulfeto intracadeia e um oligossacarídeo N-ligado do tipo complexo - biantenário (Figura 4; GILGUNN et al., 2013). No fluido seminal, as funções do PSA são para clivar as proteínas seminogelina I e II, que conduz à liquefação do sêmen (LILJA et al, 1987).

Figura 4. Ilustração da biossíntese do PSA e suas isoformas.



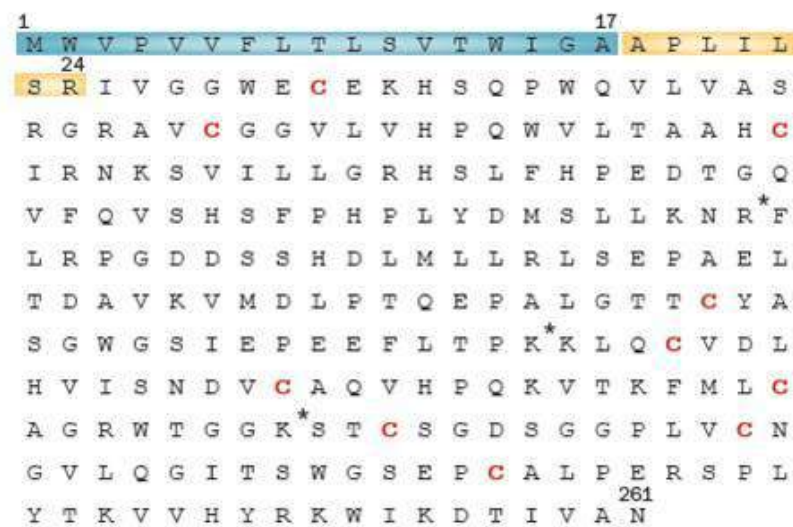
Fonte: Adaptação de GILGUNN et al., 2013.

Outras formas de proPSA também são geradas por clivagem. Isoformas com sequência líder de um, dois, quatro e cinco aminoácidos denominadas [-1]proPSA, [-2]proPSA, [-4]proPSA e [-5]proPSA, respectivamente, tem sido identificadas. Ainda no lúmen, o PSA maduro ativo é clivado em sítios específicos, gerando as isoformas BPSA (conhecida como PSA benigno) e iPSA (PSA inativo; Figura 4). Todas essas isoformas do PSA são encontradas como PSA livre circulante (fPSA), que corresponde a 16% do PSA total (VÉGVÁRI et al., 2012; SIDDIQUI; MAJID; ATHER, 2015). A maior parte do PSA circulante corresponde ao PSA complexado (cPSA), ligado a inibidores de protease principalmente alfa-1-antiquimotripsina (AQT) e alfa-2-macroglobulina (PIERA et al., 2018).

As subformas de proPSA têm sido identificadas como os constituintes predominantes do fPSA. Vários estudos tem mostrado a especificidade de proPSA

para detecção do CaP, especialmente [-2]proPSA e [-4]proPSA. O aumento da expressão de [-2]proPSA tem sido associada com o aumento da agressividade do câncer e tende a acumular-se no soro de homens com CaP (PARK et al., 2018). BPSA é uma forma inativa de PSA propensa à degradação interna nos resíduos Arg85-Phe86, Lys145-Lys146, e Lys182-Ser183 (Figura 5). BPSA é predominantemente encontrado na zona de transição prostática de homens com HBP. Pensa-se que BPSA surge da clivagem pós-tradução por proteases específicas em tecido hiperplásico de HBP (MYKOLAJCZYK et al., 2004).

Figura 5. Composição completa de aminoácidos do PSA. O PSA é sintetizado na forma de pré-proPSA, inativo, com 261 aminoácidos. A sequência de 17 aa do pré-proPSA, destacada em azul, é clivada, resultando no proPSA, com uma sequência inicial de 7 aa destacada em amarelo, que também é clivada, resultando no PSA maduro e ativo. As regiões de pontes intracadeias de cisteína são mostradas em vermelho, e os símbolos de estrela mostram os sítios de clivagem interno do PSA benigno.



Fonte: GILGUNN et al., 2013.

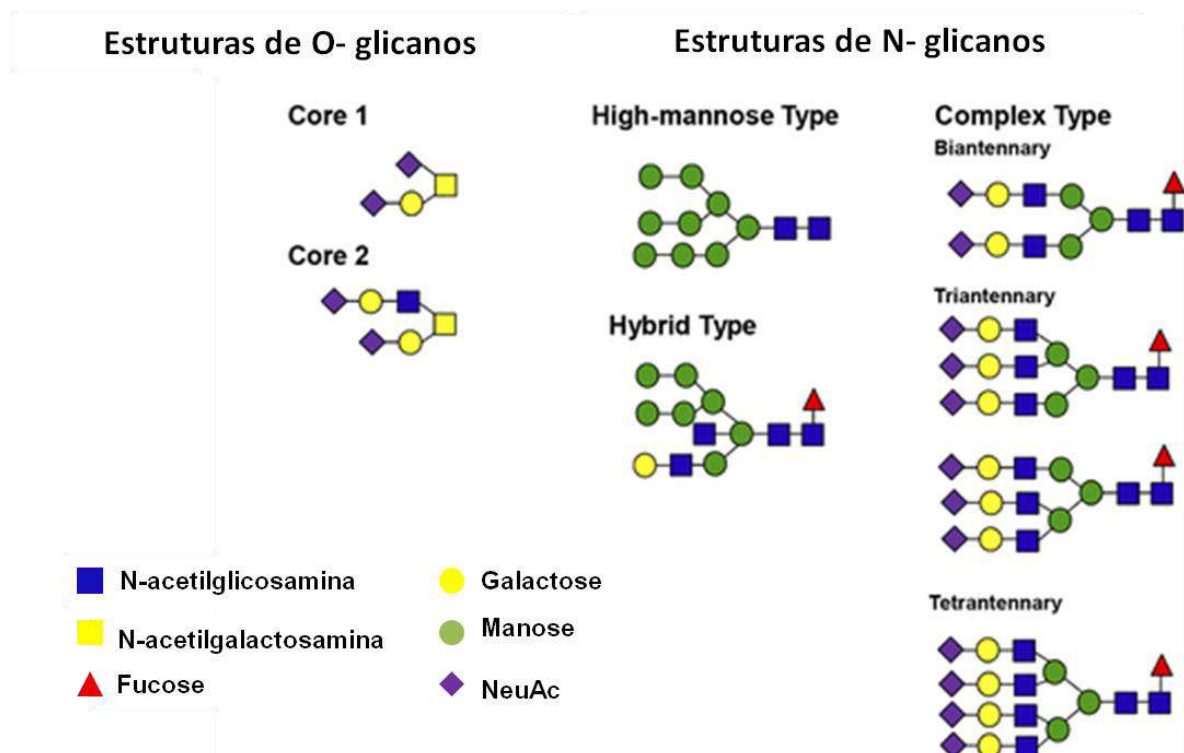
2.4 GLICOSILAÇÃO

Glicosilação é uma das modificações pós-traducionais mais comuns promovida por um sistema altamente regulado de enzimas e transportadores que adicionam covalentemente cadeias de açúcares (glicanos) a proteínas, produzindo as diversas glicoproteínas de superfície celular ou secretadas na circulação

sanguínea. Esse mecanismo faz parte do processamento secundário de proteínas nas células, exercendo um papel crítico na determinação da estrutura da proteína, função, estabilidade e proteção contra proteases (WANG et al., 2012; AEBI, 2013).

Estimativas sugerem que cerca de 70% do proteoma humano é glicosilado (VOGLMEIR et al, 2010). Os glicanos são ligados às proteínas via grupo amino (N- glicanos) ou grupo hidroxila (O- glicanos). Fucose, galactose, glicose, manose, N-acetilglicosamina, N-acetilgalactosamina, e ácido siálico são as principais unidades de formação dos N- e O- glicanos humanos (Figura 6). Os processos de O-glicosilação e N-glicosilação ocorrem durante a passagem da proteína através do retículo endoplasmático e compartimentos de Golgi. Dentro do retículo endoplasmático, carboidratos são adicionados a proteínas recém-formadas em um resíduo de aminoácido específico.

Figura 6. Exemplos de estruturas típicas de N- e O- glicanos expressas em glicoproteínas de plasma humano.



Fonte: PRESTON et al., 2013.

Na N-glicosilação, blocos pré-montados de açúcares são transferidos durante a transdução via grupo amida de um resíduo de asparagina, enquanto na O-glicosilação os açúcares são adicionados à hidroxila dos aminoácidos serina e treonina. Mais de duzentos tipos de transferases e outras proteínas reguladoras adicionais, expressos em padrões e níveis específicos em cada tecido participam da síntese de um conjunto diversificado de glicanos (BUTLER; SPEARMAN, 2014; TERMINI et al., 2017).

O conjunto de glicanos sintetizados por uma célula em condições específicas é chamado de glicoma. Porém, a constituição de um glicoma pode mudar drasticamente em resposta a mudanças sutis no ambiente celular, incluindo alterações extensas dos padrões de glicosilação, sendo característico de muitas doenças.

2.4.1 Glicosilação no Câncer de próstata

Recentes estudos no campo da glicômica têm mostrado que padrões de glicosilação anormais são uma característica fundamental da transformação maligna e progressão do câncer. Aberrações na expressão de glicanos e glicoproteínas podem ser observadas na presença do câncer, inclusive redução ou aumento na expressão de uma estrutura ou padrão particular, sugerindo que glicanos e glicoproteínas são alvos viáveis para detecção e diagnóstico do câncer (DE VROOME et al., 2018; CHANG et al., 2019).

Muitos estudos têm identificado alterações nos padrões de glicosilação que possibilitam detectar o CaP e distinguir de condições benignas. SALDOVA et al. (2011) investigaram a possibilidade de distinguir entre diferentes estágios de CaP e HBP, analisando os padrões de glicosilação em soros de homens com HBP e com CaP escore de Gleason 5 e 7. Foi encontrada maior expressão de glicanos biantenares fucosilados e ligados a α 2-3 ácido siálico nos soros oriundos de CaP em comparação com HBP, e observaram redução notável na expressão de glicanos triantenares trigalactosilados e glicanos tetrassialilados com um braço externo fucosilado, e aumento nos níveis de glicanos tetra-antenares tetrassialilados em CaP escore 7 em comparação com escore 5. Um estudo baseado em análise glicoproteômica de tecidos oriundos de CaP identificou 350 glicopeptídios, entre os

quais 17 encontravam-se alterados em CaP agressivo; e algumas glicoproteínas também mostraram-se alteradas, com aumento na expressão (ex.: periostina) e redução (ex.: monoamina oxidase.) em CaP agressivo (CHEN et al., 2013). Assim, alterações na glicosilação são sugestivos de câncer e possibilitam a identificação de diferentes estágios de CaP, que não seria possível através do teste de PSA atual.

Um estudo mais recente avaliou os níveis de glicoformas de centenas de proteínas séricas em CaP e HBP, usando cromatografia de afinidade com lectinas e espectrometria de massas. Foram observadas notáveis alterações específicas em glicoproteínas para HBP e CaP (TOTTEN et al., 2018). Outra estratégia baseada em análises glicoproteômicas investigou o valor diagnóstico de glicoproteínas encontradas na urina para discriminar entre CaP e HBP. N- e O- glicopeptídeos foram isolados e quantificados por espectrometria de massa de alta resolução, e encontraram um painel de 56 N-glicopeptídeos intactos que mostraram potencial para detectar CaP e distinguir de HBP (KAWAHARA et al., 2018).

Também têm sido descritas alterações no padrão de glicosilação de diferentes formas do PSA oriundas de CaP e HBP, a maioria delas relacionadas aos níveis de ácido siálico ligados ao PSA. A presença de resíduos de ácido siálico α 2-3 ligados ao PSA pode ser útil para discriminar entre doença maligna e benigna quando se compara os perfis de oligossacarídeos de fPSA e cPSA no soro e plasma seminal de CaP (TAJIRI; OHYAMA; WADA, 2008). Outro estudo de caracterização de glicanos presentes em subformas (F1-F5) obtidas por eletroforese bidimensional do PSA revelaram diferenças nos níveis de ácido siálico para as subformas F3 e F4, que podem distinguir o CaP de HBP. A subforma F3, que possui N-glicanos mono- e disialilados, está reduzida no CaP quando comparado à HBP, e diminui gradativamente com o estágio do câncer, enquanto a subforma F4, que aumenta gradativamente com o estágio do CaP, apresenta apenas a forma monosialilada. Os resultados indicaram redução nos níveis de ácido siálico no CaP, e a quantificação de subformas F3 pode auxiliar no diagnóstico da doença (SARRATS et al., 2010).

Outro estudo investigou e comparou os níveis de fucose e ácido siálico em N-glicanos ligados ao PSA em soros de pacientes com HBP e CaP em diferentes graus de agressividade, e encontrou uma redução significativa na expressão de fucose e um aumento de α 2,3- ácido siálico no PSA dos casos de CaP mais agressivos, que permitiu distinguir de HBP e CaP de baixo risco (LLOP et al., 2016). Também foi

desenvolvido um ensaio para identificar e quantificar as glicofomas do PSA isolado a partir da urina, o qual permite diferenciar inclusive as formas $\alpha 2,6$ - e $\alpha 2,3$ - ácido siálico- ligadas, além de outros glicopeptídios, mostrando potencial para discriminar entre CaP agressivo, CaP não agressivo e HBP (KAMMEIJER et al., 2018).

Estes estudos mostram que a expressão de padrões de glicanos diferenciados, tanto no PSA como também em outras glicoproteínas, são úteis como marcadores para o CaP e apresentam maior especificidade que o teste de PSA sérico atual.

2.5 LECTINAS

As lectinas são uma família de proteínas ligadoras de carboidratos, de ocorrência natural, que pode discriminar entre diferentes estruturas de glicanos e são, portanto, ferramentas muito úteis para glicoanálise. Estão envolvidas em eventos fisiológicos cruciais de interações proteína-carboidrato, como adesão, migração e interação celular (BROWN; WILLMENT; WHITEHEAD, 2018; VILLRINGER et al., 2018).

O termo lectina, oriundo do latim “lectus” (escolhido, selecionado), foi introduzido por BOYD e SHAPLEIG (1954), para definir um grupo de proteínas que apresentavam seletividade na interação com carboidratos. Uma definição mais aceita foi proposta por PEUMANS e VAN DAMME (1995; 1998), que as definiram como proteínas ou glicoproteínas de origem não imune que contém pelo menos um sítio não-catalítico de ligação reversível e específica a monossacarídeos ou oligossacarídeos. SHARON e LIS (2002) ampliaram esse conceito, abrangendo todas as proteínas presentes em fontes naturais, de origem não imunológica, capazes de ligarem-se a carboidratos com especificidade ou não para eritrócitos de um determinado grupo sanguíneo. A origem não imunológica das lectinas distinguem-as de anticorpos anticarboidratos, que também aglutinam células. Diferente dos anticorpos, que são estruturalmente similares, as lectinas diferenciam-se entre si pela composição aminoacídica, requerimento de metais, peso molecular e estrutura tridimensional (OBAID et al., 2015; GAUTAM et al., 2018).

A constante de associação entre lectinas e monossacarídeos varia de 10^3 a $5 \times 10^4 \text{ M}^{-1}$, e entre lectinas e oligossacarídeos, 10^4 a 10^7 M^{-1} , valores inclusos na

mesma faixa encontrada para ligações anticorpo-antígeno e enzima-substrato (SHARON; LIS, 1990; SHARON; LIS, 2002). Cada lectina liga-se a um monossacarídeo específico, oligossacarídeo ou glicoconjugados através de seus sítios de ligação que tendem a se localizar na superfície da proteína. Essa interação ocorre por ligações de hidrogênio, interações de Van der Waals e interações hidrofóbicas (CAVADA et al., 2018; MANZONI et al., 2018). No entanto, a maioria dessas interações é feita pelas ligações de hidrogênio devido a disponibilidade de grupos hidroxila nos açúcares, permitindo a formação dessas ligações entre os aminoácidos do sítio de ligação na lectina e o açúcar, além da participação de moléculas de água como pontes na formação de ligações de hidrogênio entre lectina e carboidrato.

Embora tenham sido inicialmente encontradas em plantas, as lectinas estão amplamente distribuídas entre vegetais, vírus, microrganismos e animais (COELHO et al., 2018). Lectinas tem sido detectadas em mais de 600 espécies, sendo cerca de 70 já purificadas, geralmente por cromatografia de afinidade com açúcares ou derivados imobilizados, e mais de 40 dessas lectinas estão disponíveis comercialmente (SHARON; LIS, 1990; DAN; LIU; NG, 2016).

Lectinas de leguminosas é a família de lectinas de plantas que compreende o maior número de proteínas já identificadas e melhor caracterizadas. Todas lectinas de leguminosas, embora derivadas de espécies taxonomicamente distintas, tem muitas características moleculares e propriedades físico-químicas em comum, porém com grande variedade em sua especificidade para carboidratos (Tabela 1) (RAMOS et al., 2000; GAUTAM et al., 2018). Usualmente consistem de duas ou quatro subunidades de 25-30 kDa, cada uma com um sítio de ligação a carboidrato (VAREJÃO; CORREIA; FOGUEL, 2011). Também possuem sítio de ligação a metais como os cátions divalentes Ca^{2+} e Mn^{2+} , cuja ligação é importante para estabilizar e fixar a estrutura protéica, bem como para o dobramento correto de *loops* que formam o sítio de ligação de monossacarídeos, sendo indispensáveis para a interação lectina-carboidrato (SOUZA et al., 2003).

Tribo ou sub-tribo	Espécies	Especificidade
<i>Diocleinae</i>	<i>Canavalia ensiformis</i>	Man/Glc
	<i>C. brasiliensis</i>	Man/Glc
	<i>Dioclea grandiflora</i>	Man/Glc
	<i>Cratylia floribunda</i>	Man/Glc
<i>Hedysareae</i>	<i>Onobrychis vicifolia</i>	Man/Glc
<i>Dalbergieae</i>	<i>Vatairea macrocarpa</i>	Gal
<i>Vicieae</i>	<i>Pisum sativum</i>	Man/Glc
	<i>Vicia faba</i>	Man/Glc
	<i>V. villosa-B4</i>	GalNAc
	<i>Lens culinaris</i>	Man/Glc
	<i>Lathyrus ochrus</i>	Man/Glc
<i>Genisteae</i>	<i>Crotalaria striata</i>	GalNAc
	<i>Ulex europaeus I</i>	L-Fucose
	<i>Ulex europaeus II</i>	GlcNAc
<i>Trifolieae</i>	<i>Medicago sativa</i>	Gal
	<i>M. truncatula</i>	Man/Glc
	<i>Trifolium repens</i>	Gal
<i>Glycineae</i>	<i>Glycine max</i>	GalNAc
<i>Phaseoleae</i>	<i>Phaseolus vulgaris</i>	Complex
	<i>Dolichos biflorus</i>	GalNAc
	<i>Arachis hypogaea</i>	Gal
<i>Hedysareae</i>	<i>Onobrychis vicifolia</i>	Glc/Man
<i>Shophoreae</i>	<i>Shophora japonica</i>	GalNAc
<i>Erythrineae</i>	<i>Erythrina velutina</i>	Gal
<i>Lotae</i>	<i>Lotus tetragonolobus</i>	L-Fucose
<i>Bauhinieae</i>	<i>Griffonia simplicifolia</i>	GalNAc
	<i>Bauhinia purpurea</i>	GalNAc
<i>Parkieae</i>	<i>Parkia platycephala</i>	Man/Glc

Tabela 1. Algumas lectinas pertencentes à família *Leguminosae* distribuída em várias tribos com especificidades para diferentes monossacarídeos.

Fonte: RAMOS et al., 2000.

2.5.1 Lectinas como ferramentas biotecnológicas

A disponibilidade de um grande número de lectinas e seu potencial de reconhecer diferentes carboidratos tem introduzido essas proteínas como ferramenta de reconhecimento em diversas pesquisas biotecnológicas. Graças à especificidade para mono- e oligossacarídeos expressos na superfície celular, as lectinas podem ser utilizadas para tipagem de células sanguíneas (DAS, 2015); como inseticida, ao prejudicar o desenvolvimento e mortalidade de larvas (CHEN et al., 2018); como

bactericida e fungicida (CHEN et al., 2018; MIKI et al., 2018), e vêm sendo investigadas como antivirais, por impedir a invasão e replicação viral (MAZALOVSKA; KOUOKAM, 2018). Também têm sido utilizadas em matrizes de afinidade para reconhecimento e purificação de glicoproteínas (KOCH et al., 2018) e no desenvolvimento de sistemas de liberação de drogas em mucosas (ZHOU et al., 2015).

Recentemente, as lectinas têm sido amplamente utilizadas em glicohanálise para identificar alterações na composição de glicanos e elucidar os mecanismos fisiológicos e patológicos relacionados a essas alterações, bem como as modificações em genes reguladores das enzimas que participam da glicosilação (MATSUDA et al., 2015; LLOP et al., 2016). Aumento ou redução na ramificação dos glicanos e na expressão de monossacarídeos como ácido siálico, fucose e manose em diferentes estágios de transformação tumoral são alterações comumente relacionadas à progressão do câncer e metástase (CHANG et al., 2019). O surgimento de antígenos glicosilados câncer-associado, como o antígeno Tn-siálico (Neu5Ac α 2-6GalNAc-O-Ser/Thr) também é frequentemente associado ao câncer (LLOP et al., 2016; EAVARONE et al., 2018).

A especificidade com que as lectinas reconhecem os glicanos permite a identificação de alterações tumor-específicas, levando à descoberta de novos biomarcadores glicosilados. Assim, lectinas têm sido reportadas como potenciais ferramentas de reconhecimento de alterações em glicanos relacionadas a doenças como o câncer. Um imunoenensaio utilizando a lectina de *Sambucus nigra*, SNA, e as lectinas de *Maackia amurensis* MAL-I e MAL-II foi desenvolvido para comparar a expressão de ácido siálico no PSA presente em soros de CaP e em soros não cancerosos. Os resultados do estudo sugerem que os padrões de expressão de ácido siálico do PSA são mais heterogêneos no CaP, podendo discriminar entre amostras cancerosas e não cancerosas (MEANY et al., 2009). ABBOTT e colaboradores (2010) utilizaram lectina de *Phaseolus vulgaris* (E-PHA), lectina de *Aleuria aurantia* (AAL) e lectina de *Datura stramonium* (DSL) para caracterização glicoproteômica comparativa de soros e tecidos sadios e tumorais de pacientes com câncer de ovário endometrióide e detectaram alterações tumor-específicas na glicosilação de algumas glicoproteínas, entre elas periostina, trombospondina e

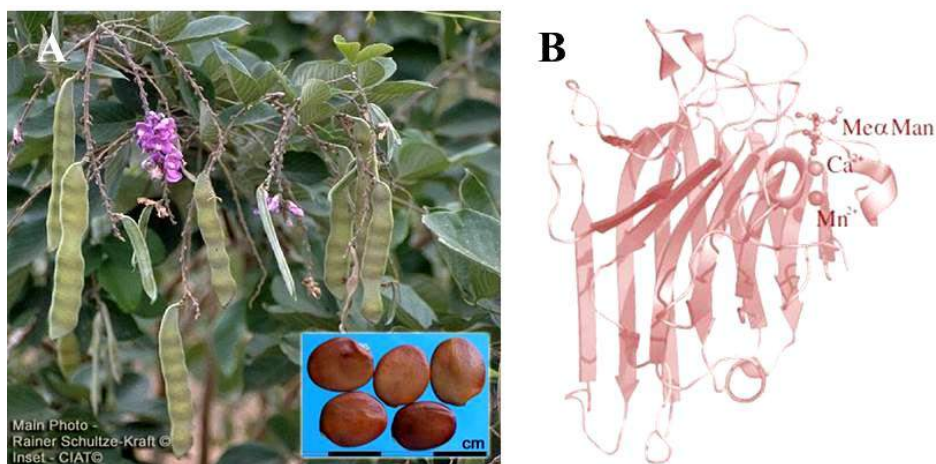
MUC5b, glicoproteínas pouco exploradas como biomarcadores, porém potenciais candidatas a marcadores glicosilados do câncer de ovário.

Outro estudo relata o desenvolvimento de um ensaio de lectina reversa baseado na captura de glicoproteínas com lectina AAL seguida da detecção com anticorpos conjugados à peroxidase como método para analisar alterações de glicoproteínas em amostras de soro de pacientes de câncer de ovário. Foi observado um aumento de haptoglobina fucosilada e que a combinação com CA 125 melhora a possibilidade diagnóstica para distinguir câncer de ovário de estágio III dos casos benignos (WU et al., 2014). Também foi desenvolvido um imunoensaio tipo sanduíche de anticorpo – lectina para analisar a glicosilação do antígeno CA 15-3 em pacientes com câncer de mama, utilizando o anticorpo anti- CA 15-3 para capturar o antígeno no soro, e a lectina concanavalina A (ConA), para analisar a glicosilação do antígeno capturado. Os resultados mostraram que o nível de glicosilação de CA 15-3 aumenta com o grau de agressividade do câncer, mostrando potencial para distinguir entre os estágios I, IIA, IIB, III, e doença benigna da mama, além de ser uma abordagem mais sensível que a detecção apenas dos níveis de CA 15-3 (CHOI et al., 2018).

2.5.2 Lectinas de *Cratylia mollis* - Cramoll

Cratylia mollis Mart. é uma forrageira nativa da região Semi-árida de Pernambuco, estado do nordeste do Brasil, popularmente conhecida como feijão camaratu ou camaratuba. Pertence a família Leguminosae, grupo *Phaseoleae* e subgrupo *Diocleinae*, que também contém o gênero *Canavalia*, ao qual pertence à espécie *Canavalia ensiformes*, de cujas sementes é obtida a lectina Concanavalina A (Con A) (SUMNER, 1919; MATTIASSON; RAMSTORP, 1984). As sementes de *C. mollis* são fontes de lectinas denominadas Cramoll (Figura 7 A e B), encontradas em quatro isoformas moleculares com especificidade variada, denominadas Cramoll 1, Cramoll 2, Cramoll 3 e Cramoll 4, além de uma preparação contendo as isoformas 1 e 4, denominada Cramoll 1, 4, similar a Con A. As isoformas mais estudadas são a Cramoll 1 e a Cramoll 1, 4, com especificidade glicose/ manose, diferentemente de Cramoll 2 e Cramoll 3, que são galactose específica (PAIVA; COELHO, 1992; CORREIA; COELHO, 1995; SANTOS et al., 2004).

Figura 7. (A) Vagens e sementes de *C. mollis*. (B) Estrutura terciária de Cramoll 1, uma lectina de semente de *C. mollis*.



Fonte: (A) <<http://www.tropicalforages.info/key/Forages/Media>> (B) SOUZA et al, 2003.

Em um estudo de caracterização utilizando cristalografia de raio-X, Cramoll 1 apresentou estrutura terciária clássica de lectinas de leguminosa. Sua estrutura primária é constituída por 236 resíduos de aminoácidos, com 82 % de homologia com a estrutura de ConA, e ambas possuem sítios de ligação ao carboidrato metil- α -D-manopiranosídeo idênticos, com o qual interagem por ligações de hidrogênio também idênticas, e sítios de ligação aos metais Ca²⁺ e Mn²⁺ (Figura 8 B). Cramoll 1 é uma lectina do grupo de especificidade glicose/ manose, sendo metil- α -D-manopiranosídeo o monossacarídeo para o qual apresenta melhor afinidade (CORREIA; COELHO, 1995). A estrutura secundária é composta exclusivamente por folhas β , todas conectadas por voltas (SOUZA et al., 2003).

Estudos desenvolvidos com as isolectinas de *C. mollis* tem mostrado seus potenciais como ferramentas biotecnológicas, principalmente Cramoll 1 e Cramoll 1,4. Algumas aplicações têm chamado a atenção, como ação antitumoral (ANDRADE et al., 2004); atividade mitogênica (MELO et al., 2011b); atividade pró-inflamatória e cicatrizante de lesões teciduais experimentais (MELO et al., 2011a); marcadores de superfície celular de tecido mamário e prostático (BELTRÃO et al., 1998; LIMA et al., 2010), anti-helmíntico (MELO et al, 2011c); composição de matriz

para purificação de glicoproteínas (SILVA et al., 2011); biomolécula de reconhecimento em biossensores eletroquímicos para detecção de glicoproteínas em diferentes sorotipos de dengue, e reconhecimento de bactérias gram-negativas (OLIVEIRA et al., 2011a; 2011b; AVELINO et al., 2014) e imunomodulação (DA SILVA et al., 2015).

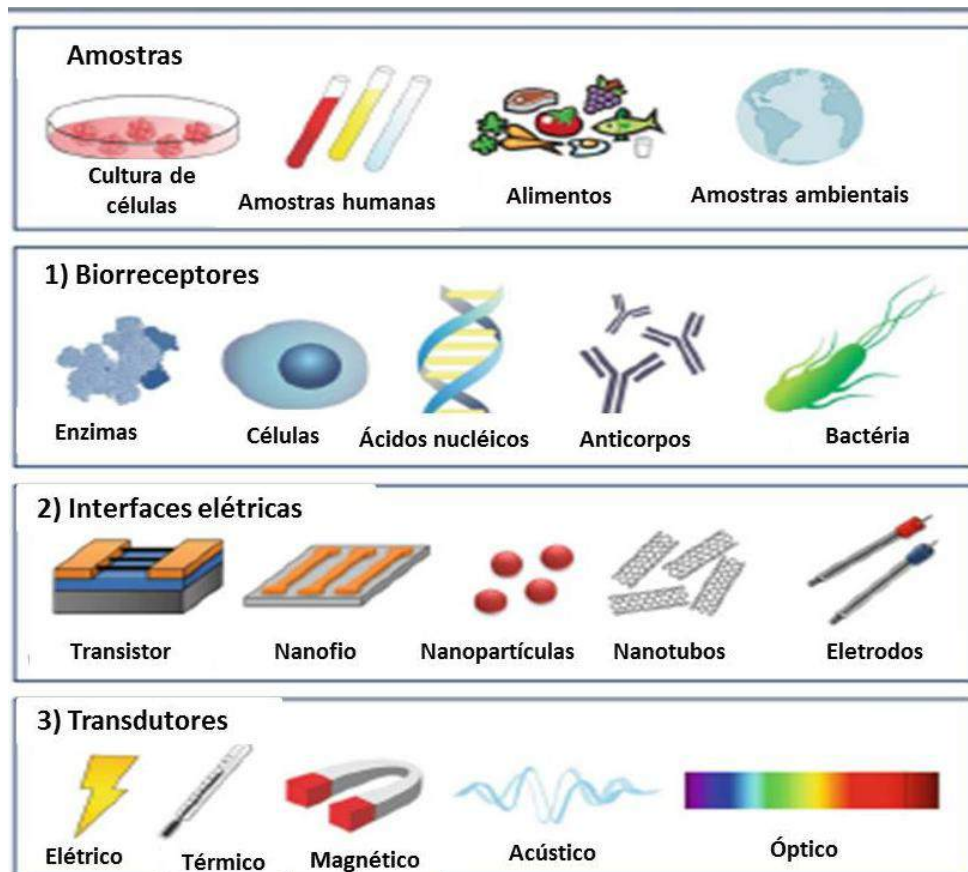
2.6 BIOSSENSORES

A importância biológica dos glicanos na detecção de doenças como o câncer tem impulsionando o desenvolvimento de novas ferramentas glicoanalíticas. As técnicas utilizadas na análise de glicanos incluem eletroforese de zona capilar, espectrometria de massa, cromatografia líquida de alta eficiência e combinações de ensaio imunossorvente com lectinas (COUZENS et al., 2014; ITO et al., 2018; YANG et al., 2018). Em comparação a essas técnicas, o uso de biossensores permite detectar anormalidades na expressão de glicanos e glicoproteínas com maior rapidez, sensibilidade e baixo custo. O desenvolvimento desses dispositivos bioanalíticos tem ganhado espaço e colaborado na elucidação de fenômenos bioquímicos e biológicos, incluindo informações sobre um analito, monitoramento e diagnóstico de patologias baseados em técnicas de eletroanálise (BERTÓK et al., 2013; BAHADIR et al., 2015).

Uma definição proposta pela União Internacional de Química Pura e Aplicada (IUPAC, do inglês *International Union of Pure and Applied Chemistry*) aponta o biossensor como um dispositivo analítico integrado capaz de fornecer informação específica quantitativa ou semi-quantitativa utilizando um elemento de reconhecimento biológico imobilizado à superfície sensora do transdutor, que identifica e converte o sinal da reação bioquímica em um sinal analiticamente útil e mensurável através de um sistema de detecção específico (KIMMEL et al., 2012; MISHRA et al., 2018). Em geral, um biossensor é composto por três elementos (Figura 8): (1) a biomolécula de reconhecimento (biorreceptor), que se liga de forma específica ao analito; (2) uma interface eletroquimicamente ativa, onde ocorrem as interações bioquímicas específicas, dando origem a um sinal; (3) um elemento transdutor, que converte a reação bioquímica específica em um sinal elétrico, amplificado por um circuito de detecção. Associado ao biossensor, tem-se um

processador de sinal (por exemplo, software de computador), para converter o sinal eletrônico em um parâmetro físico significativo, e uma interface apropriada para apresentar os resultados (LUZ; IOST; CRESPILO, 2013).

Figura 8. Componentes de um biossensor.



Fonte: LUZ; IOST; CREPILHO, 2013.

O primeiro biossensor foi desenvolvido por CLARK e LYONS em 1962, para detecção de glicose através da reação catalisada pela glicose oxidase. Eles elaboraram um sistema conhecido como eletrodo enzimático, utilizando a enzima glicose oxidase imobilizada em um filme de poliácridamida na superfície de um eletrodo de platina, e a determinação da concentração de glicose foi baseada na mensuração do consumo de oxigênio. Mais tarde, a *Yelow Spring Instrument Company* desenvolveu a tecnologia de Clark e Lyons para introdução no mercado do primeiro biossensor de glicose em 1975, baseado na detecção amperométrica do

peróxido de hidrogênio (YOO; LEE, 2010). Desde então, a tecnologia dos biossensores tem sido marcada pelo desenvolvimento de novas abordagens nas formas de detecção e na elaboração de dispositivos mais eficientes e precisos, visando os mais diversos tipos de análises.

Atualmente, os biossensores podem ser aplicados em estudos envolvendo uma grande variedade de amostras, como fluidos corporais, amostras de alimentos, culturas de células e amostras para análise ambiental. O biorreceptor ou componente biológico pode ser células, como microrganismos, e biomoléculas como receptores celulares, enzimas, anticorpos, ácidos nucleicos e lectinas (BERTÓK et al., 2013; POHANKA, 2018), que reconhecem o(s) analito(s) de interesse por meio de uma reação bioquímica, gerando alterações no microambiente que sinalizam a reação, tais como mudanças de estado de oxidação, liberação de calor, emissão de luz, variação de massa, entre outras. O transdutor converte este sinal em uma resposta mensurável do tipo corrente, potencial, frequência, etc. Com base no sistema de detecção e resposta, os transdutores podem ser classificados como eletroquímicos; térmicos ou calorimétricos, ópticos, piezoelétricos e acústicos (PERUMAL; HASHIM, 2014; GORADEL et al., 2018). Também são utilizados sistemas de detecção para leitura e processamento dos dados obtidos pelo transdutor e transformação em gráficos ou valores numéricos. Nos diversos tipos de biossensores, esses componentes são fundamentais para a construção dos dispositivos.

Os biossensores ainda podem ser classificados como catalíticos ou de afinidade, de acordo com o tipo de interação entre o elemento de reconhecimento biológico e o substrato de interesse. Os biossensores catalíticos utilizam enzima, célula, tecido, organela, e microorganismo como estrutura de reconhecimento e são caracterizados pela ocorrência de reação química com o substrato, gerando produtos (RABA et al., 2013). Os biossensores de afinidade baseiam-se na formação de complexos entre o substrato e o receptor, sem levar a transformações químicas. Entre os sistemas de bioafinidade destacam-se o imunocomplexo antígeno-anticorpo, a interação lectina-carboidrato e a hibridização de sequências de ácidos nucleicos de fita simples (SILVA et al., 2016; SOARES et al., 2018; MANDLI; AMINE, 2018).

2.6.1 Biossensores eletroquímicos

Diversos são os sistemas de transdução empregados nos biossensores, sendo os transdutores eletroquímicos os mais atrativos e predominantes em bioanálise, devido à diversidade de técnicas e materiais transdutores que colaboram para o desenvolvimento de biossensores seletivos e sensíveis (ZHU et al., 2014; HARRAD et al., 2018). Os biossensores eletroquímicos caracterizam-se pela capacidade de converter o sinal da interação biológica na interface eletródica em um sinal elétrico (TURNER, 2013).

Eletrodos de diversos materiais tais como: platina, ouro, grafite, carbono vítreo e tinta de carbono são usados na construção de biossensores eletroquímicos (MOYO; OKONKWO; AGYEI, 2012). Dentre os transdutores eletroquímicos, podem-se destacar os amperométricos, potenciométricos, condutimétricos e impedimétricos.

Os biossensores amperométricos baseiam-se na mensuração de corrente resultante da oxidação ou redução de uma espécie eletroativa, tal como a presença de grupamentos oxidáveis nas estruturas biológicas, onde o valor da corrente medida sob um potencial de escala linear é diretamente proporcional à concentração em massa do analito (espécie eletroativa) (BERTÓK et al., 2013). Nos sensores potenciométricos, mede-se o potencial ou acúmulo de carga no eletrodo de trabalho em comparação com o eletrodo de referência em uma célula eletroquímica quando o fluxo de corrente entre eles é zero ou insignificante (CHIRIZZI; MALITESTA, 2011). Os dispositivos condutimétricos medem a capacidade de uma substância ou um meio de conduzir uma corrente elétrica entre os eletrodos ou no eletrodo de referência. A concentração de carga (condutividade) é obtida através de medições da resistência da solução entre dois eletrodos (TEKAYA et al., 2014). Os biossensores impedimétricos detectam a interação do biorreceptor com o analito com base na alteração da resistência à transferência de elétrons na superfície do eletrodo (impedância), a qual aumenta após a ligação do analito (PIHÍKOVÁ et al., 2016).

No geral, biossensores eletroquímicos apresentam muitas vantagens, tais como baixo custo, facilidade de construção e mensuração, alta sensibilidade, resposta rápida, fácil manuseio para detecção de analitos e requer um volume muito

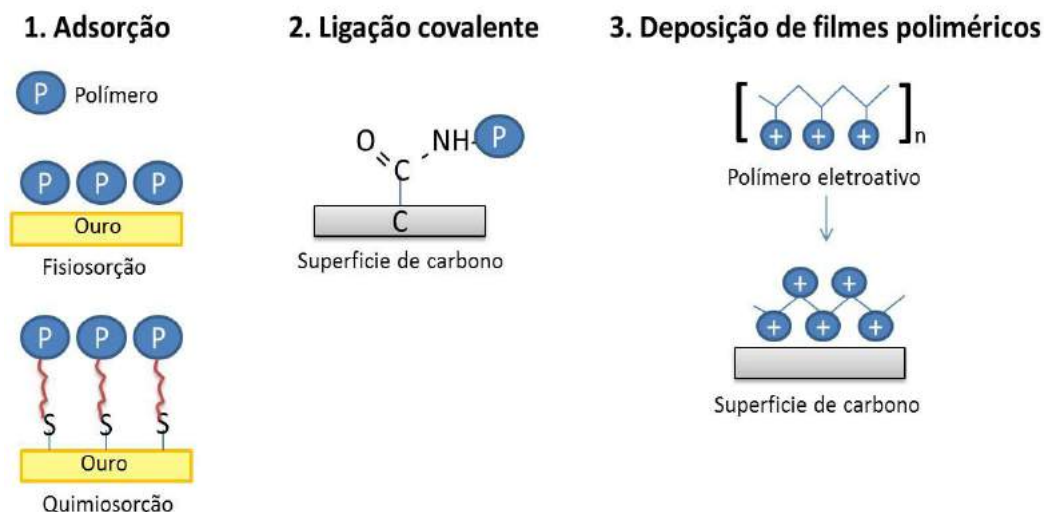
pequeno da amostra. Esses dispositivos têm sido portabilizados e produzidos em grande escala para comercialização, empregando a tecnologia do eletrodo impresso, de design flexível e possibilidade de automação, tornando-os ferramentas promissoras para o diagnóstico e monitoramento de doenças (BAHADIR et al., 2015; ARDUINI et al., 2016).

2.6.2 Eletrodos quimicamente modificados

Apesar do grande potencial analítico apresentado pelos biossensores eletroquímicos, os eletrodos utilizados na construção desses dispositivos podem apresentar algumas limitações que prejudicam as propriedades analíticas. A gradual passivação na superfície eletródica devido à adsorção dos produtos das reações de oxidorredução na interface eletrodo/ solução é uma das principais limitações (BRETT; OLIVEIRA-BRETT, 2011; RAHMAN et al., 2012). Além disso, a sensibilidade a muitos analitos pode ser reduzida em função da cinética de transferência de elétrons ser lenta na superfície desses eletrodos (TUNCER et al., 2012). Uma alternativa para minimizar tais problemas é a construção de biossensores eletroquímicos baseados em eletrodos quimicamente modificados (EQM). Muitos estudos têm empregado polímeros e nanomaterias na modificação de eletrodos de platina, ouro, carbono vítreo, entre outros (ZHU et al., 2014; BARSAN et al., 2015).

Um EQM consiste em um eletrodo base (substrato) recoberto pelo modificador químico, capaz de agregar ao eletrodo novas propriedades físico-químicas de interesse. Os principais benefícios para o emprego de eletrodos modificados na eletroanálise tem sido a possibilidade de aceleração de reações de transferência de carga e permeação seletiva de analitos na superfície. Tais processos podem melhorar a seletividade, reatividade e estabilidade de biossensores eletroquímicos como métodos analíticos (ZOHOURTALAB; RAZMI, 2018). Um modificador químico pode ser incorporado na superfície de um eletrodo pelos métodos de adsorção, ligação covalente e deposição de filmes poliméricos (Figura 9).

Figura 9. Representação esquemática para diferentes tipos de preparação de EQM.



Fonte: A autora (2017).

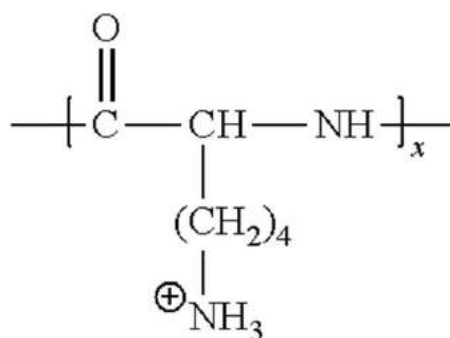
A adsorção é realizada simplesmente pela imersão do eletrodo em solução contendo o agente modificador (polímero, nanomateriais ou nanocompósitos) ou disposição dessa solução na superfície eletródica, seguida da evaporação do solvente e formação de filme através da combinação de propriedades de adsorção e atração eletrostática. Na ligação covalente, o modificador é ligado covalentemente à superfície eletródica baseado na reatividade de grupamentos funcionais, como óxidos e aminas. Na deposição de filmes, são imobilizadas camadas do polímero, geralmente eletroativo, sobre o eletrodo a partir de soluções de polímeros pré-formados, ou através da polimerização de unidades monoméricas (DAVID et al., 2018; NING et al., 2019).

Os polímeros condutores e polieletrólitos sintéticos são os mais escolhidos na construção de EQMs, pois favorecem a transferência de elétrons, a eletrocatalise, a transdução de sinal, e adição de grupamentos reativos para interação com nanoestruturas e biorreceptores. Tais propriedades melhoram o tempo de resposta, a sensibilidade e o limite de detecção dos biossensores (SILVA et al., 2015; DAVID et al., 2018). Entre os polímeros condutores mais aplicados em estudos com biossensores, estão a polianilina, o politiofeno e o polipirrol, e como polieletrólito, a poli-L-lisina, que foi utilizada neste trabalho.

2.6.3 Poli-L-Lisina

O estudo do comportamento eletroquímico dos eletrodos modificados com poliaminoácidos iônicos é de grande importância na área de eletroanálise. Um desses poliaminoácidos é a poli-L-lisina (PLL), que tem sido empregado na modificação de superfícies eletródicas. É um polímero obtido pela condensação de várias unidades de seu monômero, L-lisina, um aminoácido dotado de uma cadeia de hidrocarbonetos com cinco grupamentos $-CH_2$ e um grupo amino ($-NH_2$) na posição ϵ (Figura 10). As reações de formação do polímero ocorrem entre as unidades monoméricas da L-lisina através da formação de ligações peptídicas.

Figura 10. Estrutura molecular da poli-L-lisina.



Fonte: Disponível em <<http://www.google.com/patents/US8574384>>. Acesso em: 10/10/2017.

O polímero apresenta pK_a de 10,7; sendo catiônico em pH neutro (ANSON; SAVEANT; SHIGEHARAT, 1983 a; 1983 b). Dessa maneira, a estrutura final em pH neutro apresenta as funções peptídicas e grupos amino ativos (NH_3^+) abundantes nas cadeias laterais, que conferem várias vantagens, incluindo troca iônica e eletrocatalise, propriedades de grande interesse na área de biossensores, além da flexibilidade estrutural, biocompatibilidade, biodegradável e relativamente boa solubilidade em água (HAMANO, 2011; ZHU et al., 2017). Comercialmente, pode ser encontrado sobre diversos pesos moleculares resultantes do número de unidades matriciais utilizadas para sua confecção.

PLL pode existir em 3 tipos de conformação: α -hélice, β -folha, ou aleatória, dependendo do ambiente externo. Estudos mostram que transições conformacionais na cadeia livre de PLL em soluções podem ser induzidas por vários estímulos externos, incluindo pH, surfactantes, ânions, poliânions, solventes, calor, força de cisalhamento, os quais podem modificar suas propriedades (WANG; CHANG, 2003).

PLL tem sido utilizada na composição de nanocápsulas e vesículas de liberação controlada de genes e drogas, na imobilização celular e como agente antimicrobiano (HAMANO, 2011). A modificação de eletrodos com PLL tem sido investigada, utilizando-se diversos substratos eletródicos e técnicas de modificação. O uso de filmes de polieletrólitos na eletroanálise tem se baseado em mecanismos típicos de troca-iônica, na incorporação de pares redox, conferindo propriedades eletrocatalíticas ao eletrodo, ou ainda grupamentos ligantes capazes de interagir especificamente com o analito através de reações de complexação (ZHAO et al., 2010).

Os estudos pioneiros empregando PLL na formação de filmes sobre eletrodos foram realizados por ANSON e colaboradores (1983a; 1983b), que conseguiram formar filmes de PLL sobre eletrodo de grafite, a partir de soluções aquosas do polímero gotejadas sobre o eletrodo e aquecidos a 80°C, promovendo mudança conformacional da cadeia de PLL da forma α -helicoidal para β -laminar. Mais tarde, foram desenvolvidos filmes de PLL sobre eletrodos de outros materiais e utilizando outras metodologias, tais como eletropolimerização, deposição anódica e desenvolvimento de nanocompósitos.

JIANG et al. (2008) elaboraram um genossensor utilizando eletrodo de tinta de carbono modificado com filme de PLL obtido por eletropolimerização da L-lisina na superfície do eletrodo. ZHAO et al. (2010) e ZHANG et al. (2017) conseguiram formar filmes de PLL a partir da eletropolimerização de L-lisina na superfície de eletrodo de carbono vítreo. NILSSON et al. (2016) conseguiram depositar camadas de PLL na superfície de uma microbalança de cristal de quartzo sob condições anódicas, formando filmes reproduzíveis e uniformes. Outros trabalhos descrevem a formação de filmes de nanocompósitos de PLL com nanomateriais sobre superfícies eletródicas para o desenvolvimento de biossensores (JALIT et al., 2008; WANG et al., 2016).

2.6.4 Tecnologia do eletrodo impresso

Os biossensores eletroquímicos recebem grande crédito e preferência em relação aos demais mecanismos de transdução, devido à sua facilidade, baixo custo de instrumentação, capacidade de miniaturização e automação. Neste contexto, o uso de eletrodos impressos (SPE, do inglês *screen-printed electrode*) contribuiu significativamente, por oferecerem confiabilidade, reprodutibilidade, possibilidade de produção em massa e baixo custo.

Os SPEs geralmente são elaborados em uma configuração de três eletrodos (eletrodo de trabalho, de referência, e auxiliar), impressos em substratos de plástico, cerâmica ou papel, os quais são facilmente modificáveis com tintas comerciais ou de fabricação própria (Figura 11).

Durante o processo de impressão desses eletrodos, as tintas mais comumente utilizadas são a de prata e a de carbono. A tinta de prata é impressa na área do eletrodo de referência e no contato elétrico, enquanto os eletrodos de trabalho são geralmente impressos usando tintas de à base de grafite, ouro ou platina (Figura 11). Tintas de carbono, por exemplo, consistem em partículas de grafite, ligante polimérico e outros aditivos que auxiliam na dispersão, impressão e aderência da tinta, e são bastante utilizadas na construção de SPEs (TALEAT; KHOSHROO; MAZLOUM-ARDAKANI, 2014). Tintas à base de ouro, também são usadas na preparação de eletrodos impressos, porém menos do que o carbono devido ao seu alto custo. A composição dessas tintas pode variar dependendo do fabricante.

Uma grande vantagem dos SPEs são as várias possibilidades de modificação, fato que melhora suas características analíticas e amplia seu campo de aplicações. A modificação de SPEs é mais simples em comparação a modificação de eletrodos sólidos, os quais requerem várias etapas de preparação e secagem. No caso dos SPEs, a composição das tintas de impressão pode ser alterada pela adição de metais, polímeros, mediadores eletroquímicos como ftalocianina de cobalto, enzimas, nanomateriais, entre outros (ARDUINI et al., 2016). Os nanomateriais tais como nanopartículas de ouro e prata (NPs Au/ Ag), nanotubos de carbono, e grafeno têm sido utilizados na modificação de tintas dos eletrodos impressos, melhorando a

qualidade dos eletrodos em termos de imobilização de biomoléculas e transferência de elétrons na superfície do eletrodo. Além disso, eletrodos impressos podem ser modificados pela deposição de substâncias na superfície do eletrodo, como polímeros, nanomateriais e biomoléculas, tais como enzimas, anticorpos, DNA, lectinas e aptâmeros para detecção de analitos em amostras.

Figura 11. Esquema ilustrativo de um SPE de carbono contendo os três eletrodos (trabalho, referência e auxiliar), e abaixo SPEs de carbono, ouro e platina (da esquerda para direita).



Fonte: Disponível em <metrohm.com>. Acesso em: 17 de Jan. de 2019.

Atualmente, muitos biossensores comercialmente bem-sucedidos são baseados na tecnologia do eletrodo impresso, fazendo um mercado competitivo que tende a crescer rapidamente em todo o mundo. Os SPEs surgiram na década de 90, e desde então tem mostrado versatilidade, flexibilidade de design e materiais, produção em larga escala, possibilidade de miniaturização, instrumentação portátil, modificação por várias biomoléculas e abordagem de baixo custo para detecção em tempo real de biomoléculas, gases, pesticidas e microrganismos (RENEDO et al.,

2007). As vantagens da fabricação e portabilidade de biossensores baseados em SPEs atendem à expectativa de rápido crescimento do mercado de biossensores, especialmente na área de saúde e meio ambiente. Os principais clientes são laboratórios de pesquisa, emergências, centros de serviços de saúde e indústrias de alimentos e monitoramento ambiental (AHMED et al., 2016; SASSOLINI et al., 2019).

Além disso, o uso de SPEs facilita a obtenção das medições eletroquímicas, onde múltiplas detecções podem ser feitas em um curto intervalo de tempo, ou pelo uso de aparelhos portáteis (ex.: glicosímetro) como detectores finais. Esses avanços conduzem os SPEs para a realização de testes *point of care*, ou seja, testes projetados para ser usado no local ou perto do local onde o paciente está localizado e são realizados fora das instalações dos laboratórios clínicos (AHMED et al., 2016). Esse sistema fornece um rápido tempo de análise, baixa uso de energia, baixo (ou nenhum) uso de reagente, muito pouca ou nenhuma amostra de tratamento, alta segurança para os operadores, fácil manuseio e baixo custo.

Diversos biossensores baseados em eletrodos impressos são comercializados e estão disponíveis no mercado mundial para análise de biomoléculas, gases e íons, patógenos, marcadores cardíacos e do câncer, e hormônios para aplicações não apenas na área clínica, mas também alimentares e ambientais (BAHADIR et al., 2015). Entre estes, os biossensores de glicose são os mais difundidos mundialmente.

Grupos de pesquisa têm investigado os SPEs como plataformas promissoras na detecção de analitos e microrganismos. WARD e colaboradores (2018) utilizaram SPEs impressos em tinta de carbono como sensor para detectar células de *Staphylococcus aureus* em meios de cultura, bem como determinar a concentração das células, por espectroscopia de impedância. Outro estudo a eficácia do uso de SPEs de grafite modificados com um nanocomplexo de cobre para determinação de carbidopa em amostras biológicas (HAJIALIZADEH et al., 2018). Carbidopa é um medicamento utilizado no tratamento da doença de Parkinson. O sensor foi capaz de detectar a carbidopa, apresentando resposta linear na faixa de 0,5 a 700 $\mu\text{mol L}^{-1}$, e limite de detecção de 0,1 $\mu\text{mol L}^{-1}$.

2.6.5 Biossensores eletroquímicos baseados em lectinas

O desenvolvimento de biossensores eletroquímicos baseados em lectinas tem ganhado espaço na investigação de alterações na expressão de glicanos e glicoproteínas associadas a fenômenos fisiológicos, infecções ou doenças; na identificação de biomarcadores e microrganismos (PIHÍKOVÁ et al., 2015; YANG et al., 2016).

Vários biossensores eletroquímicos baseados em lectinas tem sido desenvolvidos para detecção e monitoramento do câncer e doenças infecciosas. A primeira tentativa para detectar a interação entre lectina e glicanos de glicoproteínas utilizando métodos eletroquímicos foi revelada por DAI et al. (2006). Foi realizado um ensaio competitivo para detectar os analitos sacarídicos galactose (Gal), N-acetil-galactosamina (GalNAc) e o antígeno T (β -D-Gal-[1 $\rightarrow$ 3]-D-GalNAc), associado ao câncer de mama, utilizando lectina de *Arachis hypogaea* (PNA) imobilizada sobre uma superfície de ouro contendo grupo carboxílico ativado ($-\text{COO}^-$), para fixação da lectina. O princípio de detecção foi baseado na competição entre os açúcares não marcados e um açúcar marcado com o nanocristal sulfeto de cádmio (CdS – 4-aminofenol- β -D-galactopiranosídeo). Após a ligação com a lectina, os CdS foram eletroquimicamente ativados e o cádmio liberado foi detectado usando voltametria de onda quadrada. Foi detectada a interação dos açúcares com a lectina, fenômeno representado por uma redução no sinal eletroquímico (corrente), que foi mais significativa para o antígeno T (DAI et al., 2006).

XUE e colaboradores (2011) desenvolveram um biossensor eletroquímico baseado na lectina Concanavalina A, utilizando eletrodo de carbono vítreo modificado com nanotubos de carbono, pelo qual conseguiram estudar e monitorar a dinâmica de expressão de glicanos na superfície de células cancerosas da linhagem k562 em resposta a uma droga. Outro biossensor eletroquímico baseado na lectina de *Sambucus nigra* I (SNA I) imobilizada sobre eletrodo impresso de ouro foi desenvolvido para detecção seletiva do antígeno STn (sialyl-Tn) associado ao câncer e discriminar amostras de soro oriundas de indivíduos saudáveis e pacientes com diferentes tipos de tumores malignos. Foi detectado um aumento na expressão

de STn em amostras de soro de carcinoma em relação aos de indivíduos saudáveis (SILVA et al., 2014).

OLIVEIRA e colaboradores (2011a) elaboraram um biossensor baseado na lectina Cramoll e nanopartículas de Fe_3O_4 sobre eletrodo de ouro para analisar as interações entre a lectina e glicoproteínas de soros de pacientes contaminados com o vírus da dengue dos sorotipos 1, 2 e 3. Através de medições eletroquímicas, foi observado heterogeneidade nos sinais eletroquímicos resultantes das interações de Cramoll com as glicoproteínas dos diferentes sorotipos, podendo ser útil no diagnóstico da dengue. O mesmo grupo desenvolveu também um biossensor eletroquímico baseado em nanopartículas de ouro e Cramoll para reconhecimento de lipopolissacarídeo de diferentes bactérias gram negativas (OLIVEIRA et al., 2011b).

Biossensores eletroquímicos baseado nas lectinas de *Lens culinaris* (LCA, do inglês *Lens culinaris agglutinin*), ConA, e lectina de gérmen de trigo (WGA, do inglês *wheat-germ agglutinin*), foram desenvolvidos para detecção e discriminação do antígeno carcinoembrionário (CEA, do inglês *Carcynoembryonic antigen*), um marcador glicoproteico associado a alguns tipos de câncer, como cólon e mama. Foram utilizados SPEs de carbono modificados com nanopartículas de ouro, onde as lectinas foram imobilizadas. Os biossensores foram eficazes na avaliação de alterações na expressão de glicanos do CEA, permitindo discriminar entre amostras de soro de pacientes saudáveis e pacientes com câncer (ZHAO et al., 2016).

Também um biossensor baseado em lectina foi desenvolvido recentemente para detecção e discriminação do antígeno Tn presente em glicoproteínas, associado ao câncer, usando a lectina de sementes de *Vicia villosa*. A lectina foi imobilizada sobre SPE de ouro, e sua ligação com as glicoproteínas em amostras de soro de pacientes foram monitoradas por espectroscopia de impedância (SILVA; RANGEL, 2017). O biossensor permitiu discriminar amostras de indivíduos saudáveis e pacientes com diferentes tipos de câncer, onde a expressão do antígeno Tn é bem relatada nas pesquisas.

Tais exemplos de biossensores baseados em lectinas para detecção e acompanhamento de doenças e agentes infecciosos, revelam o potencial e a versatilidade dessas ferramentas, estimulando o emprego dessa tecnologia no

estudo de outras doenças. Neste trabalho, foi desenvolvido um biossensor eletroquímico baseado em PLL, nanotubos de carbono e lectina Cramoll para detecção seletiva do perfil de glicoproteínas em soro humano caracterizados com diferentes níveis de PSA, associadas ao CaP, e discriminar amostras oriundas de indivíduos com HBP e CaP classificados em graus de Gleason distintos.

2.7 TÉCNICAS ELETROQUÍMICAS

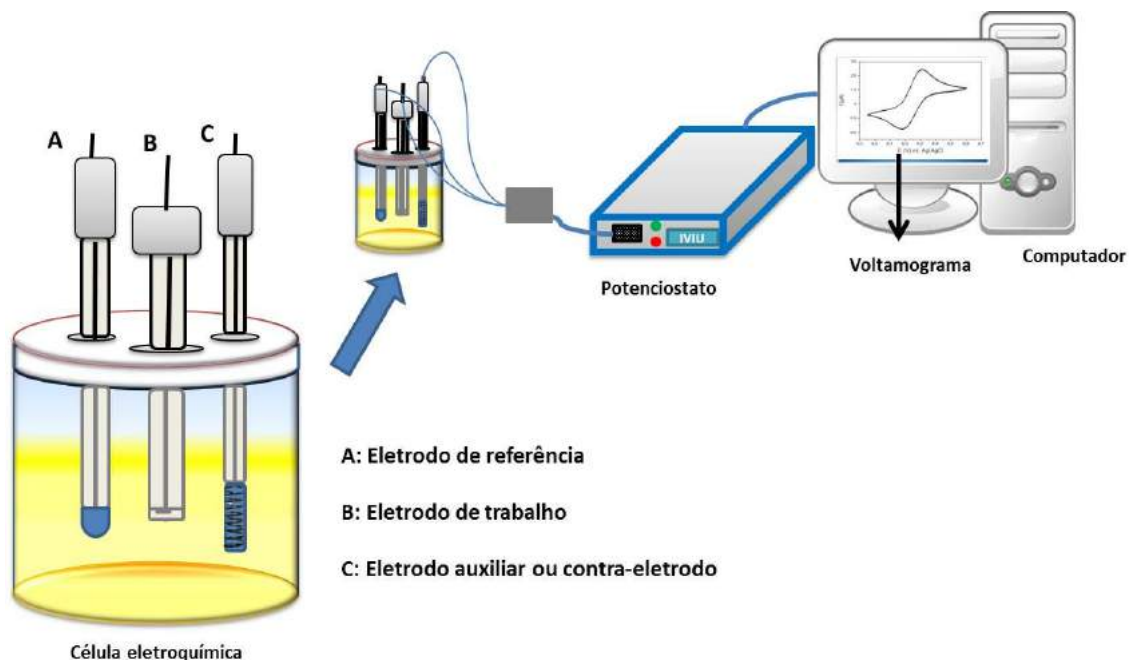
As técnicas eletroquímicas podem identificar e quantificar analitos de interesse com praticidade e eficiência a partir da relação estabelecida entre os sinais elétricos detectados e a concentração da espécie em estudo (BRETT; OLIVEIRA-BRETT, 2011). Dentre os métodos eletroquímicos existentes, a voltametria constitui importante ferramenta, uma vez que além de informações qualitativas e quantitativas, pode-se estabelecer um mecanismo de cinética de redução ou oxidação de um dado sistema de interesse (PACHECO et al., 2013).

2.7.1 Voltametria

A voltametria é uma técnica eletroquímica utilizada na análise qualitativa e quantitativa de uma espécie química eletroativa, onde as informações são obtidas a partir do registro de curvas corrente-potencial geradas durante a eletrólise da espécie eletroativa, no interior de uma célula eletroquímica. O potencial é aplicado em um eletrodo, na forma de varredura, isto é, variando a uma velocidade constante em função do tempo (t), numa determinada região que desencadeia as reações de oxidação ou redução cineticamente favoráveis da espécie eletroativa, criando-se um fluxo de elétrons na interface eletrodo-solução, a corrente elétrica (WANG, 2000).

A célula eletroquímica onde ocorre a eletrólise é normalmente constituída por uma solução contendo as espécies eletroativas e o eletrólito suporte, e três eletrodos: um eletrodo de trabalho, um eletrodo de referência e um eletrodo auxiliar ou contra-eletrodo (Figura 12).

Figura 12. Representação esquemática de um potenciostato conectado a uma célula eletroquímica.



Fonte: A autora (2017).

O eletrodo de trabalho possui área superficial reduzida e é comumente feito de um material inerte, como ouro, platina, carbono e mercúrio. Também é o mais importante, pois é nele onde o potencial é aplicado e ocorrem os fenômenos eletroquímicos que se pretendem estudar. O eletrodo de referência, por manter seu potencial constante durante as varreduras, é usado para controlar com rigor o potencial imposto ao eletrodo de trabalho. Os mais comuns eletrodos de referência utilizados são os eletrodos de calomelano saturado ($\text{Hg}/\text{Hg}_2\text{Cl}_2$) e o de prata/cloreto de prata (Ag/AgCl). O eletrodo auxiliar completa o circuito elétrico por onde circula a corrente resultante e geralmente é feito de um material condutor inerte, como platina e grafite.

A corrente passa entre o eletrodo de trabalho e o eletrodo auxiliar, sem interferir no funcionamento do eletrodo de referência (WANG, 2000; BRETT; OLIVEIRA-BRETT, 2011). O fato da área do eletrodo de trabalho ser menor faz com que este se polarize, variando o potencial de acordo com o que lhe é aplicado. O eletrodo de referência, por possuir uma área maior não se polariza, mantendo o seu

potencial constante durante as medições. A corrente gerada circula entre o eletrodo de trabalho e o eletrodo auxiliar, assegurando que a corrente resultante produzida não provoque distúrbios na função do eletrodo de referência (WANG, 2000).

Os sistemas voltamétricos fundamentam-se na utilização de potenciostatos, equipamentos que controlam o potencial aplicado ao eletrodo de trabalho e permitem a medição da intensidade de corrente (Figura 12). O potencial e a corrente resultante são registrados simultaneamente em curvas corrente vs. potencial, denominadas voltamograma.

A voltametria compreende um grupo de técnicas eletroanalíticas que avaliam os fenômenos eletroquímicos que ocorrem na interface entre a superfície do eletrodo de trabalho e a camada fina de solução adjacente a essa superfície. O parâmetro ajustado é o potencial (E), e o parâmetro medido é a corrente resultante, obtida pela transferência de elétrons durante um processo de oxirredução, que pode fornecer informações sobre modificações na superfície ou sobre o analito presente na interface. Na medição eletroquímica, o eletrodo pode atrair espécies carregadas positivamente ou negativamente, presentes na solução, que podem ou não reagir na superfície. Assim, a corrente ou velocidade das reações eletroquímicas é regulada pelos processos de transferência de massa (eletrólitos) do seio da solução para a interface do eletrodo-solução, transferência de carga (elétrons) na superfície do eletrodo, e ainda reações químicas que antecedem ou sucedem a transferência de elétron (adsorção, dessorção, protonação, etc.) (PACHECO et al., 2013).

Os métodos voltamétricos têm sido amplamente empregados na determinação de analitos, incluindo proteínas, medicamentos, e metais pesados, utilizando eletrodos com diversas possibilidades de modificação. As vantagens dessas técnicas consistem no baixo custo, sensibilidade, rapidez e versatilidade quando comparadas à técnicas cromatográficas e espectroscópicas (GUPTA et al., 2011).

2.7.2 Transporte de massa

Para que a relação com a concentração do analito do seio da solução seja mantida, o transporte de massa deve ser contínuo. Existem três formas de transporte de massa: migração, convecção e difusão. Em voltametria, as condições

experimentais são ajustadas para que os transportes por migração e convecção sejam minimizados. Na migração, os íons em solução movem-se em direção ao eletrodo pela atração ou repulsão pelo eletrodo de trabalho, onde os cátions e os ânions migram para o eletrodo de carga oposta, criando uma corrente de migração para reduzir a diferença de potencial gerada após aplicação do potencial, que prejudica as análises quantitativas. Esse transporte é minimizado pela adição de eletrólitos inertes (eletrólitos de suporte) em concentrações de 50 a 100 vezes maiores que a espécie eletroativa (WANG, 2000; PACHECO et al., 2013). No processo de convecção, as espécies carregadas são transportadas para a superfície do eletrodo por meios mecânicos, tais como agitação da solução ou do eletrodo, afetando a corrente resultante, podendo ser minimizado mantendo o sistema sem agitação (WANG, 2000; PACHECO et al., 2013).

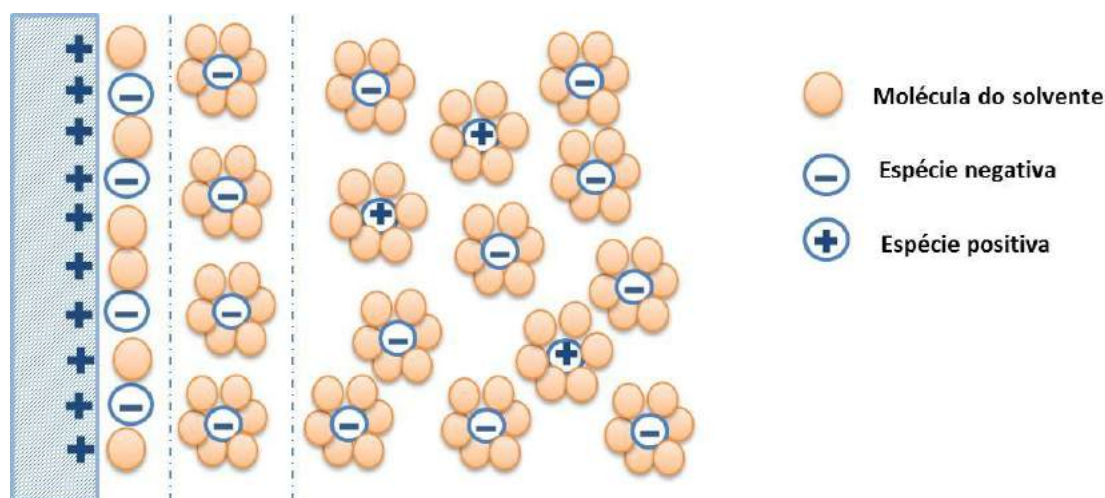
Utilizando meio com eletrólito suporte e sem agitação, o transporte de massa na voltametria é feito basicamente por difusão, que é o movimento espontâneo da espécie eletroativa devido à formação de um gradiente de concentração do analito de interesse entre a superfície do eletrodo e o seio da solução pela aplicação de um potencial no eletrodo de trabalho que leva a redução ou oxidação das espécies imediatamente vizinhas ao eletrodo (PACHECO et al., 2013).

2.7.3 Transferência de carga e Dupla camada elétrica

A interface eletrodo/solução é uma região bem diferenciada do restante da solução e pode ser representada pelo modelo de dupla camada elétrica (Figura 13). O eletrodo de trabalho carregado positivamente ou negativamente ao ser imerso numa solução de eletrólitos, a camada de solução imediatamente adjacente à superfície do eletrodo é modificada e carregada por íons de carga oposta à da superfície eletródica. A primeira camada de íons de soluto e moléculas do solvente adjacente ao eletrodo está adsorvida a superfície eletródica por força eletrostática e força de Van der Waals, mantendo os íons compactados, imobilizados na superfície. Essa região da dupla camada é conhecida como camada interna ou Plano interno de Helmholtz. Entre a camada interna e o seio da solução está a camada externa ou difusa da dupla camada ou plano externo de Helmholtz, onde se encontram espécies eletroativas solvatadas e com mobilidade, sendo atraídos pelo eletrodo, porém, não

chegam à superfície do eletrodo devido ao raio de hidratação. Esses íons da camada difusa são necessários para que exista a neutralidade na superfície, uma vez que os íons da camada interna não polarizam completamente a carga do eletrodo. Na camada difusa, a concentração da espécie química eletroativa é proporcional à concentração no seio da solução (BRETT; BRETT, 1993; PACHECO et al., 2013).

Figura 13. Representação esquemática da dupla camada elétrica.



Fonte: A autora (2017).

A transferência de elétrons que ocorre na interface eletrodo/solução gera uma corrente elétrica total composta por duas componentes: a corrente faradaica (segue a lei de Faraday) e a corrente capacitiva. A primeira é associada às reações de oxidação-redução dos eletrólitos na superfície do eletrodo proporcional a concentração desses eletrólitos no seio da solução. Já a corrente capacitiva é a necessária para manter carregada a dupla camada elétrica existente na interface eletrodo/solução, gerada pelo acúmulo de elétrons na superfície eletródica; não obedece à lei de Faraday e não é proporcional à concentração do analito (WANG, 2000; PACHECO et al., 2013). Neste contexto, os equipamentos e metodologias têm sido melhorados no sentido de reduzir a interferência da corrente não faradaica na corrente total medida. Sugere-se a medição da corrente faradaica em ambiente de baixo ruído, utilizar eletrodos de pequena área superficial e polarizados, e modos de aquisição de sinal mais apropriados (MIRCESKY et al., 2013).

2.7.4 Métodos voltamétricos

A escolha da técnica voltamétrica a ser utilizada está relacionada com o tipo e a qualidade de informação quantitativa e/ou qualitativa que se quer obter a respeito do analito ou do processo que envolve a interação entre o analito e a superfície do eletrodo de trabalho. Com esta técnica podem ser estudados processos de oxidação e redução de vários eletrólitos, processos de adsorção em diferentes materiais, e mecanismos de transferência eletrônica em superfícies de eletrodos quimicamente modificados. Há ainda a possibilidade de utilizar a técnica para efetuar modificações químicas por eletrodeposição, adsorção, entre outros (PACHECO et al., 2013).

As vantagens analíticas das várias técnicas voltamétricas incluem excelente sensibilidade, podendo alcançar limites de detecção baixíssimos (10^{-1} a 10^{-12} mol L⁻¹), variedade de solventes e eletrólitos, possibilidade de uso em diferentes variações de temperaturas, tempo reduzido de resposta (segundos), determinação simultânea de vários analitos, capacidade de determinar parâmetros cinéticos e mecânicos, e a facilidade com que as diferentes formas de onda dos potenciais podem ser geradas e obter as medições de correntes (BRETT; OLIVEIRA-BRETT, 2011). Neste trabalho, foram empregadas a voltametria cíclica e a voltametria de onda quadrada na elaboração e caracterização do biossensor.

2.7.5 Voltametria cíclica

Na voltametria cíclica (VC), são feitas varreduras de potencial vs. tempo, a partir da aplicação de uma determinada faixa de potenciais no eletrodo, gerando respostas na forma de corrente. Inicialmente o potencial é aplicado no eletrodo de trabalho de um valor no qual nenhuma redução ocorre. Com o aumento do potencial para regiões mais negativas (catódica), o eletrodo torna-se uma fonte de elétrons, favorecendo a redução das espécies eletroativas na interface eletrodo/solução, gerando um pico de corrente proporcional à concentração desta espécie, conhecido como pico de redução ou catódico. Quando o potencial já tiver atingido um valor no qual não ocorre nenhuma reação de redução, inicia-se a varredura no sentido inverso, aumentando para regiões mais positivas (anódicas) até o valor inicial

(PACHECO et al., 2013). No caso de uma reação reversível, as espécies reduzidas que foram geradas no sentido direto (e se localizarem ainda próximos à superfície do eletrodo) serão oxidadas, gerando um pico simétrico ao pico da redução, denominado pico de oxidação ou anódico (Figura 14 A e B). As varreduras de potencial direto e inverso são efetuadas em vários ciclos sucessivos observando-se a oxidação e/ou a redução e as contribuições anódicas e catódicas envolvendo as espécies eletroativas (BRETT; BRETT, 1993).

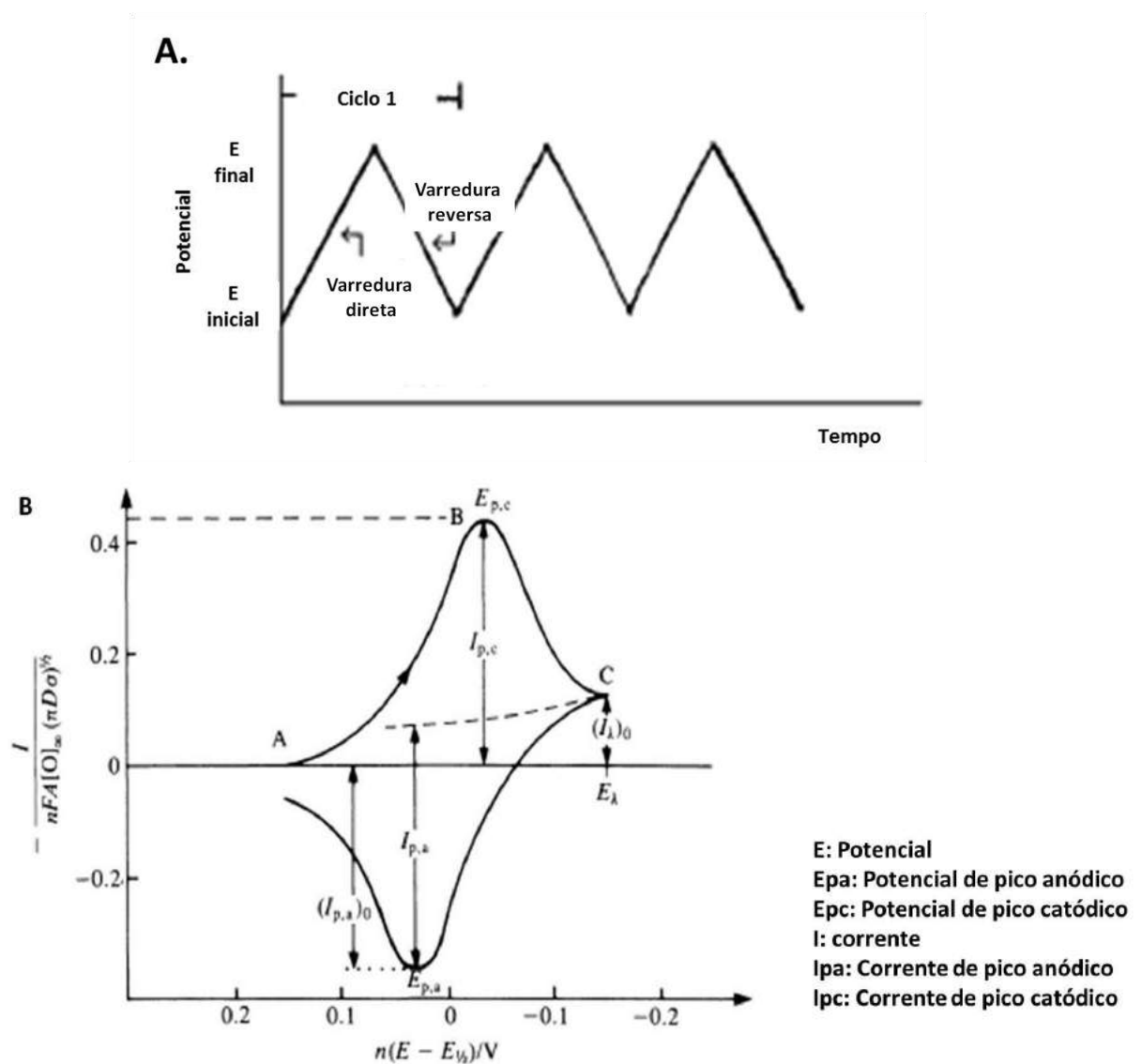
Os parâmetros mais importantes num voltamograma desta técnica são os potenciais e intensidades de corrente dos picos anódico e catódico. A presença de pares redox, ou seja, substâncias que apresentam simultaneamente potenciais de oxidação e redução podem ser trabalhadas por voltametria cíclica, que permite a avaliação das regiões redox das biomoléculas. As informações obtidas são muito úteis para descrever os sistemas com as análises de corrente e potencial de oxidação e redução, concentração das espécies, coeficientes de difusão, área dos eletrodos, cinética das reações, dentre outros, sendo bastante usada no estudo de formação e caracterização de filmes poliméricos (STRYCHARZ et al., 2011; SEBEZ et al., 2013).

Na VC, o potencial é varrido linearmente com o tempo no eletrodo de trabalho, em uma solução sem agitação, de um valor inicial a outro valor específico e, em um tempo t , o potencial é varrido no sentido inverso até o valor inicial, caracterizando um potencial em forma de triângulo. Durante a varredura do potencial, o potenciostato mede a corrente resultante, com a qual se obtém gráficos corrente *versus* o potencial aplicado (Figura 14 A e B). Dependendo da informação desejada, podem ser efetuados ciclos simples ou múltiplos. Alguns instrumentos digitais aplicam o potencial na forma de escada (*staircase*), com degraus de potenciais pequenos (da ordem de 10 mV) e tempo de duração pequeno (50 ms), onde a corrente é lida apenas no final deste intervalo.

Esse método substitui satisfatoriamente a varredura linear, uma vez que minimiza a contribuição da corrente capacitiva na corrente total. Como as etapas de potencial são pequenas, as equações para as respostas da VC *staircase* são consideradas como idênticas das provenientes da VC de varredura linear (PACHECO et al., 2013).

VC é uma técnica comumente aplicada em mensurações de biosensores enzimáticos bem conhecidos, como os sensores de glicose e colesterol. Também tem sido utilizada para detectar alvos específicos de moléculas de DNA não marcado, e para monitorar processos químicos como a morte celular e o progresso de uma reação em cadeia de polimerase. É bastante útil na caracterização de diferentes superfícies eletródicas, bem como das suas etapas de modificação (GENCONGLU; MINERICK, 2014).

Figura 14. (A) Sinal de excitação potencial-tempo na VC. (B) Voltamograma cíclico.



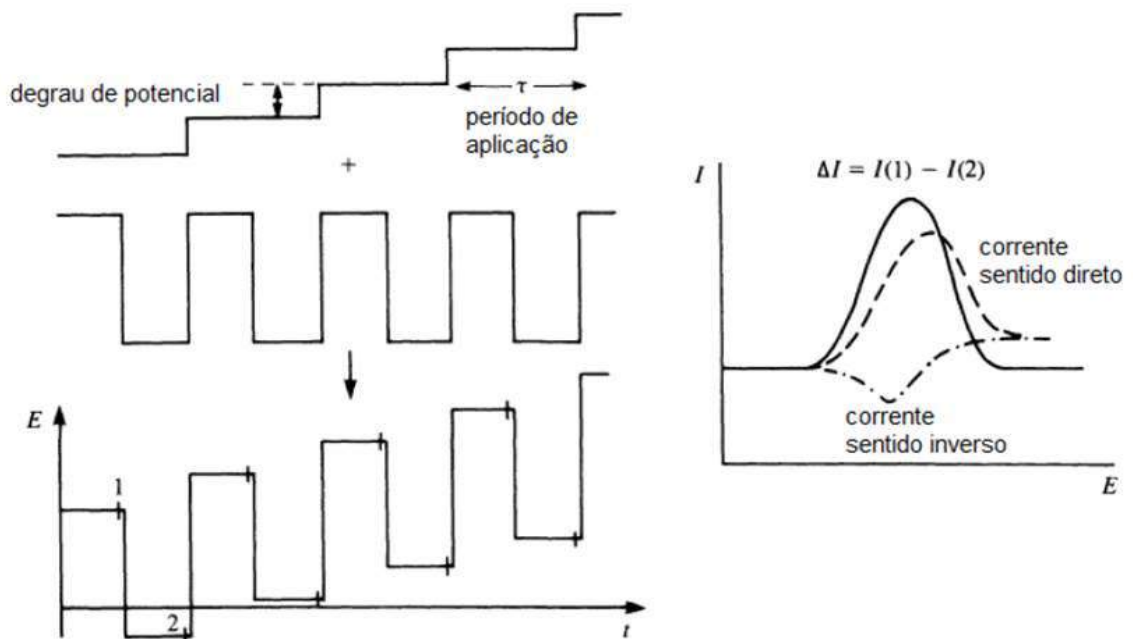
Fonte: BRETT; BRETT, 1993; WANG, 2000.

2.7.6 Voltametria de onda quadrada

É uma técnica muito conveniente que tem sido incorporada em estudos analíticos, conhecida pela rapidez e elevada sensibilidade em relação a outras técnicas voltamétricas. Na voltametria de onda quadrada, também conhecida como SWV (do inglês *square-wave voltammetry*), uma onda quadrada simétrica de amplitude E_p sobreposta a uma rampa de potencial na forma de escada (*staircase*) de amplitude E_s é aplicada ao eletrodo de trabalho (PACHECO et al., 2013).

O modo de amostragem do sinal na técnica de voltametria de onda quadrada é representado na Figura 15, onde se pode ver o perfil da variação de potencial com a forma de onda apresentada. A corrente é amostrada duas vezes, uma ao final do pulso direto, quando a direção do pulso é igual à direção da varredura, e outro ao final do pulso reverso (que ocorre no meio do “degrau” da onda *staircase*), onde a direção do pulso é contrária à direção da varredura. Esta dupla amostragem da corrente garante uma minimização da contribuição da corrente capacitiva sobre a corrente total medida. O voltamograma resultante consiste da diferença entre estas duas correntes versus a rampa de potencial aplicado (SKOOG, 2006).

Figura 15. Representação esquemática da voltametria de onda quadrada.



Fonte: PACHECO et al., 2013

Esta técnica permite a discriminação temporal da corrente capacitiva da corrente faradaica fazendo-se a amostragem da corrente perto do final da duração do pulso, uma vez que a corrente capacitiva diminui mais rapidamente que a faradaica. A diferença da corrente entre as duas amostragens é registrada em função do potencial de rampa em degraus. A voltametria de onda quadrada produz picos para processos faradaicos, cuja altura é proporcional à concentração da espécie eletroativa, e permite obter limites de detecção da ordem de 10^{-7} a 10^{-8} mol L^{-1} (WANG, 2000; SKOOG, 2006).

A maior vantagem desta técnica é a velocidade de aquisição dos dados. As frequências usadas em voltametria de onda quadrada encontram-se tipicamente na região de 1 a 100 Hz. Dentro destas faixas de frequências, as medições podem ser feitas com velocidades de varredura 100 vezes maiores ou mais que nas demais técnicas de pulso, podendo variar de 100 a 1000 mV s^{-1} . Isto diminui o tempo de análise de 3 a 5 minutos para alguns poucos segundos (3 a 10 s) sem haver perda da resolução dos picos. Além da frequência, a amplitude do pulso deve ser otimizada antes da realização das análises. O produto entre a frequência e a amplitude de pulso determina a velocidade de varredura (PACHECO et al., 2013).

As vantagens sobre outras técnicas voltamétricas, como maior sensibilidade, tempo de análise curto, configuração simples e capacidade de redução das correntes capacitivas tem incentivado a aplicação da técnica de voltametria de onda quadrada como uma alternativa viável para a determinação de compostos biologicamente importantes. Esta técnica é frequentemente utilizada em estudos de caracterização e quantificação de compostos tais como proteínas, vitaminas, fenóis, pesticidas, fungicidas, alcalóides, metais pesados e drogas (MIRCESKY et al., 2013).

2.8 TÉCNICAS DE CARACTERIZAÇÃO ESTRUTURAL E MORFOLÓGICA

Junto às técnicas de caracterização eletroquímica, a caracterização estrutural e morfológica é de grande importância para análise e comprovação de modificações estruturais e morfológicas em superfície de eletrodos, principalmente após uso de polímeros, nanomaterias e biomoléculas (OLIVEIRA et al., 2011a; SILVA et al., 2015). Neste trabalho, análises por microscopia eletrônica de varredura (MEV) e

espectroscopia no infravermelho com transformada de Fourier (FT-IR, do inglês *Fourier Transform Infrared Spectroscopy*) foram empregadas para caracterização do biossensor.

2.8.1 Espectroscopia no Infravermelho com Transformada de Fourier (FT-IR)

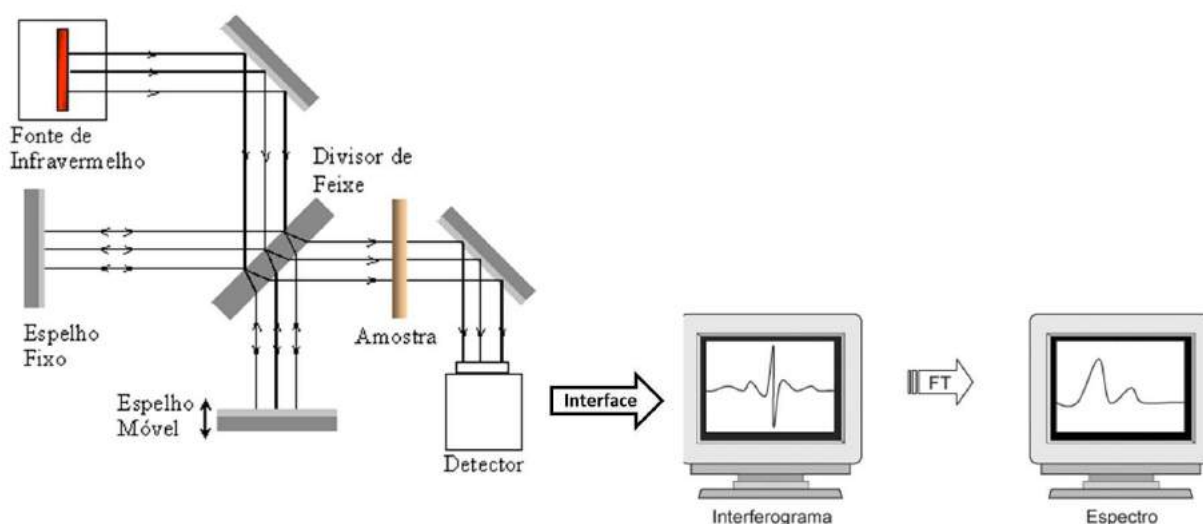
O FT-IR é uma técnica versátil que permite a determinação de propriedades químicas e estruturais de espécies orgânicas e inorgânicas, através da interação da radiação eletromagnética com a matéria, na região espectral do infravermelho. Essa região corresponde à radiação com um número de onda entre 12.800 cm^{-1} a 10 cm^{-1} , ou comprimento de onda de 780 a $1,0 \times 10^6\text{ nm}$, região do espectro eletromagnético situado entre as regiões do visível e micro-ondas.

Ao incidir em uma molécula, a radiação é absorvida pelas ligações moleculares. Para que isso aconteça, uma molécula deve sofrer variação no momento dipolo durante seu movimento rotacional ou vibracional. Assim, o campo elétrico alternado da radiação pode interagir com a molécula e causar variação na amplitude de um de seus movimentos. Se a frequência incidida for igual à frequência de vibração natural da molécula, ocorre a absorção, causando uma variação na amplitude molecular. Da mesma forma, a rotação da molécula assimétrica em torno de seus centros de massa resulta em oscilações periódicas do momento dipolo, podendo ter interação com o campo de radiação. Parte da radiação incidente é absorvida em diferentes energias, que aparecem como picos no espectro de absorção e correspondem as frequências de vibrações das ligações moleculares do material, o qual pode estar nos estados líquido, sólido ou gasoso (GRIFFITHS; DE HASETH, 2007; SMITH, 2011).

Os componentes básicos de um espectrômetro de FT-IR são uma fonte de luz na região do infravermelho, um interferômetro com um espelho móvel, um detector, um conversor analógico/digital e um computador (Figura 16). A luz da fonte do espectrofotômetro incide sobre o interferômetro, que separa a mesma em dois feixes, um incide sobre um espelho fixo e outro incide sobre um espelho móvel. Variando as distâncias percorridas pelos feixes têm-se interferências construtivas e destrutivas sequencialmente; dessa forma, o detector recebe intensidades de vibrações diferentes. Uma transformação de Fourier converte o sinal de domínio do

tempo para domínio de frequência, empregando o interferômetro de Michelson, que divide o feixe da radiação da fonte de infravermelho, fazendo com que ela reflita ao mesmo tempo a partir do espelho fixo e do outro em movimento. Assim, os feixes de luz refletidos pelo espelho móvel e fixo se sobrepõem, passam pelo material e incidem no detector. Os dados são convertidos para a forma digital por um conversor analógico/ digital e transferido para o computador, na forma de gráfico. Os dados tratados representam a relação da absorbância ou transmitância (%) da ligação dos grupos químicos vs. a frequência ou número de onda (cm^{-1}). Dependendo da amostra, o espectro pode ser medido em modo de transmitância ou de refletância (STUART, 2004; GRIFFITHS; HASETH, 2007).

Figura 16. Representação esquemática de um espectrofotômetro FT-IR.



Fonte: Adaptação de HELFER et al., 2006; <<http://www.sorocaba.unesp.br>>.

Cada molécula possui um espectro de absorção único, como uma impressão digital. Uma vez registrado o espectro de infravermelho, o próximo passo é a interpretação, baseada nas bandas de absorção, relacionadas aos grupos funcionais da molécula, conhecidos como frequência de grupos (STUART, 2004). A partir do conhecimento da localização das bandas de absorção é possível caracterizar o material. FT-IR é um excelente método para análise estrutural de filmes poliméricos e nanomateriais (SILVA et al., 2016), bem como para análises de biomoléculas,

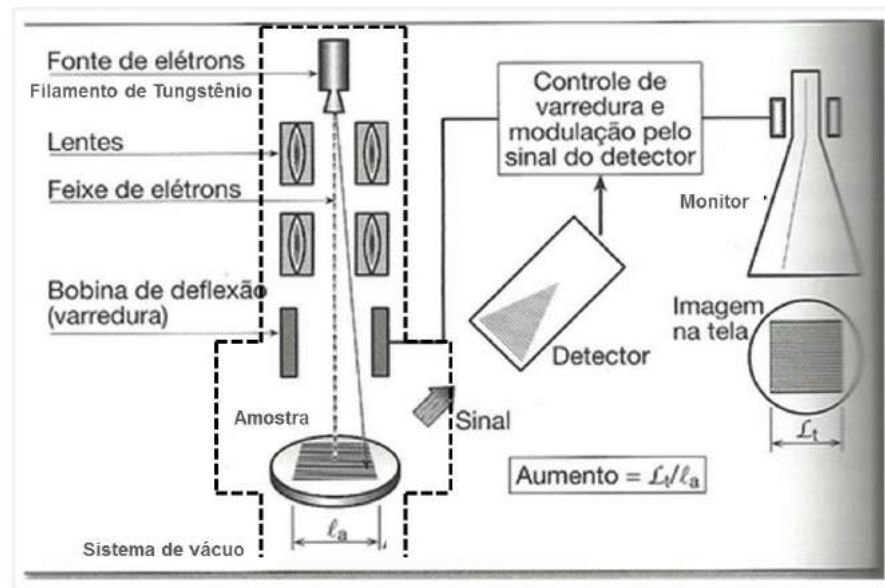
inclusive para caracterização estrutural e monitoramento de alterações bioquímicas em proteínas, polipeptídeos e membranas biológicas (JAMSHAD et al., 2015; YANG et al., 2015).

2.8.2 Microscopia Eletrônica de Varredura (MEV)

O microscópio eletrônico de varredura (MEV) é um dos mais versáteis instrumentos disponíveis para a observação e análise de características microestruturais de materiais sólidos, fornecendo com grande rapidez informações sobre a morfologia e identificação de elementos químicos da amostra. As imagens são obtidas com alta resolução, geralmente valores da ordem de 2 a 5 nm são apresentados por instrumentos comerciais, enquanto instrumentos de pesquisa avançada são capazes de alcançar uma resolução melhor que 1 nm. Outra característica importante do MEV é a aparência tridimensional da imagem das amostras, resultado direto da grande profundidade de campo (DEDAVID; GOMES; MACHADO, 2007; POWER et al., 2014).

O MEV convencional é basicamente composto por uma coluna óptico-eletrônica adaptada a uma câmara com porta-amostra aterrado, sistema de detectores e sistema de vácuo (Figura 17). O princípio do funcionamento do MEV consiste na utilização de um feixe de elétrons de pequeno diâmetro, produzido na coluna óptico-eletrônica, para explorar a superfície da amostra, ponto a ponto, por linhas sucessivas e transmitir o sinal do detector a uma tela catódica. O sinal de imagem resulta da interação do feixe incidente com a superfície da amostra. A maioria dos instrumentos usa como fonte de elétrons um filamento de tungstênio (W) aquecido, localizado no canhão eletrônico, operando numa faixa de tensões de aceleração de 1 a 50 kV. A interação do feixe com a amostra produz elétrons e fótons que podem ser coletadas por detectores adequados e convertidas em um sinal de vídeo. A imagem formada a partir do sinal captado na varredura eletrônica de uma superfície pode apresentar diferentes características, uma vez que a imagem resulta da amplificação de um sinal obtido de uma interação entre o feixe eletrônico e o material da amostra (DEDAVID; GOMES; MACHADO, 2007).

Figura 17. Esquema de operação de um MEV.



Fonte: Adaptação de COLPAERT, 2008.

A versatilidade desta técnica permite sua aplicação em diversas análises no campo da biotecnologia. O MEV, por exemplo, tem sido aplicado no desenvolvimento de sensores eletroquímicos para monitoramento das modificações da superfície eletródica (SILVA et al., 2016). Na biologia, permite estudar a morfologia, ciclo biológico e classificação taxonômica de parasitas de animais e plantas; o desenvolvimento de embriões humanos; caracterização de tecidos biológicos e acompanhamento de fenômenos de interação celular.

2.9 ENSAIOS EM MICROPLACAS BASEADOS EM LECTINAS

Diversas técnicas baseadas no uso de lectinas têm sido empregadas em glicoanálise a fim de investigar perfis de glicosilação, anormalidades e biomarcadores associados a doenças como o câncer. Entre essas técnicas, destacam-se os ensaios em microplaca (WANG et al., 2016; MARANGON et al., 2018; HIONO et al., 2019). As microplacas de titulação constituem uma ferramenta simples e versátil utilizada em diversos procedimentos laboratoriais, e aplicáveis em diferentes metodologias, como absorbância, ensaios colorimétricos e fluorimétricos,

ELISA, entre outros (WU et al., 2014; WANG et al., 2016). São placas fabricadas à base de poliestireno, material mais resistente às variações de temperatura dos ensaios, e são constituídas por múltiplos poços (mais frequente com 96 poços), onde os reagentes são depositados para detecção. A seguir, são abordados alguns ensaios em microplaca como ELISA, ELLA e ensaio fluorescente em microplaca.

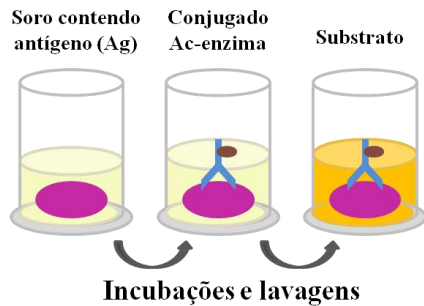
2.9.1 Ensaios ligados à enzima: ELISA e ELLA

O ensaio conhecido como ELISA (do inglês *enzyme-linked immunosorbent assay*) é o mais comum ensaio feito em microplaca de 96 poços, sendo uma técnica amplamente utilizada na detecção e quantificação de antígenos e anticorpos (OWENS et al., 2019). Laboratórios clínicos e de pesquisa, e a indústria biotecnológica em geral tem explorado o uso do ELISA na pesquisa, no diagnóstico de doenças, no controle de alimentos, e na vigilância ambiental.

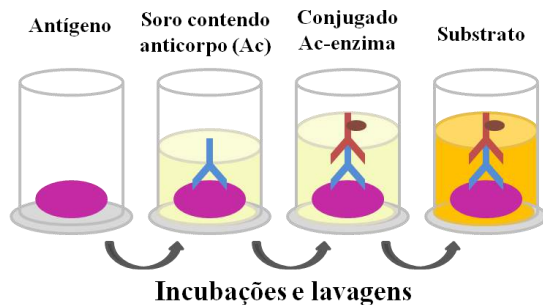
O ensaio ELISA pode ser utilizado para detectar a presença de antígenos ou anticorpos em uma amostra (ex.: soro, urina), em diferentes configuração, como observado na figura 18. Para detectar um antígeno, um anticorpo específico contra esse antígeno é adsorvido no fundo do poço da microplaca (fase sólida), para então ligar-se ao antígeno se ele estiver presente na amostra (MORIOKA et al., 2014). Para detectar um anticorpo específico numa amostra contra um determinado material antigênico, o antígeno é aderido na fase sólida, e então será reconhecido e complexado com o anticorpo presente na amostra (KUMAR; GILL, 2018). As biomoléculas não ligadas podem ser removidas do poço por etapas de lavagem. A detecção da interação antígeno-anticorpo é obtida através da adição de um segundo anticorpo ou anti-anticorpo conjugado a uma enzima, que irá reconhecer e ligar-se ao complexo antígeno-anticorpo, e posteriormente incuba-se com o substrato da enzima. O substrato é convertido em um produto mensurável colorimétrico, e então quantificado por medição de densidade óptica em um leitor de microplaca (Figura 18).

Figura 18. Representação esquemática dos tipos de ensaios ELISA para detecção de antígenos e anticorpos. Nos ensaios diretos, é feita a detecção do antígeno na amostra, enquanto nos ensaios indiretos é feita a detecção do anticorpo presente na amostra.

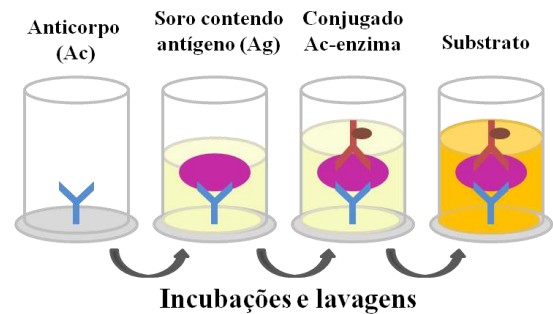
1. ELISA direto



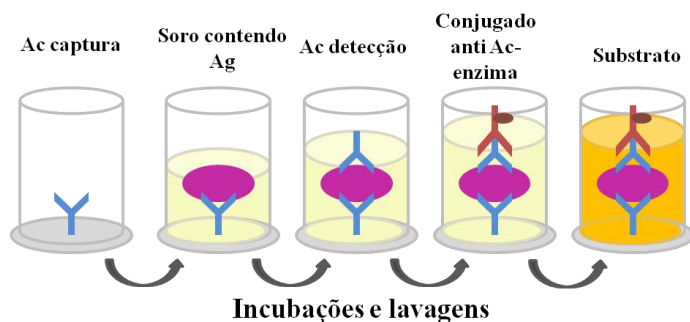
2. ELISA indireto



3. ELISA 'sanduíche' direto



4. ELISA 'sanduíche' indireto



Fonte: A autora (2017).

Assim, essa técnica tem sido de escolha na determinação do título de anticorpos purificados ou presentes em soros, bem como na avaliação quantitativa de antígenos em uma amostra, sendo frequentemente manufaturada e comercializada em formato de kit, de uso prático e fácil, contendo os reagentes e soluções a serem utilizadas nas etapas. Antígenos ou anticorpos podem ser

imobilizados passivamente na fase sólida do poço, e as biomoléculas não ligadas são removidas nas etapas de lavagem. Múltiplas amostras podem ser testadas ao mesmo tempo utilizando uma microplaca e equipamento compatível para leitura. Também permite o uso de conjugados enzimáticos ou marcadores fluorescentes que podem amplificar o sinal e melhorar o nível de detecção do ensaio. Finalmente, o sinal poderá ser detectado, e os dados coletados e analisados estatisticamente (KUMAR; GILL, 2018). O ensaio ELISA é basicamente imunológico, versátil, sensível e quantitativo, que envolve a interação específica antígeno-anticorpo para detectar anticorpos contra agentes infecciosos, antígenos virais e microbianos, e muitos biomarcadores, inclusive o PSA.

Um ensaio análogo ao ELISA que vem sendo amplamente empregado em laboratórios é o ELLA (do inglês *enzyme-linked lectin assay*), que utiliza lectinas como biomoléculas de reconhecimento conjugada a uma enzima, para detecção e quantificação de resíduos de carboidratos específicos em glicoproteínas imobilizadas na fase sólida, bem como em células e seções de tecidos, e assim identificar glicobiomarcadores associados a doenças como o câncer (COUZENS et al., 2014; LLOP et al., 2016).

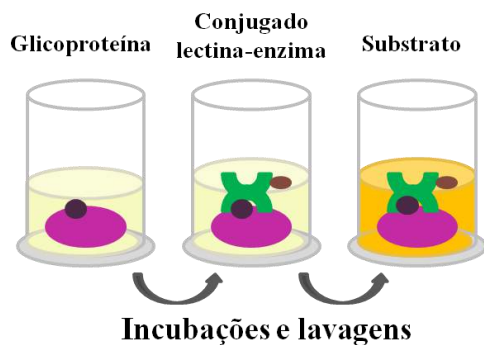
A amostra contendo glicoproteínas é adsorvida ao poço e em seguida incubada com um conjugado lectina-enzima. Após a incubação, o substrato da enzima é adicionado para desenvolvimento da reação do conjugado, formando um produto colorido de intensidade proporcional à ligação lectina-carboidrato, que pode ser determinado quantitativamente em um leitor de placa de ELISA (Figura 19).

Alguns ensaios imunológicos envolvendo a interação anticorpo-antígeno como no ELISA também empregam o uso do conjugado lectina-enzima para detecção de porções de carboidratos quando o antígeno é uma glicoproteína, formando um ensaio tipo “sanduíche”. O anticorpo é adsorvido na fase sólida, para em seguida ligar-se ao antígeno específico presente numa amostra biológica (soro, plasma, urina), e então ser incubado com o conjugado lectina-enzima, para detecção dos resíduos glicosídicos no antígeno (Figura 19). Outra abordagem consiste num ELISA reverso baseado em lectina (WU et al., 2014), onde a lectina é adsorvida ao poço, seguida da incubação com a amostra para ligação de glicoproteínas e por último, incubação com anticorpo conjugado à enzima para detecção da glicoproteínas capturada (Figura 19). Essa metodologia tem sido empregada em

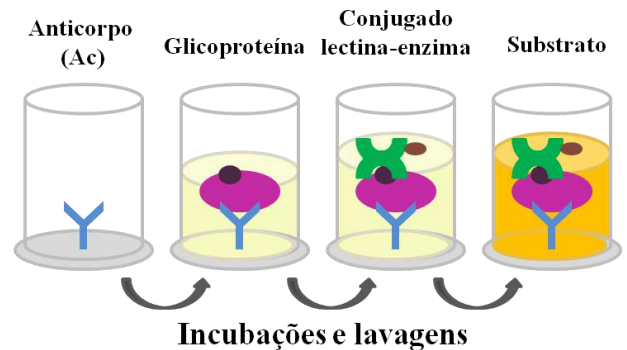
pesquisas de biomarcadores séricos para diversos tipos de câncer.

Figura 19. Representação esquemática dos tipos de ensaios ELLA para detecção de glicoproteínas presentes na amostra.

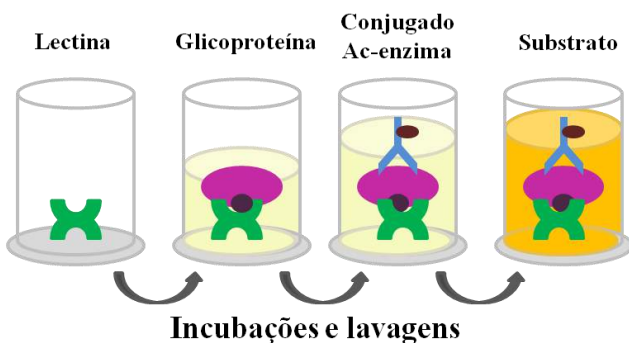
1. ELLA direto



2. ELLA 'sanduíche' direto



3. ELLA 'sanduíche' reverso



Fonte: A autora (2017).

Como foi mostrado, os métodos de coloração em ELISA e ELLA são baseados em reações enzimáticas entre a enzima do conjugado e o seu substrato associado a cromógenos incolores, que serão convertidos em produtos finais coloridos, podendo a coloração variar de acordo com a enzima e o cromógeno utilizado. A enzima mais utilizada nesses ensaios é a peroxidase isolada da raiz do rábano (*Cochlearia armoracia*), devido ao seu grau de pureza, à estabilidade que mantém mesmo após a conjugação ao anticorpo/lectina ou da interação com o substrato, e à capacidade de gerar um produto estável e detectável (RAMACHANDRAN et al., 2014). É uma enzima de natureza glicoproteica com 40 kDa de peso molecular que possui um grupo heme contendo ferro no seu sítio ativo,

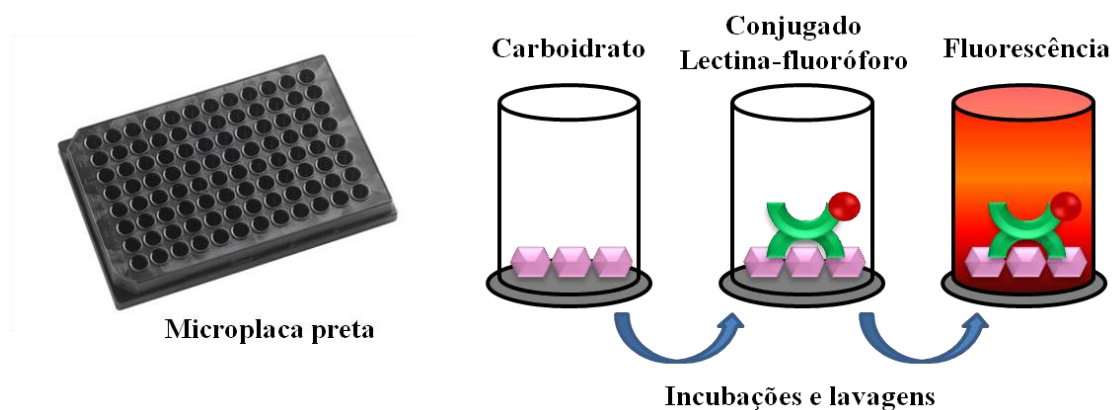
com o qual o seu substrato, peróxido de hidrogênio (H_2O_2), forma um complexo primário (VEITCH et al., 2004). O cromógeno atua como um doador de elétrons para o complexo enzima-substrato, levando à formação de água e oxigênio atômico. Em seguida, a enzima é regenerada e o cromógeno oxidado precipita como um composto colorido no local da reação.

Há vários cromógenos para a peroxidase de rábano, como por exemplo a 3,3'-diaminobenzidina tetracloridrato (DAB), que precipita como um polímero castanho e insolúvel (RAMACHANDRAN et al., 2014); o reagente de Hanker-Yates ou p-fenilenodiamino dihidroclorado, que após a oxidação precipita como um composto alaranjado (HAMILTON et al., 1999); a 3-amino-9-etilcarbazole (AEC), que forma um produto insolúvel de cor vermelha (ÇERIBASI et al., 2012), entre outros. Peroxidase tem sido muito utilizada para obtenção de conjugados com lectinas, uma vez que não prejudica a interação específica entre a lectina e seu carboidrato específico, sendo úteis na detecção específica de porções de carboidratos em superfícies celulares e glicoproteínas circulantes, não apenas em ensaios em microplaca, mas também na microscopia celular e histoquímica.

2.9.2 Ensaio fluorescente em microplaca: fluoróforos e mecanismo de fluorescência

Outro método que tem sido desenvolvido é o ensaio fluorescente em microplaca, que pode ser elaborado e analisado de maneira análoga ao ELISA e ELLA, porém baseado no uso de marcadores fluorescentes e na detecção de intensidade de fluorescência. Este ensaio é desenvolvido em microplacas pretas, que apresentam baixa fluorescência de fundo e assim aumentam a sensibilidade com o mínimo de interferências no sinal (CARVALHO et al., 2014). As análises quantitativas são monitoradas através da mensuração do sinal fluorescente gerado dentro dos poços, contendo amostras biológicas, células inteiras e proteínas isoladas, após incubação com um conjugado de anticorpo ou lectina ligado a um marcador fluorescente, por exemplo (Figura 20) (CARVALHO et al., 2014; CHINEN et al., 2015).

Figura 20. Representação esquemática de um ensaio fluorescente em microplaca para detecção de carboidrato utilizando um conjugado de lectina com um fluoróforo.



Fonte: A autora (2017).

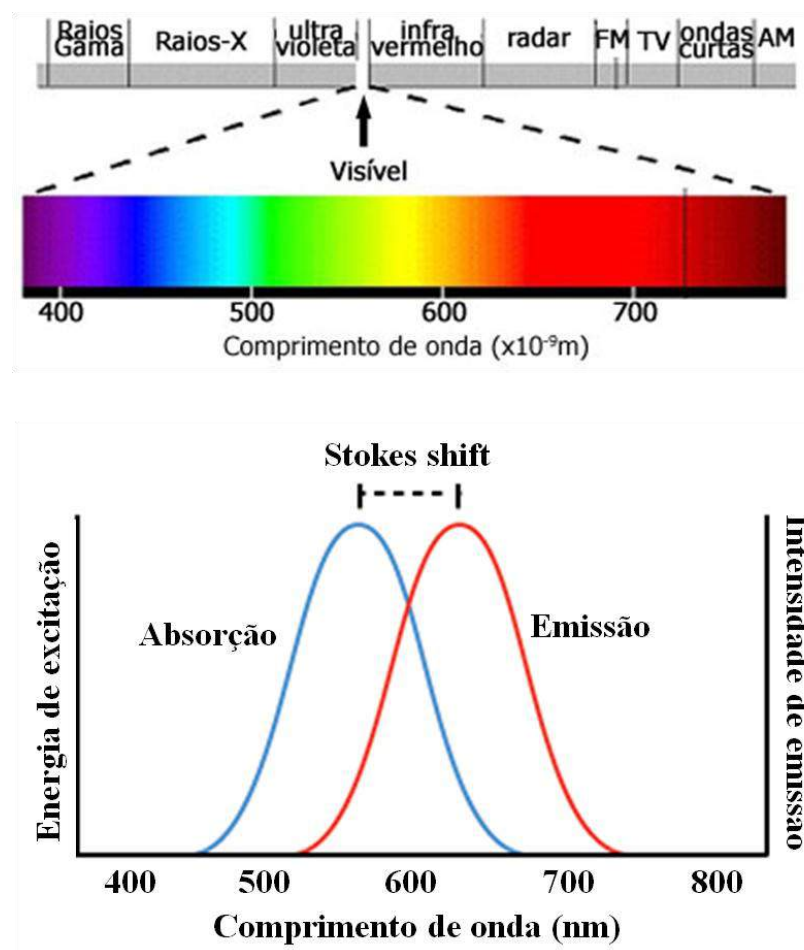
Em geral, os marcadores fluorescentes (fluoróforos) são componentes químicos ou corantes que fluorescem (emitem luz) após excitação, e devem apresentar algumas propriedades consideradas fundamentais para aplicação em bioensaios, tais como brilho (alto coeficiente de absorção molar, alto rendimento quântico e intensa fluorescência), presença de grupos funcionais para marcação específica, estabilidade, reprodutibilidade e compatibilidade com tampões, meios de cultura de células e fluidos corporais (ZHENG et al., 2014; ZHANG & WANG, 2014).

Em um ensaio fluorescente, a detecção é baseada na intensidade de fluorescência gerada quando o fluoróforo é excitado por fótons de maior energia e menor comprimento de onda. Fótons são absorvidos pelo fluoróforo, o que faz com que elétrons sejam promovidos para um nível de maior energia, ou seja, para um estado excitado. Parte dessa energia absorvida é dissipada em transições entre níveis vibracionais, e a energia restante é emitida pelo fluoróforo na forma de fótons com energia menor e um comprimento de onda de emissão maior do que a luz de excitação e fluoresce num intervalo em geral da ordem de dezenas de nanossegundos após a excitação (CHINEN et al., 2015). A intensidade de fluorescência gerada nos poços é detectada e mensurada em leitor de microplaca.

Cada fluoróforo apresenta comprimentos de onda de excitação (absorção) e emissão específicos. Embora o comprimento de onda de emissão seja diferente e independente do de excitação, a intensidade de emissão é proporcional à amplitude

do comprimento de onda de excitação (LU et al., 2016; MARTINIC et al., 2016). Outra característica específica de cada fluoróforo é o deslocamento de *Stokes*, que corresponde à distância entre os máximos do comprimento de onda de excitação e emissão (Figura 21).

Figura 21. Representação do espectro eletromagnético de luz visível e os espectros de absorção e emissão de um fluoróforo com o deslocamento de *Stokes*.



Fonte: < <https://belnaturaologa.files.wordpress.com/2012/09/raios.jpg> > e < <https://www.thermofisher.com/content/dam/LifeTech/Images/integration/Stokes-shift-675px.jpg> >

A detecção da fluorescência pode ser dificultada quando o fluoróforo apresenta o deslocamento de Stokes muito pequeno, devido aos comprimentos de onda de excitação e emissão serem próximos. Quando o deslocamento de *Stokes* é grande, torna-se fácil distinguir entre os comprimentos de onda e detectar a fluorescência emitida (CHANG et al., 2016). Os fluoróforos podem emitir na faixa visível do espectro eletromagnético (390 - 700 nm), bem como nas faixas ultravioleta

e infravermelho próximo do espectro eletromagnético (MARTINIC et al., 2016).

A disponibilidade de fluoróforos com diferentes comprimentos de onda de excitação e emissão e de equipamentos para detecção favorece o desenvolvimento de ensaios fluorescentes destinado a diversas aplicações, permitindo fazer várias detecções ao mesmo tempo utilizando leitor de microplaca. Os fluoróforos podem ser divididos em quatro grupos, de acordo com suas características: corantes orgânicos, fluoróforos biológicos, lantanídeos (III) e pontos quânticos (MARTINIC et al., 2016).

Os corantes orgânicos são compostos fluorescentes sintéticos de pequeno tamanho, sendo atrativos para bioconjugação a macromoléculas como anticorpos, avidina e biotina, sem interferir na função biológica. Estão disponíveis no mercado com diferentes espectros de excitação e emissão, alguns bem conhecidos como fluoresceína, isotiocianato de fluoresceína (FITC, do inglês *fluorescein isothiocyanate*) e rodamina (isotiocianato de tetrametil rodamina, TRITC), muito utilizados em bioconjugação (RESCH-GENGER et al., 2008).

Os fluoróforos biológicos são proteínas fluorescentes, como alofocianina, ficocianina, ficoeritrina e ficoeritrocianina, geralmente oriundas de cianobactérias e algas, e são bastante utilizadas na obtenção de imagens fluorescentes em estudos *in vivo* e como marcadores de expressão e função de proteínas intracelulares (DEDECKER et al., 2013; WALL et al., 2015). Podem ser obtidas expressão heteróloga através da introdução de plasmídeos de expressão em bactérias, células e organismos inteiros; porém, esse procedimento requer tempo e pode induzir a produção de espécies reativas do oxigênio durante a expressão da proteína (DEDECKER et al., 2013).

Os lantanídeos correspondem a uma série de quinze elementos químicos metálicos com números atômicos 57 a 71, do lantânio ao lutécio, também conhecidos como elementos terras raras, caracterizados como metais moles, prateados, extremamente reativos, formadores de cátions trivalentes (Ln III). Como a fluorescência dos íons metálicos de lantanídeos é considerada fraca devido à baixa absorção de energia pelos íons são utilizados na forma de complexos quelados com moléculas orgânicas, a fim de melhorar a intensidade de fluorescência, sendo aplicados no estudo de conformação de proteínas, na obtenção de imagens fluorescentes, entre outras aplicações (COMBY et al., 2013; HEFFERN et al., 2014).

Entre os fluoróforos existentes, os pontos quânticos apresentam propriedades físico-químicas especiais que permitem o controle das suas características espectrais durante sua síntese, e o desenvolvimento de uma gama variada de nanocristais com diferentes espectros de fluorescência (CARVALHO et al., 2014). Quando excitados, eles emitem fluorescência em um determinado comprimento de onda, que varia em função do tamanho da partícula, podendo a luz emitida variar da cor violeta para o vermelho, dependendo da composição do nanocristal, à medida que seu tamanho aumenta (RESCH-GENGER et al., 2008). Além disso, a fluorescência dos pontos quânticos é altamente fotoestável, e sua superfície fornece grupos químicos úteis para funcionalização e revestimento com biomoléculas para uso em diversas aplicações biológicas, como marcação de proteínas e bioconjugação (CARVALHO et al., 2014; CABRAL FILHO et al., 2016). Apesar dos benefícios, já foi relatada a ocorrência de toxicidade celular devido à quebra dos nanocristais, além do alto custo (VALIZADEH et al., 2012; KAUFFER et al., 2014).

2.10 NANOTECNOLOGIA

A nanotecnologia é uma área abrangente relacionada à pesquisa e ao desenvolvimento de nanoestruturas, que possuem dimensões na escala nanométrica, ou mais especificamente, pelo menos uma das dimensões menor que 100 nm. Nessa ordem, as estruturas podem apresentar propriedades especiais, diferentes daqueles apresentados em estruturas com maiores dimensões, como alta resistência mecânica e boa condutividade elétrica. A versatilidade das nanoestruturas proporciona aplicações em diversas áreas, como medicina, desenvolvimento de dispositivos óticos, supercondutores tratamento de resíduos e produção industrial (ADABI et al., 2017; SHARMA et al., 2019; TIEU et al., 2019).

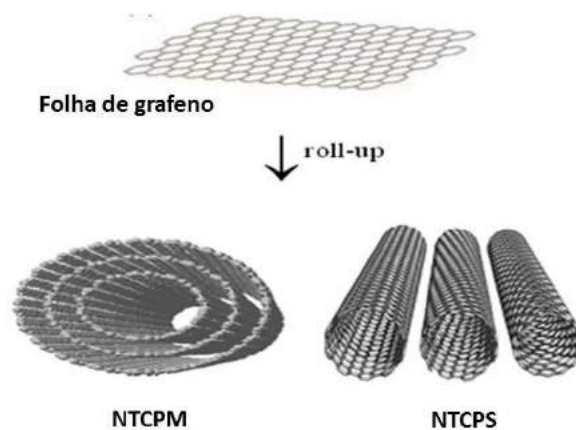
Materiais nanoestruturados têm sido introduzidos em ensaios biológicos de afinidade altamente sensíveis baseados na transdução de sinal eletrônico (CARVALHO et al., 2014; YANG et al., 2015; ADABI et al., 2017). Nanopartículas, como ouro coloidal, pontos quânticos e nanotubos de carbono são promissores no desenvolvimento de ensaios sensíveis e quantitativos devido à capacidade única de amplificação de resposta, sendo úteis em bioensaios analíticos, óticos e eletroquímicos com baixos limites de detecção, seletivos e rápidos (YANG et al.,

2015; CABRAL FILHO et al., 2016). Neste trabalho, foram utilizados nanotubos de carbono no biossensor eletroquímico e pontos quânticos como sonda no ensaio fluorescente em microplaca, ambos para investigar alterações no perfil glicoprotéico associado ao CaP.

2.10.1 Nanotubos de carbono

Nanotubos de carbono (NTC) podem ser descritos como folha(s) de grafeno enrolada(s) em torno de um cilindro imaginário, mantida por interações de Van der Waals nas camadas adjacentes. Do ponto de vista estrutural, os NTC podem ser classificados em duas formas: nanotubos de parede simples (NTCPS), formados por uma única folha de grafeno, e nanotubos de paredes múltiplas (NTCPM), que consiste em camadas concêntricas de folhas de grafeno arranjadas em torno de uma área central (Figura 22). O comprimento dos NTC pode variar de nanômetros a centímetros, porém o diâmetro varia na ordem de nanômetros, dependendo do tipo de nanotubo.

Figura 22. Nanotubos de parede simples (NTCPS) e nanotubos de paredes múltiplas (NTCPM).



Fonte: IYUKE; SIMATE, 2011.

Desde seu descobrimento em 1991, quando foi anunciada pela primeira vez a preparação dessas nanoestruturas por Sumio Iijima (1991), os NTC tem ganhado considerável interesse devido às suas propriedades mecânicas e eletrônicas,

estabilidade química e térmica. A maioria das propriedades dos NTC depende do seu diâmetro, dimensão e quiralidade dos átomos de carbono, inclusive o comportamento metálico ou semicondutor. Em geral, os nanotubos são caracterizados pela estrutura ordenada, peso ultraleve, alta resistência mecânica, condutividade elétrica e térmica e elevada área superficial (ALIM et al., 2018; BELLAMKONDA et al., 2019).

Várias técnicas têm sido desenvolvidas para fabricação de NTC, entre elas a ablação por laser, a técnica de descarga por arco de carbono e a deposição química de vapor (LAWAL et al., 2016). As duas primeiras técnicas são baseadas na condensação de átomos de carbono gerados a partir da evaporação de um precursor sólido (grafite de alta pureza). O terceiro baseia-se na decomposição de gases precursores contendo átomos de carbono, como hidrocarbonetos, sobre um catalisador (metal de transição).

Os métodos de descarga por arco e ablação por laser são mais eficientes na produção NTC em grande escala, porém requerem altas temperaturas de crescimento ($\sim 3000^{\circ}\text{C}$), complexos processos de purificação e não oferecem controle sobre a localização e orientação do material crescido ou sobre a estrutura dos nanotubos. Neste contexto, estas técnicas têm sido substituídas pela deposição química de vapor, que ocorre em temperaturas mais baixas ($< 800^{\circ}\text{C}$), que permite um maior controle do comprimento, diâmetro, alinhamento, pureza, densidade e orientação dos NTC (EATEMADI et al., 2014; LAWAL et al., 2016). Porém, aperfeiçoamentos na síntese de NTC ainda são necessários para melhorar a eficiência desses nanomateriais em termos de boa qualidade em produção de grande escala, crescimento sem a presença de defeitos estruturais e controle da quiralidade (produção controlada de nanotubos semicondutores e metálicos).

A primeira tentativa de uso de NTC no desenvolvimento de biossensores eletroquímicos foi ensaiada por BRITTO; SANTHANAM e AJAYAN (1996), a partir da observação das propriedades eletrônicas desses nanomateriais. Uma vez ancorado à superfície eletrodica, NTC pode mediar reações de transferência de elétrons entre espécies eletroativas em solução e o eletrodo, adicionando sítios oxidativos na superfície do eletrodo e aumentando a área sensora. Estas propriedades podem aumentar a cinética de transferência de elétrons na interface sensora e melhorar a

reprodutibilidade, estabilidade e a sensibilidade (LAWAL et al., 2016). Desde então, o interesse de usar NTC para desenvolvimento de biossensores eletroquímicos tem aumentado consideravelmente.

Uma grande limitação no uso de NTC é sua insolubilidade ou baixa solubilidade em solventes comuns. São extremamente hidrofóbicos e formam agregados em solventes polares. No entanto, é interessante selecionar condições que melhorem a solubilidade ou dispersão de NTC e permita sua aplicação, inclusive no desenvolvimento de biossensores eletroquímicos. A solubilidade pode ser melhorada por métodos de funcionalização, que consiste na adição covalente ou não covalente de grupamentos funcionais polares como ácidos carboxílicos (-COOH), aminas (-NH₂) e hidroxilas (-OH) na estrutura dos NTC (LAWAL et al., 2016; PRABHAVATHI; YAMUNA; JAFER, 2018). Os nanotubos funcionalizados adquirem hidrofilicidade e melhor solubilidade em muitos solventes; podem interagir com polímeros e formar nanocompósitos; e permitem a imobilização covalente e não covalente de biomoléculas (PRABHAVATHI; YAMUNA; JAFER, 2018; KANG; PARK; HÁ, 2019).

A imobilização de biomoléculas (proteína, oligonucleotídeo, carboidrato) sobre NTC é de grande interesse no desenvolvimento de superfícies sensoras e para amplificação do sinal de resposta em biossensores eletroquímicos. Biomoléculas podem ser imobilizadas por métodos de ligação não covalente, que se baseia na interação eletrostática ou hidrofóbica entre a biomolécula e o NTC, dispensando o uso de reagentes que possam afetar a estrutura e a atividade biológica (LAWAL et al., 2016).

A imobilização covalente requer interação química forte entre a biomolécula e o NTC, obtida através do uso de agentes de ligação cruzada, também chamados de *crosslinker* (BHATTACHARYA & SASMAL, 2015). Esses compostos possuem pelo menos dois grupos reativos que possam interagir covalentemente com grupamentos químicos presentes na nanoestrutura e nas biomoléculas, como carboxilas, aminas e sulfidrilas, formando uma “ponte” entre eles. Como exemplo, pode-se destacar o emprego de carbodiimidas no processo de interação entre os grupamentos carboxílicos presentes na superfície do NTC e as aminas primárias oriundas de uma proteína, e vice-versa.

O EDC [Cloreto de 1-etil-3-(3-dimetilaminopropil) carbodiimida] é comumente empregado na imobilização de biomoléculas, junto ao Sulfo-NHS (N-hidroxisulfosuccinimida), que promove estabilidade do intermediário reativo durante a reação (REZAEI et al., 2018). Grupos carboxílicos presentes em NTC são ativados pelo EDC, formando um intermediário altamente reativo e instável, a *o*-acilisouréia. Na presença de sulfo-NHS, é formado um intermediário ativo éster, que reage com as aminas primárias e forma ligação amida entre NTC e biomolécula. Outro *crosslinker* utilizado é o glutaraldeído, que possui dois grupamentos aldeídicos, um em cada extremidade livre, os quais podem interagir com aminas primárias, promovendo a formação de ligações amida e bases de Schiff com proteínas (HU et al., 2015).

Aqui, foram utilizados NTC funcionalizados com grupos carboxílicos (NTC-COOH), obtidos comercialmente, para composição da superfície sensora do biossensor eletroquímico desenvolvido e imobilização não covalente de Cramoll 1,4.

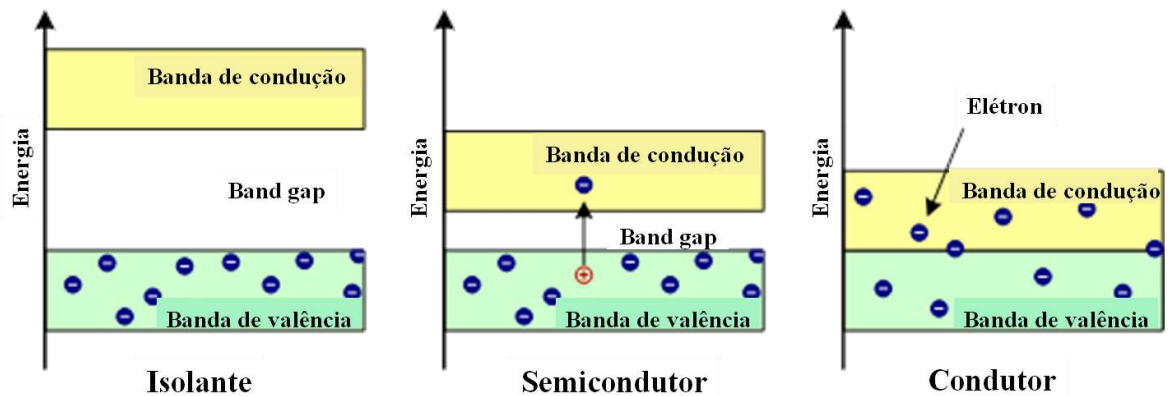
2.10.2 Pontos quânticos

Os pontos quânticos ou QDs (do inglês *Quantum dots*) são nanocristais (1 - 10 nm) fluorescentes compostos por materiais semicondutores formados por centenas a alguns milhares de átomos, que apresentam propriedades ópticas únicas e distintas de cristais da mesma composição em escala macroscópica (CARVALHO et al., 2014). QDs são formados a partir de combinações de elementos semicondutores da tabela periódica, sendo referidos como nanocristais dos grupos II-IV ou 12-16 (ex.: CdSe, CdTe), III-V ou 13-15 (ex.: InP e InAs) e IV-VI ou 14-16 (ex.: PbSe) (CHAN et al., 2002; ERWIN et al., 2005).

Os semicondutores são sólidos cristalinos que apresentam uma condutividade intermediária entre condutores e isolantes. Isso acontece porque esses materiais apresentam uma banda de valência (BV) repleta de elétrons separada de uma banda de condução (BC) por um *band gap* de energia (E_g), sendo E_g a energia mínima que os semicondutores precisam receber (fótons no ultravioleta ou visível, à temperatura ambiente têm energia suficiente para promover elétrons da BV para BC, por exemplo) para que os elétrons passem da BV para a BC, resultando em condutividade elétrica (SMITH; NIE, 2010). Em materiais isolantes, a E_g é elevada,

impedindo a passagem de elétrons para a BC, e nos materiais condutores, a BV e a BC são contínuas, favorecendo a passagem de elétrons entre as bandas (Figura 23).

Figura 23. Diferença na energia do *band gap* para materiais isolante, semicondutor e condutor.



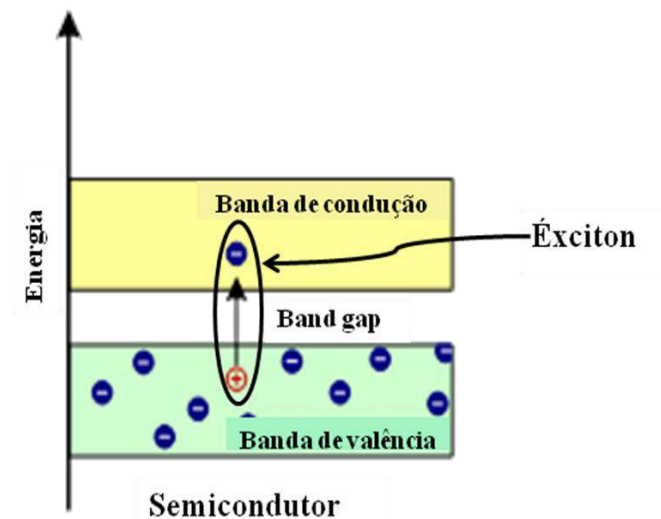
Fonte: < <https://www.halbleiter.org/images/fundamentals/conductors-insulators-semiconductors/baendermodell.gif> >.

Ao receberem energia externa através de fótons, os elétrons da BV nos semicondutores, que apresenta menor energia que a BC, passam para a BC, gerando um par elétron-buraco conhecido como éxciton (Figura 24). O éxciton possui uma vida de poucos nanossegundos e depois sofre um decaimento energético para BV denominado de recombinação excitônica, a qual resulta na emissão de fluorescência. Em escala nanométrica, os elétrons e buracos são submetidos a um forte confinamento quântico, o qual contribui para as propriedades ópticas únicas nos QDs (CUI et al., 2015; MILLO et al., 2015).

O confinamento quântico ocorre em materiais semicondutores quando uma das dimensões é fisicamente menor que o raio de Bohr do material, funcionando como uma barreira limitante do movimento dos elétrons em uma ou mais direções do material (VALIZADEH et al., 2012). Quando se tem uma estrutura tridimensional, o confinamento quântico pode ocorrer nas três dimensões do material, o movimento dos elétrons fica limitado tridimensionalmente, formando níveis de energia discretos que também podem ser ocupados por elétrons. Quando o confinamento quântico é tridimensional, a nanoestrutura é denominada QD (CARVALHO et al., 2014). Um

exemplo são as partículas de CdTe (Telureto de Cádmio), que só podem ser consideradas como QDs quando o nanocristal for menor 7,3 nm, uma vez que o raio de Bohr de CdTe é 7,3 nm (MEDEIROS NETO et al., 1991).

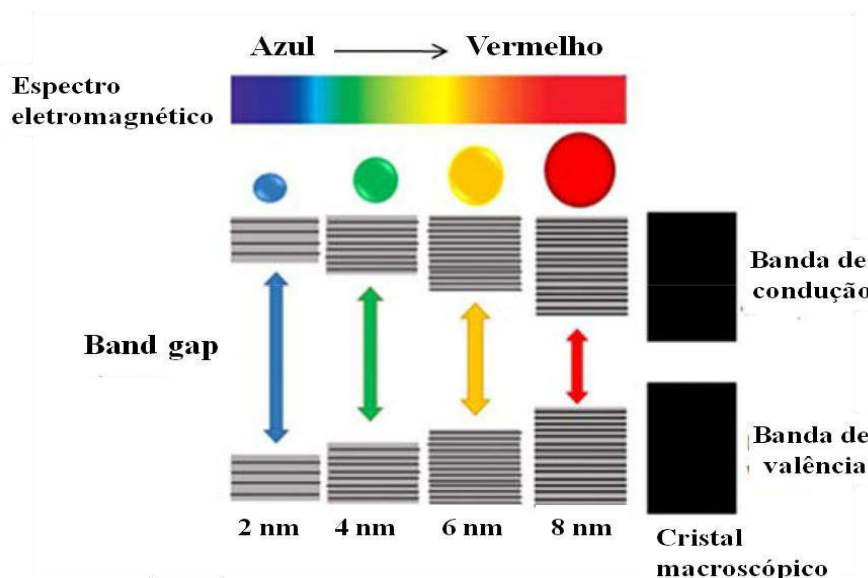
Figura 24. Esquema de formação de um éxciton.



Fonte: Adaptado de < <https://www.halbleiter.org/images/fundamentals/conductors-insulators-semiconductors/baendermodell.gif> >.

Além disso, o confinamento quântico permite o controle do comprimento de onda no espectro eletromagnético em que o QD irá emitir em função do seu tamanho, devido a alterações da E_g (Figura 25). No confinamento quântico, a E_g pode aumentar ou diminuir com o tamanho do nanocristal, sendo importante seu controle durante a síntese do nanocristal, permitindo a obtenção de QDs específicos com diferentes comprimentos de onda de excitação e emissão (MICHALET et al., 2005). Sabendo-se que a E_g é inversamente proporcional ao diâmetro da partícula e que a energia é inversamente proporcional ao comprimento de onda, pode-se observar que quanto menor a partícula, maior é a E_g , e a emissão será em menores comprimentos de onda (em direção à região do violeta), enquanto partículas maiores apresentam menor E_g e emissão em direção à região do vermelho (PROTESESCU et al., 2015).

Figura 25. Variação da luminescência e energia do *band gap* com o tamanho dos QDs.



Fonte: Adaptado de <<http://www.scielo.br/img/revistas/qn/v40n4//0100-4042-qn-40-04-0436-gf01.jpg>>.

Os QDs têm sido utilizados como marcadores fluorescentes biológicos, apresentando algumas vantagens em relação aos fluoróforos convencionais, tais como largo espectro de absorção, podendo partículas que emitem em diferentes regiões do espectro ser excitadas por uma única fonte de luz, e fotodegradação reduzida, permitindo estudos em função do tempo e com luminescência mais intensa (RESCH-GENGER et al., 2008).

A síntese de nanocristais tem passado por várias modificações. Inicialmente, os QDs eram preparados em meio orgânico e apresentavam fluorescência elevada. Mais tarde, QDs também eram preparados em meio aquoso com agentes estabilizantes, como o tioglicerol (CHAN et al., 2002; UNNI et al., 2009). Porém, a síntese aquosa pode resultar em nanocristais de baixa qualidade, com pouca fluorescência e elevada variação no tamanho (CHAN et al., 2002), devido à interferência dos defeitos de superfície, principalmente nos nanocristais de menor tamanho, onde a área superficial é maior. Esses defeitos levam à formação de níveis intermediários entre a BV e a BC, fazendo com que o elétron sofra uma perda de energia gradativa até chegar à BC, reduzindo assim a eficiência de fluorescência.

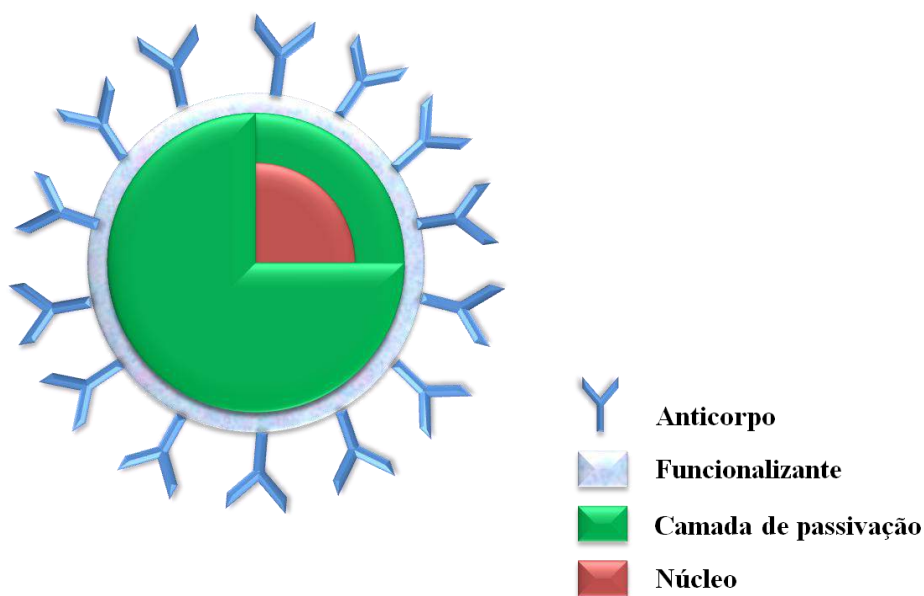
Mais tarde, pesquisadores conseguiram solucionar o problema dos defeitos de superfície quando obtiveram nanocristais de CdSe altamente fluorescentes, com estrutura de cristal quase perfeita e pequena variação de tamanho, a partir de um processo organometálico de alta temperatura, conhecido como passivação, que consiste na deposição de uma camada de cobertura superficial (casca) denominada camada de passivação, composta por um material semiconductor com um maior *band gap* do que o material do núcleo do nanocristal (CHAN et al., 2002). Um exemplo é o uso do ZnS como camada de passivação em QDs de CdSe (BARANOV et al., 2003). Apesar de o ZnS possuir maior *band gap* que o CdSe, o comprimento da ligação Zn-S é semelhante ao da ligação Cd-Se, favorecendo a formação de uma fina camada de ZnS no núcleo CdSe, formando nanocristais com estrutura denominada *core/shell* (núcleo/ casca), conforme mostrado na figura 26.

Assim, QDs apresentam uma estrutura composta por várias camadas. O núcleo determina a emissão do nanocristal, a camada de passivação determina a intensidade da emissão e a fotoestabilidade, e ainda uma camada orgânica funcionalizante, que determina a estabilidade química e atua na marcação do sistema biológico de interesse, fornecendo grupos funcionais para conjugações com biomoléculas. Os agentes estabilizantes utilizados na síntese de QDs coloidais atuam na adição de cargas aos nanocristais, evitando a aglomeração no coloide, além da presença de grupamentos contendo enxofre (ex.: grupamento tiol (-SH)), que auxiliam na formação da camada de passivação (DING et al., 2014; ZHU et al., 2014). Os QDs são considerados coloides constituídos por uma fase dispersa sólida e uma fase dispersante líquida.

No geral, QDs são sintetizados pelo método de síntese química coloidal, baseada numa reação de precipitação sob pH e/ou temperatura controlados, em água ou em solventes orgânicos (CABRAL-FILHO et al., 2016). Na síntese em meio aquoso, primeiro prepara-se uma solução aquosa contendo um sal de metal de transição (elementos do grupo 12 da tabela periódica, como o Cádmio) e um agente estabilizante, que controla o crescimento do núcleo inicial. Em seguida, é induzida a redução de um calcogênio (elementos do grupo 16, como o Telúrio e o Selênio) utilizando um agente redutor. O calcogênio reduzido é então adicionado à solução

aquosa contendo o sal de metal de transição e o agente estabilizante, e são mantidos em reação sob temperatura controlada, durante o tempo necessário para obtenção do nanocrystal num determinado tamanho. Quanto maior o tamanho, maior o tempo requerido para finalizar a síntese.

Figura 26. Esquema de um QD, destacando a estrutura *core/ shell* e a camada estabilizante/funcionalizante.



Fonte: A autora (2017).

Neste trabalho, foram utilizados como precursores para formação dos nanocristais o perclorato de cádmio ($\text{Cd}(\text{ClO}_4)_2$) e o telúrio reduzido (Te^{-2}), junto com o ácido mercaptosuccínico (AMS), o agente estabilizante da reação (CUNHA et al., 2018). Inicialmente, o $\text{Cd}(\text{ClO}_4)_2$ foi misturado com o AMS e em seguida, o pH foi ajustado para 10,5 com hidróxido de sódio (NaOH), sob agitação. O sistema foi mantido em atmosfera inerte e sob aquecimento ($\sim 110^\circ\text{C}$), e posteriormente foi adicionada uma solução aquosa contendo o telúrio reduzido através do reagente boridreto de sódio (NaBH_4) a partir do telúrio metálico (Te^0) sob atmosfera inerte e aquecimento ($\sim 110^\circ\text{C}$). O sistema agora foi mantido sob agitação constante e temperatura em torno de 110°C , durante 6 – 8h. A proporção molar utilizada na síntese foi $\text{Cd}:\text{Te}:\text{AMS} = 5:1:6$, resultando em nanopartículas de CdTe passivadas com CdS (Sulfeto de cádmio) e estabilizadas/funcionalizadas com AMS, com emissão de luz vermelha.

Entre os agentes estabilizantes mais utilizados, pode-se destacar o AMS, o ácido mercaptopropiônico (AMA), a L-cisteína (CIS) e a cisteamina (CISTM), os quais apresentam em comum o grupamento (-SH), e grupamentos funcionais como ácido carboxílico (-COOH) e amina (-NH₂) (SCHEJN et al., 2014; BAG et al., 2015; LIN et al., 2015; CABRAL-FILHO et al., 2016; DIAZ-DIESTRA et al., 2017). O enxofre auxilia na formação da camada de passivação, e o restante da molécula fornecerá cargas aos nanocristais, mantendo-os afastados em suspensão, além de participar da funcionalização dos QDs e favorecer a conjugação a biomoléculas (ex.: proteínas).

A bioconjugação de QDs é uma etapa relevante e fundamental quando se quer obter uma marcação fluorescente específica. Uma biomolécula de reconhecimento (anticorpo, oligonucleotídeo de DNA, lectina), pode ser conjugada ao QD a partir da adsorção ou ligação a grupamentos funcionais na superfície do nanocristal por meio de diferentes mecanismos. Entre os tipos de conjugação de QDs com biomoléculas pode-se destacar: adsorção não covalente; conjugação covalente utilizando etil-3-(3-dimetilaminopropil carbodiimida) (EDC) e N-hidroxisuccinimida (Sulfo-NHS); conjugação covalente utilizando glutaraldeído e interação de QDs funcionalizados com avidina/ estreptavidina com biotina (SAPSFORD et al., 2006; BIJU et al., 2010).

A adsorção é um mecanismo simples de conjugação, baseado em interações fracas do tipo eletrostáticas e hidrofóbicas entre a biomolécula e a superfície do QD, mantendo a atividade biológica da biomolécula, além de dispensar o uso de outras substâncias e ser economicamente favorável (CUNHA et al., 2018).

O EDC e o Sulfo-NHS são considerados agentes de acoplamento, os quais permitem a conjugação covalente da biomolécula ao QD através de ligações amidas. O EDC reage com grupos carboxílicos presentes na superfície do QD (ou na biomolécula), formando um intermediário instável, a *o*-acilisouréia, que precisa ser estabilizado para se obter a ligação amida no final. O Sulfo-NHS é então adicionado para reagir com *o*-acilisouréia e gerar um intermediário Sulfo-NHS éster, o qual interage com a amina primária presente na biomolécula (ou no QD), e forma ligação amida estável entre o QD e a biomolécula, concretizando a conjugação covalente via EDC/ Sulfo-NHS.

As desvantagens deste mecanismo é a necessidade de purificação do conjugado obtido, possibilidade de reação cruzada e problemas de reprodutibilidade e agregação (SAPSFORD et al., 2006)

O glutaraldeído é uma molécula que possui dois grupamentos aldeídos, um em cada extremidade, promovendo a conjugação covalente a partir da reação dos grupamentos aldeídos com grupos aminas presentes no QD e na biomolécula de interesse, formando bases de Schiff. É importante utilizar o glutaraldeído em baixas concentrações (< 1%) para evitar ligações indesejáveis e inativação das biomoléculas (JIANG et al., 2012).

A avidina e a estreptavidina são proteínas com elevada afinidade pela biotina que podem interagir com a superfície de QDs funcionalizados com grupamentos tióis e ácidos carboxílicos, e servir como ponte para conjugação de QDs com proteínas marcadas com biotina, como anticorpos biotinilados. Dessa maneira, o anticorpo biotinilado pode ficar imobilizado à superfície do QD, formando um conjugado específico para imunomarcação (SAPSFORD et al., 2006; BIJU et al., 2010).

Um método que pode ser utilizado para confirmar o sucesso da bioconjugação é o ensaio fluorescente em microplacas, através da detecção de fluorescência dos QDs conjugados introduzidos nas placas. Os QDs não conjugados não se ligam à placa, portanto são removidos durante a lavagem e não fluorescem (controle negativo). Quando conjugado a proteínas como anticorpo, este se liga à placa, e o bioconjugado permanece ligado à placa após a lavagem, havendo emissão de fluorescência (CARVALHO et al., 2014).

No caso de algumas lectinas como Con A, que não se liga à placa, é feita antes uma etapa de revestimento da placa com um polímero do carboidrato específico da lectina, e em seguida o conjugado QD-lectina é introduzido. A lectina se ligará à placa revestida com seu carboidrato específico e após a lavagem, o bioconjugado permanece ligado e emite sinal fluorescente. Para que a conjugação seja confirmada, os conjugados devem apresentar no mínimo 100% de aumento da intensidade relativa de fluorescência (FR) quando comparados ao controle, uma vez que a marcação específica de células é obtida a partir de 100%. O aumento da intensidade de fluorescência pode ser calculado utilizando a seguinte equação (CARVALHO et al., 2014):

$$\text{Fluorescência relativa (FR) (\%)} = \frac{(\text{Fluorescência do bioconjugado} - \text{Fluorescência dos controles})}{\text{Fluorescência do controle}} \times 100$$

Como demonstrado na equação, a fluorescência do bioconjugado corresponde à intensidade de fluorescência emitida pelo bioconjugado e a fluorescência dos controles corresponde à média da fluorescência emitida pelos controles (QDs e biomoléculas separados, sozinhos).

QDs conjugados a biomoléculas de reconhecimento representam um avanço no rastreamento e obtenção de imagens fluorescentes em tempo real de mecanismos intracelulares e fisiológicos, bem como na detecção específica de doenças e microrganismos. Bioconjugados formados por QDs e lectinas têm sido marcadores fluorescentes promissores no estudo de alterações na expressão de glicanos e glicoproteínas em amostras biológicas, na pesquisa de novos biomarcadores associados a doenças como o câncer e na detecção de patógenos como *Candida albicans* (ANDRADE et al., 2013; TENÓRIO et al., 2015; CUNHA et al., 2018). Aqui, QDs de CdTe foram conjugados à Cramoll 1,4 por adsorção, e empregados no ensaio fluorescente em microplaca para analisar alterações no perfil glicoproteico em amostras de soro de pacientes com HBP e CaP.

A habilidade das lectinas de ligação a carboidratos junto às propriedades ópticas dos QDs fazem desses conjugados potenciais ferramentas para microscopia, histopatologia molecular e diagnóstico de doenças. SANTOS et al., 2006 desenvolveram bioconjugados de QD - lectina para marcação de fibroadenoma mamário utilizando QDs CdS/Cd(OH)₂ conjugados com ConA, e os resultados mostraram um padrão de marcação fluorescente similar à marcação com peroxidase.

Outro trabalho relata o uso de bioconjugados do QD de CdTe com as lectinas ConA e UEA I (*Ulex europeus* agglutinin I) para investigar o padrão de expressão de carboidratos em tecidos mamários normais, fibroadenoma (benigno) e carcinoma ductal invasivo (maligno), sendo possível observar nas imagens fluorescentes diferença na distribuição de carboidratos em todos os tecidos analisados (ANDRADE et al., 2013). Bioconjugados de CdTe com ConA também foram eficientes na marcação de *C. albicans* em suspensão e biofilmes, a partir do reconhecimento de

resíduos de glicose e manose na superfície celular de *C. albicans* pela ConA (TENÓRIO et al., 2015). QD de CdTe conjugados à lectina Cramoll 1,4 também mostraram potencial para marcação de células de *C. albicans* (CUNHA et al., 2018).

Assim, bioconjugados QD - lectina são alternativas promissoras na pesquisa de microrganismos e na investigação de padrões de glicosilação e perfis glicoproteicos associados a doenças.

3 MÉTODO

3.1 OBTENÇÃO DAS AMOSTRAS SÉRICAS E LOCAIS DE ESTUDO

Amostras de soro foram obtidas de 15 pacientes com HBP e 21 com CaP, previamente confirmados por exame histopatológico do tecido prostático (2 de CaP Gleason 5; 10 de CaP Gleason 6; 6 de CaP Gleason 7 e 3 de CaP Gleason 9). Todos os doadores foram voluntários e admitidos no Hospital das Clínicas de Pernambuco, de acordo com recomendações do comitê de ética. Foram coletadas amostras de sangue venoso, as quais foram imediatamente centrifugadas a 1500 g por 5 minutos. Os respectivos soros foram separados e estocados a 20°C. Foram preparados *pools* dos soros de HBP e de cada grupo de CaP, os quais foram utilizados para analisar os perfis de glicoproteínas pelos ensaios de ligação à Cramoll 1,4.

A elaboração dos eletrodos baseados na lectina Cramoll 1,4 e os estudos eletroquímicos foram efetuados no Laboratório de Engenharia Biomédica do Departamento de Engenharia Biomédica da UFPE e no Laboratório de Eletroanalítica do Departamento de Química Fundamental, também da UFPE. As caracterizações morfológica e estrutural das superfícies eletródicas, obtidas pelas técnicas de MEV e FT-IR, respectivamente, foram realizadas no CETENE. Os bioensaios colorimétricos foram efetuados no Laboratório de Bioquímica de Proteínas do Departamento de Bioquímica da UFPE. Os ensaios fluorescentes foram realizados no Laboratório de Biofísica Química do Departamento de Biofísica da UFPE.

3.2 LECTINA CRAMOLL 1,4

3.2.1 Purificação de Cramoll 1,4

Cramoll 1,4 foi purificada a partir de sementes de *C. mollis* de acordo com protocolo estabelecido por Correia e Coelho (1995). As sementes foram coletadas na cidade de Ibimirim, localizada em Pernambuco. Um extrato salino foi preparado a partir da farinha das sementes incubada com NaCl 0,15 mol L⁻¹ (10% p/v) durante

16h sob agitação à temperatura de 4°C. O extrato foi então filtrado em gaze e centrifugado (12100 g, 15 min, 4°C). O sobrenadante foi submetido a um fracionamento salino em sulfato de amônio, e a fração selecionada (F40-60) foi dialisada em NaCl 0,15 mol L⁻¹ e depois introduzida em coluna de Sephadex G-75 (Sigma Aldrich, EUA) para purificação da lectina por afinidade. Após a aplicação da fração, foi mantido um fluxo de NaCl 0,15 mol L⁻¹ na coluna até que a mensuração da absorbância (280 nm) fosse menor que 0,1. A eluição da lectina foi efetuada com solução de D-glicose 0,3 mol L⁻¹ preparada em NaCl 0,15 mol L⁻¹.

3.2.2 Conjugação de Cramoll 1,4 com peroxidase

A conjugação foi efetuada de acordo com BELTRÃO et al. (1998). Cramoll 1,4 (1 mg mL⁻¹) foi dialisada em tampão fosfato salino (PBS; 0,1 mol L⁻¹, pH 6,8). Em seguida, metil- α -D-manopiranosose (0,1 mol L⁻¹) foi adicionada à solução de Cramoll 1,4 dializada e mantida em agitação leve por 15 minutos. Peroxidase tipo VI de rábano (3 mg mL⁻¹) foi também adicionada e a solução foi mantida em agitação suave para adição de 50 μ L de glutaraldeído (1% v/v), por gotejamento. A mistura foi mantida em repouso durante 2h à temperatura ambiente, e depois dialisada a 4°C durante 16h em tampão fosfato de sódio. O conjugado obtido foi distribuído em alíquotas de 50 μ L e estocado à -20°C.

3.2.3 Conjugação de Cramoll 1,4 a QDs de CdTe

Foi sintetizada uma dispersão coloidal aquosa de QDs vermelhos *core/shell* de CdTe/MSA de acordo com metodologia previamente descrita por CUNHA et al. (2018). Os QDs foram sintetizados pela adição de Te⁻² (obtido de telúrio metálico Te⁰ - Sigma Aldrich, EUA) em uma solução de CdCl₂ ou Cd(ClO₄)₂ (0,5 mmol L⁻¹) (Sigma Aldrich, EUA) com pH > 10, na presença de ácido 3-mercaptopossuccínico (AMS - Sigma Aldrich, EUA) como agente estabilizante. Foi utilizada uma razão molar de 5:1:6 de Cd/Te/MSA em solução de água ultrapura para obtenção dos QDs de emissão vermelha. A caracterização óptica foi realizada por espectroscopia de absorção e emissão eletrônica, utilizando um espectrofotômetro UV-Vis 1800

(Shimadzu) e um espectrômetro LS 55 (PerkinElmer, em $\lambda_{exc} = 405 \text{ nm}$), respectivamente.

Os QDs de CdTe obtidos foram conjugados a Cramoll 1,4 por adsorção em pH 7,0, de acordo com CUNHA et al (2018). Em primeiro lugar, o pH de 1 mL de CdTe QDs ($\sim 7 \mu\text{mol L}^{-1}$) foi ajustado para 7,0 usando AMS a 4,9% (p/v). Em seguida, 90 μL de Cramoll 1,4 ($3,1 \text{ mg mL}^{-1}$) foram adicionados a 910 μL de QDs para uma concentração final de $280 \mu\text{g mL}^{-1}$. O sistema foi mantido em agitação lenta à temperatura ambiente por 2h, e depois armazenado a 4°C . Após sete dias, o conjugado foi lavado com água ultrapura em quatro ciclos de centrifugação ($908 \times g$ por 6 min), a 4°C , usando filtros de ultracentrifugação Amicon (cut-off de 30 kDa; Sartorius, Alemanha), a fim de remover resíduos da síntese de QDs. Após o último ciclo, o conjugado filtrado Cramoll 1,4 – QD foi ressuspenso em água ultrapura.

3.3 ESTUDOS ELETROQUÍMICOS

3.3.1 Preparação do nanoeletrodo de Cramoll 1,4

Foi utilizado um eletrodo sólido de carbono vítreo (ECV) para preparar o nanoeletrodo de Cramoll 1,4. Antes de sua superfície ser modificada, ECV foi polido com alumina por 3 min e em seguida lavado com água ultrapura para remoção de impurezas. Solução de PLL ($0,05 \mu\text{mol L}^{-1}$) preparada em PBS ($0,01 \text{ mol L}^{-1}$, pH 7,4) foi depositada eletroquimicamente na superfície de ECV através da técnica de voltametria cíclica, na faixa de potencial de $-2,0$ à $2,0 \text{ V}$ e velocidade de varredura de $0,05 \text{ V s}^{-1}$ durante 15 ciclos. Em seguida, três camadas (15 μL) de uma suspensão de NTCPM funcionalizados com grupos carboxílicos (Dropsens, Espanha) dispersados em dimetilformamida (1 mg mL^{-1}) foram depositadas na superfície de ECV. Cramoll 1,4 ($200 \mu\text{g mL}^{-1}$) foi imobilizada na superfície modificada no nanoeletrodo durante 60 minutos em câmara úmida e temperatura ambiente (25°C). 5 μL de uma solução de glicina ($0,05 \text{ mol L}^{-1}$) preparada em água ultrapura foi depositada na superfície sensora por 60 minutos para bloqueio dos sítios inespecíficos.

Os estudos eletroquímicos foram executados utilizando um potenciostato/galvanostato portátil Ivium Compact Stat (Eindhoven, Holanda) conectado a um microcomputador e controlado pelo software IviumSoft. Foi utilizado um sistema trieletródico, composto pelo ECV como eletrodo de trabalho, um eletrodo de platina helicoidal como eletrodo auxiliar, e um eletrodo de Ag/AgCl como eletrodo de referência. As etapas de modificações do biossensor foram monitoradas por VC. As análises foram executadas em uma faixa de potencial de -0,1 a 0,5 V, velocidade de varredura de $0,05 \text{ V s}^{-1}$, em presença de $0,005 \text{ mol L}^{-1}$ de $\text{K}_3\text{Fe}(\text{CN})_6 / \text{K}_4\text{Fe}(\text{CN})_6$ preparada em solução de KCl ($0,1 \text{ mol L}^{-1}$).

3.3.2 Resposta analítica do nanoeletrodo de Cramoll 1,4

O desempenho analítico do nanoeletrodo foi avaliado através de incubação com soluções de fetuína ($10 \text{ }\mu\text{L}$) em diferentes concentrações, durante 20 minutos em câmara úmida e temperatura ambiente. As respostas foram monitoradas por SWV usando uma solução de $\text{K}_3\text{Fe}(\text{CN})_6 / \text{K}_4\text{Fe}(\text{CN})_6$ ($0,005 \text{ M}$) como sonda redox, faixa de potencial de 0 à 0,4 V e frequência de 10 Hz. A detecção de fetuína pela lectina foi avaliada pelo percentual de diminuição da corrente ($\Delta I\%$) na leitura de SWV antes e após a incubação com fetuína. *Pools* de soros de pacientes de HBP e CaP diluídos na proporção de 1:30 em tampão PBS ($0,01 \text{ mol L}^{-1}$, pH 7,4) foram utilizados para avaliar as respostas analíticas. Todas as medidas foram executadas em triplicata.

3.3.3 SPE baseado em Cramoll 1,4 para detecção voltamétrica de fetuína

Cramoll 1,4 ($300 \text{ }\mu\text{g mL}^{-1}$) foi imobilizada na superfície de SPE de carbono modificados com NTCPM (Dropsens, Espanha) por gotejamento de $10 \text{ }\mu\text{L}$ na superfície e manutenção em temperatura ambiente durante 60 minutos. Em seguida, $10 \text{ }\mu\text{L}$ de uma solução de glicina ($0,075 \text{ mol L}^{-1}$), preparada em água ultrapura, foram pipetados na superfície do eletrodo e mantidos por 60 minutos para bloquear os sítios inespecíficos. A resposta analítica foi analisada através da incubação com $10 \text{ }\mu\text{L}$ de fetuína em diferentes concentrações durante 20 minutos em temperatura ambiente. VC e SWV foram as técnicas utilizadas para caracterizar a modificação da

superfície do eletrodo e monitorar a resposta analítica na presença de fetuína, usando solução de $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ ($0,005 \text{ mol L}^{-1}$) preparada em solução de KCl ($0,1 \text{ mol L}^{-1}$) como sonda redox. VC foi realizada em uma faixa de potencial de -0,2 a 0,5 V e velocidade de varredura de $0,05 \text{ V s}^{-1}$. As mensurações de SWV foram realizadas em uma faixa de potencial de 0,1 a 0,4 V e frequência de 10,0 Hz. A detecção da afinidade da lectina foi padronizada usando a diminuição da corrente (ΔI) nas medidas de VC e SWV antes e após a incubação com fetuína. VC também foi utilizada para analisar a difusão eletrônica da superfície modificada de SPE.

3.4 BIOENSAIOS COLORIMÉTRICOS COM CRAMOLL 1,4

3.4.1 ELLA

Pools séricos foram diluídos em PBS ($0,01 \text{ mol L}^{-1}$, pH 7,2) e alíquotas de 50 μL de cada *pool* foram pipetadas em poços de microplacas de poliestireno (96 poços), em triplicata, e então as microplacas foram incubadas à 4°C , *overnight*. Os poços foram lavados três vezes com 200 μL de PBS contendo Tween 20 (0,1%). Foram adicionados 250 μL de BSA a 1% para bloquear os sítios de ligação não específicos, e a microplaca foi incubada a 4°C por 2h. Os poços foram lavados como descrito anteriormente. Uma alíquota de 100 μL de Cramoll 1,4 - peroxidase ($50 \mu\text{g mL}^{-1}$) foi adicionada nos poços e incubada a 4°C , 2h. Os poços foram novamente lavados. *O*-fenilenodiamina dihidroclorada foi utilizado como substrato cromogênico para peroxidase. Em resumo, foi adicionado 50 μL de 10 mmol L^{-1} de tampão citrato (pH 6,0) contendo 0,08% (p/v) de *O*-fenilenodiamina dihidroclorada e peróxido de hidrogênio em cada poço, e a reação colorimétrica prosseguiu por 30 min, quando foi parada pela adição de 100 μL de solução de ácido cítrico 20 mmol L^{-1} . A absorvância de cada poço foi medida a 490 nm em um leitor automático de microplacas (Epoch - Biotek, EUA). Os mesmos procedimentos também foram realizados com solução de fetuína (1 mg mL^{-1}) no lugar dos *pools*, a fim de analisar a eficácia da conjugação e melhorar as condições analíticas.

3.4.2 ELISA

Poços de microplacas de poliestireno (96 poços) foram revestidos com 50 μL de solução de anticorpo monoclonal anti-PSA total, clone 5A11E9 (Sigma, EUA) na concentração de 1 $\mu\text{g mL}^{-1}$, em triplicata, e incubados a 4°C, *overnight*. Os poços foram lavados três vezes com 200 μL de PBS contendo 0,1% de Tween 20. Depois, 200 μL de solução de BSA (1% p/v) em PBS foram utilizados para bloqueio dos sítios inespecíficos, à 4°C por 2h. Em seguida, as microplacas foram lavadas como descrito anteriormente. Para evitar a ligação da lectina por afinidade aos resíduos de carboidratos ligados ao anticorpo anti-PSA total presente nos poços, a superfície foi tratada com 150 μL de tampão periodato de sódio preparado em NaCl (0,15 mol L^{-1}) acetato de sódio (0,10 mol L^{-1} , pH 5,5) a 4°C por 1h.

Após o tratamento, as placas foram lavadas como antes, e 50 μL de *pools* de soro (1:10 em PBS) ou de solução de PSA recombinante humano à 1 $\mu\text{g mL}^{-1}$ (Sigma Aldrich, EUA) foram adicionados a cada poço e incubados a 4°C por 2h. As placas foram lavadas como antes e 100 μL de solução Cramoll 1,4 - peroxidase (50 $\mu\text{g mL}^{-1}$) foram adicionados a cada poço com posterior incubação a 4°C por 2h. Após as etapas de lavagem, foram adicionados 50 μL de tampão citrato fosfato (0,01 mol L^{-1} , pH 6,0) contendo 0,08% (p/v) de *O*-fenilenodiamina dihidroclorada e peróxido de hidrogênio. A reação colorimétrica prosseguiu por 30 minutos, quando 100 μL de solução de ácido cítrico 0,02 mol L^{-1} foram adicionados para interromper a reação. A absorbância foi medida a 490 nm em um leitor automático de microplacas (Epoch - Biotek, EUA).

3.5 BIOENSAIOS FLUORESCENTES COM CRAMOLL 1,4

3.5.1 Marcação de células de *C. albicans* com Cramoll 1,4 – QD

A parede celular da levedura *C. albicans* contém carboidratos, como β -glucanos, quitina e proteínas contendo resíduos de manose (TENÓRIO et al., 2015). Assim, *C. albicans* foi utilizada para confirmar a eficiência da conjugação de Cramoll 1,4 aos QDs em manter a afinidade biológica da lectina, uma vez que Cramoll 1,4

reconhece especificamente resíduos de α -D-manose e α -D-glicose. As células de *C. albicans* (ATCC 10231) foram cultivadas em caldo Sabouraud dextrose por 24 horas a 37°C sob agitação constante a 75 rpm, de acordo com TENÓRIO et al. (2015). O caldo contendo as células da levedura foi centrifugado (1680 x g por 2 min; MiniSpin - Eppendorf) e ressuspensão em NaCl 0,15 mol L⁻¹. A suspensão celular foi incubada com 100 mL de conjugado na proporção de 1:1 por 1h a temperatura ambiente sob agitação lenta. Em seguida, a amostra foi centrifugada a 1200 x g por 30 s para remover os conjugados não ligados às células, e ressuspensão em 100 μ L de NaCl 0,15 mol L⁻¹.

Para confirmar a especificidade do conjugado Cramoll 1,4 - QD, foi realizado um ensaio de inibição incubando o conjugado com metil- α -D-manopiranosídeo (0,4 mol L⁻¹) por 30 minutos antes da incubação com as células. A marcação celular foi avaliada por microscopia de fluorescência (DMI 4000B, Leica), a fim de obter informações sobre a localização espacial das moléculas alvo.

3.5.2 Ensaio fluorescente em microplaca com revestimento de manana

Este método foi empregado para avaliar a eficiência da conjugação de lectinas específicas para manose e QDs, com base na presença ou ausência de sinais de fluorescência da amostra (CARVALHO et al., 2014). 50 μ L de PBS contendo 200 μ g mL⁻¹ de manana (manana de *Saccharomyces cerevisiae*, Sigma Aldrich, EUA) foram colocados em poços de microplaca de poliestireno (microplaca OptiplatF HB preta de 96 poços - PerkinElmer), em triplicata, e incubados a 37°C em uma incubadora (banho-maria, câmara úmida), por 3h. Manana é um polissacarídeo linear de manose encontrado em plantas e fungos, útil para confirmar a eficácia da conjugação e a manutenção dos sítios de ligação da lectina para reconhecimento de carboidratos. Depois, a placa foi lavada três vezes com 100 μ L de PBS (10 mmol L⁻¹).

As placas foram incubadas com Cramoll 1,4 - QD a 4°C por 2h, a fim de avaliar o reconhecimento de manana pela lectina e a especificidade do sinal fluorescente. As placas foram lavadas como descrito anteriormente e as medições de fluorescência foram realizadas usando um Leitor de Placas WALLAC 1420 com o software Victor2 (PerkinElmer). O filtro de passagem da banda de excitação foi o

F405, e o filtro de passagem de banda de emissão foi o F595. O poço contendo manana sem o conjugado foi utilizado como controle. A intensidade do sinal foi obtida através da Intensidade Relativa de Fluorescência (RF), calculada pela equação: $RF = [(Conjugado\ FL - controle\ FL) / controle\ FL] \times 100\%$. O conjugado FL é a intensidade média de fluorescência dos conjugados. O controle FL é o sinal médio detectado no controle.

3.5.3 Ensaio fluorescente em microplaca baseado em Cramoll 1,4 - QD

Foram preparadas soluções de fetuína (1 mg mL^{-1}) e PSA ($1\text{ }\mu\text{g mL}^{-1}$) em PBS, e alíquotas de 50 μL foram pipetadas em triplicata em poços de microplaca OptiplatF HB preta de 96 poços, seguido de incubação a 4°C , *overnight*. Os poços foram lavados três vezes com 100 μL de PBS. Os sítios inespecíficos de ligação foram bloqueados com a adição de 100 μL de BSA a 1% (p/v) em PBS, e a microplaca foi incubada a 4°C por 2h. Os poços foram lavados como descrito anteriormente. Em seguida, 100 μL de Cramoll 1,4 - QD ($280\text{ }\mu\text{g mL}^{-1}$) foram adicionados aos poços e incubados a 4°C por 2h. Depois, os poços foram lavados como descrito anteriormente e as medições de fluorescência foram realizadas usando um Leitor de Placas WALLAC 1420 com o software Victor2 (PerkinElmer). O filtro de passagem da banda de excitação foi o F405 e o filtro de passagem da banda de emissão foi o F595. O poço com fetuína ou PSA sem bioconjugado foi considerado como controle. A intensidade do sinal foi obtida através do RF, calculado conforme descrito em 3.4.4.

3.6 ENSAIO DE INIBIÇÃO

Para confirmação de que os resultados obtidos estão relacionados à especificidade da lectina, foram realizados ensaios de inibição da ligação à lectina. Os conjugados Cramoll 1,4 – peroxidase e – QD foram previamente incubados com metil- α -D-manopiranosídeo (400 mmol L^{-1}) a temperatura ambiente por 30 minutos, para depois serem incubados com fetuína ou manana no ELLA, ELISA e nos ensaios fluorescentes. Conjugados inibidos também foram utilizados como controle na análise da eficiência da conjugação.

3.7 ANÁLISE ESTATÍSTICA

Os resultados obtidos nos ensaios eletroquímicos e colorimétricos relacionados à interação entre Cramoll 1,4 e glicoproteínas dos *pools* dos soros de CaP e HBP foram comparados utilizando o teste t-student não pareado e o ANOVA (fator único).

4 RESULTADOS

4.1 ARTIGO 1 - A COMPARATIVE STUDY OF GLYCOSYLATION CHANGES IN SERUM PROTEINS OF PROSTATE CANCER BY LECTIN-BINDING ASSAYS USING CRAMOLL 1,4

Research Article em preparação para ser submetido ao periódico *Clinica Chimica Acta*

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A comparative study of glycosylation changes in serum proteins of prostate cancer by lectin-binding assays using Cramoll 1,4

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ABSTRACT

Background: Changes of glycosylation in serum proteins has been investigated for the detection and discrimination of prostate cancer (PCa) and benign prostate hyperplasia (BPH). Two lectin-binding assays were developed using the lectin Cramoll 1,4 and equipment available in routine biomedical laboratories to determine the changes in serum glycoproteins of PCa and BPH.

Methods: Enzyme-linked lectin-binding assay (ELLA) and enzyme-linked immussorbent lectin assay (ELISA) with colorimetric, detection and a fluorescent microplate assay on microplate were devised using the lectin from *Cratylia mollis* seeds (Cramoll 1,4, specific for mannose/glucose residues) as probe to assess glycosylation changes in pools of serum samples from patients with PCa (Gleason degree 5, 6, 7, and 9) or BPH. Cramoll 1,4 was conjugated to peroxidase and quantum dots of CdTe in order to form the colorimetric and fluorescent probes, respectively. An enzyme-linked immunosorbent lectin assay is being developed to investigate changes in PSA glycosylation in serum from PCa or HBP.

Results: First, the optimal conditions of these assays were improved for serum glycoproteins. The conjugates peroxidase - Cramoll 1,4 and QDs - Cramoll 1,4 were efficiently produced. Cramoll 1,4 showed more affinity to glycoproteins from PCa patients compared with glycoproteins from patients with BPH in the ELLA. The Cramoll 1, 4 binding increased with the PCa degree.

Conclusions: Changes in glycosylation of serum glycoproteins were identified using 50 μ L of serum, allowing the detection of PCa and its discrimination with BPH patients.

Keywords: benign prostate hyperplasia (PCa); Cramoll 1,4; enzyme-linked lectin-binding assay (ELLA); fluorescent lectin assay on microplate; prostate cancer; quantum dots.

1. Introduction

The serum concentration of prostatic specific antigen (PSA) has been measured for diagnosis of prostate cancer (PCa) and monitoring patient response to treatment. PSA concentrations when detected in blood from healthy men, are commonly low (< 4 ng mL⁻¹) and may elevate until 10⁴ ng mL⁻¹ in men with PCa [1]. However, PSA test shows a poor specificity for PCa diagnosis, since noncancerous prostatic diseases such as benign prostatic hyperplasia (BPH) also can elevate serum levels of PSA to 4 ng/mL or higher [2]. Thus, screening methods that would be more reliable for PCa detection and to distinguish BPH, low-grade Gleason PCa and high-grade Gleason PCa have been developed based in the analysis changes of glycosylation tumor-associated.

Protein glycosylation of proteins is observed in half or all serum proteins and alterations in glycosylation have been associated to malignant transformation and cancer progression, being potential tools to identify various types of cancer [3]. Aberrant glycosylation of proteins has been observed in PCa tissues with a distinct pattern of the BPH and normal tissue [4]. Similarly, changes in the glycans on serum proteins, including PSA, have been identified in PCa compared with BPH, but these studies have used technologies only available in specialized glycoproteomic laboratories, such as mass spectrometry, affinity chromatography and affinity electrophoresis.

Most of these studies were performed using lectins as probes to recognize carbohydrate portions linked to proteins. Other methods more practical, with accessible instrumentation, named enzyme-linked lectin-binding assays (ELLA) have been developed for detecting changes of glycosylation with accuracy and flexibility [5, 6]. The identification of glycosylation changes in PCa may provide greater specificity than the PSA test. Here in, a simple enzyme-linked lectin-binding assay (ELLA) using the lectin Cramoll 1,4 from *Cratylia mollis* seeds, specific for mannose/glucose, were developed in-house and the optimal conditions were improved to analyze the glycoprotein profiles in PCa and HBP pool sera. It was also developed a lectin–antibody sandwich immunoassay to evaluate glycosylation changes in PSA from PCa. Anti-total PSA monoclonal antibody was used to capture PSA, and Cramoll 1,4 to recognize glycan portions of PSA.

A fluorescent microplate assay was also developed using Cramoll 1,4 conjugated to quantum dot (QDs) of CdTe as fluorescent marker for detection. This approach is advantageous due to easily conjugation lectin-QD, low cost for QD synthesis and dispenses the use of colorimetric substrates for enzymatic reaction, reducing the steps and the time-consuming compared with ELLA and lectin–antibody sandwich immunoassay. Cramoll 1,4 probes showed greater reactivity to glycoproteins from PCa pool sera compared with BPH, increasing with the aggressivity of PCa. The glycoprotein fetuin was used in some tests in order to standardize the optimal conditions of the assays and evaluate the lectin-binding activity, since Cramoll 1,4 has high affinity to bind fetuin.

2. Materials and methods

2.1 Human serum samples

Individual serum samples were obtained from patients with biopsy-confirmed prostate cancer (2 of PCa Gleason 5; 10 of PCa Gleason 6; 6 of PCa Gleason 7 and 3 of PCa Gleason 9) and 15 patients with biopsy-confirmed benign prostate hyperplasia. Total PSA concentrations of the BPH and PCa groups were matched so that the great majority (90%) had total PSA concentrations between 4 and 10 ng mL⁻¹. Serum pools of BPH sera and each group of PCa sera were prepared and used for glycoprotein profile analysis by Cramoll 1,4 - binding assays.

2.2 Reagents

The 96-well microplates were from Greiner Bio One (Kremsmunster, Áustria). Total PSA monoclonal antibody (clone 5A11E9), recombinant human PSA, bovine serum albumin (BSA) peroxidase from horseradish type VI (HRP), *o*-phenylenediamine dihydrochloride (OPD), hydrogen peroxide (H₂O₂) and methyl α -D-mannopyranoside were obtained from Sigma Aldrich (St. Louis, MO). Glutaraldehyde (25 % v/v) was obtained from Vetec (São Paulo, BR). Cramoll 1,4 was obtained in Laboratório de Bioquímica de Proteínas (Departamento de Bioquímica, UFPE), according to protocol established by Correia and Coelho (1995) [7].

2.3 HRP-Cramoll 1,4 conjugation

The conjugation of Cramoll 1,4 to peroxidase was performed as described in Beltrão et al. (1998) [8]. Cramoll 1,4 (1 mg mL^{-1}) was dialyzed against 0.1 M sodium phosphate buffer, pH 6.8. After, 0.1 M methyl- α -D-mannoside was added to Cramoll 1,4 dialyzed solution and maintained with smooth stirring for 15 min. Subsequently, HRP (3 mg mL^{-1}) was added and the solution was gently stirred for dropwise addition of 50 μL of 1% (v/v) glutaraldehyde aqueous solution. The reaction mixture was kept at rest for 2h at room temperature and then dialyzed at 4°C for 16h in 0.01 mmol L^{-1} sodium phosphate buffer, pH 6.8, containing 150 mmol L^{-1} NaCl. HRP - Cramoll 1,4 was distributed in aliquots of 50 μL and stored at -20°C .

2.4 Enzyme linked lectin-binding assay (ELLA)

Pool serum samples were diluted 1:10 in PBS (10 mM sodium phosphate, pH 7.2, containing 150 mmol L^{-1} NaCl) and aliquots of 50 μL of each pool were pipetted in triplicate onto 96-well microplates, which were incubated at 4°C , overnight. The wells were washed three times with 200 μL of PBS containing 0.1% Tween 20. Unspecific binding sites were blocked with the addition of 250 μL of 1% BSA in PBS, and the microplate was incubated at 4°C for 2h. The wells were washed as described before. A sample of 100 μL of HRP - Cramoll 1,4 ($50 \mu\text{g mL}^{-1}$) was added in wells and incubated at 4°C , 2h. After, the wells were washed four times with 200 μL of PBS containing 0.1% Tween 20. OPD was used as a substrate for peroxidase. Briefly, 50 μL of 10 mmol L^{-1} citrate phosphate pH 6.0 containing 0.08% (w/v) of OPD and H_2O_2 were added and the colorimetric reaction allowed to proceed for 30 min. The reaction was stopped with 100 μL of 20 mmol L^{-1} citric acid solution. The absorbance of the wells was measured at 490 nm with an automatic microplate reader (Epoch – Biotek, USA). The same procedures were also performed with fetuin solution (1 mg mL^{-1}) in the place of pool sera, in order to analyse the conjugation efficiency and to improve the optimal conditions.

2.5 Lectin-antibody sandwich immunoassay

Microplates were coated with 50 μL of the PSA monoclonal antibody at a concentration of 1 $\mu\text{g mL}^{-1}$ in triplicate and incubated at 4°C overnight. The wells were washed three times with 200 μL of PBS containing 0.1% Tween 20. After, 200 μL of PBS with 1% BSA was used for blocking at 4°C for 2 h. Next, microplates were washed as described before. To prevent binding of the lectin to the carbohydrate residues on the PSA antibody coating the wells, the surface was treated with 150 μL of sodium periodate buffer prepared in 150 mmol L^{-1} NaCl and 100 mmol L^{-1} sodium acetate (pH 5.5) at 4°C for 1h. Following treatment, the plates were washed as before and 50 μL of pool serum 1:10 PBS or PSA (1 $\mu\text{g mL}^{-1}$) was added to each well and incubated at 4°C for 2h. Plates were washed as before and 100 μL of HRP - Cramoll 1,4 solution (50 $\mu\text{g mL}^{-1}$) was added to each well plate for incubation at 4°C for 2h. After wash steps, 50 μL of 10 mM citrate phosphate pH 6.0 containing 0.08% (w/v) of OPD and H_2O_2 were added and the colorimetric reaction allowed to proceed for 30 min. Finally, 100 μL of 20 mmol L^{-1} citric acid solution was added to stop the reaction and the absorbance of the wells was measured at 490 nm with an automatic microplate reader (Epoch – Biotek, USA).

2.6 Synthesis and characterization of CdTe QDs

Aqueous colloidal dispersion of red CdTe/MSA core/shell QDs were synthesized according to previously reported method [9]. Briefly, QDs were prepared by addition of Te^{2-} (obtained from metallic tellurium – Sigma Aldrich) in a 0.5 mmol L^{-1} CdCl_2 or $\text{Cd}(\text{ClO}_4)_2$ (Sigma Aldrich) solution with pH > 10 in the presence of 3-mercaptop succinic acid (MSA – Sigma Aldrich) as stabilizing agent. It was used a 5:1:6 molar ratio of Cd/Te/MSA in ultrapure water solution for red emission QDs. Optical characterization was performed by electronic absorption and emission spectroscopies using a UV-Vis 1800 (Shimadzu) spectrophotometer and a LS 55 spectrometer (PerkinElmer, at $\lambda_{\text{exc}} = 405 \text{ nm}$), respectively.

2.7 QD-Cramoll 1, 4 conjugation

Obtained CdTe QDs were conjugated with Cramoll 1,4 by adsorption at pH 7.0 according to Cunha et al (2018) [10]. Firstly, the pH of 1 mL of CdTe QDs ($\sim 7 \mu\text{M}$) was adjusted to 7.0 using MSA at 4.9% (w/v). Then, 90 μL of Cramoll 1,4 (3.1 mg mL^{-1}) was added to 910 μL of QDs adjusted for a final concentration of $280 \mu\text{g mL}^{-1}$. The system was maintained by slow stirring at room temperature for 2h and stored at 4°C . After seven days, the conjugate was washed with ultrapure water using Amicon ultracentrifugal filters (cut-off 30 kDa; Sartorius, Germany) in order to remove residues from the QDs synthesis in four centrifugation cycles ($908 \times g$ for 6 min), at 4°C . After the last cycle, the filtrated conjugate was resuspended in ultrapure water.

2.8 Labeling Candida albicans cells with QD-Cramoll 1,4

C. albicans yeast cell wall contains carbohydrates such as β -glucans, chitin, and mannoproteins [11]. Thus, *C. albicans* was used to confirm the conjugation efficiency of QD-Cramoll 1,4 and maintenance of the carbohydrate recognition ability of the lectin, since Cramoll 1,4 recognize specifically to α -D-mannose and α -D-glucose residues. Cells from *C. albicans* (ATCC 10231) were cultivated according to Tenório et al (2015) [11], in Sabouraud dextrose broth for 24h at 37°C under constant shaking at 75 rpm. The broth containing yeast cells was centrifuged ($1680 \times g$ for 2 min; MiniSpin – Eppendorf) and resuspended in $150 \text{ mmol L}^{-1} \text{ NaCl}$. Cell suspension was incubated with 100 μL of conjugate at a ratio of 1:1 for 1h at room temperature under slow stirring. Next, the sample was centrifuged at $1200 \times g$ for 30 s to remove conjugates that did not bind to cells, and resuspended in 100 μL of $150 \text{ mmol L}^{-1} \text{ NaCl}$. To confirm the specificity of conjugate, an inhibition assay was performed incubating the conjugate with methyl- α -D-mannopyranoside (400 mmol L^{-1}) for 30 min before incubation with the cells. Cell labeling was evaluated by fluorescence microscopy (DMI 4000B, Leica) in order to obtain information about spatial localization of the target molecules.

2.9 Fluorescent microplate assay with mannan coating

This method was employed to evaluate the conjugation efficiency of QDs with mannose-specific lectins based on the presence or absence of sample fluorescence signals [12]. Then, 50 μL of PBS containing 200 $\mu\text{g mL}^{-1}$ of mannan (mannan from *Saccharomyces cerevisiae*, Sigma) was placed in a polystyrene microplate (black 96-well OptiplatF HB microplate – PerkinElmer) in triplicate and incubated at 37°C in an incubator (water bath, humid chamber), for 3h. Mannan is a plant/fungal linear polysaccharide of mannose and is useful to confirm the conjugation efficiency and the maintenance of the lectin binding sites to recognize mannose residues. After, the plate was washed three times with 100 μL of 10 mmol L^{-1} PBS.

Plates were incubated with QD-Cramoll 1,4 at conjugate 4°C for 2h in order to evaluate the lectin-mannan recognition and the specificity of the fluorescent signal. The plates were washed as described before, and the fluorescence measurements were performed using a WALLAC 1420 Plate Reader with the software Victor₂ (PerkinElmer). The excitation band pass filter was the F405 and the emission band pass filter was the F595. The well containing mannan without QD-Cramoll 1,4 was used as control. The signal intensity was obtained through the Relative Fluorescence Intensity (RF), calculated by the equation: $\text{RF} = [(\text{Conjugate FL} - \text{control FL}) / \text{control FL}] \times 100 \%$. FL conjugate is the average fluorescence intensity of the bioconjugates. FL control is the average control detected signal.

2.10 Fluorescent microplate assay based on QD-Cramoll 1,4

Fetuin (1 mg mL^{-1}) or PSA (1 $\mu\text{g mL}^{-1}$) solutions were prepared in PBS and aliquots of 50 μL were pipetted in triplicate onto black 96-well OptiplatF HB microplate, which was incubated at 4°C, overnight. The wells were washed three times with 100 μL of PBS. Unspecific binding sites were blocked with the addition of 100 μL of 1% (w/v) BSA in PBS, and the microplate was incubated at 4°C for 2h. The wells were washed as described before. Then 100 μL of QD-Cramoll 1,4 (280 $\mu\text{g mL}^{-1}$) was added in wells and incubated at 4°C for 2h. After, the wells were washed as described before and the fluorescence measurements were performed using a WALLAC 1420 Plate Reader with the software Victor₂ (PerkinElmer). The excitation

band pass filter was the F405 and the emission band pass filter was the F595. The fetuin or PSA without bioconjugate was used as control. The signal intensity was obtained through the RF, calculated as described in 2.8.

2.11 Lectin inhibition assay

In order to prove that labeling results were due to the lectin specificity, lectin binding inhibition assays were accomplished by incubating HRP- and QD- Cramoll 1,4 with 400 mmol L⁻¹ methyl α -D-mannopyranoside at room temperature for 30 min prior to their incubation with fetuin or mannan in the ELLA, lectin-antibody sandwich immunoassay and fluorescent microplate assays. Inhibited conjugates were also used as control in the analysis of conjugation efficiency.

2.12 Statistical analysis

Wells containing all the reagents but no serum (blank wells) were prepared for each experiment. The data obtained for the binding between Cramoll 1,4 and glycoproteins from PCa and BPH were compared using an unpaired student t-test and ANOVA (single factor).

3. Results

3.1 Establishment of ELLA

Tumorigenesis, malignant transformation and cancer progression modify the activity of enzyme glycosylation altering the expression and structure of glycans in the glycoproteins free and bound to the cell membrane. The identification of changes in glycoproteins has contributed to distinguish cancerous from non cancerous conditions. The carbohydrate-binding specificity of lectins allows the recognition of distinct glycoprotein patterns expressed in human samples. Several lectin-based techniques have detected glycosylation changes that provide to discriminate among different grades of PCa and BPH with more reliability and security than PSA levels. In this work, the proposed ELLA was used to detect the glycoprotein change profiles

present using a new set of serum samples from patients with BPH and with PCa Gleason scores 5, 6, 7 and 9, all prepared at a 1:10 dilution; total PSA started concentration at 4 – 10 ng mL⁻¹.

A range of conditions were explored: temperature, washes, incubation times and conjugated concentration (data not shown). The final ELLA was based on a colorimetric detection system. Standardization and optimal conditions were obtained using fetuin, a glycoprotein with a high content of mannose residues to which Cramoll 1,4 has showed elevate affinity. The assay showed detection signal in the presence of fetuin, with Cramoll 1,4 concentrations in the range of 0.5 – 200 µg mL⁻¹ (Fig. 1). Pre-incubation of the lectin with methyl α-D-mannopyranoside resulted in a decrease in Cramoll 1,4 binding, consistent with competitive inhibition of lectin binding reported in literature [6]. The colorimetric signal is specific to Cramoll 1,4 – carbohydrate binding.

--- Please insert Fig. 1 ---

The assay was tested with pools of serum samples from PCa (Gleason 5, 6, 7 e 9) and BPH at a range concentrations of Cramoll 1,4 (0,5 – 100 µg mL⁻¹), as showed in figure 2. An increasing absorbance is observed when the lectin concentration enhanced (Fig. 2A). The incubation of the samples with HRP-Cramoll 1,4 resulted in a Cramoll 1,4 binding to serum glycoproteins, showing an increasing signal for PCa groups correlated with malignancy and cancer degree, as observed for Cramoll 1,4 at concentration of 50 µg mL⁻¹ (Fig. 2B) and at concentration of 100 µg mL⁻¹ (Fig. 2C). Colorimetric signals were positively correlated with Gleason degree, suggesting the potential of this ELLA to predict aggressive PCa. The statistical analysis using ANOVA showed that the responses are not significantly different among themselves, and paired t-test did not show significant difference among the BPH and PCa.

--- Please insert Fig. 2 ---

The assay also gave a linear detection signal for BPH pools serum diluted serially (1:5; 1:10 and 1:25) (Fig. 3A). The reproducibility of the assay was tested

using the same pool serum on two separate days and the inter-assay variability was found to be less than 10% (Fig. 3B).

--- Please insert Fig. 3 ---

Linearity was also observed for PCa pools serum diluted serially (1:5; 1:10 and 1:25) (Fig. 4A). The reproducibility of the assay was tested using the same pool serum on two separate days and the inter-assay variability was found to be less than 10% (Fig. 4B).

--- Please insert Fig. 4 ---

3.2 PSA glycosylation in serum samples from PCa and BPH by lectin-antibody sandwich immunoassay

The detection of glycosylation changes in PSA and other glycoproteins are promise for PCa diagnosis. The lectin-antibody sandwich immunoassay was also developed to detect the changes in PSA glycosylation using serum samples, all prepared at a 1:5 dilution; initial total PSA concentration of 4 – 10 ng mL⁻¹. A range of conditions were explored: temperature, washes, incubation times and conjugated concentration (data not shown). The final immunoassay was based on a colorimetric detection system. A significant detection signal in the presence of PSA was observed when compared without PSA or inhibited conjugate (Fig. 5). The assay was tested with pool serum samples from BPH and PCa, showing a greater absorbance signal for BPH, when compared with absorbance for PCa pool. It suggests that PSA can express glycosylation change distints for BPH and PCa. Studies report that PSA glycans isolated from tumoral cell and PCa sera showed tri- and tetraantennary structures non-detected in BPH [13], as well as a decrease in PSA core fucosylation were also related for PCa [14].

--- Please insert Fig. 5 ---

3.3 QD optical characterization

QDs – CdTe synthesized were characterized by emission and absorption spectra at excitation of 405 nm. It was observed a maximum emission at 607 nm with a full width at a half maximum (FWHM) of about 50 nm (Fig. 6A), and an absorption spectrum at 320 nm (Fig. 6B).

--- Please insert Fig. 6 ---

3.4 Evaluating the QD-Cramoll 1,4 conjugation

An intense fluorescence was observed on *C. albicans* cell surface (Fig. 7A) after incubation with QD-Cramoll 1,4. Non fluorescence was observed when *C. albicans* was incubated with inhibited conjugate (Fig. 7B). These events show that the inhibitor (methyl α -D-mannopyranoside) occupies Cramoll 1,4 binding sites, preventing subsequent binding of QD-Cramoll 1,4 to the fungal cell. This observation also shows a specific QD-Cramoll 1,4 labeling to *C. albicans* cell wall. Carbohydrates, mainly consisting of glucose, N-acetylglucosamine and mannose residues represent approximately 80 to 90% of fungal cell wall composition [15]. This high content of glucose and mannose represents an expressive number of suitable sites available for QD-Cramoll 1,4 binding. Fluorescence images also show that QDs-Cramoll 1,4 were successfully bound to most of the cells.

--- Please insert Fig. 7 ---

3.5 Confirmation of QD-Cramoll 1,4 conjugation by Fluorescent Microplate Assay with mannan coating

The fluorescent microplate assay with mannan coating results are presented as the average of the fluorescent signal of triplicate wells for controls and conjugates. Mannan was used as control, and the conjugates of QD-Cramoll 1,4 non-inhibited and inhibited with methyl α -D-mannopyranoside were tested (Fig. 8). The data

analysis was carried out according to Carvalho et al. [12], which affirm that the bioconjugation process is efficient when the bioconjugates show a relative fluorescence (RF) higher than 100%. RF results for QD-Cramoll 1,4 was 561.84%. The pre-incubation of conjugate with methyl α -D-mannopyranoside reduced the RF to 30.71%, showing that the conjugation was efficient, since the pre-incubation with inhibitor occupied the binding sites of Cramoll 1,4, preventing the binding to mannan.

--- Please insert Fig. 8 ---

3.6 Fluorescent Microplate Assay for fetuin and PSA detection

A fluorescent microplate assay has been established for detection and discrimination of PCa and BPH in serum samples. In order to standardize and improve the optimal conditions of the assay, some preliminary experiments have been performed using the glycoproteins fetuin and PSA. The fluorescence microplate assay results are presented as the average of the fluorescence signal of triplicate wells for controls and conjugates. The results indicate the binding of QD-Cramoll 1,4 to fetuin and PSA; the bioconjugation was efficient after ten days and improved over time [9]. For example, the average signal of fluorescence intensities obtained for fetuin/ QD-Cramoll 1,4 system after ten days of bioconjugation was 2049, increasing to 9577 after forty days; and the average signal obtained for PSA/ QD-Cramoll 1,4 was of 60455 after sixty days of bioconjugation (Table 1).

4. Discussion

In this study, an ELLA, a lectin-antibody sandwich immunoassay and a fluorescent microplate assay have been developed based on lectin Cramoll 1,4, which was used to assess the glycosylation of serum glycoproteins and PSA. Pools of BPH and PCa Gleason 5; 6; 7 and 9 sera were prepared with patient sera with PSA in the “greyzone”; range of 4 - 10 ng mL⁻¹ was used in the assay. Previous results showed an increase in the binding of Cramoll 1,4 in glycoprotein sera from PCa compared with BPH, correlated with the Gleason degree [16].

Other studies have shown glycosylation changes in PCa glycoproteins compared with BPH, using tissues, urine, semen and sera from PCa and BPH patients. The glycosylation of serum glycoproteins has also been studied in other lectin based approaches. A differential Cramoll 1,4 binding to glycans located on tissue surfaces from normal, hyperplastic and malignant human prostate was observed [4]. The analysis of lectin ConA-binding serum glycoproteins by mass spectrometry revealed differential glycan expressions in PCa compared with BPH, suggesting an increase in the binding of lectin ConA in PCa [17]. An elevated fucosylation was also observed in glycoproteins in PCa sera [18].

Table 1. Evaluation of the fluorescence intensity signals of the conjugates after thirty days and sixty days of conjugation.

Assay	Signal average after 30 days from the conjugation	Signal average after 60 days from the conjugation
Fetuin*	275	285
Fetuin/ QD - Cramoll 1,4	9577	27315
PSA*	297	285
PSA/ QD - Cramoll 1,4	8043	60455

*Negative controls.

Saldiva et al. [19] also found high expression of biantennary fucosylated and connected to $\alpha 2$ -3 sialic acid in PCa sera compared to BPH; a significant reduction of triantennary trigalactosylated glycans and tetrasialylated glycans with an outer arm fucosylated, and increased levels of tetra-antennary tetrasialylated glycans in PCa score 7 compared to PCa score 5.

Patients with PCa Gleason scores 7 or higher are at increased risk of metastasis and recurrence after therapy [20], becoming attractive methods premature predictors for high-grade of Gleason. Studies report quantitative alteration

increase in serum glycoprotein levels from PCa patients that tend to rise correlated with the Gleason degree, helping to predict aggressiveness and prognosis of cancer. Some glycoproteins including periostin, cartilage oligomeric matrix protein and membrane primary amine oxidase were identified with quantitative changes associated to aggressive PCa in tissues [21]. Increased level of glycans in haptoglobin serum was also reported in PCa compared with BPH and healthy individuals [22]. Analyses by mass spectrometry also demonstrated an increase of total fucosylated di-, tri- and tetraantennary glycans of haptoglobin in the sera of PCa patients correlated with Gleason score [23]. Antibodies anti-oligomannose including IgG^{Man9} and IgM^{Man9} are glycoprotein floating in human plasma related to glyco-epitope cryptics high-mannose expressed in tumoral tissues. These autoantibodies were widely present in the sera of men with BPH, as well as those with cancer, but significantly increase in the subjects with the largest volumes of high Gleason grade cancer [24].

It is clear at the start of this study that methodologies compatible with equipment available in the majority of biomedical science laboratories needed to be developed to enable studies of the glycosylation of glycoproteins with larger number of patient samples. This straight forward ELLA/ lectin-antibody sandwich immunoassay/ Fluorescent microplate assay systems is of sufficient sensitivity to allow the assessment of the glycosylation of serum glycoproteins. The systems use equipment and skills that are available in most laboratories.

Those data suggest changes in glycoprotein profiles related to each PCa degree that may detect and discriminate BPH using the proposed ELLA based in Cramoll 1,4. This lectin recognizes with high affinity mannose residues, frequently found in high-mannose type and complex or hybrid type N-glycans linked to proteins. These changes may be detected and in future studies cooperate in the characterization of glycosylation in diseases conditions, prognosis and therapies.

5. Conclusions

We have proposed new approaches based in lectin Cramoll 1,4 probing glycosylation changes in serum glycoproteins of patients with PCa compared with BPH which may provide the detection of PSA glycosylation changes in PCa and the

correlation with cancer degree. The overall results showed that the developed methods are efficient strategies to target glycidic residues, and to assist in the elucidation of malignant processes related to changes in carbohydrates using cells and serum samples.

Acknowledgements

The Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) is acknowledged for fellowships (LCBBC, AF and MTSC) and grants. The authors are also grateful to the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and the Fundação de Amparo à Ciência e Tecnologia do Estado de Pernambuco (FACEPE).

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Figure captions

Fig. 1. Absorbance signals of Cramoll 1,4 ($0.5 - 200 \mu\text{g mL}^{-1}$) binding to fetuin (1 mg mL^{-1}). *A*: All assays contain fetuin and HRP-Cramoll 1,4, except in II and IV (without fetuin). In I, HRP-Cramoll 1,4 was previously incubated with methyl α -D-mannopyranose for inhibition. Thus, inhibited conjugated showed a reduced signal in the presence of fetuin (I). A similar signal was also detected in the presence of PBS and BSA without fetuin (II). High response was observed in III from fetuin – HRP - Cramoll 1,4 non-inhibited binding. The curve IV resulted of the signals from the conjugated alone in the plate, which adheres to polystyrene. *B*: Illustration of colorimetric signal after OPD reaction.

Fig. 2. Absorbance signals of Cramoll 1,4 binding to pool serum samples diluted 1:10 in PBS (I – BPH; II – PCa 5; III – PCa 6; IV – PCa 7; V – PCa 9). *A*: An increasing absorbance is observed when the lectin concentration enhanced ($10; 25; 50$ and $100 \mu\text{g mL}^{-1}$). *B*: Absorbance for Cramoll 1,4 at concentration of $50 \mu\text{g mL}^{-1}$. *C*: Absorbance for Cramoll 1,4 at concentration of $100 \mu\text{g mL}^{-1}$.

Fig. 3. Linearity and reproducibility of BPH pool serum. *A*: Linearity when BPH pool serum was serially diluted ($r = 0,974$; $y = 0,431 + 2,175 * x$). *B*: Linearity and reproducibility of the system were observed when the same sample was serially diluted and evaluated on two separate days.

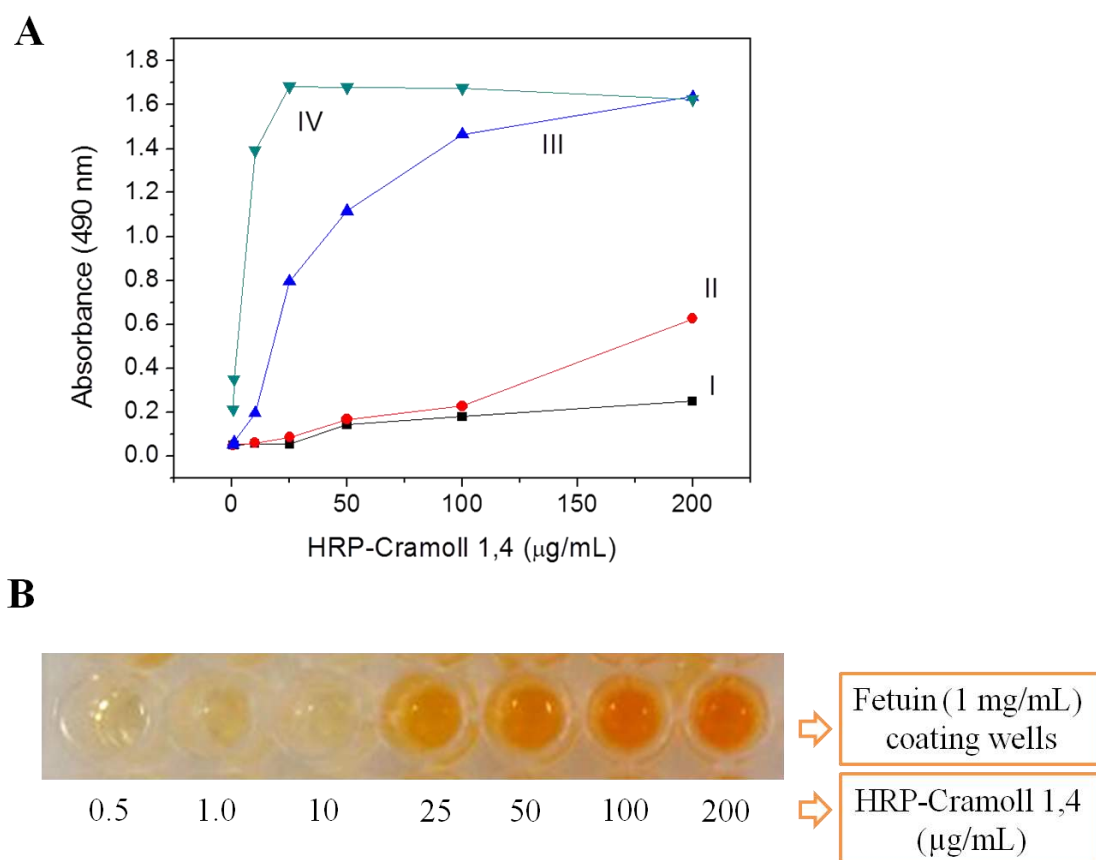
Fig. 4. Linearity and reproducibility of PCa pool serum. *A*: Linearity when PCa pool serum was serially diluted ($r = 0,964$; $y = 0,686 + 4,471 * x$). *B*: Linearity and reproducibility of the system were observed when the same sample was serially diluted and evaluated on two separate days.

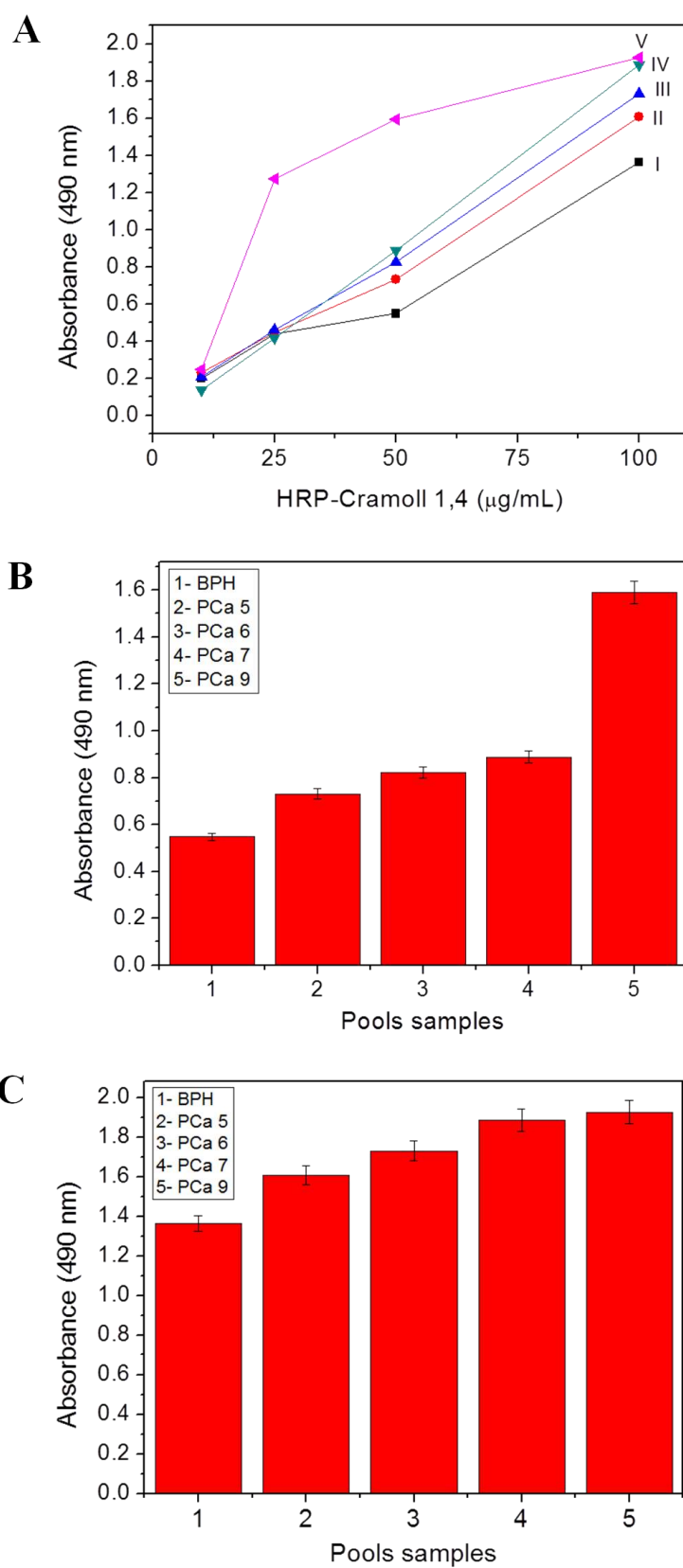
Fig. 5. Lectin-antibody sandwich immunoassay absorbance signals of Cramoll 1,4 binding to recombinant PSA (rPSA), BPH pool and PCa pool. The plot shows the significant response of Cramoll 1,4-PSA binding when compared to the signal detected in the absence of PSA or inhibited conjugate, as well as the PSA binding response of BPH and PCa pools. Absorbances show a significative response to BPH pool when compared to PSA and PCa pool.

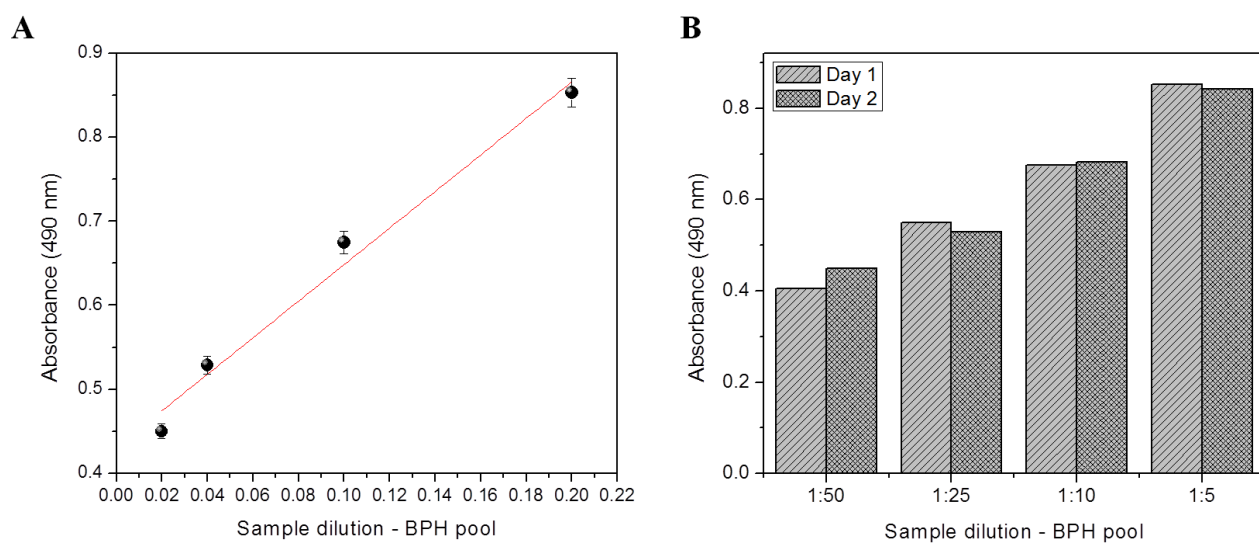
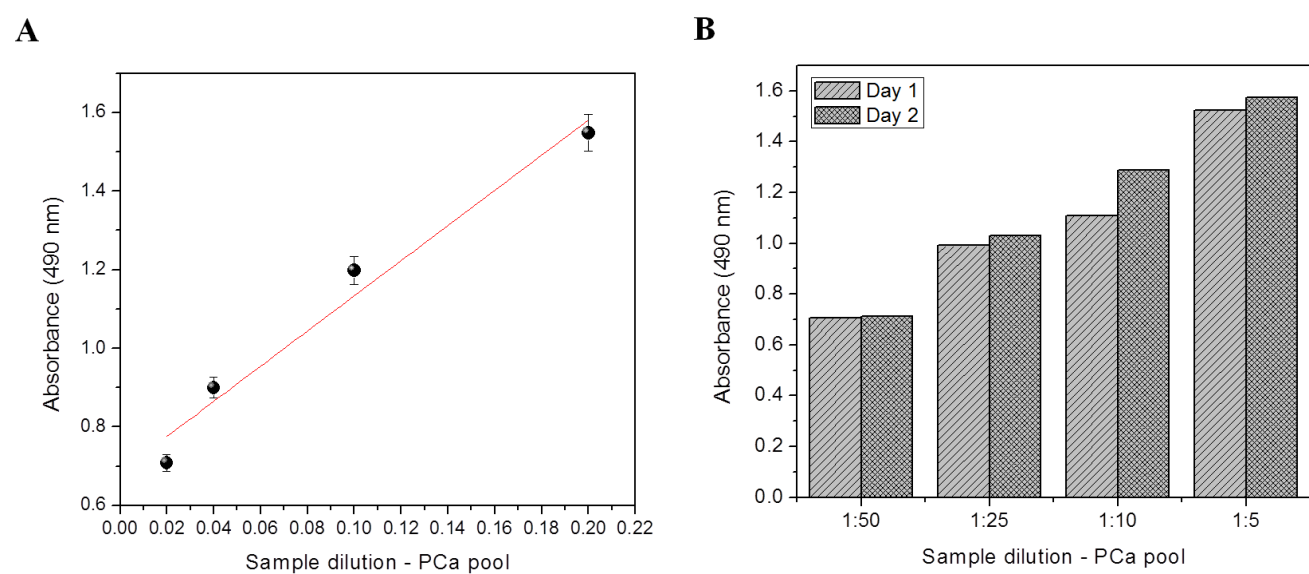
Fig. 6. Spectra of emission (A) and absorption (B) of synthesized QDs-CdTe.

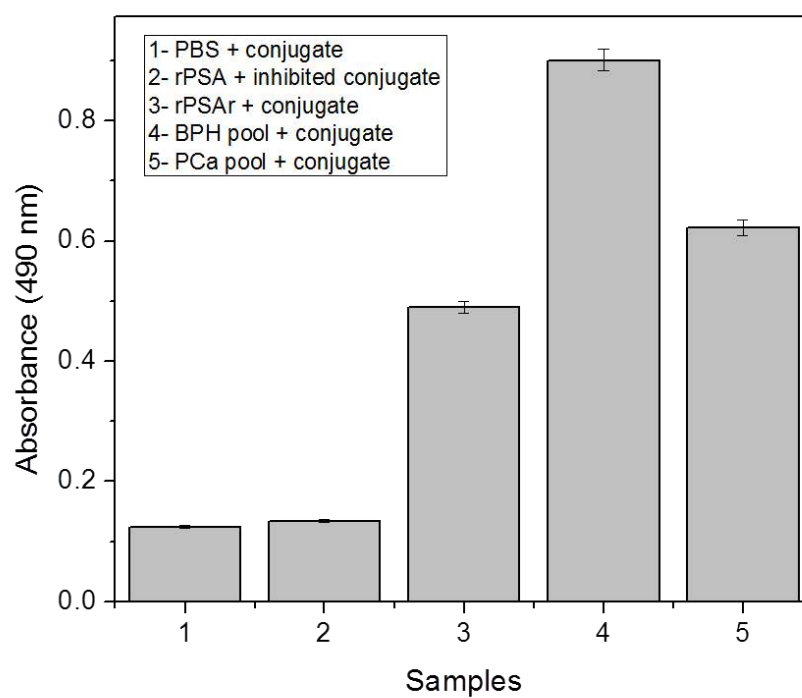
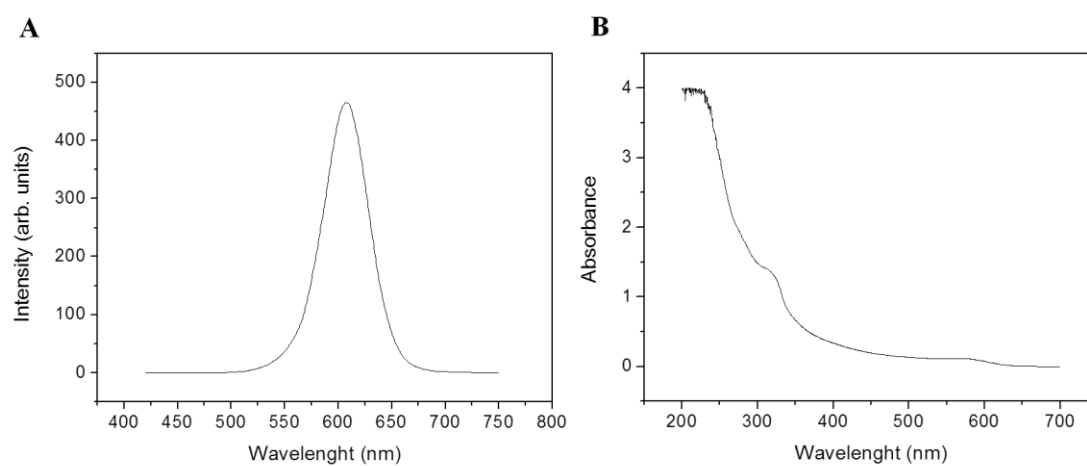
Fig. 7. Fluorescent images of *Candida albicans* cell suspensions labeled with QD-Cramoll 1,4 (A) and inhibited conjugate (B).

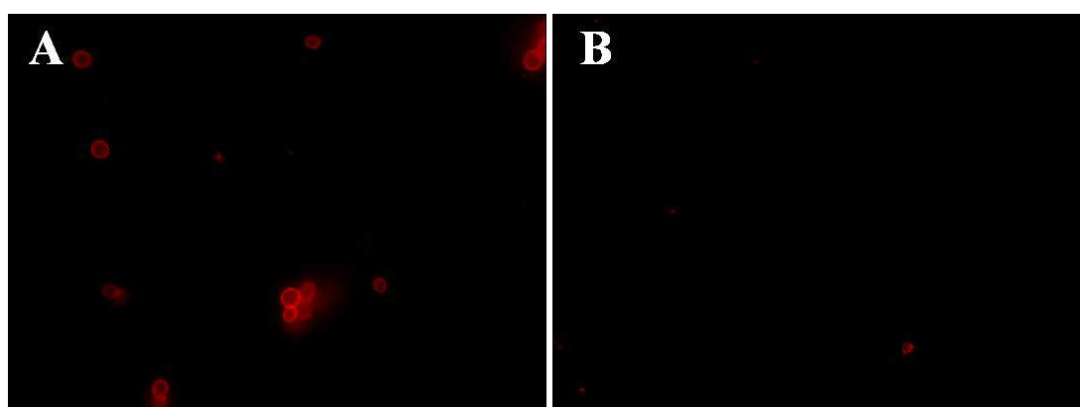
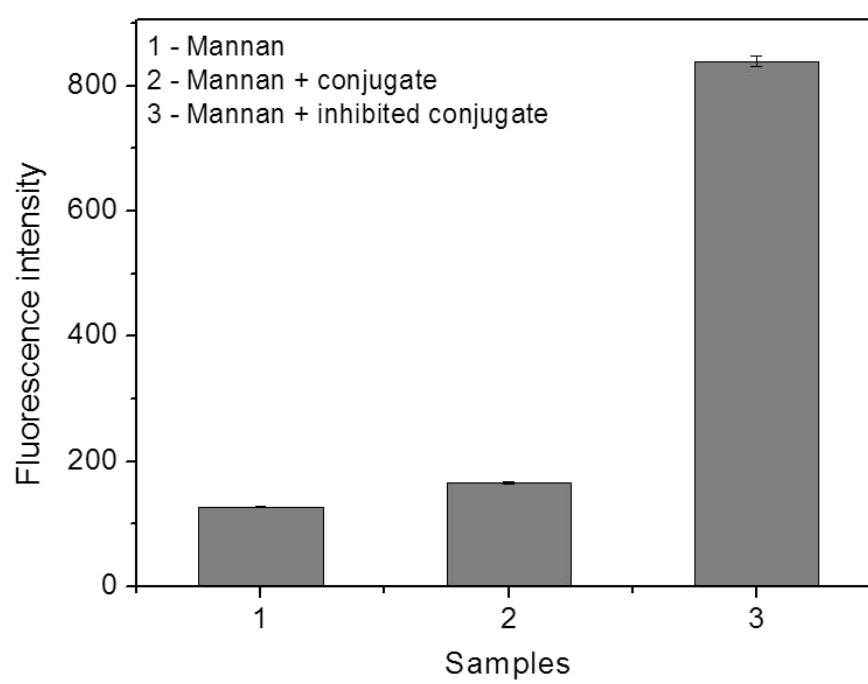
Fig. 8. Fluorescence microplate assay results showing the signal fluorescence intensities from conjugate – mannan binding.

**Fig. 1**

**Fig. 2**

**Fig. 3****Fig. 4**

**Fig. 5****Fig. 6**

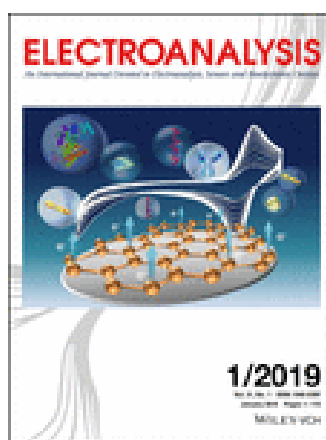
**Fig. 7****Fig. 8**

4.2 ARTIGO 2 – CRAMOLL-BASED SCREEN PRINTED ELECTRODE FOR VOLTAMMETRIC DETECTION OF FETUIN

Short Communication em preparação para ser submetido ao periódico

Electroanalysis

Fator de impacto: 2,851; Qualis CBI: B 1



Cramoll-based screen printed electrode for voltammetric detection of fetuin

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ABSTRACT

For convenient and fast measurement of serum glycoproteins, a portable lectin-based biosensor with screen-printed carbon electrode carbon nanotubes-modified was developed for electrochemical detection of fetuin. Firstly, the lectin Cramoll was immobilized onto the electrode surface, followed by glycine blocking and incubation with fetuin. An electron diffusion study of this surface was investigated submitting the electrode to different scan rates. The biosensor was also tested by cyclic voltammetry and square wave voltammetry to evaluate the relationship between the response current and fetuin concentration. A proportional increase in peak currents was observed according to the scan rates, suggesting that the reaction on the sensor surface is diffusional. The results also showed a detection linear range at 5 to 20 $\mu\text{g mL}^{-1}$ ($r = 0.985$, $p < 0.001$) for the determination of fetuin with the low detection limit of 3.14 $\mu\text{g mL}^{-1}$. The merits of the Cramoll-based biosensor are a promise for fetuin assays, as well as for detection of other glycoproteins for biomedical diagnostics.

Keywords: electron diffusion, fetuin, lectin-based biosensor.

Mammalian fetuins constitute a family of circulating glycoproteins with inhibitor of ectopic calcification capacity, produced by hepatocytes, and members of the cystatin superfamily of cysteine protease inhibitors [1]. In humans, lower serum fetuin levels lead to vascular calcification process and inflammation, increasing cardiovascular risk and mortality. When fetuin serum levels are elevated, it is correlated with severe disease in atherosclerosis patients [2]. Clinical assays of fetuin are required to diagnose and monitoring diseases as hepatocellular carcinoma, atherosclerosis, and disorders associated to calcium phosphate metabolism. Plasma fetuin measurements are normally made with commercial fetuin ELISA kits, but alterations in fetuin glycosylation status related to diseases may reflect differences in the specificity of ELISA methods [3].

As fetuin, many serum glycoproteins have been used as diagnostic tools because glycosylation changes are correlated with diseases. Thus, there are critical needs for developing glycotecnologies non expensive and simples methods for glycoproteins analysis. Lectin-based biosensors can be an attractive strategy for glycoproteins sensing, which are sensitive, rapid, simple and cost-effective. Lectins are proteins from natural sources that exhibit specific and selective hability to bind free or bound carbohydrate moieties. Among the characterized lectins, Cramoll 1,4 is a lectin isolated from seeds of the leguminous *Cratylia mollis*, specific for mannose and glucose residues, showing also high affinity to glycoproteins, as fetuin and ovoalbumin [4]. It has been used in the development of biosensors for probing microorganisms, carbohydrates, glycoproteins and glycosylation changes related to diseases [5, 6, 7].

For diverser types of biosensing applications, electrochemical biosensors have been eployed widely during the last decade, because can offer advantages such as diversity of electrodes, designs and materials, use of nanomaterials, and easy immobilization of proteins. Here in, we presented a new Cramoll-based electrochemical biosensor to evaluate fetuin detection and quantification through the interaction with the lectin. Taking advantage of their low-cost and operational convenience, multi-walled carbon nanotubes modified screen-printed carbon electrodes (MWNT-SPCEs, Metrohm, USA) were used on the transducer surface. In general, biosensing processes require multiple immobilization and incubation steps, and analyses of different concentrations of the analyte [7]. When traditional solid

electrodes are used, a single electrode is repeatedly used and requires washing and polishing steps before each experiment. SPCEs eliminate this inconvenience, since different electrodes can be used for each concentration of the analyte, and facilitate duplicate or triplicate analysis. In addition, SPCEs are portables, small size, and can be fabricated already modified with nanoparticles as MWNTs, which contribute in the signal amplification and immobilization of biomolecules [8]. MWNTs have been emerged as new sensing materials, since possess high surface area and can improve the sensitivity and response time of biosensors.

Cramoll 1,4 ($300 \mu\text{g mL}^{-1}$) was immobilized on the MWNTs-SPCEs by dripping 10 μL onto the surface and maintaining at room temperature during 60 min. After, 10 μL of the glycine solution (0.075 mol L^{-1}), prepared in ultrapure water, was pipette on the electrode surface for 60 min in order to blocking the nonspecific sites, as showed in the figure 1. The analytical response of the Cramoll-based biosensor was evaluated through the incubation with 10 μL of fetuin at different concentrations during 20 min at room temperature. Cyclic voltammetry (CV) and square wave voltammetry (SWV) were used to characterize electrode surface modification and to monitor the analytical response to fetuin by using $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ (0.005 mol L^{-1}) as redox probe, prepared in KCl solution (0.1 mol L^{-1}). The CV analysis was performed in a potential range of -0.2 at 0.5 V at scan rate of 0.05 mV s^{-1} , and SWV analysis was carried out in a potential range of 0.1 – 0.4 V at frequency of 10.0 Hz. The detection of lectin affinity was standardized by using the decrease of current (ΔI) in the CV and SWV measurements before and after fetuin incubation. CV was also used to perform the electron diffusion of the modified SPCE surface.

Figure 2 shows the electrochemical characterization of modification steps on the electrode surface. The analysis of voltammograms after the immobilization of Cramoll 1,4 (curve II) onto MWNT-SPCE exhibits a reduction in the current amplitude when compared as the naked electrode (curve I), due to the insulating nature of the biomolecule. After blocking of the free reactive sites with glycine (curve III), a slight reduction in the redox peaks amplitude was observed. SILVA et al. (2016) [7] developed a nanoelectrode of Cramoll 1,4 for fetuin detection, based on traditional glassy carbon electrode modified with the polymer poly-L-lysine, followed by addition of carboxylated multi-walled carbon nanotubes, prior the immobilization of Cramoll 1,4. More time and preparation steps were required for this biosensor development,

when compared with Cramoll 1,4-based SPCE, which is already modified with MWNT during its fabrication.

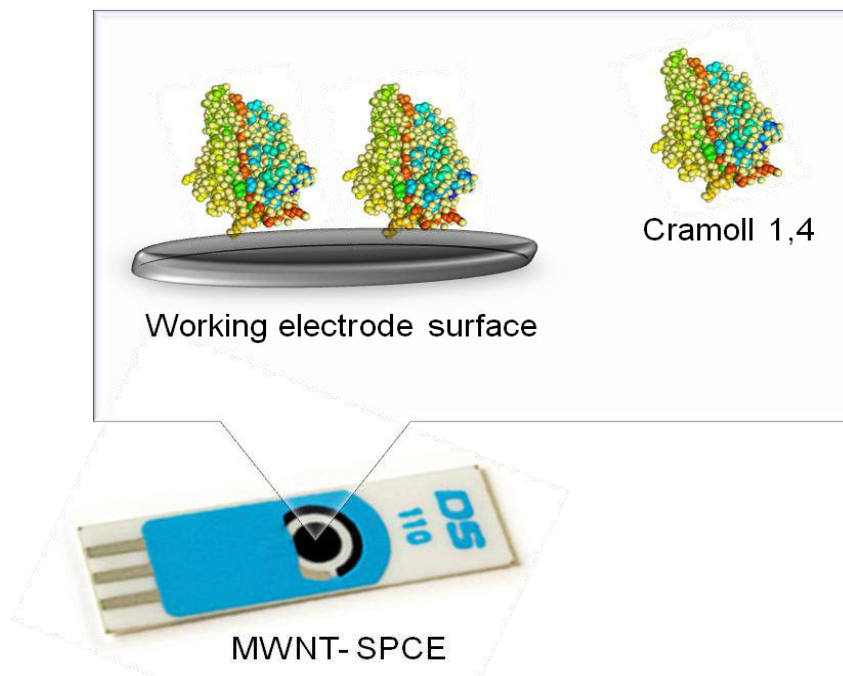


Figure 1. Disposable MWNT – SPCE modified with Cramoll 1,4 for fetuin detection.

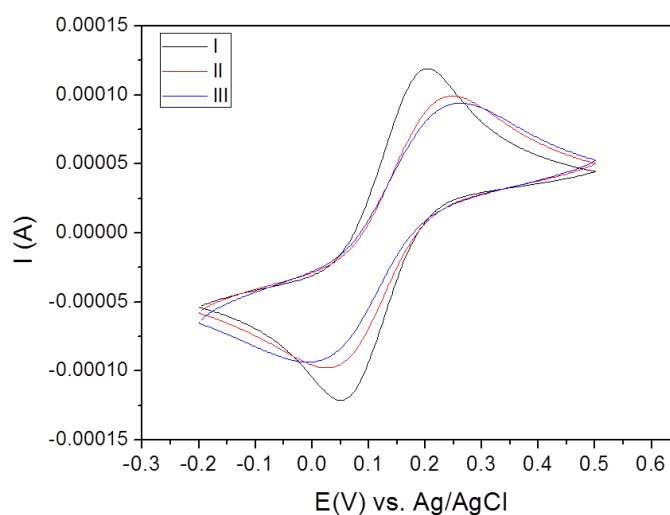


Figure 2. CVs of the stepwise modification of the Cramoll 1,4-based SPCE: (I) MWNT-SPCE; (II) Cramoll 1,4/ MWNT-SPCE; (III) glycine/ Cramoll 1,4/ MWNT-SPCE. Measurements performed in $K_3Fe(CN)_6/K_4Fe(CN)_6$ (0.005 mol L^{-1}) prepared in KCl solution (0.1 mol L^{-1}).

The electron diffusion study of the glycine/ Cramoll 1,4/ MWNT-SPCE surface was investigated after incubation with fetuin ($15 \mu\text{g mL}^{-1}$). The electrode was

submitted to different scan rates. As can observe in figure 3A, the voltammogramas exhibit a proportional increase in both cathodic and anodic peak currents, according to the scan rate (10; 15; 25; 35 mV s^{-1}). The cathodic peak currents were plotted versus square root of scan rates (figure 3B), resulting in a linear regression equation: $I \text{ (A)} = 7.51 \cdot v^{1/2} + 20.8$ ($r = 1.0$). These results suggest that the mass transport on the sensor interface were controlled by diffusion, in agreement with a reversible system [9].

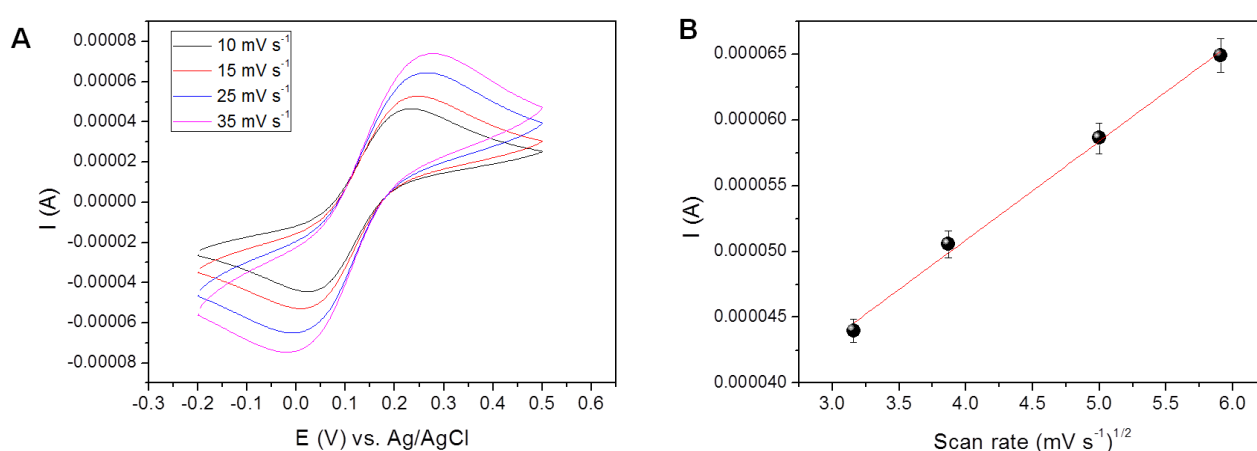


Figure 3. (A) Voltammetric curves of the fetuin/ glycine/ Cramoll 1,4/ MWNT-SPCE under different scan rates (10; 15; 25 and 35 mV s^{-1}). (B) Plots of cathodic peak currents vs. square roots of the scan rates. All the measurements were performed in $\text{K}_3\text{Fe}(\text{CN})_6 / \text{K}_4\text{Fe}(\text{CN})_6$ (0.005 mol L^{-1}) prepared in KCl solution (0.1 mol L^{-1}).

In this study, fetuin was chosen to evaluate the biological affinity of Cramoll 1,4. Cramoll 1,4-based SPCEs were incubated with 10 μL of fetuin solutions at different concentrations (5; 10; 15 and 20 $\mu\text{g mL}^{-1}$) during 20 min. The electrodes were submitted to CV and SWV measurements. Figure 4A and B shows the voltammogram profiles of CV and SWV, respectively, with the step modifications of MWNT-SPCE, and a reduction in the peak currents after incubation of fetuin (15 $\mu\text{g mL}^{-1}$), indicating the Cramoll 1,4 – fetuin interaction on electrode surface, and maintenance of carbohydrate-binding activity of the lectin.

The results were examined in terms of current variation (ΔI), being $\Delta I = I_{(\text{SPEC})} - I_{(\text{fetuin})}$, where $I_{(\text{SPEC})}$ is the current value of the bare electrode, and $I_{(\text{fetuin})}$ is the current value of the biosensor after exposure to fetuin. The analytical responses showed a linear enhance in the ΔI values with the increase of fetuin concentrations

(Figure 5). The curve revealed the linear regression equation: $\Delta I [\text{fetuin}] = 0.124 \cdot [\text{fetuin}] + 3.94$, ($r = 0,9855$, $n = 4$, $p < 0.001$). This result confirms the greater affinity of the Cramoll 1,4 in detection of the glycoproteins for characterization of serum glycoproteins (Lima et al., 1997; Oliveira et al., 2008). The limit of detection (LOD) was statically determinate as $\text{LOD} = 3.3\text{RSD}_{\text{blank}}/b$, where $\text{RSD}_{\text{blank}}$ is the RSD of the blank and b is the slope calculated of the analytical curve. The LOD obtained by this biosensor for fetuin was $3.14 \mu\text{g mL}^{-1}$. Others electrochemical lectin-based biosensors for fetuin detection have showed lower LOD [10, 11]. However, these systems require impedimetric techniques for measurements, which involve more complex analyses than SWV techniques.

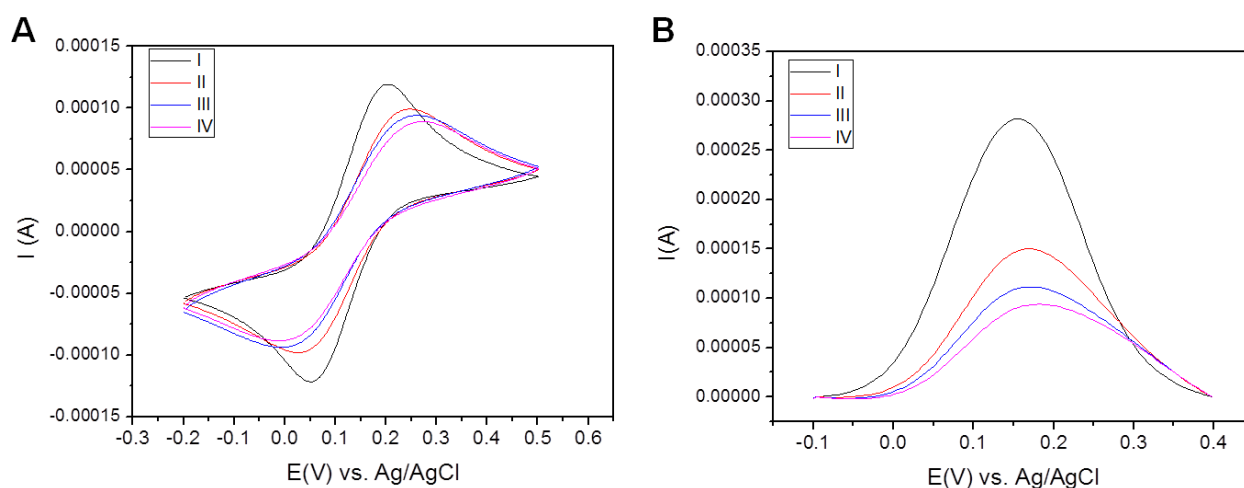


Figure 4. (A) CV curves of the (I) MWNT-SPCE; (II) Cramoll 1,4/ MWNT-SPCE; (III) glycine/ Cramoll 1,4/ MWNT-SPCE and (IV) fetuin/ glycine/ Cramoll 1,4/ MWNT-SPCE. (B) SWV curves of the I, II, III and IV. All the measurements were performed in $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ (0.005 mol L^{-1}) prepared in KCl solution (0.1 mol L^{-1}).

Cramoll 1,4-based SPCE showed to be sensitive to different concentrations of fetuin, showing also that the reaction on the electrode surface are controlled by diffusion. This system provides a potential tool for fetuin detection and quantification, can be tested with other glycoproteins binders to Cramoll 1,4. Due to the portable and disposable characteristics, the proposed biosensor will be used as a potential platform to perform onsite analysis.

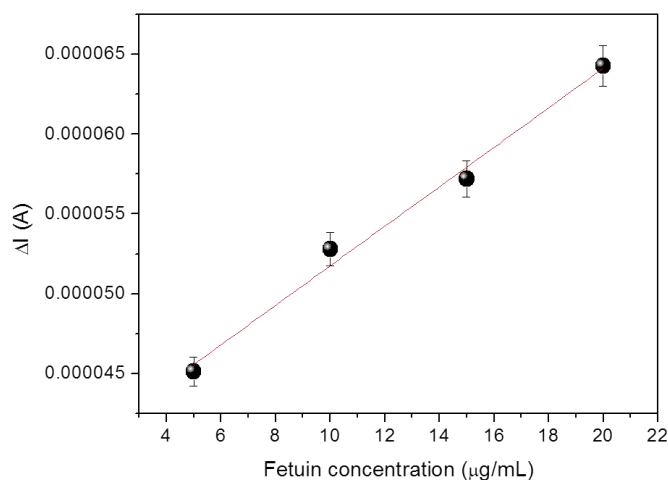


Figure 5. Analytical curve of the Cramoll 1,4-based SPCE for different fetuin concentrations (5 – 20 $\mu\text{g mL}^{-1}$) obtained by SWV measurements in $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ (0.005 mol L^{-1}) prepared in KCl solution (0.1 mol L^{-1}).

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5 CONCLUSÕES

- O biossensor eletroquímico baseado em Cramoll 1,4 desenvolvido em ECV modificado com PLL e NTC-COOH foi eficiente para quantificar a glicoproteína fetuína, alcançando baixo limite de detecção;
- O presente biossensor também foi útil para detectar e distinguir os perfis de glicosilação em amostras séricas oriundas de pacientes com HBP e CaP em diferentes escores de Gleason;
- As caracterizações da superfície eletródica por técnicas eletroquímicas, MEV e FT-IR mostraram a formação do filme de PLL e NTC-COOH na superfície do eletrodo;
- As técnicas de CV e SWV foram eficientes na análise das etapas de modificação do eletrodo e no estudo analítico para detecção das biomoléculas;
- O uso da técnica de eletrodeposição de PLL sobre ECV colaborou para manutenção e estabilidade do filme de PLL obtido;
- A associação de NTC-COOH ao filme de PLL proporcionou um aumento na área eletroativa, melhora da condutividade elétrica promovida pelos nanotubos de carbono e aumento da estabilidade da superfície;
- Os grupos carboxílicos presentes na estrutura dos NTC-COOH auxiliaram na imobilização da lectina Cramoll 1,4;
- As concentrações de PLL, NTC-COOH e Cramoll 1,4 foram otimizadas, bem como velocidade de varredura e tempo de imobilização;
- O biossensor proposto exibiu boa sensibilidade e reprodutibilidade, com resposta de corrente eletroquímica linear para fetuína ($r = 0.994$, $p < 0.001$). Também apresentou ótima estabilidade e reprodutibilidade para fetuína, sendo considerado um sistema estável, reprodutível e confiável para análise de glicoproteínas;
- O biossensor foi bastante seletivo para fetuína na presença dos interferentes: glicose, galactose, frutose, fucose e BSA;
- As respostas eletroquímicas obtidas para as amostras séricas normais, de HBP e CaP escores 6, 7 e 9 foram estatisticamente diferentes entre si, sendo evidenciado um aumento da resposta com o grau do tumor, mostrando a utilidade do biossensor baseado em Cramoll para detectar alterações de glicosilação associadas ao câncer de próstata.

- Em relação ao biossensor de Cramoll 1,4 desenvolvido em eletrodo impresso de carbono, foram necessárias menos etapas de modificação e lavagem da superfície quando comparado ao biossensor desenvolvido em ECV, uma vez que a tinta de carbono da superfície já contém nanotubos de carbono. As etapas de modificação com PLL e NTC-COOH foram dispensadas, e Cramoll 1,4 foi imobilizada diretamente na superfície do eletrodo impresso, sem nenhuma modificação prévia. Assim, o uso do eletrodo impresso simplificou o processo de preparação do biossensor de Cramoll 1,4;
- O estudo de velocidade de varredura na superfície do eletrodo impresso modificado mostrou que o transporte de massa na interface do eletrodo é controlado por difusão, caracterizando um sistema reversível;
- A detecção eletroquímica da interação de Cramoll 1,4 com fetuína na superfície do eletrodo impresso de carbono foi linear nas concentrações de 5 a 20 $\mu\text{g mL}^{-1}$;
- Cramoll 1,4 foi conjugada covalentemente à peroxidase;
- Estudos da conjugação feitos por ELLA, imunoensaio tipo sanduíche de anticorpo – lectina (ELISA) e respectivos ensaios de inibição de carboidratos com detecção colorimétrica em 490 nm indicaram que a peroxidase foi conjugada eficientemente à lectina Cramoll 1,4;
- As etapas e condições analíticas dos ensaios colorimétricos ELLA e ELISA foram definidas;
- O conjugado peroxidase-Cramoll 1,4 conseguiu identificar e diferenciar os perfis glicoproteicos em soros de pacientes com CaP e HBP através do reconhecimento e ligação a glicoproteínas circulantes nos soros, usando o ensaio ELLA;
- O ensaio ELLA mostrou linearidade e reprodutibilidade frente a diluições dos *pools* de soros de HBP e CaP;
- Os QDs de CdTe funcionalizados com AMS foram sintetizados na cor vermelha, e foram conjugados à Cramoll 1,4, mantendo a emissão praticamente inalterada;
- Os testes de confirmação da conjugação por marcação de *C. albicans*, ensaio fluorescente em microplaca e inibição com carboidratos indicaram que os QDs foram conjugados eficientemente com a lectina Cramoll 1,4.

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APÊNDICE A – ARTIGO PUBLICADO NA REVISTA ADVANCES IN RESEARCH

Electrochemical biosensing strategies to detect serum glycobiomarkers

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Electrochemical Biosensing Strategies to Detect Serum Glycobiomarkers

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Authors' contributions

This work was carried out in collaboration between all authors. Author PMSS managed the literature search, wrote the manuscript and developed the figures. Authors LCBBC and MTSC designed, supervised and managed the study performed. All authors read and approved the final manuscript.

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ABSTRACT

Changes in glycosylation profiles have emerged as indicators of diseases. Altered glycans and glycoproteins secreted by pathological tissues are found in human serum and are potential glycobiomarkers for early diagnostic and prognostic of diseases such as inflammation, infection and cancer. To obtain serum samples is a simple procedure and minimally invasive; thus the detection of glycobiomarkers in serum is attractive for clinical applications. Electrochemical biosensors are a friendly strategy for rapid, easy and highly sensitive measuring of glycans and glycoprotein biomarkers. The use of lectins as biorecognition elements in glycobiosensors has provided a specific detection and profiling of glycans linked to glycoproteins. Electrochemical glycobiosensors based on lectin interactions employed to characterize glycan profiles in serum glycoproteins constitute a promising tool in diagnostic and monitoring of diseases.

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1. INTRODUCTION

Glycosylation is a post-translational modification occurring in more than half of human proteome and glycans have important roles in physiological functions and diseases. Changes in glycosylation profiles are frequently observed on cell surfaces and serum glycoproteins in pathological processes, such as immune disorders, infectious diseases and cancer. Evaluations by chromatographic techniques, mass spectrometry, among other approaches in tissues, cells, biological fluids and serum samples reveal quantitative and structural alterations in glycans in diseases when compared with healthy samples [1,2]. Then, the correlation of altered glycans with diseases makes them glycan biomarkers of choice for clinical diagnosis and monitoring of prognosis [3].

The diagnostic of diseases and monitoring during treatment through detection of glycan biomarkers present in bloodstream are attractive in terms of low invasiveness, agility and facility to follow glycan modifications. Analytical techniques based on Enzyme Linked Immunosorbent Assay (ELISA) and other immunoassays are most used to detect and measure serological glycoproteins in clinical laboratories. In addition, the technology of biosensing has been applied successfully to identify and quantify glycan biomarkers with high specificity and selectivity, based on biorecognition events connected to a system of transduction [4]. Biosensors can be constructed using a variety of transducer types, including optical, piezoelectric and electrochemical, to generate the results. Electrochemical biosensors have been detached since they provide rapid diagnostic by a simple manipulation and low cost, overcoming some limitations showed in other technologies.

Lectins, a group of sugar-binding proteins have been largely employed for biorecognition of sugars in binding assays. Lectins recognize carbohydrates with specificity for monosaccharides or oligosaccharides, free or bound to a glycosylation site, and may distinguish the linkage type. The lectin-carbohydrate binding provides quantitative and qualitative information about expression of glycans in biological samples, being valuable tools to characterize and identify changes in secreted glycoproteins [5]. Techniques based on chromatography, electrophoresis, immunoassays

and biosensors use lectins as biorecognition agents. Lectin biosensors contain a sensing platform with immobilized lectins to detect glycan biomarkers in serum samples through lectin-glycan interaction with high specificity [6]. Various groups of research have developed lectin biosensors for cancer, infectious diseases and virus or bacterial glycan detections. Lectins of different sources and specificity groups are employed in biosensing due to their versatility and diversity.

Isoforms from a seed lectin of *Cratylia mollis*, denominated Cramoll, belongs to the specificity group of mannose/glucose such as Concanavalin A, Con A [7]. Cramoll biosensors have demonstrated potential to recognize profiles of glycoproteins of different dengue serotypes in human serum [8,9] and glycans of bacterial lipopolysaccharides [10], through electrochemical methods, representing a good alternative for detection of diseases. This review introduces some advances in the lectin biosensor area directed for detection of glycan biomarkers to help in the assays of early diagnostic and monitoring of diseases.

2. GLYCOSYLATION

Glycosylation is the most common post-translational modification of proteins and has fundamental importance in biological processes in eukaryotic organisms. It is predicted that nearly 80% of the human plasma proteins are glycosylated [11], besides of glycans that cover cell membranes. Glycosylation reactions involve the covalent attachment of glycan chains to specific amino acid moieties of proteins during and after translation of polypeptide chains in order to form glycoproteins. The glycosylation profile of a protein is determined by a wide group of enzymes known as glycosyltransferases and glycosidases, residents in the endoplasmic reticulum and Golgi apparatus, which catalyze the extension and transfer of glycan chains to glycosylation site of the protein [12]. They constitute specific groups to a cell type, tissue and organism, resulting in tissue- or cell-specific differences in glycosylation between sites of the same protein [13].

Glycans are linked to human proteins via two pathways, *N*- and *O*-glycosylation, with various branching points. *O*-glycosylation is characterized by the attachment of *O*-Linked

glycans through hydroxyl group of serine (Ser) or threonine (Thr) residues, starting with the addition of N-acetyl-galactosamine (GalNAc-O-Ser/Thr) transferred by an N-acetyl-galactosaminyltransferase in the Golgi apparatus [14]. After, specific transferases elongate different types of core structures, including mucin-type O-linked glycans (core 1, 2, 3, 4; as well as T, TF and Tn antigens), O-linked GlcNAc and O-linked Fuc (Fig. 1). In the N-glycosylation, N-linked glycans are attached to the amidic nitrogen of asparagine (Asn) residues within the Asn-X-Ser/Thr glycosylation site, being X different of the proline [15]. N-glycosylation starts in endoplasmic reticulum, through the synthesis of a N-glycan precursor (Glc3Man9GlcNAc2) from a dolichol phosphate to the Asn site of nascent polypeptide chain. This N-glycan precursor originates the pentasaccharide tri-mannosyl core (Man3GlcNAc2) that is prolonged

to generate three subtypes of mature N-glycans: high-mannose, complex and hybrid (Fig. 1). The tri-mannosyl core cleaved by glycosidases is transferred to Golgi apparatus; there other monosaccharides added by specific transferases prolong glycan chains generating a complex branching. The resulting structures originate the subtypes of complex N-glycans and hybrid N-glycans (Fig. 1) [15,16]. In addition, the subtype high-mannose N-glycans contains only mannose residues linked to the tri-mannosyl core [16]. Studies report the complex type N-glycans as the most abundant in human serum, while hybrid and high-mannose types are rare. The monosaccharide residues mannose (Man), galactose (Gal), fucose (Fuc), N-acethyl-galactosamine (GalNAc), N-acethyl-glucosamine (GlcNAc) and sialic acid (SA) or neuraminic acid (NeuNAc) compound the N- and O-glycan chains attached to human proteins [15,16].

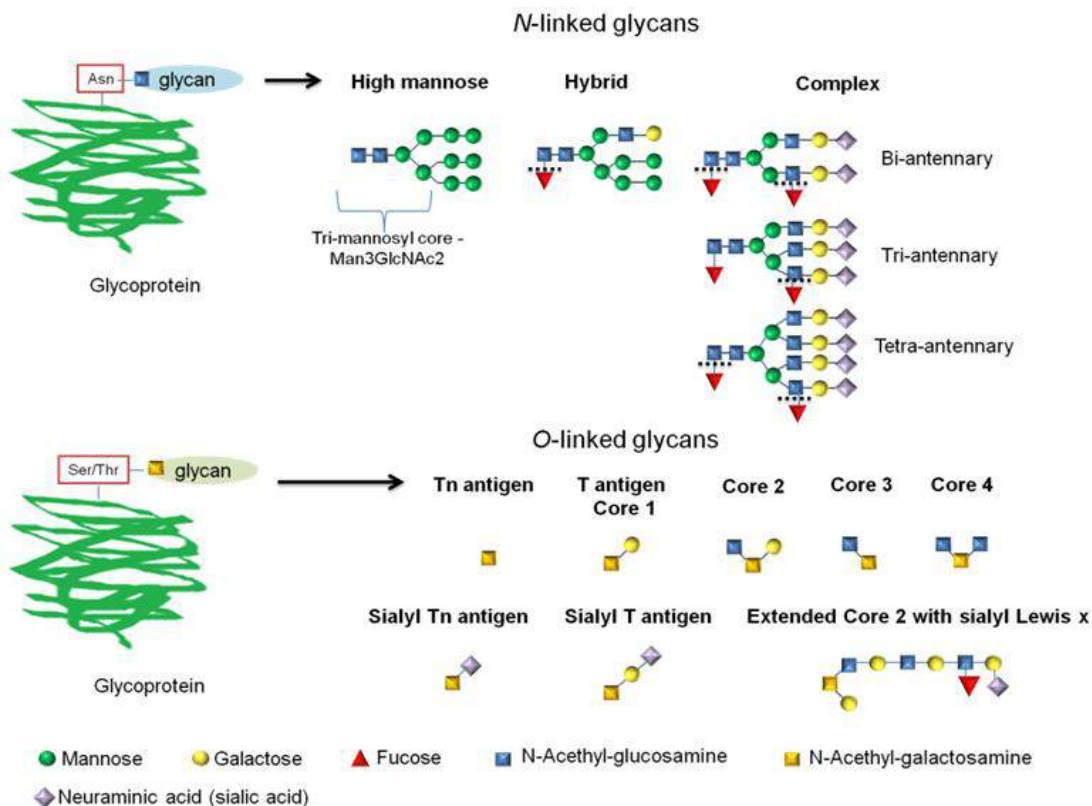


Fig. 1. Types of N- and O- linked glycans found in human serum

N-linked glycans bind to Asn residue in the nascent polypeptide chain into endoplasmic reticulum. The schematic representation shows the three types of N-linked glycans: high mannose, hybrid and complex type, with the common core structure present in all N-glycans. O-linked glycans bind through Ser or Thr residues present in the protein, starting with the addition of GalNAc. O-glycans can be extended with other monosaccharide residues and generate different core structures, and antigens highly expressed in cancer, such as Lewis antigen, T, Tn and sialyl Tn antigens

The heterogeneity of glycan structures controlled by enzymes contribute to the varied of functions that glycosylation carry out in cells and tissues. Glycosylation plays relevant influence in the synthesis, processing and function of proteins, including folding, stability of tertiary structure, protection against action of proteases, increased serum half-life of proteins and reduction of nonspecific protein-protein interactions [17-19]. Moreover, glycosylation mediates a regulation role in many biological processes involving the interactions cell-cell and cell-matrix, such as cell proliferation, cell recognition, adhesion, host-pathogen recognition, receptor binding, signaling, fertilization, inflammation and immune responses [19,20].

3. GLYCANS ARE POTENTIAL GLYCOBIOMARKERS IN DISEASES

The activities of glycosylation enzymes are dependent and modulated by cellular dynamics. Changes in cellular environment and physiology often due to diseases, affect enzyme activities, resulting in aberrant glycosylation of proteins [21]. Disturbed glycosylation has been linked to various pathologies such as infection, chronic inflammations, immunological and genetic disturbs, cancer and metastasis [12,21,22]. This involves changing of glycosylation sites, increase or decrease of the site numbers, modification in the chemical composition or type of glycan, extra branching of glycans and quantitative alteration of specific glycoprotein on cell surface and secreted in bloodstream [23,24].

There is strong evidence that these glycosylation changes in serum glycome are a good way to identify potential glycobiomarkers of diagnostic and progression of pathological states. Serum glycoproteins such as immunoglobulins, fetuin, haptoglobin, transferrin, alpha-fetoprotein, and other acute phase proteins showed glycosylation changes in response to inflammatory and immune diseases. Immunoglobulin G contains complex bi-antennary *N*-glycans that have a significant decrease in galactosylation for patients with inflammatory arthritis [25,26]. The glycan profiling in the serum of liver fibrosis patients and infected by hepatitis B virus and hepatitis C virus revealed a significant decrease of *N*-glycans bi-antennary and tri-antennary as glycobiomarkers to monitor the progress of fibrosis [27]. Multi-branched glycans highly sialylated were detected in elevated levels in serum of ulcerative colitis patients correlated with disease degree [28]. Alpha 1-6-linked arm

monogalactosylated and core fucosylated bi-antennary *N*-glycans were reduced in serum glycoproteins from subjects with diabetes mellitus type 2 and provide an alternative of serological glycobiomarker [29]. Glycan profiles of whole serum of patients with autoimmune pancreatitis showed elevated levels of a galactosyl and monogalactosyl bi-antennary glycans, being potential biomarkers of the disease [30].

Studies have evaluated the presence of aberrant glycoforms and quantitative levels of glycosylation in patients with cancer when compared with benign disease and healthy individuals to identify cancer glycobiomarkers. Aberrant glycosylation patterns in proteins from cancer cells, tissues and serum commonly involve altered sialylation and fucosylation, change in glycan size and branching, and the presence of Lewis antigens and truncated *O*-glycans [12]. Lewis antigens (Fig. 2) are frequently expressed on membrane glycoproteins of several cancer cells. Cholangiocarcinoma cells expressed high levels of sialyl Lewis a (Neu5Ac α 2-3Gal β 1-3(Fuc α 1-4)GlcNAc) and glycoprotein mucin 5AC was elevated in tissues and serum from cholangiocarcinoma patients [31,32]. Serological *N*-glycome in breast cancer patients showed increased levels of sialyl Lewis x antigen (Neu5Ac α 2-3Gal β 1-4(Fuc α 1-3)GlcNAc) and an increased sialylation [33]. An increase of β 1-6GlcNAc branches in *N*-glycans added by *N*-acetylglucosaminyltransferase V (GnT-V) is observed in breast and ovarian cancer [23,34]. Glycans of prostate-specific antigen isolated from tumoral cell and sera showed tri- and tetra-antennary structures of *N*-glycans [35]. In cancer cell-surface, *O*-linked glycans of mucin-type glycoproteins are often defective, resulting in the expression of truncated *O*-linked glycans denominated of T (Gal β 1-3-GalNAc α -O-Ser/Thr) and Tn (GalNAc α 1-O-Ser/Thr) antigens (Fig. 1), as observed in breast and colon cancer [23,36]. T/Tn antigen quantitative assays detected cancer without previous biopsy using highly purified T antigen for determination of anti-T immunoglobulins in serum samples [37].

4. SERUM GLYCOPROTEINS AS BIOMARKERS OF DISEASES

Clinical assays using body fluids such as serum, plasma, urine and saliva are minimally invasive, easily handled, with short time response, being preferred for detection and prognostic evaluation

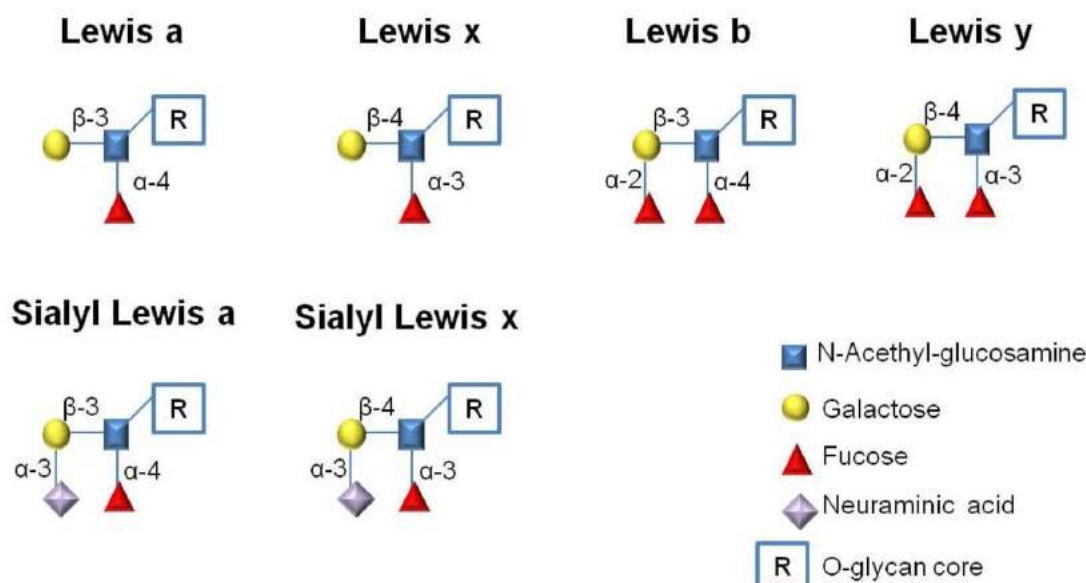


Fig. 2. Terminal Lewis and sialylated Lewis antigens expressed by human tumors
The Lewis antigens can be characterized by the presence of Gal β -3 linkage (Lewis a and Lewis b) or Gal β -4 linkage (Lewis x and Lewis y). The sialylation in the galactose residue can originate sialyl Lewis a and sialyl Lewis x antigens

of various diseases. Glycoproteins secreted in the bloodstream are a considerable part of serum biomarkers and they might show differential quantitative and qualitative level between healthy and pathological samples [3,38]. Some glycoproteins are well known serologic biomarkers for cancer diagnostic that may be detected by laboratory clinical assays, such as the carbohydrate antigens (CA) CA-125 for ovarian cancer, CA15-3 for breast cancer, carcinoembryonic antigen (CEA) for colorectal cancer, alpha-fetoprotein (AFP) for hepatocellular carcinoma (HCC) and prostate-specific antigen (PSA) for prostate cancer (Fig. 3). The early diagnosis of infectious diseases based on serum glycoproteins is also possible. Dengue virus nonstructural 1 (NS1) antigen is a test commercially available to detect dengue infection. NS1 antigen is a glycoprotein present in elevated levels in serum of infected individuals before that specific antibodies emerge [39].

Current tendencies evaluate changes in the glycosylation patterns of serologic biomarkers as potential candidates for highly specific glycobiomarkers, for early detection and staging of various types of cancers. Researches appoint an increase of core-fucosylation levels of serum AFP of patients with HCC as an indicator more specific for HCC staging [40]. Altered profiles of fucosylation and sialylation in PSA glycans are

reported for prostate cancer and fucosylated PSA showed to have potential to substitute the PSA test in the differentiation of aggressive tumors from non-aggressive prostate tumors [41]. Neoglycoforms of CEA in cancer identified as bi-antennary, tri-antennary and tetra-antennary glycans with residues of sialic acid and fucose can improve tumor diagnosis and staging [42]. Haptoglobin is an acute phase glycoprotein produced by the liver and its concentration is elevated significantly in many diseases, including hepatic inflammation, hepatitis and various types of cancer [43,44]. Studies on altered glycosylation of serum haptoglobin show high levels of fucosylation in haptoglobin associated with cancer; fucosylated haptoglobin (Fuc-Hpt) is reported as a biomarker for diagnostic and prognosis of pancreatic cancer and colorectal cancer [45]. Elevated levels of serum Fuc-Hpt also have been associated with prostate cancer. An increase of fucosylated bi-, tri- and tetra-antennaries glycans of serum haptoglobin was observed in prostate cancer patients, correlated with Gleason score [46].

Analytical techniques have allowed the search and characterization of neoglycoforms present in the bloodstream. The structural characterization of glycans by techniques, such as liquid chromatography and mass spectrometry provides detailed information about composition

and quantity of carbohydrate residues [47]. However, they require long time of analysis, previous treatment of samples, and technical preparation for manipulation, complex and expensive; then, they are not viable for direct diagnostic of diseases and monitoring of patients in clinical treatment. Besides, human serum and plasma are considered complex samples containing a mixture of proteins, some very abundant (more than mg/mL) such as albumin, that can mask other proteins expressed in low concentrations (ng/mL and pg/mL range), mainly biomarkers that appear in early diseases.

Techniques based on specific binding assays using biorecognition agents (eg. antibody, lectin) bind a glycoprotein biomarker or glycan chains linked to protein, measuring these molecules at very low concentrations in human serum and plasma (Fig. 3) with specificity and selectivity [45,48]. Immunoassays as ELISA commercially available and lectin-based assays detect and measure specific serum glycoproteins in research and clinical applications to diagnostic of diseases [48,49]. Lectins immobilized onto inert supports and incubated with serum sample serve to analyse a glycoprotein, such as observed in disposable printed lectin arrays; or lectins

employed in enzyme-linked lectin assay unravel glycosylation profiling of a glycoprotein immobilized on a surface. However, these techniques frequently require significant sample volumes and several steps for obtaining results. Biosensors based on binding assays have emerged as a rapid, simple and sensitive strategy for serological glycoprotein measurements [50,51].

5. BIOSENSORS: A STRATEGY FOR DETECTION OF GLYCOPROTEIN BIOMARKERS

Biosensors are analytical devices capable of detecting and measuring a specific analyte using a biological recognition element contacted with a transducer, that converts the recognition event into a signal analytically useful and measurable [52]. A biosensor contains a biorecognition element (bioreceptor) that binds specifically to analyte of interest; a transducer element that detects signals (current, voltage, changes of mass, light or temperature) resultant of the interaction bioreceptor-analyte and converts in an electric signal. Besides, the transducer connected with an appropriate interface process electric signals and display measurable results [53] (Fig. 4).

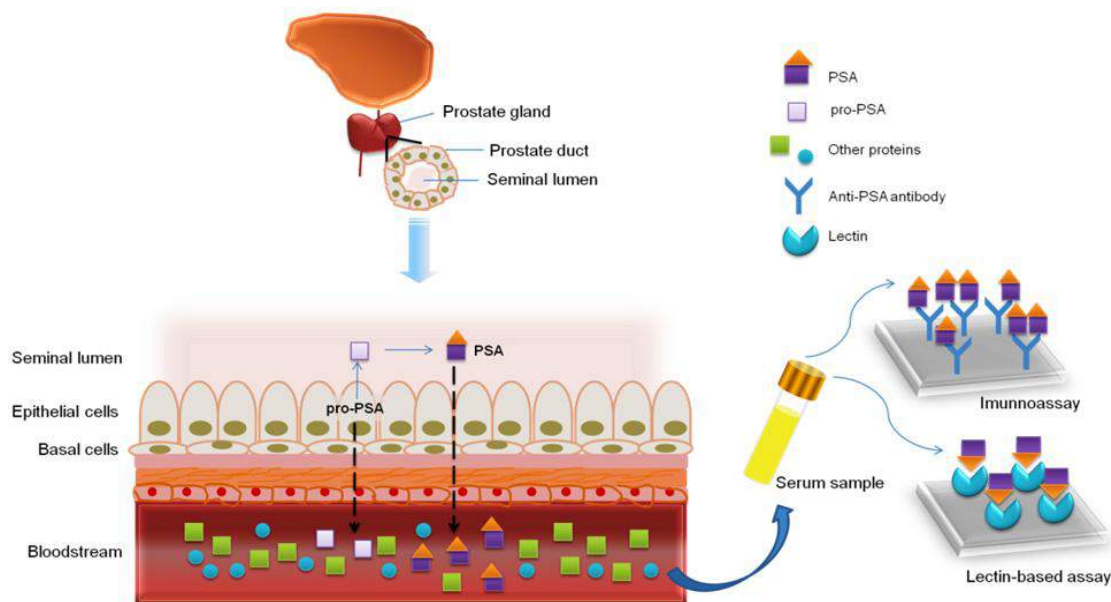


Fig. 3. Schematic representation of secretion from specific prostate biomarker, prostate-specific antigen (PSA), in bloodstream and assays commonly used for specific detection
The prostate gland secretes PSA in the seminal lumen and small concentrations arrive in the bloodstream. In hyperplastic and cancerous conditions, quantitative alterations in the PSA concentration and changes of PSA glycosylation are useful for detection of these diseases. This biomarker analyzed in serum samples through binding assays using antibodies or lectins determine the concentration of PSA and glycan profiles in PSA structure

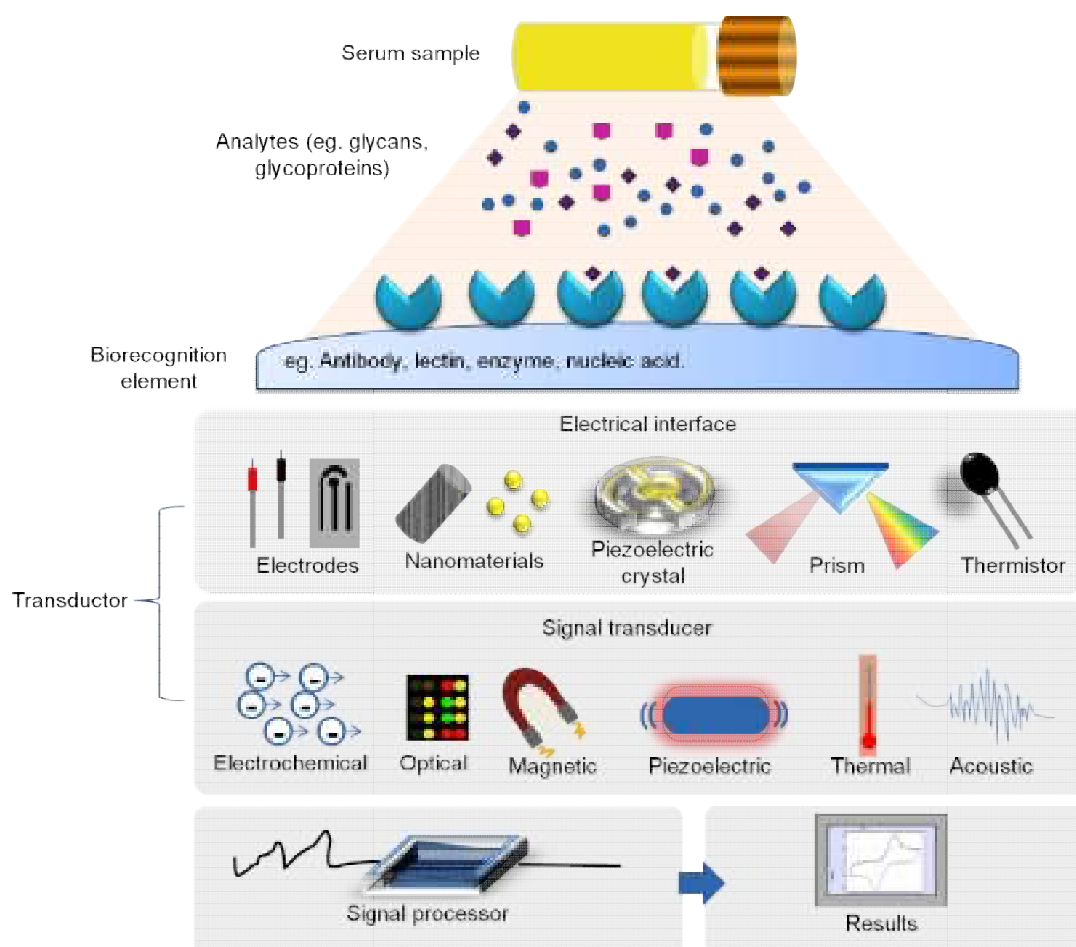


Fig. 4. Schematic representation of a biosensor with a biorecognition element immobilized onto the solid surface in direct contact with an electrical interface of the transducer and a signal processor

The biorecognition element interacts with a specific analyte present in serum sample. The transducer detects the interaction signal converting it to an electrical signal measurable, displaying result

According to type of interaction bioreceptor-analyte, the analytical devices are classified as catalytic or affinity biosensors. Catalytic biosensors based on enzyme activity generate a product [54]. Affinity biosensors involve binding event between the bioreceptor and analyte without chemical transformations, such as antigen-antibody, nucleic acid hybridization and interaction lectin-carbohydrates [53,55]. Biosensors are also classified in relation to the signal detected by transducer (Fig. 4). They can be an electrochemical biosensor, which detect current, voltage, potential or impedance; an optical biosensor, which measure the intensity of the light emitted; a piezoelectric or acoustic biosensor, that detect variation of mass, pressure or elasticity, and a thermal biosensor, which detect changes in temperature [53]. This variety

of mechanisms favors a range of designs and applications of biosensors. Electrochemical biosensors are more attractive and predominant in bioanalysis, due to diversity of electrical interfaces and techniques for measurement that collaborate for high selectivity and sensibility; these sensors are of easy construction, portability, possible miniaturization and low cost [56].

Clark and Lyons developed the first biosensor in 1962, for detection of glucose through glucose oxidase enzyme. They elaborated a system known as enzyme electrode, using glucose oxidase immobilized on polyacrylamide film, on a platinum electrode surface; determination of glucose concentration was based on measurement of oxygen consumed [57]. After,

the Yellow Spring Instrument Company developed the Clark and Lyons's technology to introduce in the market the first biosensor of glucose in 1975 [58]. Since then, the biosensor technology has developed different approaches in the detection mode and elaboration of more efficient devices, aiming the various types of analysis [58]. Biosensor technologies focus in the fabrication of self-contained, portable, miniaturized devices and at the same time, in conservation of good analytical properties, such as speed, high sensitivity and selectivity. Biosensors are then of friendly application to diverse areas: environmental monitoring, biosafety and assays for clinical applications [59].

Biosensors or glycobiosensors used to detect glycans and glycoproteins perform analysis of human serum [55]. Different of the complex approaches such as glycoproteomic techniques, which require sophisticated instrumentation and significant volume of sample, biosensor advances result in easy detection and measuring of glycans or glycoproteins in minimal volume of sample, simple manipulation, short-time response, high sensitivity and specificity, and friendly to use outside of a laboratory [59,60]. These possibilities are attractive for development of point-of-care tests useful in early diagnostic, staging and monitoring diseases such as cancer. The detection of biomarkers using glycobiosensors frequently use antibodies and/or lectins. A variety of available glycobiosensors uses antibodies directed to protein as biorecognition element for specific detection and measurement of serum glycoprotein biomarkers [56]. Lectin glycobiosensors developed for characterization of the glycosylation patterns of glycans on serum glycoproteins correlate to diseases, such as degree of sialylation and fucosylation [60,61].

6. LECTINS AS BIORECOGNITION ELEMENTS

The term lectin is derived from the Latin word "lectus" (chosen, selected) [62], and was introduced to define a group of proteins that showed selectivity in the interaction with carbohydrates. Lectins are sugar-binding proteins of non-immune origin that exhibit specific recognition and reversible bind to free carbohydrates and link to glycoconjugates (eg. glycoprotein and glycolipid). They are involved in crucial physiological events of protein-carbohydrate interactions, such as adhesion and

cell migration [63,64]. Glycobiosensors frequently use lectins as biorecognition elements.

The association constant between lectin and monosaccharides ranges from 10^3 to $(5 \times 10^4) \text{ M}^{-1}$ and between lectins and oligosaccharides, 10^4 to 10^7 M^{-1} , included values in the same range found for antibody-antigen bindings and enzyme-substrates [65,66]. Lectins bind to a specific monosaccharide or oligosaccharide through their binding sites via hydrogen bonds, Van der Waals interactions, and hydrophobic interactions [66,67]. According to the specificity, lectins are classified in five main groups: mannose and/or glucose, N-acetyl-glucosamine, N-acetyl-galactosamine/galactose, L-fucose and sialic acids [68].

Lectins are distributed among viruses, microorganisms and animals although they were initially found in plants [69-71]. The majority of available lectins, obtained from leguminous plants, comprise the largest number of already characterized proteins, such as seed lectin from *Canavalia ensiformis*, Concanavalin A (Con A), soybean agglutinin (SBA) and *Cratylia mollis* seed lectin (Cramoll). Leguminous lectins, although derived from taxonomically distinct species, have molecular characteristics and physicochemical properties in common, such as Con A and Cramoll, and they exhibit a great variety of specificity for carbohydrates [67,72].

Lectins have been used in glycoanalytical areas, including analysis of glycosylation profiles and detection of glycobiomarkers in serological samples based on lectin-carbohydrate interaction. They are widely used in the characterization of glycans linked to glycoproteins for comparative analysis between healthy and pathological conditions and identification of aberrant glycan profiles as potential glycobiomarkers [24,45]. According to their specificity, lectins can detect alterations in degree of sialylation [73], fucosylation [46], galactosylation [74], mannosylation [75] and the presence of cancer-associated antigens, like sialyl-Tn (STn) antigen, Lewis antigens [4,76], and other changes associated with diseases. However, the main limitation of lectins is their ability of recognizing the same carbohydrate in different substrates, and do not evidence the origin of the alteration. Besides, a lectin can bind to similar carbohydrates belonging to the same group of specificity, reducing the directed recognition in glycoanalysis.

Glycobiosensors have employed various lectin-based models to configure the lectin-glycan interactions, sometimes assisted by antibody and nanomaterials to improve the selectivity and signal amplification. Lectins directly immobilized onto a solid support do recognize glycoproteins by glycan portion, in according to specificity, detecting similar glycans in different glycoproteins. Following this model, a lectin-based glycobiosensor for profiling of the STn antigen in serum samples was developed by immobilizing the lectin *Sambucus nigra* agglutinin type I (SNA-I) on electrode surface which showed potential to discriminate between cancer and healthy conditions [4]. The inverted configuration based on the immobilization of glycan or glycoprotein followed through binding of lectin also is available. A biosensor based on D-glucose was successful used to evaluate Con A with a limit detection of 1.0 pM [77]. This configuration is useful to search lectins in a mixture of proteins.

The two-step sandwich model involves a lectin and an antibody, both to interact with the glycoprotein biomarker. In this case, antibody or lectin immobilized can recognize the glycoprotein, and in the second step, a lectin or secondary antibody added complete the sandwich. A plastic sensor chip based on the

technique surface-plasmon field-enhanced fluorescence spectrometry used to detect GalNAc β 1-4GlcNAc-linked prostate-specific antigen applied anti-PSA antibody to capture PSA and the lectin *Wisteria floribunda* agglutinin (WFA) to profile the glycan in serum from patients with benign prostate hyperplasia and prostate cancer patients [74]. The use of the anti-PSA antibody allowed the characterization of glycosylation profile from the specific glycoprotein target present in serum samples. Other sandwich system based on lectin or antibody confined on surface does recognize the glycobiomarker, followed by the addition of lectins coated nanomaterials. The latter compounds are attractive since they increase the amount of secondary biorecognition element and promote the signal amplification. A highly sensitive and selective lectin-based glycol-biosensor developed for comparative analysis of mannose and sialic acid levels on normal and cancer cells used a sandwich system formed by lectins immobilized to detect glycans on cell surface, followed by addition of lectins linked to gold nanoparticles (AuNp) [78]. These strategies provide a characterization of glycosylation profiles on glycoprotein biomarkers with high sensitivity, which is useful in clinical diagnostics, including early detection and staging of cancer and monitoring of patients during treatment.

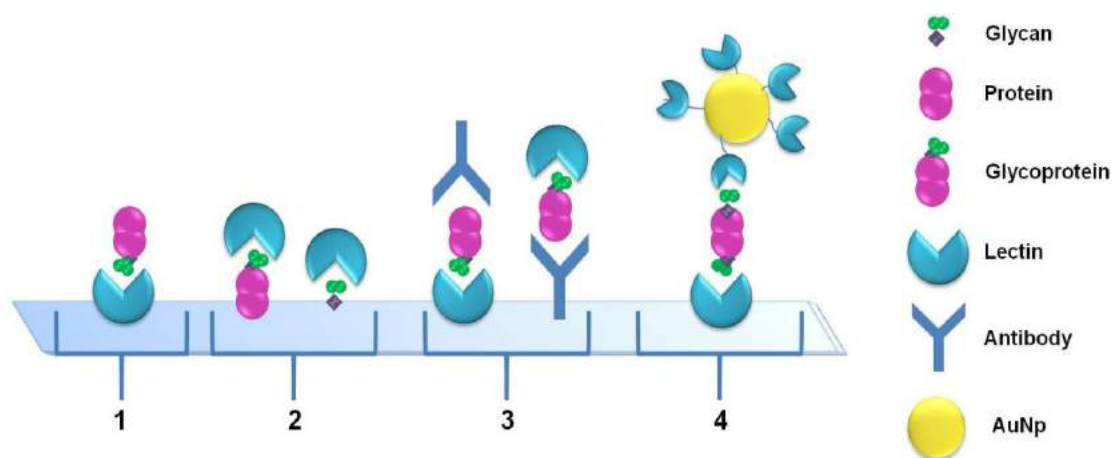


Fig. 5. Configurations of lectin-based assay in glycobiensing. Direct detection by immobilized lectin (1), reverse detection by incubated lectin (2), lectin-antibody sandwich assay (3) and lectin-conjugate sandwich assay with gold nanoparticles using one lectin type or two different lectins (AuNp) (4)

Lectins are useful for detection of glycans free or linked to proteins. The association with antibody allows the analysis of glycans from a glycoprotein, a more specific assay. Nanomaterials provide a broad field to conjugate lectins, increasing glycan detection

7. ELECTROCHEMICAL GLYCIBIO-SENSORS

Electrochemical methods are the most attractive for sensing biorecognition events due to simple instrumentation, friendly analytical performance and diversity of methods and electrodes available for specific applications [79]. Biosensors based on electrochemical detection are widely applied to glycoanalysis in the field of glycomics to analyze cleaved glycans, together to separation techniques (liquid chromatography, electrophoresis or mass spectrometry) [80]. Currently, developed electrochemical glycobiosensors using a lectin as biorecognition element detect changes in the degree of sialylation, fucosylation, galactosylation and mannosylation in cell surfaces and serological samples [81]. Characterization of glycosylation profile from a sample or a specific glycoprotein by electrochemical methods used glycobiosensors [82,83].

Different electrochemical methods are available for glycobiosensors, including electrochemical impedance spectroscopy (EIS) and voltammetric techniques, such as differential pulse voltammetry (DPV) and square wave voltammetry (SWV) [84-86]. Among these, EIS is widely used in glycobiosensors as an effective method for detection of the biorecognition lectin-glycan on the electrode surface. The measurement applies a small alternating current amplitude on the electrode interface and subsequent detection of change in impedance during binding events. When the binding event happens, a change in impedance is detected in the presence of a redox probe (eg. electrolyte solution of ferricyanide and ferrocyanide), and the change in electron transfer resistance (RCT) is measured. The change in RCT is due to blocking of charge transfer on the electrode surface in the presence of coating, immobilized biomolecules and biorecognition complex [87]. EIS measurements are transformed in an equivalent circuit and a Nyquist plot to obtain information about RCT and lectin-glycan binding. EIS provides a rapid, simple, highly sensitive and label-free detection of glycans in low concentrations, eliminating the use of labels commonly required in non-electrochemical techniques [85]. Voltammetric techniques, also used for label-free detection of lectin-glycan binding, in general, are based on the application of a voltage range in the electrode interface, that in the presence of a redox probe, promote a redox reaction and subsequent generation of a

current flow [86]. The electrode surface modification with biomolecules and biorecognition complex changes interfacial electron transfer reaction of the redox probe, resulting in changes of current. The current variation is measured and quantitative information about the target glycans obtained, more rapidly than EIS method. The voltammetric techniques more used for detection of biorecognition events are DPV and SWV due to high sensitivity, quickness (3 to 10 s is necessary for measurement) and minimization of capacitive interference [86].

The first electrochemical biosensor using electrode surface modified with lectins was reported by La Belle and coworkers for detection of cancer-associated T antigen (β -D-Gal-[1 $\rightarrow$ 3]-D-GalNAc) [84]. The N-acetyl-galactosamine/galactose specific plant lectin, peanut agglutinin (PNA) was immobilized onto a gold electrode surface previously modified with a mixed self-assembly monolayer, followed by the incubation with nanocrystal CdS-tagged 4-aminophenyl- α -D-galactopyranoside sugar and target sugars (GalNAc, Gal and T antigen) to establish a competitive assay. The lectin-sugar recognition monitored by square-wave voltammetric stripping current peaks, decrease with the bind of target glycan. Current peaks decrease for target sugars, in the affinity order T antigen > Gal > GalNAc, achieving a detection of 0.1 μ M for T antigen, 1.0 μ M for Gal and 2.7 μ M for GalNAc. T antigen is a known glycobiomarker for different types of cancer and this electrochemical biosensor based on PNA lectin demonstrated potential to detect and distinguish the T antigen of other similar ones, being a sensitive and simple technique for quantification of T antigen in human serum samples.

EIS analysis requires more steps of processing data; however, this method promoted the detection of very small concentrations of glycobiomarker. An ultrasensitive label-free glycobiosensor reported for measurement of sialylated glycoproteins used the EIS method [83]. A lectin from *Sambucus nigra* (SNA I) specific for sialic acid was covalently attached to a mixed self-assembled monolayer (SAM) consisting of 11-mercaptoundecanoic acid and 6-mercaptohexanol on a gold electrode. The biosensor detected glycoproteins fetuin and asialofetuin through the fraction of sialic acid, with a limit of detection of 0.33 fM for fetuin and 0.54 fM for asialofetuin. Its potential to detect sialylated glycoproteins with very low limit of

detection is attractive for clinical applications in the search of sialylated glycobiomarkers of diseases.

Other label-free biosensor based on EIS developed for detection of cancer glycobiomarker alpha-fetoprotein (AFP) and discrimination of glycosylation profile of AFP in serum samples used lectins as biorecognition elements [81]. First, a lectin biosensor for detection of AFP was mounted through attachment of the wheat-germ agglutinin (WGA) lectin to single-wall carbon nanotubes (SWCN) onto a screen-printed carbon electrode (SPCE). The biosensor used for AFP binding achieved a low limit of detection of 0.1 ng/L. Moreover, lectin biosensors fabricated with different lectins characterized glycosylation profile of N-glycan linked to AFP and discriminate between serum samples of cancer patients and healthy subjects. The lectins WGA (GlcNAc-specific), LCA (mannose-specific), Con A (mannose-specific), SNA (sialic acid-specific) and DAS (LacNAc-specific) were immobilized onto different SWCN-modified SPCE and the changes in charge transfer resistance were monitored. The results suggest an increase of core fucosylation and α 2-6 sialylation in AFP of cancer when compared with healthy serum, being a potential tool for label-free profiling of glycan expression in serum samples and early diagnosis.

A Con A-based biosensor was reported as a strategy for electrochemical detection of abnormal glycoproteins from serum of patients infected with dengue virus serotypes 1, 2 and 3 [82]. This biosensor based on gold electrode modified with phospholipid membrane and Con A to form a lipid-Con A surface, was incubated with serum from patients. Electrochemical characterization performed by voltammetry and EIS recorded decreases in the response of current of the electrodes and notable increases of RCT after incubation with infected sera by dengue virus serotypes 1, 2 and 3. These findings indicate the binding of serum glycoproteins to Con A through its mannose-binding sites, revealing that the biosensor was able to recognize dengue serotypes and are useful for detection of dengue infections.

8. ELECTROCHEMICAL GLYCOBIO-SENSORS BASED ON CRAMOLL 1,4 LECTIN

Cramoll 1,4 is a legume lectin isolated from *Cratylia mollis* seeds that has been employed as

biorecognition element in glycobiosensors. *C. mollis* is an occurrent native forage in the semiarid region of Brazil, popularly known as camaratu bean; it belongs to the same Fabaceae family of *Canavalia ensiformis*, the natural source of Con A lectin [7,88]. *C. mollis* seeds are source of four molecular isoforms of lectins with diverse specificity denominated Cramoll -1, -2, -3 and -4, and a preparation containing the 1 and 4 isoforms, known Cramoll 1,4. Cramoll 1, 2 and 4 are glucose/mannose-specific lectins; Cramoll 3 is a galactose specific lectin [7,89]. Cramoll 1 showed classic tertiary structure of legume lectins through X-ray crystallography. Its primary structure consists of 236 amino acid residues with 82% homology with Con A structure, and both have identical carbohydrate-binding sites to the methyl- α -D-mannopyranose, with which they interact by hydrogen bond, and binding sites for Ca^{2+} and Mn^{2+} metals. The secondary structure is composed exclusively of three β sheets; all connected by turns [67].

Cramoll -1 and -1,4 demonstrated its potential for various biotechnological applications such as antitumor action [90]; mitogenic activity [91]; pro-inflammatory and healing of experimental tissue lesions [92] and anthelmintic [93]. The potential of this lectin to recognize glycans and glycoproteins is a valuable tool for detection changes in glycosylation of diseases through tissues and serum samples. Cramoll 1 showed a higher potential to mark neoplastic mammary tissues [94] and Cramoll 1,4 revealed more intense staining in hyperplasia prostate tissues when compared to prostate carcinoma tissues [1], being a potential probe for histochemical studies. An affinity column of Cramoll 1 coupled to Sepharose CL4B was an efficient matrix to isolate serum glycoproteins, such as lecithin-cholesterol acyltransferase [95].

Cramoll 1,4 has been explored in the recognition of serum glycoproteins from patients contaminated with different serotypes of dengue virus using an electrochemical biosensor, a very promising field. Firstly, it was reported the ability of a Cramoll 1,4 biosensor for sensitive detection of glycoproteins in solution [96]. A mix of Cramoll 1,4 with gold nanoparticles (AuNp) and polyvinylbutyral (PVB) was immobilized onto the surface of gold electrodes; the lectin-modified electrode was incubated with solutions containing ovalbumin. Electrochemical analysis by voltammetric and EIS detected lectin-ovalbumin interactions in the electrode surface, confirming that the immobilization process of

Cramoll 1,4 did not affect lectin recognition sites. In this case, the lectin recognized oligomannose glycan chains linked to ovoalbumin. Cramoll 1,4 biosensors also were developed to detect dengue serotypes. A gold electrode modified with Cramoll 1,4, Fe₃O₄ nanoparticles and PVB was applied to evaluate the binding with fetuin in solutions and serum glycoproteins from patients infected with dengue serotypes 1, 2 and 3 (DS1, DS2 and DS3) [8]. EIS and voltametric measurements registered the binding of Cramoll 1,4 with fetuin and serum glycoproteins of DS1, DS2 and DS3, showing a differential response for each serotype and higher interaction with glycoproteins from serum contaminated by DS2. Similar approach discriminated serum glycoproteins from DS1, DS2 and DS3; also detected different stages of infection using sera of patients with dengue fever and dengue hemorrhagic fever. An electrochemical biosensor was constructed using a gold electrode, which surface was modified with a nanocomposite of AuNPs-polyaniline (PANI) and Cramoll 1,4, subsequently exposed to different sera [9]. Voltammetric and EIS data showed that Cramoll 1,4 biosensor discriminates the different serotypes and stages of dengue infection. Thus, Cramoll 1,4 is a promisor tool in researches for biosensing of serum glycoprotein profiles associated to diseases and this lectin preparation is a potential candidate for recognition of glycobiomarkers.

9. CONCLUSION

Glycosylation is a rich code of information related to physiological and pathological cellular events. Many changes in glycan profiles significantly correlate with diseases and their detection provide early diagnostic, staging and prognostic with high sensitivity. Glycobiomarkers detected in serum samples and measured through techniques more friendly can provide rapid results. Lectins, as sugar-binding proteins recognizing alterations in glycosylation profiles are useful in glycoanalytical techniques, among these, biosensing. Glycobiosensors based on electrochemical detection is a good strategy for development of simple, rapid, portable and sensitive devices detecting glycans in minimal volume of serum samples to search glycobiomarkers in clinical diagnostic.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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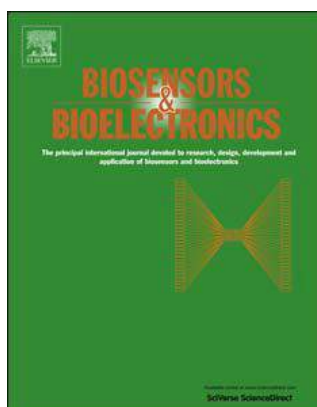
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APÊNDICE B – ARTIGO PUBLICADO NA REVISTA BIOSENSORS AND BIOELECTRONICS

***Cratylia mollis* lectin nanoelectrode for differential diagnostic of prostate cancer and benign prostatic hyperplasia based on label-free detection**

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Cratylia mollis lectin nanoelectrode for differential diagnostic of prostate cancer and benign prostatic hyperplasia based on label-free detection

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ABSTRACT

The research for new biomarkers of cancer has studied the role of fetuin glycoprotein on the metastatic disease diagnosis. *Cratylia mollis* is a lectin with high finity to fetuin, and used here to differentiate prostate cancer and benign prostatic hyperplasia. A label-free electrochemical nanosensor based on assembled carboxylated carbon nanotubes (COOH-CNTs) and poly-L-lysine (PLL) film was developed and applied to serum samples of prostate cancer positive for Gleason score. The electrode analytical response to fetuin in PBS samples, obtained by square wave voltammetry, exhibited a linear range from 0.5 to 25 $\mu\text{g mL}^{-1}$, with a high correlation coefficient ($r=0.994$, $p<0.001$) and low limit of detection (0.017 $\mu\text{g mL}^{-1}$). The lectin nanoelectrode showed a good repeatability (1.24% RSD) and reproducibility (4.24% RSD). A pool of serum samples from prostate cancer patients with known the Gleason score were tested showing a significant statistically correlation. Thus, the lectin nanoelectrode was able to distinguish the degree of staging prostate cancer, providing the diagnostic differentiation of benign and malign hyperplasia. To the best of our knowledge, it is the first biosensor for this application using a lectin.

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1. Introduction

Prostate cancer (PCa) is the second most common cause of cancer death in men, after lung cancer, in worldwide (Siegel et al., 2014). The Prostate-specific antigen (PSA) is the gold-standard marker for PCa diagnostic used in clinical routine (Heidenreich et al., 2014). However, the PCa screening based on PSA levels has been associated to high false-positive rates (Moyer, 2012). The early staging of PCa facilitates the appropriate treatment, mainly for patients with high Gleason score that are more likely to progress for metastasis (Pierorazio et al., 2013). Despite extensive efforts, the lack of reliable methods for prediction of progression beyond early-stage disease and paucity of treatment options for patients with bone metastasis results in many patients with localized disease subjected to aggressive treatment with sequelae including incontinence and impotence (Schover et al., 2002). Thus, identification of biomarkers to improve the accuracy of clinical assessment of PCa constitutes a great challenge.

The fetuin glycoprotein has been studied as a new potential

biomarker for pancreatic and liver cancers and tested in metastasis of tumor cells (Sakwe et al., 2010; Yi et al., 2009). Fetuin acts as an extracellular adhesive protein that conjointly with annexins induces cell adhesion and growth (Drake et al., 2006; Rho et al., 2009). Due to great importance as biomarker, different fetuin assays have emerged using auto antibodies against fetuin, like enzyme-linked immunosorbent assay-based in vitro diagnostic procedure (Vashist et al., 2015a), sandwich immunoassay using agarose-3-aminopropyltriethoxysilane modified microtiter plate (Vashist et al., 2015b) and surface plasmon resonance biosensor (Vashist et al., 2012; 2014). Fetuin is a glycoprotein, then the great interaction with specific lectins has been strategically explored by some authors. Bertok et al., (2013) developed a biosensor using the *Sambucus nigra* lectin for the determination of sialylated fetuin glycoproteins and asialofetuin in PBS; Oliveira et al., (2011) demonstrated that *Cratylia mollis* lectin (Cramoll) strongly interacted with fetuin using an impedimetric biosensor.

To improve accuracy of PCa diagnostic on metastatic phase, Mintz et al. (2015) published the first report associating fetuin as a potential marker for prostate cancer and demonstrated its prognostic utility as indicator for metastasis and aggressiveness of tumor. Herein, Cramoll was immobilized on a nanostructured electrode to successfully distinguish positive prostate cancer and

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benign prostate hyperplasia (BPH) patients. This found correlated with Gleason score showing a significant concordance. To our knowledge, this is the first report of a biosensor using *Cratylia mollis* lectin to differentiate PCA and benign hyperplasia.

Electrochemical approaches like biosensors are of particular interest as transducers due to their ease mass production, portable and low cost (Bertok et al., 2013). They also provide a friendly instrumentation, and a great compatibility with portable systems suitable for point-of-care testing (Ahmed et al., 2016). Some electrochemical biosensors based on impedance spectroscopy using lectins as marker for fetuin detection have been developed (La Belle et al., 2007; Oliveira et al., 2011; Bertok et al., 2013). Although, these biosensors had advantages of non-requirement of a tracer or label, allowing a rapid detection, they require more careful in measurements due to frequent noises and misinterpretations when compared to recent methods of electrochemical label-free immunosensors, such as square-wave voltammetry (SWV) technique. This method is based on staircase of potential pulse superimposed on a staircase potential signal with the latter centred between a cathodic and anodic pulse of the same amplitude, allowing to discriminate the capacitive current with high sensitivity (Liu et al., 2010).

The use of nanomaterials for biosensors based in film, particularly carbon nanotubes (CNTs) have attracted considerable attention due to provide a high surface-volume ratio, faster electron transfer and label-free responses (Gomes-Filho et al., 2013). Synergic effect between CNTs and polymers was explored to make the nanostructured films more stable with higher electrocatalytic activity (Silva et al., 2014). Additionally, CNTs are easily functionalized by chemical or thermal reactions (Ma et al., 2010), promoting irreversible processes of biomolecules immobilization on electrode surface (Freitas et al., 2014). Herein, the poly-L-lysine (PLL), a cationic polymer rich in amino groups was used to electrostatically attach carboxylated CNT (COOH-CNT) in order to form a stable nanostructured film (COOH-CNT) with a high electroactive area. The nanohybrid compound of charged polymeric matrix on the electrode surface was obtained by electrodeposition techniques, providing functional groups to immobilize the carbohydrate-specific lectin. A label-free electrochemical biosensor developed to prostate tumor allowed to distinguish the glycoprotein profile in human serum.

2. Material and methods

2.1. Reagents

PLL, fetuin, bovine serum albumin (BSA), galactose and fucose were obtained from Sigma Aldrich (St. Louis, USA). Multi-walled carbon nanotubes functionalized with carboxylic groups (COOH-CNTs) (average diameter of ~ 10 nm, average length of 1–2 μm and 95% purity) were acquired from DropSens (Oviedo, ESP). Cramoll 1,4 (preparation containing isoforms 1 and 4 of *C. mollis* lectin) were obtained from the Protein Biochemistry Laboratory (UFPE) (Recife, BRA), according to protocol established by Correia and Coelho (1995). Fructose, glucose, glycine, dimethylformamide (DMF), potassium ferrocyanide ($\text{K}_4\text{Fe}(\text{CN})_6$) and potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$) were purchased from Vetek (São Paulo, BRA). The other reagents used were of analytical grade. The phosphate buffer saline (PBS) solution (0.01 mol L^{-1} , pH 7.4) was utilized in the experiments for sample dilution. The ultrapure water obtained from a water purification system Milli-Q (Billerica, USA) ($18 \text{ M}\Omega$) was utilized to prepare all solutions.

The serum samples were obtained from BPH and PCA patients that were confirmed via histopathological examination of prostate tissue. PCA patients were previously stratified by biopsy in the

Gleason score of the 6, 7 and 9. All voluntary donors were admitted to the University Hospital of the UFPE (Recife, BRA), in accordance with the recommendations of the ethics committee. Venous blood samples were collected and immediately centrifuged at $1500 \times g$ for 5 min. The samples were stored at -20°C while it is not in use.

2.2. Apparatus

The electrochemical studies were performed by using a potentiostat/galvanostat portable Ivium Compact Stat (Eindhoven, NLD) connected to a microcomputer and controlled by IviumSoft software. It was used a three electrode system, comprising a glassy carbon electrode (GCE) ($\varnothing=3 \text{ mm}$) as the working electrode, a helical platinum wire as counter electrode and Ag/AgCl (KCl saturated) as the reference electrode.

The morphological characterization of the sensor surface was performed by the Scanning Electron Microscopy (SEM) technique by using a Philips XL-30 FEG microscope (Eindhoven, NDL). The measurements were recorded at acceleration voltage of 10 kV in low-vacuum mode and working distance of 1 μm . The structural characterization was accomplished with Fourier Transform Infrared in the Attenuated Total Reflectance mode (ATR FT-IR) using the Bruker IFS 66 model FT-IR spectrometer (Billerica, USA). Spectra were acquired at 4000 cm^{-1} – 500 cm^{-1} , through a glassy carbon disc ($\sim 0.5 \text{ cm}$ diameter) that was adapted to the electrochemical cell for further modifications.

2.3. Preparation of the nanoelectrode

Prior to modification, the GCE was polished with alumina powder (1 and $0.5 \mu\text{m}$) for 3 min until obtaining a mirror-like surface. In order to remove any impurities, cleaned electrode was carefully rinsed with ultrapure water. After this step, the PLL ($0.05 \mu\text{mol L}^{-1}$) prepared in PBS solution (0.01 mol L^{-1} , pH 7.4) was electrochemically deposited on the GCE surface through the CV technique in the potential range -2.0 to 2.0 V at scan rate of 0.05 V s^{-1} for 15 cycles. Then, three layers of the COOH-CNT solution ($15 \mu\text{L}$) was deposited onto the GCE surface modified with PLL. The COOH-CNT solution consisted of 1 mg of COOH-CNT dispersed in 1 mL of DMF and sonicated in an ultrasonic bath for 2 h. Each layer was dried at 40°C for evaporation of the solvent.

2.4. Immobilization of Cramoll 1,4 on the nanoelectrode

Cramoll 1,4 ($200 \mu\text{g mL}^{-1}$) was immobilized on the nanoelectrode during 60 min. The working electrode was maintained in a moist chamber at room temperature ($\sim 25^\circ\text{C}$). Finally, $5 \mu\text{L}$ of the glycine solution (0.05 mol L^{-1}), prepared in ultrapure water, was pipetted on the sensor surface for 60 min in order to blocking the nonspecific sites. The Fig. 1 shows the stepwise of the biosensor preparation.

2.5. Analytical response of the Cramoll 1,4 nanoelectrode

The analytical performance of the as-prepared nanoelectrode was evaluated through the incubation with $10 \mu\text{L}$ of fetuin at different concentrations. The samples diluted in PBS solution (0.01 mol L^{-1} , pH 7.4) were placed during 20 min in a moist chamber at room temperature. The analytical responses were monitored by SWV in a label-free assay by using $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ (0.005 mol L^{-1}) as redox probe. The SWV analysis was carried out in a potential range of 0.0 – 0.4 V at frequency of 10.0 Hz .

The detection of lectin affinity was standardized by using the percentual decrease of current ($\Delta\%$) in the SWV measurements

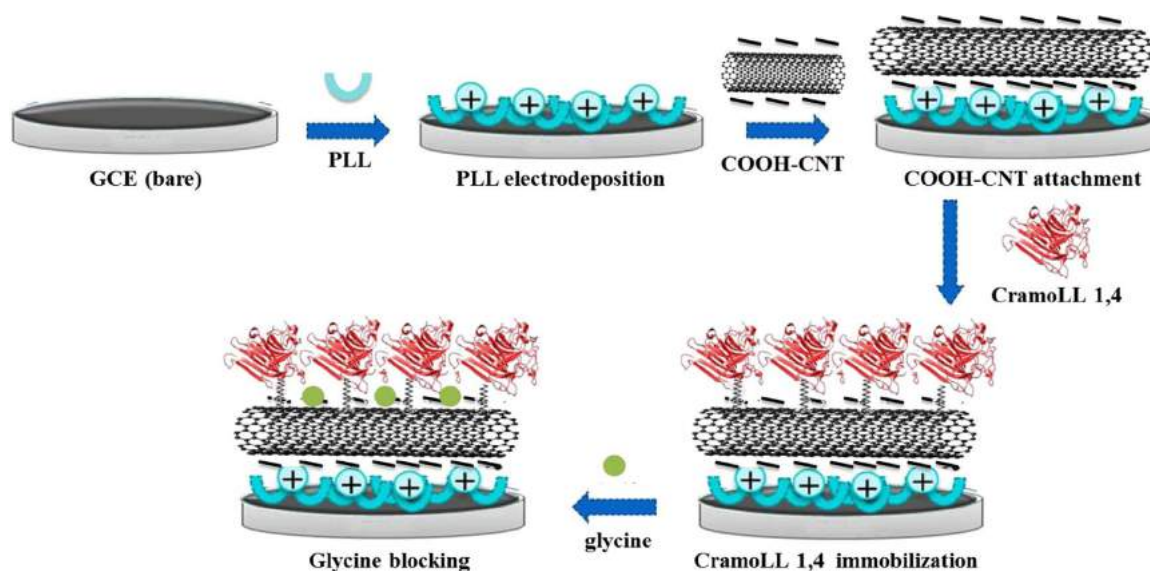


Fig. 1. Schematic illustration of the stepwise modification of the CramoLL 1,4 lectin nanoelectrode.

before and after carbohydrate incubation. A pool of serum of BPH and PCa patients diluted 1:30 in PBS solution (0.01 mol L^{-1} , pH 7.4) was used to evaluate the analytical responses. All measurements were carried out in triplicate.

The stepwise of the biosensor modifications were accomplished through CV measurements. The analysis was carried out in a potential range of -0.1 to 0.5 V , at 0.05 V s^{-1} scan rate, in presence of 0.005 mol L^{-1} of the $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ prepared in KCl solution (0.1 mol L^{-1}).

3. Results and discussion

3.1. Characterization of the nanoelectrode

In order to characterize the interaction of the COOH-CNTs with PLL as nanosurface support and the CramoLL 1,4 immobilization, comparative spectra of the modified GCE were evaluated by FT-IR analyses (Fig. 2). PLL spectrum (curve I) exhibits two peaks at 3406 and 3271 cm^{-1} characteristic of the primary $-\text{NH}_2$ of the polymer. Another peak at 3063 cm^{-1} is attributed to the $-\text{NH}_2^+$ groups due to protonation of $-\text{NH}_2$ of PLL in PBS solution (0.01 mol L^{-1} , pH 7.4), taking account that pKa of polymer is found at 10.7 (Barth, 2007; Wang et al., 2012). The peaks at 1612 and 1525 cm^{-1} are ascribed to the amide I ($\text{C}=\text{O}$ stretching vibration) and II ($\text{N}-\text{H}$ bending vibration and $\text{C}-\text{N}$ stretching vibration) bands

of the polypeptide PLL (Barth, 2007). The spectrum of COOH-CNT (curve II) shows a peak at 3320 cm^{-1} indicator of $-\text{OH}$ groups; a peak at 1655 cm^{-1} , resulting from molecular elongation of $\text{C}=\text{O}$ group, and another peak at 1002 cm^{-1} attributed to the $\text{C}-\text{O}$ stretching vibration. These peaks are typically related to the carboxylic groups of the CNTs (Silva et al., 2014). The spectrum of COOH-CNT/PLL (curve III) exhibits a peak at 3335 cm^{-1} and other peaks at 1670 cm^{-1} , 1514 cm^{-1} and 1019 cm^{-1} , similar to COOH-CNT spectrum, confirming that nanotubes were anchored onto the PLL. Cationic polymers, such as PLL have been often used for the electrostatic interaction and ionic interaction between the positively charged polymer and COO^- groups of the CNT (Jiang et al., 2008; Fujigaya and Nakashima, 2015). Regarding the immobilization of CramoLL 1,4, the spectrum revealed two peaks at 3373 cm^{-1} and 3265 cm^{-1} attributed to primary $-\text{NH}_2$ and the peaks at 1621 cm^{-1} and 1521 cm^{-1} characteristic of the amide I ($\text{C}=\text{O}$ stretching vibration) and II ($\text{N}-\text{H}$ bending vibration and $\text{C}-\text{N}$ stretching vibration) bands of CramoLL 1,4 and COOH-CNT interaction.

The stepwise modification of GCE surface was also characterized by SEM technique. Firstly, Fig. 3a shows GCE surface after electrodeposition of the PLL film with a thin layer of the polymer homogeneously distributed on the sensor surface. The micrograph of Fig. 3b exhibited an irregular surface formed by abundant spaghetti-like filamentous structures attributed to the COOH-CNT presence (Jiang et al., 2008). The deposition of carbon nanotubes on PLL/GCE surface showed a porous three-dimensional nanostructured film (Xue et al., 2011).

In order to evaluate the operational stability of COOH-CNT/PLL film, the modified GCE was submitted to 20 consecutive voltammetric cycles in $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ (0.005 mol L^{-1}). The relative standard deviation (RSD) estimated for current peak values of COOH-CNT/PLL was found at 0.6% showing a good stability of nanostructured film. To evaluate the role of PLL in assure to CNT binding, a control experiment was carried without PLL film. It was found a RSD of approximately 2.7% demonstrating that COOH-CNT/PLL was around four times more electrochemically stable than COOH-CNT straight on the electrode surface. This result can be ascribed to electrostatic interaction between cationic PLL and carboxylic groups of nanotubes (Jiang et al., 2008).

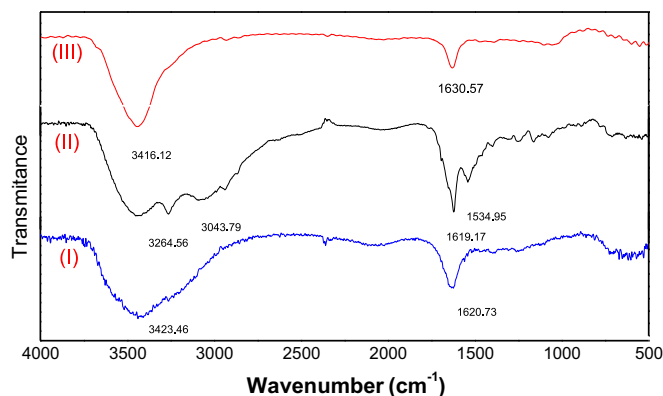


Fig. 2. FTIR spectra of the GCE modified with (I) COOH-CNT, (II) PLL and (III) COOH-CNT/PLL.

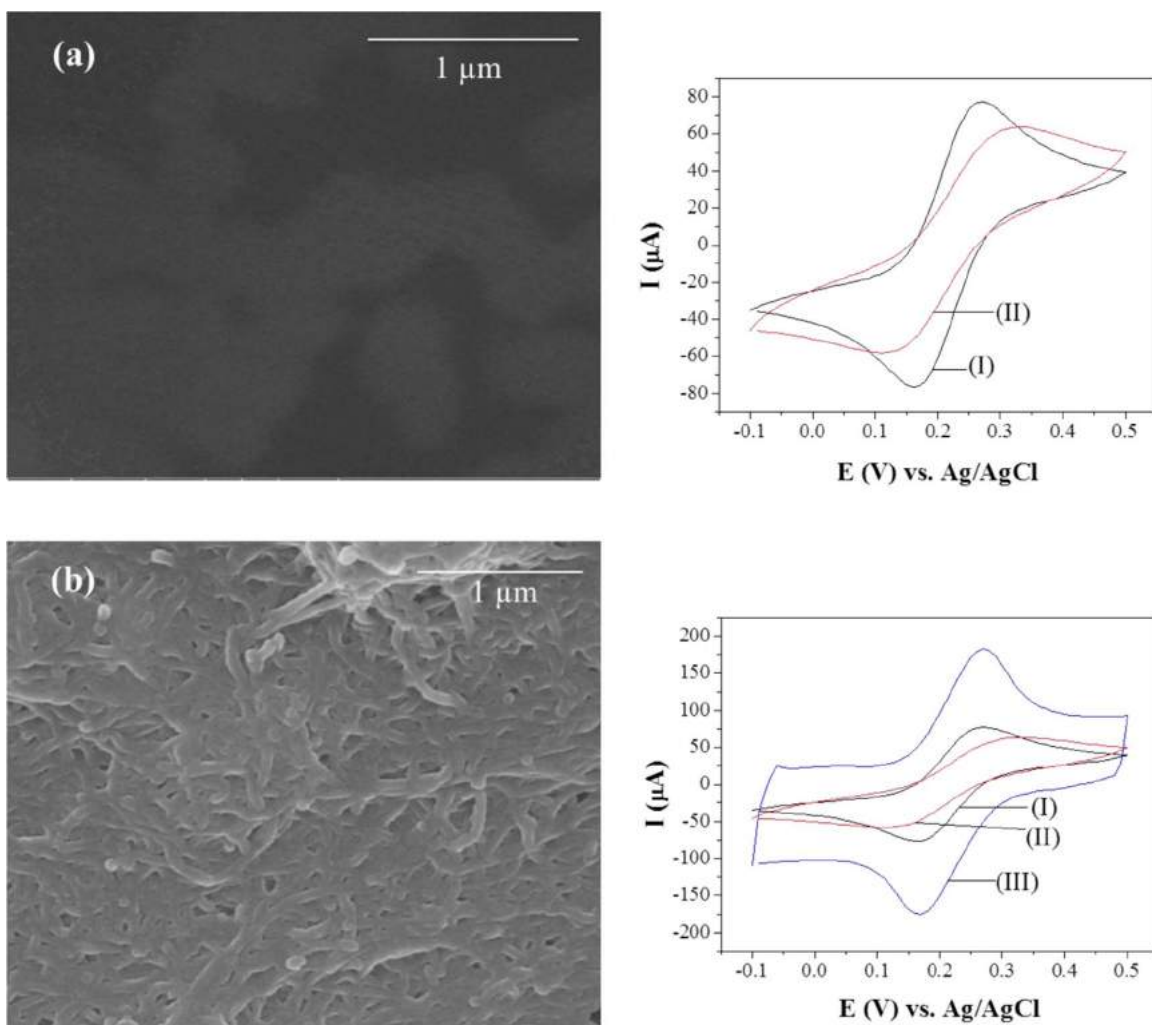


Fig. 3. SEM images and CVs of the surface (a) PLL/ GCE and (b) CNT/PLL/GCE. In a) and b), (I) GCE after cleaning; (II) PLL/GCE; (III) COOH-CNT/PLL/GCE. Measurements performed in $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ (0.005 mol L^{-1}) prepared in KCl solution (0.1 mol L^{-1}).

3.2. Electrochemical characterization of lectin nanoelectrode

Fig. 4a shows the electrochemical characterization of construction steps of the biosensing platform. The monitoring of modifications from the electrode surface was performed by using CV technique in $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ as redox probe. The analysis of redox peaks of voltammograms after modification of GCE with PLL ($0.05 \mu\text{mol L}^{-1}$) (curve II) exhibits a reduction in the current amplitude when compared as the cleaned electrode (curve I). The electroactive area of GCE (0.096 cm^2), calculated according to Randles-Sevcik equation (Bard and Faulkner, 2001), showed a decrease after the PLL film (0.0079 cm^2). This result is attributed to the non-conducting nature of the polymer (Tsai et al., 2011), which confirms the electrodeposition of PLL on the sensor surface. After deposition of COOH-CNT on the PLL film (curve III), the voltammogram showed an increase in electroactive area (0.225 cm^2), that can be attributed to higher electron transfer promoted by the increase of conductivity due to COOH-CNTs (Zhu et al., 2012).

The immobilization of the Cramoll 1,4 onto COOH-CNT/PLL film is represented by a reduction in the amplitude of redox peaks in voltammograms (curve IV). The insulating nature of the biomolecule promotes a decreased of 11.5% of electroactive area from the electrode (0.202 cm^2). After blocking of the free reactive sites with glycine (curve V), it also was observed a slight reduction in charge transfer on the electrode surface.

The electron diffusion study of the COOH-CNT/PLL nanosurface

with Cramoll 1,4 immobilized was investigated submitting the electrode to different scan rates. As can see in Fig. 4b, the voltammograms registered in a $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ (0.005 mol L^{-1}) exhibit a proportional increase in both cathodic and anodic peak currents (I_{pa} and I_{pc} , respectively) according to the scan rate ($10\text{--}120 \text{ mV s}^{-1}$). The I_{pa} and I_{pc} were directly proportional to the square root of scan rates (Fig. 4b - inset) with the following linear regression equations: $I_{pa} (\mu\text{A}) = 31.69 \cdot v^{1/2} - 57.27$ ($r = 0.997$) and $I_{pc} (\mu\text{A}) = -29.73 \cdot v^{1/2} + 50.23$ ($r = 0.995$). These results suggest that the reactions on the sensor interface were controlled by mass transport (surface diffusion), in agreement with the quasi-reversible system (Kang et al., 2009).

3.3. Experimental optimization

In order to achieve the optimal performance of the COOH-CNT/PLL film, the influence of PLL concentrations ($0.0001 \mu\text{mol L}^{-1}$ to $0.2 \mu\text{mol L}^{-1}$) was evaluated. In this study, a proportional increase in the amplitude of the peak currents in relation to PLL concentrations was found until reach a maximal response at $0.05 \mu\text{mol L}^{-1}$ of polymer (Fig. S1a - Supplementary data). Then, the COOH-CNTs concentrations (0.5 mg mL^{-1} to 3.5 mg mL^{-1}) deposited on the PLL film were investigated. The current peaks increased until the concentration of 1.5 mg mL^{-1} of nanotube (Fig. S1b - Supplementary data). After this point, the current decreased indicating a saturation of free reactive groups of polymer in the

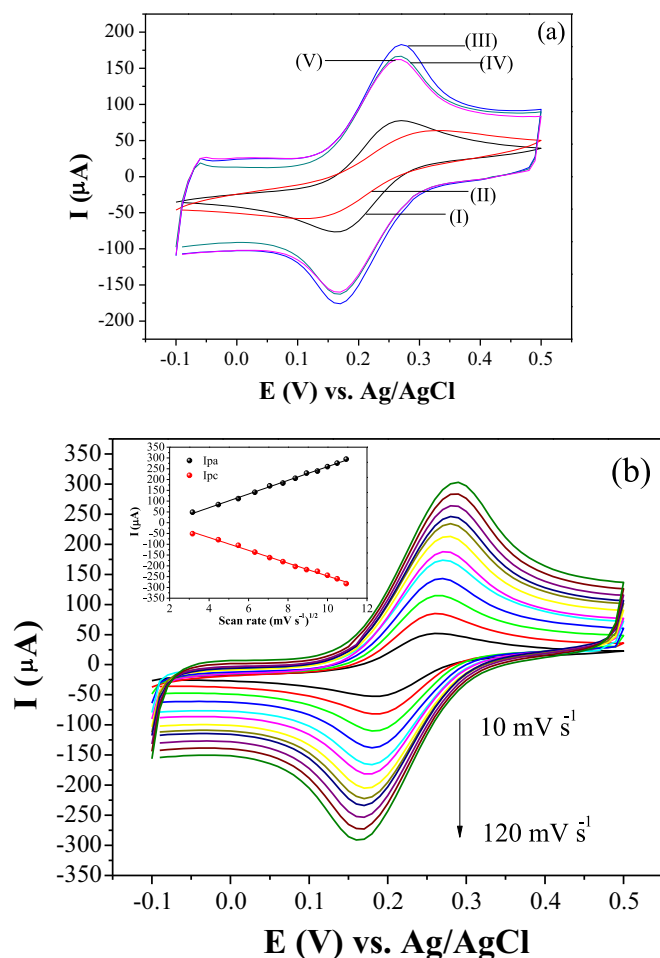


Fig. 4. (a) CVs of the stepwise modification of the lectin nanoelectrode: (I) GCE; (II) PLL/GCE; (III) COOH-CNT/PLL/GCE; (IV) Cramoll 1,4/COOH-CNT/PLL/GCE; (V) glycine/Cramoll 1,4/COOH-CNT/PLL/GCE; (b) Voltammetric profile of the Cramoll 1,4/COOH-CNT/PLL/GCE under different scan rates (10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110 and 120 mV s^{-1}) (inset: plots of the I_{pa} and I_{pc} vs. square roots of the scan rates). All the measurements were performed in $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ (0.005 mol L^{-1}) prepared in KCl solution (0.1 mol L^{-1}).

sensor surface.

The amount of the Cramoll 1,4 immobilized on the COOH-CNT/PLL film is a limiting aspect in the biosensing platform to obtain a good analytical sensitivity. Thus, the different concentrations (1–200 $\mu\text{g mL}^{-1}$) of Cramoll 1,4 immobilized on the nanosurface were evaluated (Fig. S1c - Supplementary data). The electrode response showed a proportional increase of current value in relation to Cramoll 1,4 concentrations with a maximal lectin-nanosurface interaction at 200 $\mu\text{g mL}^{-1}$.

3.4. Analytical response of the lectin sensor to fetuin

The activity of Cramoll 1,4 was analysed through the correlation of the electrochemical measurements at different glycans concentrations. In this study, fetuin was chosen to evaluate the carbohydrate specific of Cramoll 1,4. Different electrodes were incubated with 10 μL of fetuin solutions at specific concentrations during 20 min. Then, the electrodes were submitted to SWV measurements in $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ solution (0.005 mol L^{-1}) prepared in KCl (0.1 mol L^{-1}). The label-free SWV detection is based on interfacial resistance to electron transfer of the $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ probe after successive incubations of the sensor surface with fetuin solution. The results were examined in terms of relative current variation ($\Delta I\%$) according to the following

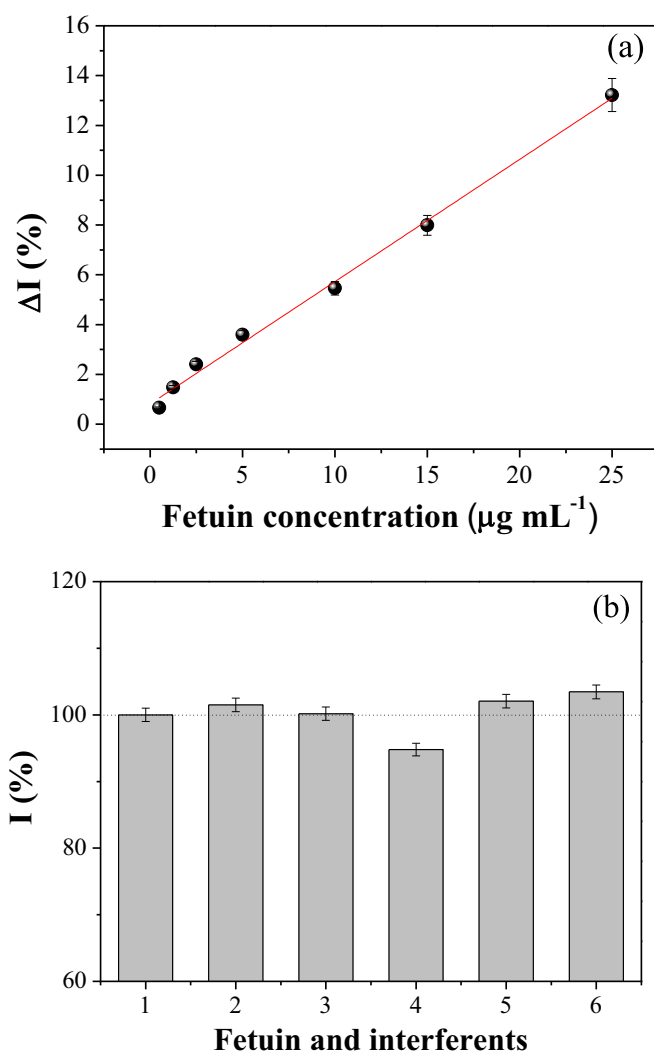


Fig. 5. (a) Analytical curve of the Cramoll 1,4 lectin nanoelectrode for different fetuin concentrations (0.5–25 $\mu\text{g mL}^{-1}$) obtained by SWV measurements in $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ (0.005 mol L^{-1}) prepared in KCl (0.1 mol L^{-1}); (b) Electrochemical response of the Cramoll 1,4 lectin nanoelectrode for interferent molecules: (1) fetuin (25 $\mu\text{g mL}^{-1}$); (2) glucose (500 $\mu\text{g mL}^{-1}$); (3) galactose (500 $\mu\text{g mL}^{-1}$); (4) fructose (500 $\mu\text{g mL}^{-1}$); (5) fucose (500 $\mu\text{g mL}^{-1}$) and (6) BSA (5 g L^{-1}).

equation: $\Delta I\% = [I_{(\text{Cramoll } 1,4)} - I_{(\text{Cramoll } 1,4\text{-glycoprotein})}] / I_{(\text{Cramoll } 1,4)} \times 100$, where $I_{(\text{Cramoll } 1,4)}$ is the current value of the biosensor as-prepared and $I_{(\text{Cramoll } 1,4\text{-glycoprotein})}$ is the current value after exposure to glycoprotein. The analytical responses of the biosensor showed a linear enhance in the $\Delta I\%$ with the augment of fetuin concentrations (Fig. 5a). The curve revealed the following linear regression equation: $\Delta I_{(\text{fetuin})} = 0.49[\text{fetuin}] + 0.81$ ($r = 0.994$, $p < 0.001$, $n = 7$). The analytical sensibility of the nanoelectrode for fetuin was of 2460 $\mu\text{A M}^{-1} \text{cm}^{-2}$. The major analytical sensibility to fetuin confirms the greater affinity of the Cramoll 1,4 in detection of the glycoproteins for characterization of serum glycoproteins (Lima et al., 1997; Oliveira et al., 2008). The limit of detection (LOD) was statically determinate as: $3.3\text{RSD}_{\text{blank}}/b$, where $\text{RSD}_{\text{blank}}$ is the RSD of the blank and b is the slope calculated of the analytical curve. The LOD achieved by nanoelectrode for fetuin was 0.017 $\mu\text{g mL}^{-1}$ that was found higher than lower LOD described biosensor for fetuin glycoprotein (Bertok et al., 2013; Kluková et al., 2014), although this biosensor has used the SNA I lectin from *Sambucus nigra*. On the other hand, fetuin biosensors with similar or lower LOD described require impedimetric responses

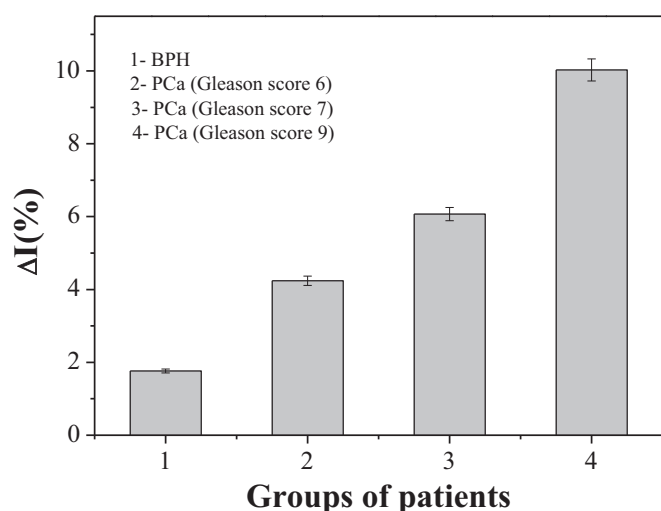


Fig. 6. Electrochemical response of the Cramoll 1,4 lectin nanoelectrode for serum samples of patients with (1) BPH, (2) PCa (Gleason score 6), (3) PCa (Gleason score 7) and (4) PCa (Gleason score 9). Measurements obtained by SWV assays performed in $\text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ (0.005 mol L^{-1}) solution prepared in KCl (0.1 mol L^{-1}).

(Oliveira et al., 2011), which involve more sophisticated and complicated analyses than SWV techniques.

3.5. Reproducibility, repeatability, long-term stability and interference study

The reproducibility and repeatability are crucial parameters to evaluate the operational performance of the biosensor. The reproducibility was estimated through the response of 8 different electrodes incubated with fetuin ($5 \mu\text{g mL}^{-1}$) under optimal conditions. The nanoelectrodes showed an acceptable RSD of 4.24% ($< 5\%$). The repeatability of the nanoelectrode, pre-incubated with fetuin ($5 \mu\text{g mL}^{-1}$), was obtained through 20 successive measurements with an interval of 1 min. The nanoelectrode exhibited an excellent repeatability with an RSD of 1.24% ($< 5\%$).

The long-term stability of the lectin nanoelectrode was tested using six electrodes. The electrodes were incubated with a solution of fetuin ($5 \mu\text{g mL}^{-1}$) under optimal conditions in the intervals of 1, 3, 5, 7 and 15 days. The nanoelectrode during the study was maintained in a moist chamber at 4°C . The SWV responses revealed a good stability showing that 79.55% of the analytical response was preserved after 15 days.

Prior to application in assays with serum samples, an interference study was performed by addition of pattern with several species in order to investigate the specificity of the biosensor. The nanoelectrodes were incubated with $10 \mu\text{L}$ of solution containing only fetuin ($25 \mu\text{g mL}^{-1}$), as control, and a fetuin solution ($25 \mu\text{g mL}^{-1}$) plus interferences. Five interferences were selected and among these BSA, an abundant protein present in serum sample, and monosaccharides which can be found in bloodstream and glycoproteins. The interferences on the solutions were found at final concentrations: $500 \mu\text{g mL}^{-1}$ glucose, $500 \mu\text{g mL}^{-1}$ galactose, $500 \mu\text{g mL}^{-1}$ fructose, $500 \mu\text{g mL}^{-1}$ fucose and 5 g L^{-1} BSA. The degree of interference was determined considering the response of current for control sample as 100%. Calculated recoveries were $105.1 \pm 1.01\%$ for glucose, $100.18 \pm 1.00\%$ for galactose, $94.8 \pm 0.95\%$ for fructose, $102.06 \pm 1.02\%$ for fucose and $103.45 \pm 1.03\%$ for BSA (Fig. 5b), with average value of $101.12 \pm 1.00\%$. The results obtained revealed a low degree of interference (less than 10%) on nanoelectrode response, being higher for fructose.

3.6. Glycoprotein detection in serum samples with lectin nanoelectrode

The proposed lectin nanoelectrode was applied to detection of glycoproteins in sera of patients with BPH and PCa confirmed via histopathological examination. The Cramoll 1,4 nanoelectrode was incubated with pools of serum samples diluted 1:30 in PBS and then submitted to SWV analysis in $0.005 \text{ mol L}^{-1} \text{K}_3\text{Fe}(\text{CN})_6/\text{K}_4\text{Fe}(\text{CN})_6$ prepared in $0.1 \text{ mol L}^{-1} \text{KCl}$. The results in Fig. 6 show that the Cramoll 1,4 nanoelectrode was able to screen glycosylation profiles of patients with BPH and PCa. The analytical response exhibited a proportional increase in $\Delta I\%$ value for PCa samples with Gleason score of 6, 7 and 9. The increase in electrochemical response was statistically significant for all observed samples according to ANOVA test ($p < 0.02$). The data analysed by Student *t*-test, at a 95% confidence level, show highly significant difference between all PCa samples (scores 6, 7 and 9) and BPH sample ($p < 0.01$).

Quantitative alterations of glycoproteins in sera of PCa patients analysed by mass spectrometry have demonstrated that glycan levels tend to rise with the Gleason score and predict the prognosis of cancer (Drake et al., 2006; Saldova et al., 2011). Patients with PCa Gleason scores 7 or higher to this have increased risk of metastasis and recurrence after therapy (Pierorazio et al., 2013). This proposed nanoelectrode showed an excellent performance and is the first report using Cramoll lectin to distinguish benign hyperplasia and Gleason scores in serum samples. However, studies that are more detailed associated with immunoassay in human serum for PCa diagnostic concentrations are necessary to indicate this system as an alternative approach to apply in clinical routine.

4. Conclusions

Cramoll 1,4 lectin nanoelectrode based on COOH-CNT and PLL showed to be sensitive to differentiate serum glycoprotein profiles of patients with BPH and PCa advanced Gleason score, providing a potential tool for PCa staging. However, additional studies with a higher number of samples and correlation with other techniques are required to validate these important finding, taking account that PCa is a multifactorial disease.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2016.05.004>.

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APÊNDICE C – ARTIGO PUBLICADO NA REVISTA CLINICA CHIMICA ACTA**Glycosylation Products in Prostate Diseases**

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Review

Glycosylation products in prostate diseases

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ABSTRACT

Although prostate cancer is notable for its high incidence and mortality in men worldwide, its identification remains a challenge. Biomarkers have been useful tools for the specific detection of prostate cancer. Unfortunately, benign prostate diseases cause similar alterations in screening assays thus reducing the potential for early and specific diagnosis. Changes in glycan and glycoprotein expression have often been associated with the onset and progression of cancer. Abnormal glycans and glycoproteins have been reported as new biomarkers of prostate metabolism that can distinguish benign prostate disease and cancer in non-aggressive and aggressive stages. Carbohydrate-binding proteins known as lectins have been valuable tools to detect these changes, investigate potential biomarkers and improve our understanding aberrant glycosylation in cancer. Here we review progress in elucidating prostate disease and discuss the roles of glycans in the differential detection of benign and cancerous prostate disease. We also summarize the lectin-based tools for detecting glycosylation changes.

1. Introduction

Currently, thousands of people worldwide are diagnosed with various types of cancer. Cancer is a group of diseases characterized by the growth and multiplication of abnormal cells, and if not controlled, can easily lead to death. Among the cancers that affect the male population, prostate cancer is the second most common, surpassed only by lung cancer [1], and the second most frequent cause of cancer death in men in the United States, according to estimates by the American Cancer Society [2].

The latest global estimate also pointed to prostate cancer (PCa) as the second most common cancer in men, with about 13.5% of incidence in 2018. Incidence rates of this cancer remain high in the world's highest income regions, including North America, Western and Northern Europe, and Australia-New Zealand, while mortality rates from PCa tend to be higher in low average income areas, and parts of South America, the Caribbean as well as Sub-Saharan Africa. The very high incidence rates observed in developed countries are partly due to

the widespread dissemination of prostate-specific antigen (PSA) screening and the high longevity of the population in those countries [3].

Cancer mortality can be reduced by early diagnosis and treatment. PCa screening and diagnosis is based on the PSA test and clinical evaluation by digital rectal exam. When abnormalities suggestive of cancer are identified, the diagnosis is confirmed by prostate biopsy. PSA is a glycoprotein synthesized by prostatic cells, considered the prostate tumor marker. Normally, this is secreted into the blood at very low concentrations ($0 \leq 4 \text{ ng mL}^{-1}$), however it can increase and reach quite high concentrations in individuals with prostate cancer [4]. The use of the PSA test for prostate cancer diagnosis is controversial, since benign prostate diseases, such as benign prostatic hyperplasia (BPH) and prostatitis can also be associated with increased serum PSA levels ($> 4 \text{ ng mL}^{-1}$), while a significant incidence of cancer has also been reported at reduced levels of PSA [4], hindering the diagnosis of prostate cancer and benign prostate diseases. Thus, many cases are sent unnecessarily for biopsy, highlighting the poor sensitivity and

Abbreviations: AQT, alpha-1-antichymotrypsin; Asn, asparagine; BPH, Benign prostatic hyperplasia; BPSA, Benign PSA; cPSA, complexed PSA; DRE, Digital rectal exam; EIS, Electrochemical impedance spectroscopy; FDA, Food and Drug Administration; fPSA, free circulating PSA; IARC, International Agency for Research on Cancer; KLK, Human kallikrein; LTA, *Lotus tetragonolobus* agglutinin; MAA, *Maackia amurensis* agglutinin; PCa, Prostate cancer; PSA, Prostate specific antigen; Ser, serine; SNA, *Sambucus nigra* agglutinin; Thr, threonine; TRUS, Transrectal ultrasound; WHO, World Health Organization

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specificity of the PSA test for the diagnosis of the disease [5]. Recent research has focused on the development of alternative methods to identify new, more sensitive and specific markers for prostate cancer.

Methods based on glycosylation pattern analysis represent new approaches for the detection of several types of cancer, including prostate cancer. Changes in the glycosylation of free glycoproteins and on cell surfaces are often observed in cancer progression, resulting in specific glycosylation profiles that can be identified, quantified and used as cancer biomarkers [6]. PSA, for example, is found in the bloodstream in different forms and patterns of glycosylation, which commonly change in prostate cancer [7]. In addition to PSA, other glycoproteins derived from prostatic tissue, serum and seminal fluid from individuals with prostatic tumors have revealed significant changes in the glycosylation pattern that allow distinguishing between BPH and prostate cancer [8,9].

Lectins are natural and non-immune proteins with carbohydrate-binding sites, interacting with carbohydrates in a specific and reversible way. They have been widely used in the development of diagnostic methods to investigate disease-related glycosylation patterns, which can detect glycosylation alterations in circulating and cell-surface glycoproteins and assist in cancer diagnosis in the early stages of development [10,11]. Studies of glycosylation patterns in serum glycoproteins have employed highly accurate and sensitive techniques coupled to lectins, such as affinity chromatography, mass spectrometry, histochemistry, enzymatic immunoassays, point-of-care testing and biosensors [12,13].

In this review, we highlight advancements in the diagnosis of prostate diseases related to biomarkers, focusing on glycosylation changes and their role in identifying potential new targets based on altered glycans and glycoproteins. The differential detection of benign and malignant prostate diseases may be obtained by profiling these biomarkers. We also discuss the role and progress of lectin-based analysis for detection of glycosylation changes.

2. Prostate diseases

2.1. Benign

Benign diseases of the prostate represent an important health problem mainly for aging men through their associated symptoms and complications. The prostate is a walnut-sized gland that is part of the male reproductive system, located in the lower abdomen, below the bladder and surrounding the urethra at the neck of the bladder. This gland produces a fluid that nourishes and protects the sperm contained in the semen and is composed primarily of acid phosphatase, citric acid, fibrinolysin, PSA, proteolytic enzymes, and zinc. While size of the prostate is approximately that of a ping-pong ball, it may be much larger in elderly men due to a benign growth of the gland that occurs in most men from adulthood [14]. Prostatitis and benign prostatic hyperplasia or enlarged prostate, are benign diseases that affect the prostate [15].

Prostatitis is a commonly painful condition associated with bacterial infection or inflammation of the prostate gland (Fig. 1), which can affect men of any age, most commonly men younger than age 50. This condition affects about two million patients each year in the United States. Four types of prostatitis have been identified: chronic prostatitis or chronic pelvic pain syndrome (CPPS), acute bacterial prostatitis, chronic bacterial prostatitis and asymptomatic inflammatory prostatitis. The symptoms can vary depending on the cause and individual factors [16,17].

The symptoms of CPPS can involve pain or discomfort in some areas including the lower abdomen, between the scrotum and anus, the penis and the lower back; pain during or after ejaculation and urination; alteration in urinary frequency and a weak or interrupted urine stream [18]. The symptoms of acute bacterial prostatitis are severe and require immediate medical care. These include urinary frequency and urgency;

nocturia; fever; pain in the genital area, lower abdomen and during urination; urinary blockage; nausea; vomiting and urinary tract infection. In the case of chronic bacterial prostatitis, the symptoms are similar to acute bacterial prostatitis; however, it is not severe and generally occurs after treatment of acute bacterial prostatitis and urinary tract infection [19]. The diagnoses of prostatitis are based on personal and family medical history, a physical exam such as digital rectal exam, and laboratory tests such as urinalysis, semen analysis, blood tests, transrectal ultrasound and biopsy. The treatment can vary according to prostatitis type, and includes antibiotics, anti-inflammatory drugs and other medications to decrease the symptoms [16,20]. In asymptomatic inflammatory prostatitis, men do not show symptoms, with the diagnoses obtained through testing for other urinary tract or reproductive disorders; this condition does not require treatment.

Benign prostatic hyperplasia (BPH) is a non-cancerous condition commonly associated with aging, which is more prevalent in men older than 50, reaching more than half of the male population in the seventh decade of life and almost all in the eighth decade [21,22]. This condition is characterized by benign enlargement of the prostate gland resulting from progressive hyperplasia that begins around age 25 and continues during the man's life (Fig. 1). Evidence suggests that the increase in levels of androgen hormones and estrogen within the prostate associated with aging men induces prostate cell growth and tissue hyperplasia, leading to obstruction of the bladder neck or urethra, making it difficult to urinate and causing incomplete emptying of the bladder with urinary retention [21,23]. Symptoms suggestive of BPH are urinary frequency and urgency, nocturia, a weak or an interrupted urine stream, urinary retention, difficulties to start a urine stream, urinary incontinence and pain during ejaculation and urination.

Some conditions are related as predisposing factors for BPH development, like age of 40 years and older, family history, obesity and circulatory diseases [24]. Treatment includes medication to decrease symptoms or surgical therapy when the symptoms are severe. Evaluation of the patient with BPH is performed through anamnesis with application of the prostate symptom score, physical analysis with digital rectal exam, laboratory evaluation through serum PSA, urinalysis and renal function, as well as imaging and urodynamics [23,25].

2.2. Cancerous

More than any other type, PCa is considered a cancer of senior citizens, occurring mainly in elderly men. About 62% of the world's diagnosed cases occur in men aged 65 years and older. Another risk factor to be considered is ethnicity, since PCa is approximately twice as frequent in Afro-descendants as in men of other races [26]. Genetic studies suggest that family predisposition may account for 5–10% of cases. Having a father or sibling with PCa, especially before 60 years of age, increases the chance of men developing the disease by more than twice. The risk is higher for men with several affected relatives, particularly if their relatives were young when cancer was found [27–29]. Other risk factors are investigated in relation to this disease, such as genetic mutation, diet, obesity, smoking, inflammation in the prostate, sexually transmitted infections and vasectomy [29,30].

Most PCa originate in prostate glandular cells, called adenocarcinoma; some can grow and spread quickly to other organs, but most grow slowly. Autopsy studies show that many elderly men and some young men who died of other diseases had PCa which never affected them during their lives. In its early stages, PCa usually causes no symptoms, a fact that makes early detection of the disease difficult, especially in individuals who do not perform screening tests. In more advanced disease, tumor growth often presses and obstructs the urethra (Fig. 2), causing the characteristic symptoms: weak or disrupted urine flow; inability to urinate or difficulty controlling urine flow; urinary frequency and urgency, especially at night; presence of blood in the urine and pain or burning on urination [31]. Being in an advanced state, PCa can spread through the body, reaching other organs and

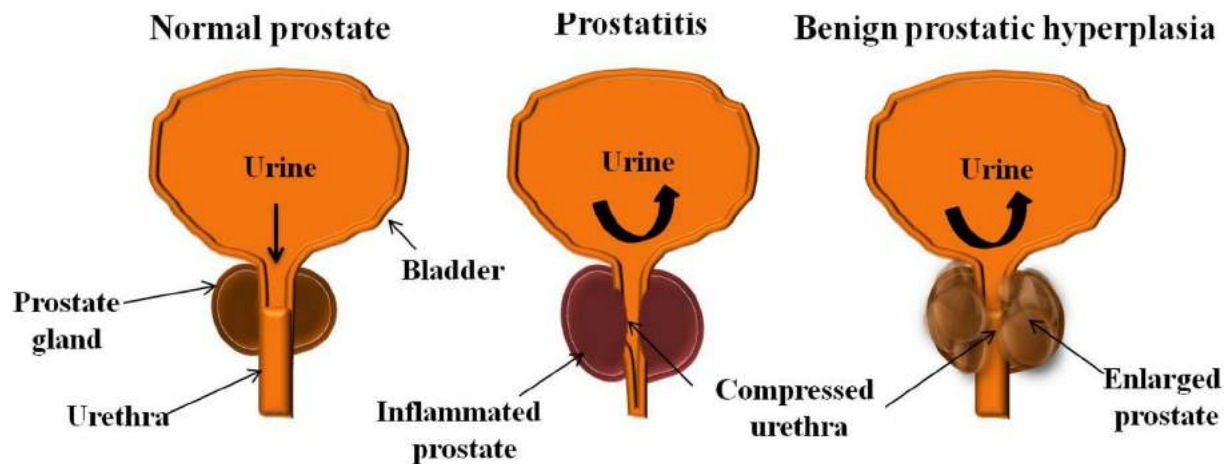


Fig. 1. Representation of normal prostate gland, prostatitis and benign prostatic hyperplasia.

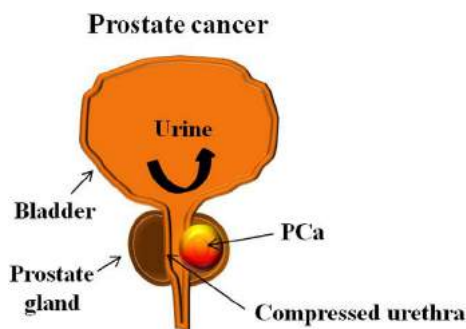


Fig. 2. Prostate gland with cancer tumor, PCa, compressing the urethra.

especially bones, including the spine, hip, ribs, femur, among other areas. Spinal pain in an individual at the age of risk or spontaneous fracture of the femur without any trauma may be caused by spread of the tumor [32].

2.2.1. PCa diagnosis

The diagnosis of PCa is made through screening by digital rectal exam and blood PSA test on an annual basis, beginning at age 50. In men belonging to risk groups (Afro-descendants or a relative diagnosed with PCa before the age of 65), the screening should begin at age 45, and at age 40 in men with several relatives already diagnosed with PCa at an early age [2,32].

PSA is a glycoprotein produced in prostate tissue and secreted into seminal fluid, being also found in serum and urine. The PSA test is routinely utilized as a prostate tumor marker for PCa screening. Most of the PSA produced is present in the seminal fluid at relatively high concentrations ($0.5\text{--}5\text{ mg mL}^{-1}$) and only a small fraction is released into the bloodstream, where the concentration varies from 0 to 4 ng mL^{-1} [33]. In healthy subjects, serum PSA levels are commonly $\leq 0.1\text{ ng mL}^{-1}$ and are considered normal until 4 ng mL^{-1} . However, any alteration in prostate gland architecture allows for higher concentrations of PSA to enter circulation. Values above 10 ng mL^{-1} suggest strong evidence of cancer; values between 4 and 10 ng mL^{-1} are classified into the diagnostic gray-zone category, not allowing a clear differentiation between PCa and other pathologies. Thus, biopsy is indicated for PSA levels above 4 ng mL^{-1} [34].

Although the PSA test revolutionized cancer testing since its first approval in 1986 by the US Food and Drug Administration (FDA), its clinical specificity for detecting PCa is questionable. Other prostate diseases such as BPH and prostatitis also cause increased PSA in circulation, and there are even cases of PCa that occur when PSA levels are considered normal [35]. Studies have reported a risk of 6.6–26.9% of

PCa incidence when PSA levels are $\leq 4\text{ ng mL}^{-1}$ [35]. These factors confirm the limitations in terms of sensitivity and specificity of the PSA test for PCa diagnosis. Considering a cutoff point at 4.0 ng mL^{-1} , the PSA test presents an estimated sensitivity of 71% and a specificity of 46% [36]. Studies estimate that its positive predictive value is around 28%, which means that about 72% of patients with altered PSA are submitted to unnecessary biopsies [33,36].

When total PSA is below 4 ng mL^{-1} , or between 4 and 10 ng mL^{-1} , it is recommended to consider the free/total PSA ratio, which is inversely proportional to prostate and tumor volume, and the Gleason score. Cutoff values can vary with age range, being considered values of 10–25%. Results below cutoff values are considered suggestive of PCa, and above are suggestive of BPH [36,37]. Thus, the % free/total PSA ratio has shown greater sensitivity and specificity when compared with the PSA test and can decrease the number of unnecessary biopsies [38].

Studies also indicate that PSA expression is not tissue specific. The antigen has already been detected by immunohistochemistry in the endometrium; secretions from male periurethral and anal glands, breast tumors, lung adenocarcinoma, among other tumors [39]. Considering this statement and the other factors mentioned, it is important to associate the PSA test with other assays, such as digital rectal exam, for cancer detection.

The digital rectal exam (DRE) has been recommended because of the increased possibility of PCa diagnosis, especially in patients with normal levels of PSA. The presence of alterations observed by DRE is a strong indication for biopsy. Studies show that up to a fifth of patients with PCa show alterations only in the DRE, and among these, a third have PCa, even with normal PSA [36,40]. DRE sensitivity ranges from 48% to 59% and specificity from 89 to 92%. Positive predictive value is estimated between 28% and 40%. Despite the recommendation to perform the examination at each visit, the adherence to clinical practice of DRE is about 20%, which is considered low [36,40,41]. If there are abnormalities in digital rectal exam or PSA levels are equal to or $> 4\text{ ng mL}^{-1}$, or both, a histopathological exam of tissue obtained through prostate biopsy is indicated [36].

Transrectal ultrasound-guided (TRUS) prostate biopsy is the indicated method to confirm diagnoses of PCa when the patient shows abnormal PSA levels and/or a palpable alteration through DRE. This procedure involves insertion of an ultrasound probe about the size of a finger into the rectum, followed by biopsy with a spring-driven needle core biopsy device, or biopsy gun, for obtaining prostatic tissue for histological evaluation. The prostate cells collected by biopsy are analyzed and compared to normal prostate cells. The higher the degrees of abnormality found in prostate biopsy cells in relation to normal cells, the more aggressive is the cancer, and its spread will be faster. Following histological analysis, PCa is graded using the modified

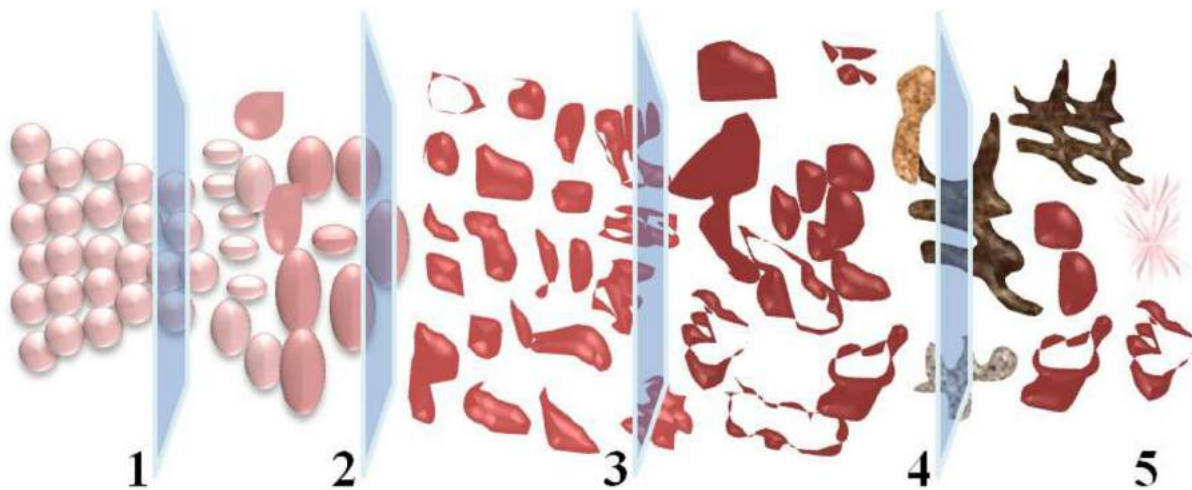


Fig. 3. Representation of prostate cancer cells in the Gleason grading system.

Gleason system.

The Gleason system [42] describes the degree of tumor aggressiveness [35]. Scoring of the Gleason system scale is based on histological patterns ranging from grades 1 to 5 (Fig. 3), with grade 1 being the least aggressive form and grade 5 representing the anaplastic tumor. To obtain the total Gleason score the two most frequent areas of the tumor are graded from 1 to 5 and the results are added, ranging from 2 to 10. The lower the score, the less aggressive the tumor is and the better the patient's prognosis, and vice versa. However, the evolution of histological diagnosis and treatment of PCa led to revisions of the Gleason system, which resulted in some modifications. This more recent classification does not assign scores from 2 to 5, and some patterns previously classified as score 6 are now classified as 7, and the tumors are classified into three levels: 6, 7, and 8–10.

Despite these changes, the Gleason system has some deficiencies, such as the final classification does not recognize that the tumors 3 + 4 and 4 + 3 are grade 7, as well as 8 and 9–10 scores show very different prognoses. Another deficiency is the lowest score to be considered is equal to 6, although the scale starts at grade 2, suggesting the cancer is more aggressive than it is, and arousing expectations and fear of the diagnosis by patients [43]. In 2013, a new Gleason classification system was proposed in an attempt to minimize these deficiencies. It consisted of five separate groups in grades from 1 to 5: the grade 1 group (Gleason score < 6), grade 2 group (Gleason grade 3 + 4 = 7), grade 3 group (Gleason grade 4 + 3 = 7), grade 4 group (Gleason grade 8); and grade 5 group (Gleason Degrees 9–10) [43]. In this way, more suitable classification and treatments can be obtained, mainly of low-grade PCa.

Despite allowing for definitive diagnosis of PCa, studies have shown that in up to a third of cases, cancer is not detected in the initial biopsy [44]. When the first biopsy is negative, a second biopsy is recommended, which when negative also reduces the possibility of development and diagnosis of PCa [41].

3. PSA biochemistry

PSA (or human kallikrein 3; KLK3) is a 237-amino acid serine protease with chymotrypsin-like activity, androgen regulation, and belongs to the kallikrein family. In contrast to the rodent tissue kallikrein (KLK) gene families, the human KLK gene family seems to consist of just three genes: KLK1 (encoding tissue kallikrein), KLK2 (encoding glandular kallikrein), and KLK3 (encoding PSA), which are encoded by a cluster of genes located in a region of 300 kb on human chromosome 19q13.4 [45–48]. PSA is synthesized by the prostate ductal and acinar epithelium, being initially translated as a prepropeptide with a 17-amino acid leader sequence that is cotranslationally cleaved during passage

through the secretory pathway, producing an enzymatically inactive protein precursor (proPSA with 244 amino acids) named [–7]proPSA. This precursor contains an extra 24 amino acids that are activated by other kallikreins (hK2 or hK4), which cleave proPSA between the arginine at position 7 and the isoleucine at position 8, such that the N-terminal amino acid of the mature active PSA protein is isoleucine, yielding a 28–32 kDa mature active protease [49,50] consisting of five intrachain disulphide bonds and a single N-linked glycan [8,51–53].

PSA is highly abundant in the seminal fluid, present at concentrations of 0.5 mg ml^{-1} and greater [48,54], synthesized by both healthy and diseased prostate tissue which cleaves the proteins semenogelin I and II, which leads to the liquefaction of the semen [55].

It is important to note that truncated forms of proPSA could be also generated by cleavage. Isoforms with leader peptide variants of one, two, four, and five amino acids, named [–1]proPSA, [–2]proPSA, [–4]proPSA, and [–5]proPSA, respectively, have been identified [56]. Still in the lumen, mature active PSA is cleaved at specific sites, generating the BPSA isoforms (known as benign PSA) and iPSA (inactive PSA). All of these PSA isoforms are found as free circulating PSA (fPSA), which corresponds to 16% of total PSA [60]. Most of the circulating PSA corresponds to complexed PSA (cPSA) bound to protease inhibitors, mainly alpha-1-antichymotrypsin (AQT) and alpha-2-macroglobulin [57]. Fig. 4 represents PSA and the biosynthesis of its isoforms.

ProPSA subforms have been identified as the predominant constituents of fPSA. Several studies have shown the specificity of proPSA for detection of prostate cancer, especially [–2]proPSA and [–4]proPSA. Increased expression of [–2]proPSA has been associated with increased cancer aggressiveness and tends to accumulate in the serum of men with PCa [58]. Benign PSA (BPSA) is a distinct, inactive form of PSA that is prone to internal degradation at amino acid sites Arg85-Phe86, Lys145-Lys146, and Lys182-Ser183 (Fig. 5). BPSA is predominantly found in the prostate transition zone of men with BPH; in addition, it is thought to arise from post-translational cleavage by specific proteases in hyperplastic BPH tissue [59].

The emergence of the importance of PSA for the detection of prostate cancer and its potential use as a biomarker for residual or recurrent disease has been reported [60]. Those authors showed that patients with PCa had high serum levels of PSA; in addition, they observed that PSA increased with advanced clinical disease, proportional to tumor volume, and its levels in serum reached undetectable values after radical prostatectomy [61]. After three decades, PSA continues to be the most widely used oncological biomarker in medicine and is used as a screening method to detect prostate cancer [62]. Although the PSA test has previously been considered the gold standard biomarker for PCa, recent research shows that screening based on PSA does not efficiently

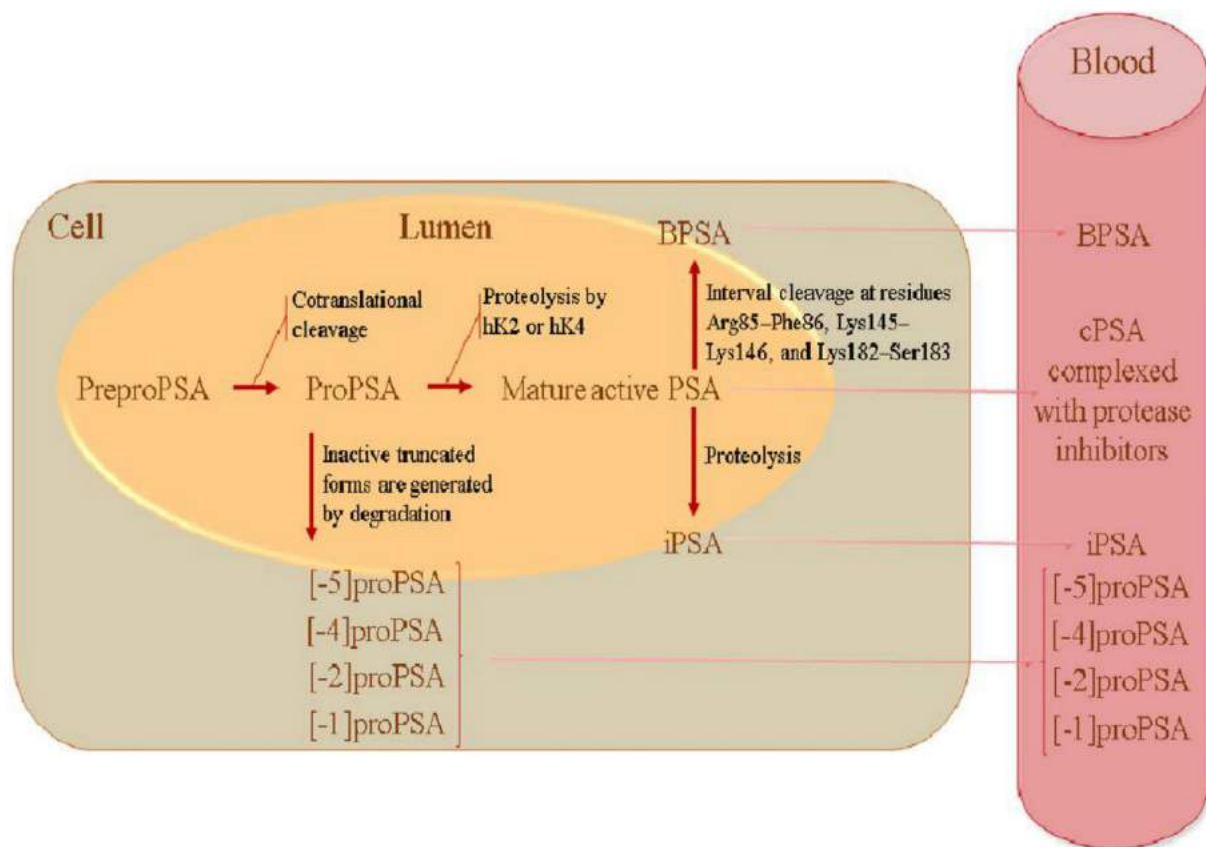


Fig. 4. PSA and biosynthesis of its isoforms. PSA is synthesized as a prepropeptide with 261 amino acids, which is cleaved by cotranslation in the cell to generate [−7]proPSA, an inactive precursor protein. Cleavage within the propeptide yields inactive, truncated variants of PSA. In the lumen, hK2 acts as the major activating enzyme, cleaving [−7]proPSA into mature, active PSA with 237 amino acids. Additional isoforms are cleaved at specific sites, generating BPSA and iPSA. The majority of the mature, active PSA that enters into circulation rapidly binds to protease inhibitors, primarily α 1-antichymotrypsin. The remaining, unbound PSA circulates as free PSA.

detect PCa. In view of this, the serum PSA test is complemented with parameters such as PSA isoform-specific tests, PSA kinetics, and PSA density. It is important to note that the specificity and sensitivity of PSA as a diagnostic tool have improved markedly with an increased understanding of the molecular isoforms of PSA [8].

Several studies have illustrated the cancer specificity of proPSA and highlighted [−2]proPSA and [−4]proPSA as important isoforms for the detection of prostate cancer [63–65]. The distinct biochemical properties of [−2]proPSA and [−4]proPSA result in enhanced stability in cancerous tissues, as these isoforms cannot be converted by hK2 in mature PSA. So, increased [−2]proPSA expression could be directly linked with more aggressive cancers [8].

In essence, PSA is an organ-specific biomarker of the prostate, but it cannot be considered a cancer-specific biomarker. Thus, many groups of researchers are proposing the association of glycans with PSA and its tumor specificity for clinical diagnosis.

4. Glycosylation

Glycosylation is an enzymatic process in which carbohydrate molecules known as glycans become attached to other biological molecules such as proteins and lipids, thus affecting different biological pathways. These processes require glycans for a range of purposes, including cell adhesion, endocytosis processes, molecular trafficking and clearance, receptor activation, and signal transduction [66]. Regarding glycans and proteins, the produced glycoproteins are often disposed on cell surface or secreted into the bloodstream. This mechanism is part of the secondary processing of proteins in cells, displaying a critical role in determining protein structure, function, stability, and protection

against proteases [67,68].

It is conservatively estimated that around 2% of all genes in the human genome encode for proteins involved in various aspects of glycosylation and that half of all cellular proteins are directly glycosylated in some form [69,70]. Another estimate reports the human proteome as 70% glycosylated [71]. Glycans are attached to proteins via amino groups (N-glycans) or hydroxyl groups (O-glycans); fucose, galactose, glucose, mannose, N-acetylglucosamine, N-acetylgalactosamine, and sialic acid are the major building blocks of human N- and O-glycans [67,72] (Fig. 6).

The structural complexity of glycoproteins arises from several key factors, including various combinations in which monosaccharide subunits can be linked together. Factors such as the anomeric position of the linkage (α or β configuration), the presence and degree of branching, the presence of more than one glycosylation site on each individual protein, and modifications made by non-carbohydrate substituents (such as sulphate, phosphate, and acetate) can be considered [8,73].

In the glycosylation process, a series of glycoenzymes, such as glycotransferases and glycosidases, act upon a protein as it passes through the endoplasmic reticulum to the Golgi apparatus. The structural modifications made by these glycoenzymes affect cell-specific glycan expression patterns. Glycans have numerous functions and can be involved in the correct conformation and folding of the protein. They can affect the above-mentioned characteristics (structure, function, stability, and protection against proteases), in addition to serving as recognition motifs for lectins (carbohydrate-binding proteins) [8].

Briefly, in N-glycosylation pre-assembled blocks of sugars are transferred during the amide group transduction of an asparagine

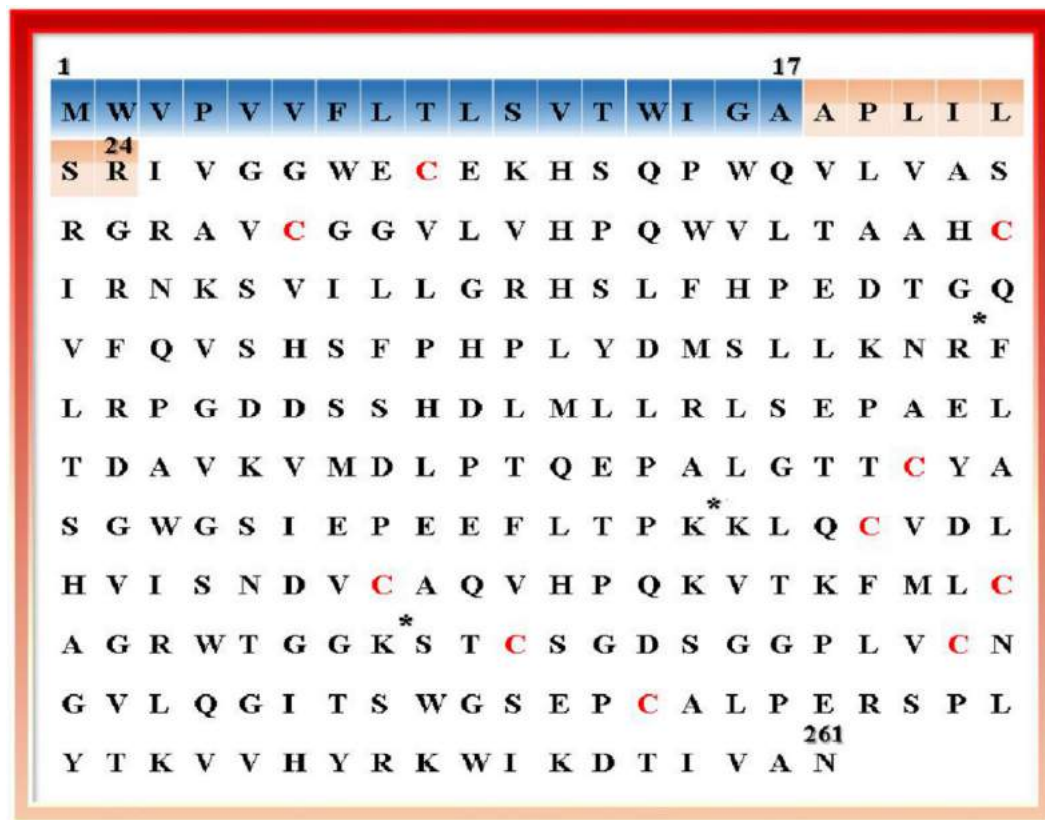


Fig. 5. Complete amino acid composition of PSA with highlighted cleavage points. The 17-amino acid leader sequence is highlighted in blue and the inactive, truncated cleavage region is highlighted in beige. Intrachain cysteine bonds are shown in red, and star symbols denote benign PSA internal cleavage sites. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

residue, whereas in *O*-glycosylation the sugars are added to the hydroxyl of the amino acids serine and threonine. > 200 types of transferases and other additional regulatory proteins, expressed in specific patterns and levels in each tissue, participate in the synthesis of a diverse set of glycans [74,75].

O-glycosylation is a covalent post-translational modification in which monosaccharides are transferred to amino acid residues of serine (Ser) and/or threonine (Thr) by an *O*-glycosidic bond. The synthesis of *O*-glycans occurs in the Golgi apparatus and is mostly started by the activity of polypeptide *N*-acetylgalactosamine-transferases that link a single *N*-acetylgalactosamine residue to Ser or Thr, thus forming the antigen. In contrast to this glycan type, which is termed mucin-type *O*-glycans, a wide range of non- mucin *O*-glycans exist, namely α -linked *O*-fucose, β -linked *O*-xylose, α -linked *O*-mannose, β -linked *O*-*N*-acetylglucosamine, α - or β -linked *O*-galactose, and α - or β -linked *O*-glucose glycans [76].

In contrast to *O*-glycosylation, *N*-glycosylation occurs during translation of target proteins by the addition of glycan structures to the amino group of asparagine (Asn) residues at the consensus motif asparagine-X-serine/threonine, in which X is any amino acid except proline. Instead of step-by-step addition of single sugar residues, *N*-glycosylation starts with synthesis of a dolichol-bound oligosaccharide precursor in the endoplasmic reticulum, consisting of 14 sugar moieties including mannose 9 residues. Then, this oligosaccharide is transferred to an Asn residue within the nascent polypeptide by the oligosaccharyl-transferase protein complex. After the transference, the correct folding and secretion of the glycoprotein depends on trimming of the glycan precursor in the Golgi, resulting in high mannose glycans. Removal of part of the mannose 9 residues by Golgi mannosidases is the prerequisite of formation of complex or hybrid di-, tri- or tetra-antennary glycans. These can then undergo a variety of extensive modifications

including sialylation, fucosylation, addition of galactose, *N*-acetylglucosamine, among others, resulting in highly complex and heterogeneous structures [76].

Glycome can be conceived as the set of synthesized glycans by a cell under specific conditions. However, the constitution of a glycome can change dramatically in response to subtle changes in the cellular environment, including extensive changes in glycosylation patterns that characterize many diseases [77].

4.1. Glycosylation in prostate cancer

In cancer, glycans are implicated in a range of purposes, including cell dissociation and cell invasion, cell-to-cell interaction, and cell signaling through cell-matrix interaction [78]. Glycans have been demonstrated to play a role in endothelial cells by ensuring the survival of endothelial cells, regulating vascular permeability, and influencing the connection of blood and lymphatic vessels. In addition, glycosylation is also implicated in immune modulation and cancer metastasis [6,66].

The elucidation of the molecular isoforms of PSA, as it concerns PCa, has enhanced the utility of PSA testing for PCa detection. However, still no single test exists that can distinguish between significant and insignificant disease. Recent advances in the field of glycobiology have shown that aberrant glycosylation patterns are a fundamental characteristic of tumorigenesis, including reduction or increase in expression of a particular structure or pattern, suggesting that modified glycoproteins with tumor-specific glycan moieties are viable targets for cancer detection and diagnosis [8,79].

Specific targeting of the single site of PSA glycosylation for structural characterization of the constituent *N*-glycans as potential disease and stage-specific tumor biomarkers has been a longstanding goal. Multiple studies have the aim to distinguish PCa and BPH. For example,

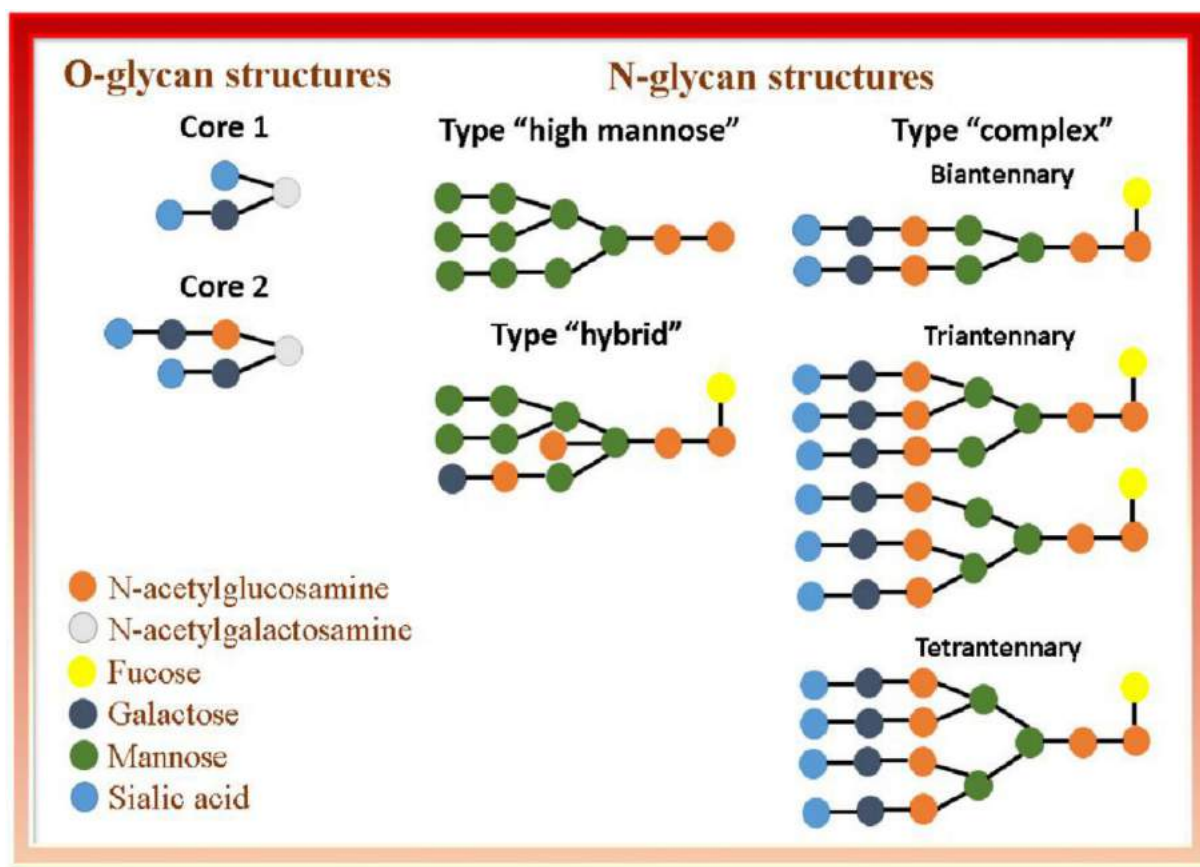


Fig. 6. Typical structures of N- and O-glycans expressed in glycoproteins in human serum.

the possibility has been investigated of distinguishing different stages of PCa and BPH by analyzing the glycosylation patterns in sera from men with BPH and with PCa in Gleason scores 5 and 7. Also, greater expression of fucosylated and biantennary glycans linked to α 2,3 sialic acid has been studied in PCa-derived sera compared to BPH. In addition, a remarkable reduction has been observed in the expression of trigalactosylated and tetrasialylated tri-antennary glycans with a fucosylated outer arm, and increased levels of tetrasialylated tetra-antennary glycans at PCa score 7 in comparison with score 5 [80].

A study based on glycoprotein analysis of tissues from PCa identified 350 glycopeptides, in this case 17 were altered in aggressive PCa; some glycoproteins were also altered, with increased expression (perioestin) and reduction (monoamine oxidase) in aggressive PCa [81]. It is possible to observe that alterations in glycosylation are suggestive of cancer and enable the identification of different stages of PCa, which would not be possible through the PSA test.

Changes in the glycosylation pattern of different forms of PSA from PCa and BPH have also been described, most of them related to sialic acid levels. The presence of α 2,3 sialic acid residues linked to PSA may be useful to discriminate between malignant and benign diseases when comparing the oligosaccharide profiles of fPSA and cPSA in serum and PCa seminal plasma [82]. Another study characterizing glycans present in isoforms (F1-F5) obtained by two-dimensional PSA electrophoresis revealed differences in the degree of sialylation for F3 and F4 isoforms that can distinguish CaP from BPH. F3, which has mono- and disialylated N-glycans, is reduced in PCa when compared to BPH, and decreases gradually with the stage of cancer, while F4, which gradually increases with the PCa stage, presents only the monosialylated form. The results indicated a reduction in sialic acid levels in PCa; the quantification of F3 can aid in the diagnosis of the disease [83].

Another study investigated and compared the levels of fucose and sialic acid in PSA-linked N-glycans in sera of patients with BPH and PCa

having different degrees of aggressiveness. They found a significant reduction in fucose expression and an increase in α 2,3 sialic acid in the PSA of the more aggressive PCa cases; the results allowed authors to distinguish BPH and PCa considered of low risk [7].

These investigations show that the expression of differentiated glycan patterns in both PSA and other glycoproteins may be useful markers for PCa and could offer better specificity than the current serum PSA test.

5. Lectins as biotechnological tools

Lectins are naturally occurring carbohydrate-binding proteins which are able to discriminate different glycan structures and, thus, are useful tools for glycoanalysis. They are involved in crucial physiological events of protein-carbohydrate interactions, such as cell adhesion, migration and interaction [84,85]. Although initially found in plants, lectins are widely distributed among viruses, microorganisms and animals [86]. Unlike antibodies, which are structurally similar, lectins are differentiated by amino acid composition, metal requirement, molecular weight and three-dimensional structure [87].

The association constant of lectins with monosaccharides ranges from 10^3 to $5 \times 10^4 \text{ M}^{-1}$, while among lectins with oligosaccharides, 10^4 to 10^7 M^{-1} , values included in the same range found for antibody-antigen and enzyme-substrate [88,89] associations. Each lectin binds to a specific monosaccharide, oligosaccharide or glycoconjugate through its binding sites that tend to be located on the surface of the protein. This interaction occurs by hydrogen bonds, electrostatic, hydrophobic and Van der Waals interactions [87,89,90]. However, most of these interactions are made by hydrogen bonds due to the availability of hydroxyl groups in the sugars, allowing for the formation of these bonds among amino acids of the binding site in the lectin and carbohydrate. In addition, water molecules participate as bridges in the formation of

hydrogen bonds between the protein and its ligand.

The great variety of lectins and their potential to recognize different carbohydrates have stimulated the use of these proteins as recognition tools in many biotechnological studies [91]. Since there is specificity for mono- and oligosaccharides expressed on the cell surface, lectins can be used for typing blood cells [92] and also, as insecticides, as they impair the development and cause mortality of larvae [93]. Some of these proteins present antimicrobial action, for example, acting as antibacterial and antifungal agents [94] and as antivirals by preventing viral invasion and replication [95]. They have also been used in affinity matrices for recognition and purification of glycoproteins [96] and in the development of drug delivery systems in mucous membranes [97].

Lectins have been widely used in glycoanalysis to identify changes in glycan composition and to elucidate the physiological and pathological mechanisms related to these alterations, as well as the modifications in regulatory genes of enzymes that participate in the glycosylation process [7,98]. An increase or reduction in branching of glycans and expression of monosaccharides such as sialic acid, fucose and mannose at different stages of tumor transformation, or even the appearance of cancer-associated glycosylated antigens, such as the sialyl-Tn antigen (Neu5Ac α 2-6GalNAc-O-Ser/Thr), are changes commonly related to cancer progression and metastasis [7,99–101]. The specificity by which lectins recognize the glycans allows for the identification of tumor-specific alterations, leading to the discovery of new glycosylated biomarkers.

Thus, lectins have been reported as potential recognition tools for disease-related glycans, such as those affecting the prostate. For example, the glycosylation profile of PSA in seminal fluid can be studied using three lectins with different specificity: SNA (*Sambucus nigra* agglutinin type I) with specificity for α -(2,6)-terminal sialic acid, LTA (*Lotus tetragonolobus* agglutinin) which binds to α -L-fucose and MAA (*Maackia amurensis* agglutinin II) recognizing α -(2,3)-terminal sialic acid. After incubating the previously mentioned lectins in the immunosensor with attached PSA, a high electrochemical impedance spectroscopy (EIS) response was observed with LTA and SNA when compared to MAA, suggesting higher concentrations of the binding sugars of LTA and SNA lectins in PSA glycan [102]. In another study, α -2,3 sialic acid linkage was found only on PSA of cancerous origin; this finding was made using MAA lectin in an immunoassay [103]. The glycoprotein fetuin has been applied as an analyte for a biosensor constructed with Cramoll, a glucose- and mannose-specific lectin, extracted from seeds of a leguminous plant, *Cratylia mollis*. Cramoll, when immobilized on a nanostructured electrode, has been shown to be able to differentiate prostate cancer and benign prostatic hyperplasia [104]. Therefore, it is necessary to search for new lectins that may detect aberrant glycans expressed in pathology that affect the prostate; this class of proteins is ubiquitous in nature and can be obtained by easy techniques, in addition to being able to detect subtle changes that may help diagnosis.

6. Conclusion

PSA is an N-glycosylated protein that has been used for diagnosis and monitoring of PCa. However, PSA levels are unable to distinguish aggressive from non-aggressive PCa and other benign prostatic diseases. Altered glycosylation may be expressed in abnormal cells and accompany many diseases, including tumor progression. These changes can be identified as potential glycan biomarkers by comparing the glycosylation profiles between tumor and normal tissues or biofluids at early disease stages, which can potentially improve diagnosis, treatment and patient survival. The identification of potential new biomarkers has been facilitated by glycan-based techniques. Glycans and glycoproteins show specific indications for PCa diagnosis, aggressiveness and prognosis. PSA and other glycoproteins produced in PCa express aberrant glycosylation, which could be used to distinguish between healthy and diseased prostate. The ability of lectins to detect different types of

glycan biomarkers has encouraged the development of lectin-based strategies for selective capture of these glycotargets for cancer in tissues and biofluids. However, confirmatory studies from technical to biological perspectives are required for development of clinically diagnostic, quantitative assays based on glycan biomarker detection.

Declaration of Competing Interest

The authors declare that no competing interests exist.

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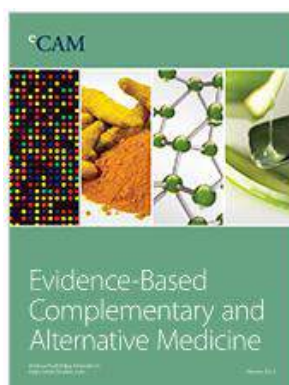
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**APÊNDICE D – ARTIGO PUBLICADO NA REVISTA EVIDENCE-BASED
COMPLEMENTARY AND ALTERNATIVE MEDICINE**

**Lectins, interconnecting proteins with biotechnological/ pharmacological and
therapeutic applications**

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Review Article

Lectins, Interconnecting Proteins with Biotechnological/Pharmacological and Therapeutic Applications

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Lectins are proteins extensively used in biomedical applications with property to recognize carbohydrates through carbohydrate-binding sites, which identify glycans attached to cell surfaces, glycoconjugates, or free sugars, detecting abnormal cells and biomarkers related to diseases. These lectin abilities promoted interesting results in experimental treatments of immunological diseases, wounds, and cancer. Lectins obtained from virus, microorganisms, algae, animals, and plants were reported as modulators and tool markers in vivo and in vitro; these molecules also play a role in the induction of mitosis and immune responses, contributing for resolution of infections and inflammations. Lectins revealed healing effect through induction of reepithelialization and cicatrization of wounds. Some lectins have been efficient agents against virus, fungi, bacteria, and helminths at low concentrations. Lectin-mediated bioadhesion has been an interesting characteristic for development of drug delivery systems. Lectin histochemistry and lectin-based biosensors are useful to detect transformed tissues and biomarkers related to disease occurrence; antitumor lectins reported are promising for cancer therapy. Here, we address lectins from distinct sources with some biological effect and biotechnological potential in the diagnosis and therapeutic of diseases, highlighting many advances in this growing field.

1. Introduction

Considering the diverse diseases and infectious agents that affect the human species and their consequences, the biotechnological field has searched biorecognition molecules from natural or recombinant sources with diagnostic and therapeutic potential. The key for efficient detection, treatment, and healing of pathological conditions is the biorecognition event. The identification of carbohydrate moieties in cell surface and glycoconjugates has been performed with the use of lectins, a heterogeneous group of proteins (or glycoproteins) of nonimmune origin that bind carbohydrates through molecular sites, with high affinity and specificity [1]. These particular lectin sites interact with mono- or oligosaccharides

through no covalent linkage involving hydrogen bonds, van der Waals and hydrophobic interactions, with reversibility, high specificity, and no catalytic or immune activity [2, 3]. Lectins have been isolated from distinct sources such as viruses, bacteria, fungi, algae, animals, and plants [1]; they show specificity to distinct carbohydrates, such as mannose, sialic acid, fucose, N-acetylglucosamine, galactose/N-acetylgalactosamine, complex glycans, and glycoproteins [4, 5]. Lectins recognize carbohydrates and glycoconjugates in cells, tissue sections, and biological fluids, being valuable tools in biotechnology, including diagnosis, and pharmacological and therapeutic applications [6–8].

Some lectins mediate the infection mechanism through the interaction of viral lectins with glycan chains on surface of

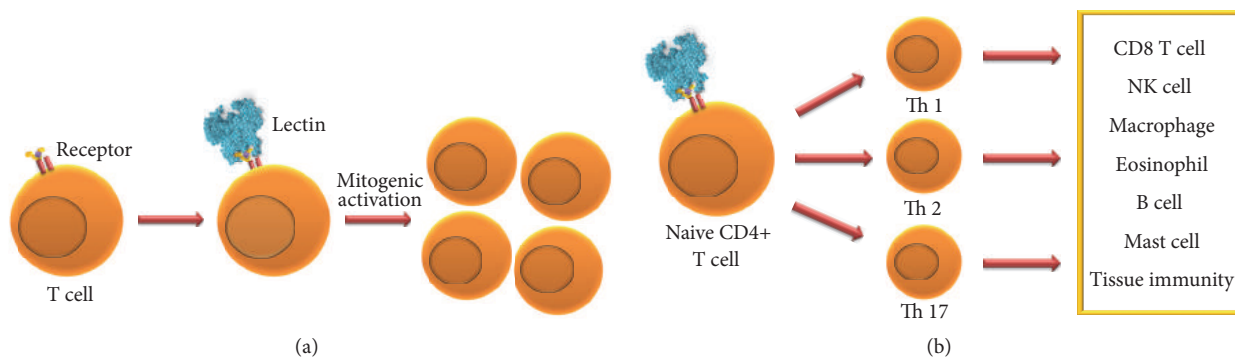


FIGURE 1: Lectins induced in vitro mitogenic activation of T cells (a) and stimulated in vivo Th1, Th2, and Th17 responses (b).

host cells, resulting in the virus entrance into cell [30]. Sialic acid-specific lectins such as influenza virus hemagglutinin were used to search antiviral drugs and inhibitors that can remove or block the sialic acid site in host cells in order to prevent the binding [31]. Lectins from bacteria play a role in the bacterial virulence through the binding between bacterial lectins and specific carbohydrate moieties of host cells, being an important factor for recognition and adhesion [32, 33]. Strong anti-HIV (human immunodeficiency virus) activity in vitro has been described to bacterial lectins [34–36]. Various fungal lectins distributed among mushrooms, microfungi, and yeasts exhibited many physiological effects and biomedical applications. Similar to bacterial lectins, the interaction between fungal lectins and host glycans has an important role in the infection process of fungi [37]. Fungal lectins revealed immunomodulatory, mitogenic, antiproliferative, antitumoral, antiviral, and antimicrobial activities [5, 38].

A large number of algal lectins have also attracted considerable attention for biomedical applications, including anti-HIV, antitumoral, antimicrobial, anti-inflammatory, and antinociceptive activities [39]. Animal lectins play a role in various physiological processes, such as metastasis of cancer, apoptosis pathways, and immunomodulation [40, 41]. Many lectins isolated from animal tissues were investigated as apoptotic agents, immunomodulatory, antiviral therapy, and anticancer drug targets [41, 42]. Plant lectins purified and characterized from distinct plant families and tissues including seeds, barks, leaves, roots, fruits, and flowers exhibited various molecular features, structures, and carbohydrate specificities [43]. The Leguminosae family has the largest group of well-characterized legume lectins, which are interesting due to a variety of carbohydrate specificity and greater availability in nature [44]. In general, a wide range of biological applications has been attributed to plant lectins, such as mediators of inflammatory and immune response; antiviral, antibacterial, antifungal, and antihelminthic agents, healing effect, drug delivery, histochemical markers, biosensing of diseases, and antitumoral activities [3, 45, 46]. This review discusses representative lectins from distinct natural sources, highlighting some biological effects in vivo and in vitro and their potential for diagnosis and therapeutic applications.

2. Lectin Induced Mechanisms of Immunological and Inflammatory Responses

Immunological and inflammatory responses play a role in the protection of an organism against an invasive agent and transformed cells. The immune system acts through two ways known as innate and adaptive immune responses, activated by a group of cells and molecules that promote the inactivation or destruction of aggressive agent [47]. There are neutrophils, eosinophils, basophils, and monocytes/macrophages, with specific functions and ability to produce and release molecules named cytokines, which modulate the activation of immune cells, inflammation, and humoral response. The search for biomolecules that can modulate (up or down) mechanisms of immune response is attractive for adjustment of immune conditions and therapeutic applications in diverse immune response-related diseases and infections.

Several lectins from distinct sources showed immunomodulatory effects, such as mitogenic activity (Figure 1(a)) and induction of T helper type 1 (Th1), type 2 (Th2), or type 17 (Th17) responses (Figure 1(b)). The pivotal step to start immunomodulatory activities of lectins is the binding of lectins to glycans targets on cell surface, which have the role of lectin-receptors [48]. Lectin binding can induce the immune response through mediators, such as second messengers released from the membrane. For example, diacylglycerol and inositol 1,4,5-triphosphate generated through the hydrolysis of phosphatidylinositol 4,5-diphosphate; increase in cytosolic Ca^{2+} levels; release of cytokines specifics; binding to receptors distributed in stimulatory regions or domains; cascade started by linkage to TCR receptor; and further other mechanisms incompletely elucidated [49, 50].

Fungal lectins have been highlighted as potent modulators of immune response. TML-1 and TML-2 are lectins from the mushroom *Tricholoma mongolicum*, which induced the Th1 response through the activation of macrophages from lectin-treated mice, and stimulated the production of nitrite ion and cytokine tumor necrosis factor (TNF) by macrophages. Additionally, both lectins promoted in vivo activation of mouse macrophages, triggering the inhibition of tumor cell growth [51]. These effects may result from

the lectin binding to the glycan determinants on mouse macrophages surface, modulating the immune response. A lectin purified from the edible mushroom *Volvariella volvacea*, named VVL, induced the proliferation of murine splenic lymphocytes and stimulated the transcriptional expression of interleukin 2 (IL-2) and interferon γ (INF γ) in vitro, stimulating the Th1 response [52]. Similar activity was evidenced by a mushroom lectin from *Ganoderma capense*, which induced high proliferative response in mouse splenocytes [53]. The lectin isolated from another edible mushroom *Pleurotus citrinopileatus* showed in vitro mitogenic activity on mouse splenocytes, with the maximal stimulation at 2 μ M of lectin concentration [54]. These lectins could have some similarity with fungal immunomodulatory proteins, such as Fip-vvo, in the slight N-terminal sequence, which may be related to this proliferative response [52, 53].

Immunomodulatory activities of *Agaricus bisporus* lectin (ABL) from edible mushroom acted on innate and adaptive immune responses in vivo and in vitro but showed an inhibitory and antiproliferative effect on macrophages. ABL downregulated in vitro nitric oxide (NO) production by mouse peritoneal macrophages after lipopolysaccharide stimuli and inhibited mononuclear cell proliferation under different conditions. Orally administered ABL to BALB/c mice also induced inhibition of NO production by peritoneal macrophages and stimulated a tumor development. The interaction of ABL-macrophages occurs through the terminal residues of T antigen and sialyl-T antigen in mucin-type O-glycans exposed on macrophages, and this interaction can modify the Akt signaling pathway. ABL binding can block Akt phosphorylation, resulting in the inhibition of NO production, and cytokine production by macrophages [55]. Unlike this, the oral treatment with ABL promoted a reduction of the tumor growth and attenuated symptoms of experimental autoimmune encephalomyelitis in Wistar rats [55].

A mycelial lectin from *Aspergillus nidulans* microfungi induced an increase of NO production, and INF γ and IL-6 levels also enhanced in splenocyte cultures from treated Swiss albino mice groups. Additionally, the lectin promoted an antianaphylactic effect and prevented Arthus reaction in vivo; a therapeutic potential against ulcerative colitis was observed in rats pretreated with the lectin via intraperitoneal injection that showed better recovery comparing with posttreated rats. The high specificity of this lectin for N-acetylgalactosamine (GalNAc) residues on epithelial cells could trigger the therapeutic effect [56].

Seaweed lectins are widely studied due to attractive biological activities. The mitogenic activity for T lymphocytes from mouse spleen was firstly reported to a lectin from the red seaweed *Carpopeltis flabellate* (Carnin) [57]. The lectin from green seaweed *Caulerpa cupressoides* (CcL) showed antinociceptive and anti-inflammatory activities on zymosan-induced arthritis in rat temporomandibular joint. Rats treated with CcL reduced until 89.5% zymosan-induced hypernociception and leukocyte influx until 98.5%. The lectin also lowers the expression levels of IL-1 β and TNF α compared with nontreated group, being a probable way for

antinociception and anti-inflammation effects lectin induced [58].

Animal lectins present in different tissues from insects, fishes, and mammals, among others, play important roles in immune system regulation, being applied as potent immunomodulators. Mitogenic activity was reported to a D-galactose-binding lectin from *Musca domestica* pupae. Proliferation of mouse splenocytes was stimulated in the presence of the lectin in vitro with maximal activity at a concentration of 20 μ g/mL [59]. A rhamnose-specific lectin isolated from ovaries of the grass carp fish (*Ctenopharyngodon idellus*) showed immunostimulatory effect, including proliferative response in murine splenocytes and peritoneal exudates cells. Grass carp lectin also induced IL-2 and INF γ expression in treated splenocytes [60]. A lectin also was isolated from roe of the grass carp and similarly to lectin from grass carp ovaries showed mitogenic activity on murine splenocytes and stimulated the phagocytic activity of sea bream macrophages [61]. A mannose-binding lectin from the ovary of cobia fish (*Rachycentron canadum*) also exhibited mitogenic activity on mouse splenocytes, at 14 μ M [62]. Another mannose-binding lectin, isolated from serum of cobia named *Rachycentron canadum* lectin (RcaL), was also reported as immunomodulatory agent. The mitogenic response and cytokine production in splenocytes from mice in vitro treated with RcaL were evaluated. A high proliferation index to treated cells and induction of high levels of IL-2 and IL-6 production were observed; RcaL was a potential mitogenic agent [63]. RcaL induced Th1 response in treated cultures of mice splenocytes through INF- γ and NO production without cytotoxicity [64]. A mannose-specific lectin from serum of *Oreochromis niloticus* (Nile tilapia fish) denominated OniL demonstrated in vitro Th1 response induction on mice splenocytes. OniL also stimulated high levels of INF γ production and low levels of IL-10 and nitrite, without cytotoxic effect [65].

Galectins are a family of β -galactoside-binding lectins occurring in animals and play important roles in diverse immunomodulatory processes. Galectin-1 revealed a regulatory role in the thymocyte maturation through the interaction with O-glycans on immature cortical thymocyte surface [66]. In addition, galectin-1 induced apoptosis of immature thymocytes by activation of a p53 pathway [67] and inhibited cell growth, as observed to human leukemia T cells [68]. It is suggested that galectin-1 induced apoptosis through the regulation of intracellular signals, such as activation of AP-1 transcription factor, downregulation of Bcl-2, and activation of caspases [69]. Galectin-3 behaved as a mitogenic and anti-apoptotic agent on human leukemia T cells with apoptosis induced by Fas receptor ligation and by staurosporine [70]. The high sequence similarity of galectin-3 with an antiapoptotic protein family named Bcl-2, including the presence of the NWGR motif, highly conserved in Bcl-2, suggests that galectin-3 can modulate the Bcl-2 pathway and inhibit apoptosis [70]. Galectin-3 also prevents the apoptosis by protection of the mitochondrial membrane and inhibition of reactive oxygen species production [69]. Moreover, galectin-3 acts as a chemotactic agent to monocytes and macrophages by a G-protein pathway [69].

A group of C-type lectins known as macrophage galactose-type lectin (MGL) are commonly glycan-binding receptors on dendritic cells and macrophages from human immune system that participate in immune response steps, as pathogen recognition, endocytosis, and presentation of antigens to T cells. In the activation of human T cells, MGL recognize Tn antigens on the CD45 of effector T cells, triggering reduction in the phosphatase activity of CD45, inhibition of T cell proliferation and inflammatory cytokines production, and T cell apoptosis [71]. Human MGL can enhance IL-10 production by dendritic cells and induce the proliferation of regulatory T cells and CD8+ T cell responses [71].

Many mechanisms of immunomodulation have been attributed to plant lectins. Phytohemagglutinin (PHA) is an N-acetylgalactosamine specific lectin from red kidney bean (*Phaseolus vulgaris*) and one of the first lectins identified as mitogenic agent to lymphocytes [72]. Concanavalin A (Con A) is a mannose/glucose specific lectin isolated from *Canavalia ensiformis* seeds and is among some lectins used as models in lectin-carbohydrate interaction studies. Con A has been reported as mitogenic agent on CD4+ T cells and as antitumoral. Autophagic pathway induced by Con A is beginning when the lectin binds to the mannose residues on the cell membrane, internalized through clathrin-mediated endocytosis to the mitochondria, altering its membrane permeability and inducing the mitochondria autophagy; tumoral cells suffer the cell death [73]. Con A is commonly used as positive control in the evaluation of immunomodulation involving other lectins.

Immunomodulatory activity reported to Korean mistletoe (*Viscum album*) lectin (KML) showed a stimulatory effect on expression of cytokines IL-3, IL-23, and TNF α and in the intracellular reactive oxygen species (ROS) generation, while inhibiting events induced by lipopolysaccharide, such as the production of IL-10 and NO [74]. Agglutinin from *Abrus precatorius* has been reported as an inducer of Th1 immune response through splenocyte activation and induction of IL-2, IFN γ , and TNF α cytokine production. Native and denatured forms of *A. precatorius* agglutinin [75] also induced NK-cell activation and thymocyte proliferation. ConBr, a mannose-binding lectin from *Canavalia brasiliensis* seeds, demonstrated mitogenic activity on in vitro splenocytes showed upregulation in the IL-2, IL-6, and IFN γ expression and a decrease in IL-10 expression [76].

Isoforms of mannose-binding lectins purified from *Cratylia mollis* leguminous seeds, Cramoll 1 and Cramoll 1,4, demonstrated high potential to stimulate human T lymphocyte mitogen in vitro [77]. Cramoll 1,4 and Con A also showed high mitogenic activity in vivo in splenocytes from mice previously inoculated and were potential inducers of cytokines IL-2, IL-6, and IFN γ release, as well as NO production, stimulating the Th1 response [78]. It is suggested that its mitogenic activity on T lymphocytes is induced by transmembrane signals started with the Cramoll binding to glycans of the cell surface, and the proliferation of splenocytes may be induced by a TCR-dependent mechanism [78]. Another study reported the potential of Cramoll 1,4 to stimulate rat spleen lymphocytes activation in vivo through

a significant increase of cytosolic Ca²⁺ and ROS Cramoll 1,4-induced. In this case, the production of ROS may be induced by NADPH oxidase and to have relation with the increase of Ca²⁺ release. Cramoll 1,4 also promoted an increase in IL-1 β levels and stimulated the Th2 response [79]. Immunomodulatory activity of Cramoll 1,4, Con A, and PHA was demonstrated in experimental cultures of treated mice lymphocytes by the induction of Th1 response, showing NO suppression, high production of IFN γ , low production of IL-10, and anti-inflammatory activity [80]. Cramoll 1,4 also stimulated cytokine releases in Th 17 pathway, inducing the production of IL-6, IL-17A, IL-22, and IL-23 as well as immunologic memory in cultured splenocytes [81]. Immunomodulatory effects of Cramoll 1,4 and recombinant Cramoll (rCramoll) were observed on cell culture of mice peritoneal exudates infected and noninfected with *Staphylococcus aureus*. The lectins induced the production of IL-1 β and IFN γ , reducing the expression of TNF α and IL-6 in *S. aureus* infected cells. NO and ROS production enhanced, and phagocytic activity of *S. aureus* increased. Both lectins stimulate phagocytic activity and production of proinflammatory cytokines by activation of intracellular signaling cascades [82].

A lectin purified from taro (*Colocasia esculenta*), the tarin, promoted proliferation of mouse splenocytes in vitro and in vivo. Optimum in vitro cellular proliferation was observed in mouse splenocytes treated with 500 ng of tarin and total in vivo proliferation was 3.3-fold higher than control group [83]. A lectin from *Ziziphus oenoplia* Rhamnaceae, *Z. oenoplia* seed lectin (ZOSL), showed potential anti-allergic and anti-inflammatory effects by prevention of Arthus reaction and anaphylactic shock in vivo in Wistar albino rats after oral administration of ZOSL (200 mg/kg of b.w.) [84].

Some lectins isolated from edible tissues of plants have immunomodulatory activity. Garlic (*Allium sativum*) lectins ASA I and ASA II revealed a mitogenic effect in human peripheral blood lymphocytes, murine splenocytes, and thymocytes and stimulated in vitro histamine release from leukocytes in atopic patients when compared with nonatopic individuals [85]. A mannose-specific lectin from onion (*Allium cepa* agglutinin, ACA) also showed mitogenic activity in murine thymocytes, as well as a high production of IFN γ and IL-2. An increase in the release of NO and in the production of cytokines TNF α and IL-12 by murine macrophage cell line (RAW264.7) and rat peritoneal macrophages was observed after 24 h of the lectin treatment, showing an inductor effect on Th1-type immune response, in vitro [86]. Banana (*Musa acuminata*) lectin (BanLec) showed in vivo immunomodulatory effect. BanLec was orally administered to mice and after seven days, mouse peripheral blood showed an increased level in IL-10, IL-17, and TNF α and a reduction of IL-6 and IFN γ . In addition, CD4+ T cells enhanced while the CD8+ T cell population reduced in mouse thymus [87].

These findings show the importance of lectin-based recognitions to regulate inflammatory processes and immune responses with their potential for biotechnological applications, to understand immune mechanisms and therapeutic tools for immunological disorders and diseases.

3. Antifungal and Antiparasitic Activities of Lectins

Recent studies have demonstrated the potential of lectins from different origin and carbohydrate specificities as antifungal and antiparasitic agents. Plant lectins investigated for antifungal potential, mainly against phytopathogenic species, have most reported antifungal effects binding to hyphae, causing inhibition of growth and prevention of spore germination. For example, a lectin isolated from *Myracrodruon urundeuva* heartwood was able to inhibit in more than 50% the mycelial growth of *Fusarium oxysporum*, *F. decemcellulare*, and *F. fusarioides* [26]. *Fusarium* growth was also impaired by a galactose-specific lectin isolated from *Bauhinia monandra* secondary roots, with highest effect (30% inhibition) on *F. solani* [45]. Inhibitory effects of lectins on growth of phytopathogens from other genera were also reported. Lectins from *Phaseolus vulgaris* seeds inhibited the growth of *Coprinus comatus* and *Rhizoctonia solani* [88] as well as *Valsa mali* [89]. A mannan-specific lectin from *Ophioglossum pedunculatum* roots strongly damaged the growth of *Sclerotium rolfsii* at 40 µg/mL [90].

Microgramma vacciniifolia rhizome lectin had its carbohydrate-binding ability inhibited by glycosylated molecules from *F. oxysporum* f. sp. *lycopersici* mycelia [91]. These authors then suggested that the interaction between this lectin and fungal carbohydrates might be involved in the growth inhibitory property detected against races 1, 2, and 3 of this phytopathogen. However, the carbohydrate-binding sites of lectins are not always involved in the antifungal action. An example is the lectin from *Astragalus mongholicus* roots, which was active against *F. oxysporum*, *Colletotrichum* sp., *Drechslera turcia*, and mainly *Botrytis cinerea*. The addition of lactose and galactose (inhibitors of hemagglutinating activity of this lectin) in the assay did not interfere with antifungal effect [92].

A chitin-binding lectin from *Setcreasea purpurea* rhizome inhibited the germination of *R. solani*, *Sclerotinia sclerotiorum*, *Penicillium italicum*, and *Helminthosporium maydis* spores with minimal inhibitory concentrations ranging from 48.1 to 96.2 µg/mL [93]. In the same way, jackin and frutackin, two chitin-binding lectins from the genus *Artocarpus*, inhibited the germination of *Fusarium moniliforme* spores at a concentration of 2.25 mg/mL [94]. The authors of both works also reported that these lectins impaired the development of hyphae; the mycelia formed were not able to produce spores, and the chitin-binding property is probably involved in the fungistatic action. Interestingly, the antifungal property of *S. purpurea* lectin remained even after heat-treatment of the protein at 75°C [93].

Antifungal lectins have specificity regarding fungal species; mannose/glucose-binding lectin from *Capsium frutescens* var. *fasciculatum* seeds was able to inhibit the spore germination and hyphal growth of *Aspergillus flavus* and *Fusarium moniliforme* but showed no effect on *F. graminearum*, *F. solani*, *Physalospora piricola*, and *B. cinerea* [95].

Some plant lectins were used in plant transgenic researches; this is the case of lunatin, a glycosylated and metal-dependent lectin isolated from *Phaseolus lunatus* seeds,

which showed potent antifungal activity against *Sclerotium rolfsii*, *P. piricola*, *F. oxysporum*, and *B. cinerea* [96]. Another example is the lectin from rhizome of *Ophiopogon japonicus*, with antifungal activity against *Gibberella saubinetii* and *R. solani*, showing minimal inhibitory concentrations of 0.06 and 0.05 mg/mL, respectively. This lectin is able to interact with glycans containing mannose [Man-α(1,3,1,6)-mannotriose, Man-α(1,3)-Man, Man-α(1,6)-Man, Man-α(1,2)-Man, Meα-D-man, and D-mannose] and its carbohydrate-binding sites shown to be very structurally similar to those from monocot lectins [97]. Transgenic plants expressing antifungal lectins were already effectively developed and tested under laboratory conditions. Transgenic rice plants expressing a stable monomeric mutant variant of *Allium sativum* leaf lectin exhibited reduced sensitiveness to sheath blight disease caused by *R. solani*, in comparison with nontransformed plants [98].

Fungi that are human and animal pathogens are also affected by antifungal lectins. *Helianthus annuus* seed lectin inhibited the growth and altered membrane permeability of *Candida tropicalis*, *Candida parapsilosis*, *Candida albicans*, and *Pichia membranifaciens*. This protein was also able to induce the formation of pseudohyphae and the production of reactive oxygen species in *C. tropicalis* [99]. A lectin isolated from *Cladonia verticillaris* lichen was able to inhibit the growth (35%) of dermatophyte *Trichophyton mentagrophytes* [100]. Klafke et al. [101] evaluated the potential of *Con A* and *Abelmoschus esculentus*, *Mucuna pruriens*, and *Clitoria fairchildiana* lectins against *Candida albicans*, *C. tropicalis*, *C. parapsilosis*, *Cryptococcus gattii*, *Cryptococcus neoformans*, *Malassezia pachydermatis*, *Rhodotorula* sp., and *Trichosporon* sp.; however, only *C. parapsilosis* growth was inhibited by these lectins. Pinheiro et al. [102] reported antifungal effect of *Talisia esculenta* lectin on arthroconidial forms of the dermatophyte *Microsporum canis* obtained from hairs of infected animals. Authors proved that the antifungal mechanism involved the carbohydrate-binding sites of this protein.

Antifungal lectins obtained from other organisms, such as the mussel *Crenomytilus grayanus*, contain a lectin able to inhibit germination of conidia from several *Aspergillus* strains [103]. A galactose/N-acetylgalactosamine-specific lectin from the mussel *Mytilus trossulus* impaired conidia germination of species belonging to *Fusarium*, *Trichoderma*, *Haematonectria*, *Aspergillus*, and *Alternaria* genera [103, 104].

The relationship between structure and antifungal activity of lectins is also target of researches. The lectin from the plant *Pinellia ternata* f. *angustata* contains two domains called PTADOM1 and PTA-DOM2, each one with a mannose-binding site. This lectin as well as the two separate domains (expressed in *Escherichia coli*) showed antifungal activity toward the phytopathogens *Alternaria alternata* and *Bipolaris sorokiniana* and the dermatophyte *Curvularia lunata*. The whole lectin showed higher activity than two separated domains, an expected result, since both domains exert antifungal action [105].

The effects of lectins on human and animal parasites investigated under different approaches include the determination of parasitocidal action, prevention of infection, and study of the involvement from carbohydrate-receptor

interactions on the infective process. The lectins Con A and ricin promoted tegumental damage (basal vacuolation and swelling of the basal membrane invaginations) in adult worms of *Schistosoma mansoni* (schistosomiasis causer), which were prevented when assays were performed in presence of carbohydrates that inhibit these lectins [106]. Con A, *Triticum vulgaris*, and *Glycine max* lectins interfered with migration pattern of *Strongyloides ratti* (rat threadworm) along a sodium chloride gradient, which indicates the involvement of carbohydrate moieties in the chemosensory activity of labial sensilla in this nematode [107]. Lectins from *Coprinopsis cinerea*, *Aleuria aurantia*, and *Laccaria bicolor* showed larvostatic effect on *Haemonchus contortus* (Barber's pole worm), resulting in arresting at L1 phase; the lectin from *Marasmius oreades* promoted larval death [108]. Cramoll 1,4 was evaluated for in vivo antihelminthic activity using mice infected with *S. mansoni*; the treatment with this lectin led to decrease in the number of excreted eggs, recovered adult worms, and liver granulomas [109].

Plant lectins prevented the infection of intestinal epithelium of *Psetta maxima* fish by the myxozoan *Enteromyxum scophthalmi*. Con A and *Glycine max* lectins inhibited attachment and invasion of *E. scophthalmi* to the intestinal epithelium; authors suggested that these lectins acted by blocking *N*-acetylgalactosamine, galactose, mannose, and/or glucose residues that are important in the interaction between the parasite and intestinal cells [22]. A lectin isolated from *Synadenium carinatum* latex reduced the infection of murine macrophages by *Leishmania amazonensis* [110]. Authors also reported that the lectin showed no cytotoxicity to mammalian host cells and that the macrophages treated with the lectin showed increased expression of cytokines IL-12, IL-1, and TNF α .

Lectins also evaluated the use in control of host and vectors from parasites and virus. The *Microgramma vacciniifolia* rhizome lectin at 100 $\mu\text{g}/\text{mL}$ was able to promote death of *Biomphalaria glabrata* (intermediate host of *S. mansoni*) embryos and adults; in addition, the snails treated with the lectin laid a few number of eggs, among which several showed malformations [111]. The lectins from *Cratylia floribunda* (CFL) and *Dioclea guianensis* (Dgui) were also able to promote death of *B. glabrata* adult snails [112]. Larvicidal activity of lectins from *Myracrodruon urundeuva* bark, heartwood, and leaves against the mosquito *Aedes aegypti* (vector of the virus that causes dengue fever, chikungunya, and Zika virus fever) was reported [113, 114]. In addition, lectins isolated from whole seeds and seed cake of *Moringa oleifera* demonstrated larvicidal, ovicidal, and oviposition-stimulant effects on *A. aegypti*, being considered important candidates for using in control of mosquito population, including in traps for egg capture [115–118].

4. Lectins for Healing Applications

Many researchers have reported healing effect induced by lectins. The healing is the process of tissue repair after trauma, in which a monitored group of cells and molecules trigger ordered phases to result in anatomical and functional restoration of injured tissues [119]. The repair in response to

injury includes sequential phases of hemostasis, inflammatory phase, tissue formation (proliferation), and remodeling of extracellular matrix (tissue maturation). Firstly, coagulation factors and platelets promote the blood coagulation in the damaged tissue. Inflammatory cells as neutrophils and macrophages phagocyte damaged cells and extracellular matrix; thus, a new tissue starts its regeneration and finally the scar formation [120]. The role of lectins as healing agents is not completely clear; however, lectins may influence the immune response, production of cytokines, inflammatory response, and cell antiproliferative effect during the healing process [8]. Lectins have promoted healing effect in cutaneous wounds and modification of scarring process, with great results and therapeutic potential.

Antiproliferative effect of an edible mushroom *Agaricus bisporus* lectin (ABL) in a model of wound healing was evaluated on human ocular fibroblasts in vitro in order to test the control of scar formation [121]. Ocular fibroblast proliferation was inhibited until 40% and collagen lattice contraction was completely abolished at 100 $\mu\text{g}/\text{mL}$ of ABL. These effects indicate the potential of ABL to modulate the healing process and scar formation in human ocular tissue. ABL may inhibit the proliferation by the influence of growth factors, as epidermal growth factor and insulin. It was also proposed that ABL is internalized after the linkage and accumulates around the nucleus where it may block nuclear localizing sequence- (NLS-) dependent uptake of protein into the nucleus [121, 122].

A lectin isolated from the marine red algae *Bryothamnion seaforthii* (BSL) demonstrated prohealing potential on skin wounds in mice [123]. Induced wounds in dorsal thoracic region of mice were submitted to topical treatment with BSL. During the treatment, BSL showed proinflammatory effect and stimulated reduction of the wound areas. After 7 and 12 days, treatment with BSL promoted the synthesis of collagen by fibroblasts and active presence of young skin annexes, promoting the restructuration of the luminal epithelium and an effective wound closure. Here, BSL plays an immunomodulatory effect on immune cells during inflammatory and proliferative phases, where BSL demonstrated stimulatory activity on the migration of polymorphonuclear cells to injured site, and activation of fibroblasts, resulting in the prohealing effect [123].

Galectins 3 and 7 play important role in reepithelialization of wounds, according to study conducted in models of mice corneal wound healing [124]. Significant reduction in reepithelialization of wounds was observed in galectin 3-deficient ($\text{gal}3^{-/-}$) mice lineage when compared with wild-type lineage ($\text{gal}3^{+/+}$). In the in vitro healing assay the presence of galectin-3 stimulated reepithelialization of cornea wounds in $\text{gal}3^{+/+}$ lineage, but not in $\text{gal}3^{-/-}$, probably due to its deficiency of galectin 7. On the other hand, galectin 7 induced reepithelialization of cornea wounds in $\text{gal}3^{-/-}$ and $\text{gal}3^{+/+}$. These results represent a potential application of $\text{gal}3$ and $\text{gal}7$ for treatment of wounds. Members of galectin family have showed possibility of mediating cell-matrix interactions, mainly galectin 3, which has been expressed in inflammatory cells and fibroblasts and located at sites of corneal epithelial

cell-matrix adhesion and may stimulate cell-matrix interaction and cell migration in the wound healing process [124].

Mannose-binding lectin (MBL) replacement therapy was reported as a healing strategy of a radiation-induced chronic ulcer [125]. In this study, a patient with an insufficient level of MBL and a chronic radiation-induced ulcer after treatment of breast cancer was observed, which even after 15 months of conservative treatment and plastic surgery a satisfactory healing was not obtained. Thus, an experimental intravenous therapy with human plasma-derived MBL was carried out for 6 weeks; after the treatment, a complete healing was obtained. MBL is a component of innate immunity and its role in the elimination of microorganisms and modulation of immune response could contribute to wound healing.

D-mannose-binding lectin from *Artocarpus integrifolia* (jackfruit) seeds known as KM+ or artocarpin promoted wound healing on rabbit corneal epithelium [126]. Firstly, lesion of 6.0 mm diameter area was induced on the cornea of both eyes of experimental and control groups. The lesions of groups were treated with KM+ and buffer, respectively; the wound areas were monitored by fluorescein staining. KM+ enhanced the neutrophil influx into the wound area and may stimulate the production of cytokines, contributing for corneal epithelium healing [126].

Prohealing potential on skin wounds in mice was showed by the native lectin of orchid tree *Bauhinia variegata* (nBVL) and its recombinant isoform (rBVL-1) [127]. Dorsal skin wounds were induced surgically in mice, followed by topical lectin treatment for 12 days. Both lectins promoted wound closure in treated animals, and all skin layers were restructured. It is suggested that the prohealing effects of nBVL and rBVL-1 are due to stimulatory potential of these lectins for mitosis of resident cells such as macrophages and mast cells, triggering the release of cytokines and the recruitment of neutrophils into the wound area. These prohealing properties are attractive for therapy applications involving skin wounds.

Cramoll 1,4 has been reported as healing agent [8]. In order to analyze the cicatricial power, the lectin was applied to topical treatment of cutaneous wounds surgically induced in normal and immunocompromised albino Swiss mice [128]. Treated animals showed more edema formation and recruitment of more polymorphonuclear cells at the wounds. The prevalence of polymorphonuclear cells induced by Cramoll 1,4 is important to remove cell debris and microorganisms in the wound, favoring the healing [128]. Cramoll 1,4 also induced efficiently the granulation phase, collagen fiber deposition, and no incidence of microorganisms in all treated wounds, resulting in wound closure and repair faster than control groups. A parallel study conducted in female albino Swiss mice with skin wound surgically induced included daily treatment with Cramoll 1,4 (5 and 10 $\mu\text{g}/100\ \mu\text{l}$) at 2nd, 7th, and 12th days. Macroscopic and histological aspects of treated groups were compared with a positive control group treated with Con A (10 $\mu\text{g}/100\ \mu\text{l}$) and a negative control group administered with 150 mM NaCl. In the 12th day, wound closure, complete reepithelialization, a great deposition, and organization of collagen fibers and formation of cutaneous annexes were observed in wounds treated with lectins; no wound closure and partial

reepithelialization were visualized in negative control group (Figure 2). Another study related the occurrence of gradual healing process induced by a hydrogel containing Cramoll 1,4 on experimental second-degree burns in rats [129]. On the 7th day of treatment, treated group showed higher edema, exudates, and necrosis. With more 7 days, tissue reepithelialization and moderate autolysis were observed. With more two weeks, tissue epithelialization was completed; and in the 35th day was observed a modeled dense collagen. The potential for healing of cutaneous wounds and thermal burns has been related to immunomodulatory profile of Cramoll 1,4 described in other studies, including proinflammatory action in polymorphonuclear cells, induction of cytokines release, and proliferation of fibroblasts.

A lectin from *Eugenia malaccensis* seeds (EmaL) induced healing of cutaneous wounds in mice [130]. Surgical wounds produced in the skin followed by daily treatment with topical administration of EmaL reduced the intensity of inflammatory signals such as edema and hyperemia. However, higher reepithelialization with well-organized collagen fibers than control group was observed. EmaL was efficient to induce the repairing of cutaneous wounds and can be useful for therapeutic applications.

Parkia pendula seed lectin showed a potential healing effect on cutaneous wounds in normal and immunocompromised mice [131]. The lectin was daily topically administered in wounds produced in the dorsal region of mice. Histopathological analysis revealed edema and hyperemia during inflammatory period; no bacterium proliferation and complete wound closure were observed in normal and immunocompromised groups treated with this lectin.

Previous studies with plant lectins demonstrated their potential to stimulate the production of metalloproteinase-9 (MMP-9) that participates in different steps of wound healing, as chemoattractive factors for inflammatory cells, inducers of cytokines release, and synthesis of collagen [132]. Healing impaired is a relevant therapeutic problem in tissue repair, mainly for patients with diabetes and other diseases, which suffer from chronic wounds and require more care for cicatrization. In this context, some lectins are prohealing natural source molecules, very efficient for induction of faster reepithelialization and cicatrization.

5. Lectins for Drug Delivery

The therapies using chemical agents have some barriers, mainly regarding the need of increasing dosages and action of metabolism, which reduce the effectiveness of treatment. Systems for delivering drugs to a specific target may constitute interesting and effective strategies to troubleshoot these problems and minimize negative side effects [133]. The controlled deliverance techniques, such as liposomes, nanosuspensions, and bioadhesive systems, provide an adequate release rate and duration, producing the desired effect; however they have a main disadvantage of nonspecificity to substrate [134]. On the other hand, the lectin-mediated bioadhesion constitutes specific establishing interactions with receptor-like structures in cell membrane, binding directly to target cells [135].

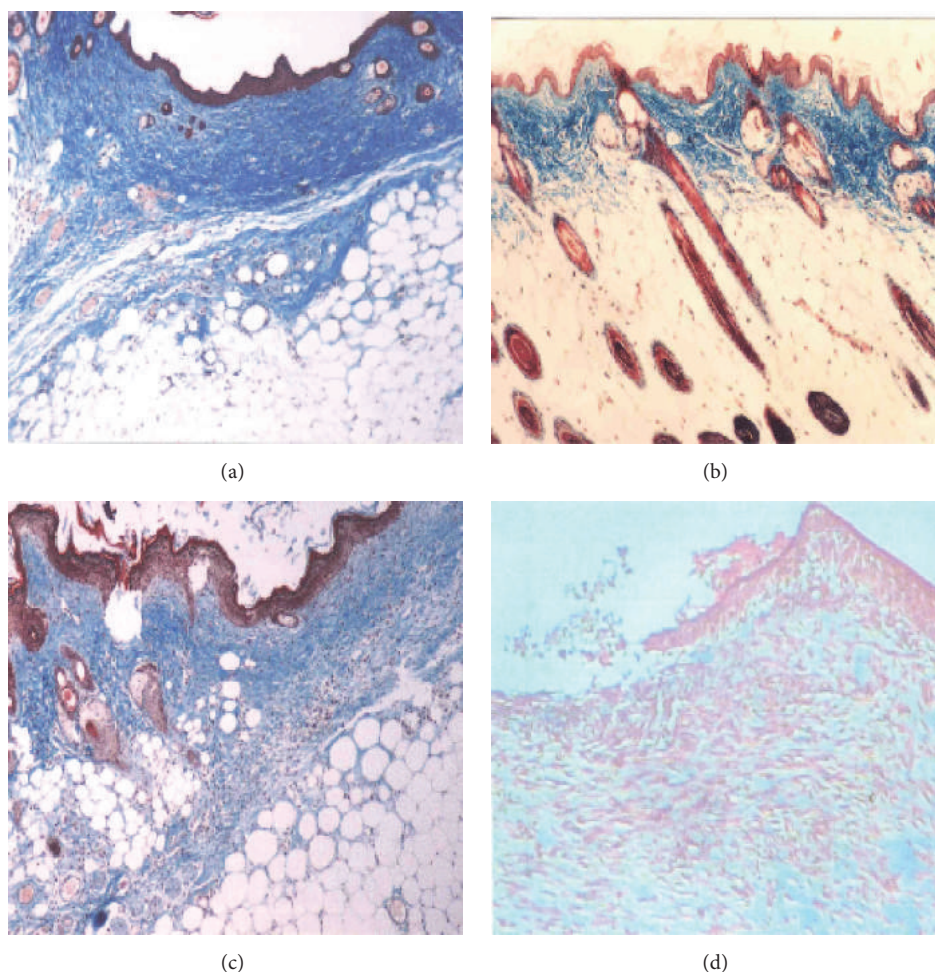


FIGURE 2: Histologic micrographs of wounds treated with Cramoll 1,4 and Con A in the 12th day in female albino Swiss mice. A total wound closure, reepithelialization, and deposition of collagen fibers are observed in Cramoll 1,4 treated group with $5 \mu\text{g}/100 \mu\text{l}$ (a), Cramoll 1,4 treated group with $10 \mu\text{g}/100 \mu\text{l}$ (b), and Con A ($10 \mu\text{g}/100 \mu\text{l}$) treated group (c). The formation of well-developed cutaneous annexes is present in Cramoll 1,4 ($10 \mu\text{g}/100 \mu\text{l}$) treated group (b). Incomplete reepithelialization without wound closure observed for control negative group administered with 150 mM NaCl (d).

Since a set of cell surface proteins and lipids are glycosylated, they can operate as lectin binding sites. Different cell types generally express glycoconjugates that differ in the glycosylation patterns, as with tumor cells compared with their normal counterparts (Figure 3). In this sense, lectins can interact differently with distinct cells and may act as carriers of drugs specifically to desired cells and tissues [136]. To be a potential tool for using in drug delivery lectins should be of avid binding, low toxicity, and site-specific molecules. Figure 4 outlines the mechanism of bioadhesion by lectins.

Lectins may interact on cell surface or be internalized via endocytosis mediated by receptors. These molecules not only allow a target specific attachment, but also can promote a drug uptake actively mediated by the cell [137]. Leong et al. [138] reported that the oral administration of insulin entrapped into surface-lectin-functionalized microparticles extended duration of the hypoglycemic effect, up to 12–24 h, in diabetic rats regarding the free insulin. Neutsch et al. [139] showed that the covalent surface modification of microparticles containing a gemcitabine derivative with

wheat germ agglutinin (WGA) resulted in enhancing of binding duration on urothelial cells; bound microparticles were able to withstand the extensive washout and improved antiproliferative activity [139].

WGA covalently coupled with nanoparticles for carrying thymopentin [140]. After conjugated with nanoparticles, the lectin retained its specific carbohydrate-binding activity. In addition, the increase in WGA content on nanoparticles enhanced oral uptake of thymopentin by improving absorption of nanoparticles and protecting thymopentin against degradation. Nanoparticles coupled with WGA allowed investigation for targeted delivery of β -galactosidase to the intestinal mucosa in Wistar rats. Fluorometric methods showed that nanoparticles adhered to intestinal mucosa for prolonged period (6.7 h), corresponding to 6.9-fold higher than nanoparticles without lectin. Then, authors stated that WGA-nanoparticles are promising candidates for efficient mucosal drug delivery to treat lactose intolerance [141].

Liposomes modified with Con A had its degree of membrane adhesion significantly increased in comparison

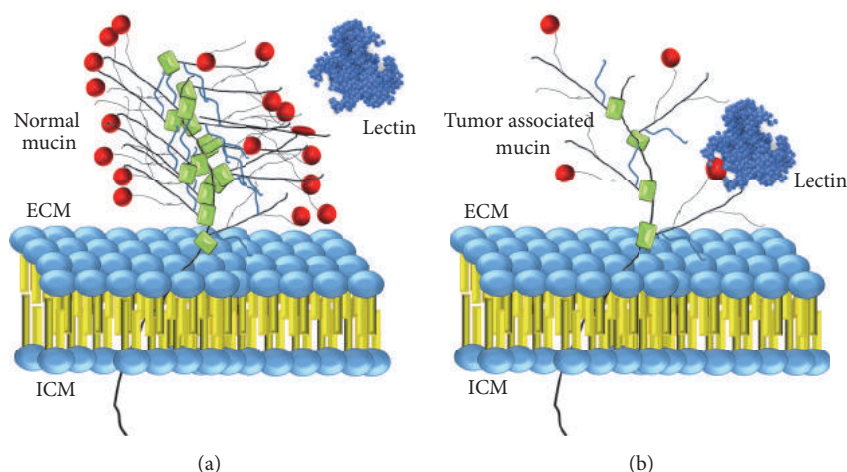


FIGURE 3: Differential interaction of lectins with cells expressing diverse glycosylation pattern. Mucins constitute a glycosylated protein family with high molecular weight, expressed by epithelial tissues. In normal cells (a) the mucin is extensively glycosylated and more than 50% of its molecular mass corresponds to oligosaccharide chains, which may be difficult or impair the interaction of lectins and the carbohydrate residues from mucin. On the other hand, in its tumor counterparts (b), the mucin generally has fewer oligosaccharide side chains, which may facilitate the binding between lectins and glycosylated sites. ECM = extracellular medium; ICM = intracellular medium.

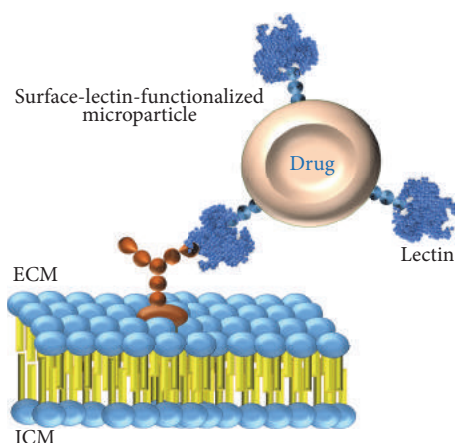


FIGURE 4: Mechanism of lectin bioadhesion. Glycoconjugates on cell surface (glycoproteins or glycolipids) can operate as lectin binding sites. ECM = extracellular medium; ICM = intracellular medium.

with liposome without lectin [133]. This adhesion followed by fusion of vesicles was also higher for Con A conjugated liposomes than for unmodified liposomes. Additionally, Con A conjugated microspheres showed high attachment rate (83.7%) in comparison with nonconjugated microspheres (16.7%) and was able to control the release of the drug amoxicillin trihydrate in simulated gastrointestinal fluids [142].

Floating-mucoadhesive microparticles containing ethylcellulose and chitosan were loaded with clarithromycin and conjugated with Con A to form a lectin-drug carrier complex against *Helicobacter pylori*. The conjugation did not interfere with the buoyancy and release of clarithromycin from microspheres using a mucus diffusion model. About 53% and 40% of drug were released from unconjugated and conjugated microspheres, respectively, within 12 h. Lectin conjugation improved mucoadhesion and interaction with

porcine gastric mucin regarding unconjugated microspheres [143]. Lectins from *Pisum sativum* seeds were encapsulated in alginate microbeads for oral drug delivery against hepatocellular carcinoma; results showed that the release of lectins from microbeads depended on a variety of factors including microbead forming carriers and the amount of encapsulated lectins [144].

Acosta et al. [145] investigated the ricin B-chain (RTB) plant lectin, which corresponds to the nontoxic carbohydrate-binding B subunit of ricin AB toxin from *Ricinus communis*, as a promising carrier for human lysosomal enzymes. Authors genetically fused RTB with human α -L-iduronidase (IDUA), a lysosomal enzyme that degrades glycosaminoglycans. The product of this fusion RTB: IDUA retained both lectin selectivity and enzyme activity and treated human fibroblasts from normal and iduronidase-deficient individuals. The results showed that RTB: IDUA was

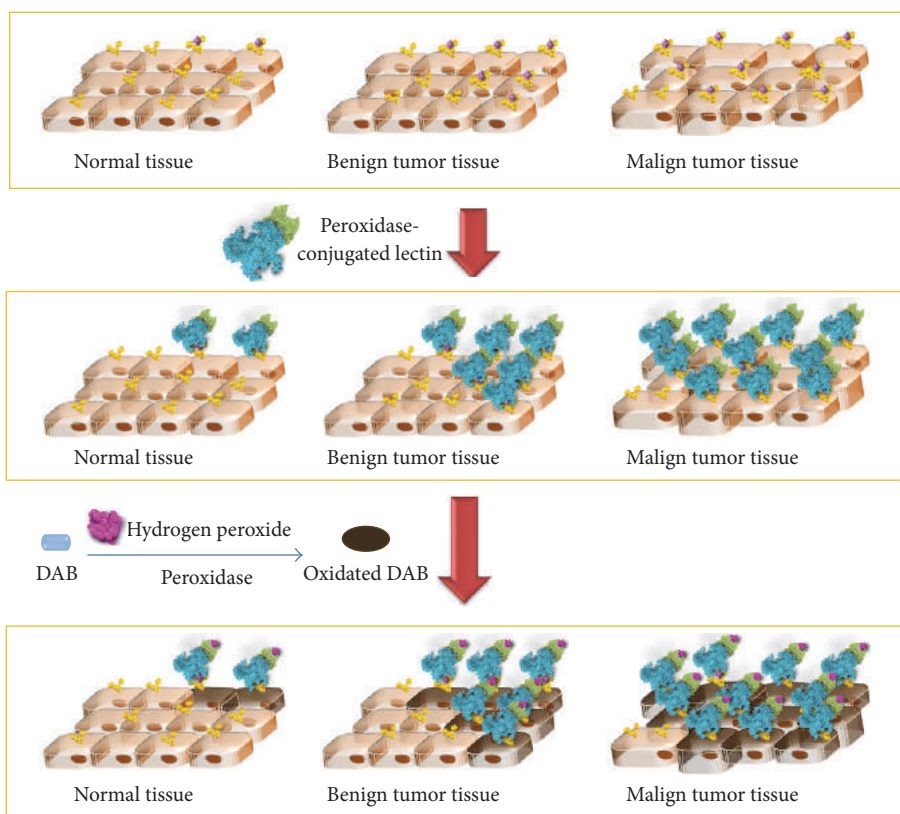


FIGURE 5: Schematic representation of lectin histochemistry using peroxidase-conjugated lectins. In this hypothetical case, the lectin binds glycan moieties more expressed on normal tissues, which suffer a modification in their structure (e.g., sialylation or fucosylation) in benign and malign tumors tissues. Thus, the lectin binding increased in transformed tissues. The DAB reagent in the presence of peroxidase and hydrogen peroxide was converted to DAB oxidized that precipitates as a brown product and allows visualization of lectin binding.

efficiently endocytosed into human fibroblasts and able to correct the disease phenotype of mucopolysaccharidosis in fibroblasts under in vitro conditions.

Liposomes covered with *Bauhinia purpurea* agglutinin were evaluated as a drug delivery system to treat human prostate cancer. The liposomes containing the lectin were able to bind DU145-cells in mice and suppress the growth of the cells [146].

6. Lectins as Histochemical Markers

The glycan moieties covering cell surfaces are involved in many physiological and pathological processes related to cell. Disturbances in cell environment related to diseases frequently trigger changes in glycans, such as fucosylation, sialylation, abnormalities in glycan structure, and uncommon glycans [147]. Inflammation, infections, immunological disorders, and neoplasia have been associated with glycan changes [148, 149]. In this context, lectin abilities to bind carbohydrates are useful to investigate changes in the expression of glycans on cells in tissue surfaces. Histochemical analysis using conjugated lectins as potential markers for altered glycans may show differential binding patterns to normal and transformed tissues [150]. Generally, lectin histochemistry uses peroxidase-conjugated lectin followed by addition of diaminobenzidine (DAB) and hydrogen peroxidase for

visualization of binding, as a hypothetical example in Figure 5. This technique has been an approach for research, diagnosis, and prognosis of human diseases signaled by altered cells in tissues, such as cancer.

Cramoll isoforms demonstrated specific binding patterns to normal and transformed human tissues. Normal and transformed (infiltrating duct carcinoma and fibroadenoma) mammary tissue sections were incubated with Cramoll 1 and Con A conjugated to peroxidase; the lectin binding patterns were visualized after interaction with DAB and hydrogen peroxidase. Cramoll 1 marked neoplastic tissues more intensely than normal tissues, similarly to Con A [151]. Cramoll 1,4 and Cramoll 3 both conjugated to peroxidase were evaluated as histochemical markers of normal, hyperplastic, and carcinoma tissue samples from human prostate; staining patterns were compared with Con A and peanut agglutinin (PNA) results [46]. Differential binding patterns were observed among normal, hyperplastic, and carcinoma tissues. Cramoll 1,4 and Con A showed more intense binding in hyperplastic than normal samples and a distinct binding pattern in carcinoma tissues, reducing the staining with the gravity of tumor. Cramoll 3 and PNA showed an increase of staining degree from normal to carcinoma tissues. Cramoll 1, Cramoll 1,4, and Cramoll 3 are potential histochemical markers for normal and transformed mammary and prostate tissues.

Helix pomatia agglutinin (HPA) histochemical analysis detected cancer and metastatic cells in tissues. Oligosaccharides on aggressive human breast cancer were marked by HPA histochemistry [152]. After the histochemical lectin staining, oligosaccharides were released from lectin in order to investigate some correlation between HPA binding oligosaccharides and breast cancer. A high level of monosialylated oligosaccharide HPA binding expressed in breast cancer specimens was observed, showing a positive correlation between HPA binding and aggressiveness of breast cancer. A relation between HPA binding and metastasis was reported in histochemical analysis of cutaneous malignant melanoma [153]. The lectin stained tissue sections detected primary cutaneous malignant melanoma and a positive correlation between HPA binding and metastasis. Apparently, HPA recognized N-acetylgalactosamine or N-acetylglucosamine residues on the cells and these carbohydrates have some relation with metastasis formation in malignant melanoma, being useful as histochemical marker.

In order to characterize glycosylation changes related to metastasis of breast and colon cancer cells, a histochemical analysis with HPA and selectins was performed [154]. The glycoprofiling binding of human breast and colon cancer cells, metastasizing or nonmetastasizing, was analyzed by histochemistry using lectins, among these HPA, E-selectin, and P-selectin. HPA bound metastasizing breast and colon cancer cells, while it did not bind nonmetastasizing cells. An increase of selectin ligands on metastatic colon cancer cells was observed through E-selectin binding. P-selectin binding was more intense in metastasizing breast cancer than nonmetastasizing ones. Thus, the lectin binding properties are also useful to detect metastatic cancer cells.

Parkia pendula lectin (PpeL) conjugated to horseradish peroxidase was evaluated as histochemical marker for characterization of meningothelial tumor tissue [155]. PpeL showed differential staining pattern that allowed identifying the meningothelial subtype. In addition, a preferential PpeL binding to cytoplasmatic glycans was observed. These results suggest the potential of PpeL as histochemical marker useful for meningothelial tumor characterization and diagnosis.

Ulex europaeus agglutinin I (UEA-I) was indicated as candidate to prognostic marker in ovarian cancer tissues [156]. UEA-I showed a differential staining pattern related to tumor stage. Tumors with high malignancy stained by UEA-I; since this lectin is fucose-specific, it was suggested that fucose residues increase with the degree of ovarian cancer. Another study analyzed the expression of tumor-associated carbohydrate antigen to in situ breast ductal carcinoma using lectin histochemistry to detect carbohydrates [157]. The plant lectins *Griffonia simplicifolia* lectin-I (GS-I) and *Vicia villosa* agglutinin (VVA) were used for breast cancer tissue staining and results correlated to prognostic factors such as tumor size and grade as well as expression of other markers. For both lectins, more intense staining in specimens with nuclear grades II and III than nuclear grade I was observed, indicating a positive relation between expression of carbohydrate antigen GS-I and VVA-binding and more aggressive ductal carcinoma in situ.

The potential of recombinant and native frutalin, α -D-galactose-binding plant lectin from *Artocarpus incisa* seeds to marker human prostate tumor, was evaluated [158]. Prostate carcinoma and benign prostate hyperplasia tissues were analyzed using the lectins conjugated with anti-frutalin polyclonal antibody to react with a complex biotinylated anti-rabbit IgG and streptavidin-conjugated peroxidase, followed by the addition of DAB for visualization of lectin staining. Native frutalin showed a preferential binding for carcinoma cells compared with hyperplastic cells; recombinant frutalin marked only carcinoma cells, showing heterogeneous binding patterns. Both lectins are useful for histochemical detection of prostate tumor.

A study with human gastric cancer demonstrated association between lectin binding and metastasis formation by lectin histochemistry [159]. *Maackia amurensis* leucoagglutinin (MAL) histochemical staining was performed in order to analyze the level expression of α 2, 3-linked sialic acid residues in gastric cancer samples and their association with metastatic potential of one cell line. High levels of α 2, 3-linked sialic acid residues on gastric cancer cells were evidenced by MAL, as well as relation with potential of invasion and metastasis. MAL is an efficient histochemical marker to human gastric cancer metastasis.

Con A and UEA-I were used for histochemical analysis of parotid gland mucoepidermoid carcinoma (MEC) [160]. MEC tissues of the parotid gland previously classified as low, intermediate, and high grade were incubated with Con A and UEA-I conjugated with horseradish peroxidase. Differential binding patterns for Con A and UEA-I staining were visualized. Con A binding was observed in all grades of MEC tissues, but ductal cells of high and intermediate grades were less stained. UEA-I bound intensively MEC tissues in low grade, moderately the cells in intermediate grade, and weakly MEC cells in high grade.

Lectin histochemistry has also detected fungal species infecting human tissues binding carbohydrates of their cell wall surface. Con A, UEA-I, WGA, and PNA, conjugated with horseradish peroxidase, recognized the presence of glucose/mannose, D-galactose, L-fucose, and N-acetyl D-glucosamine on the cell wall surfaces of *Aspergillus* species in human brain and lung specimens, following the visualization with DAB and hydrogen peroxide. Specimen tissues were obtained in patient autopsy diagnosed post-mortem with invasive aspergillosis. The expression of methyl- α -D-mannoside and N-acetyl D-glucosamine detected in *Aspergillus* species by Con A and WGA staining showed the presence of fungal structures in infected specimens of brain and lung tissues [161].

Generally, lectin histochemistry uses enzyme-lectin conjugated in order to reveal the lectin binding. Glycoconjugates present in normal and transformed tissues have also been characterized by quantum dot-lectin histochemistry [162]. Con A and UEA-I were conjugated with quantum dots to bind glycoconjugates on breast tissues in accordance with specificities (α -D-mannose and L-fucose residues, respectively). The results revealed a differential expression and distribution of sugar residues in normal and transformed breast tissues, showing distinct binding patterns.

Lectin histochemistry is an attractive approach to mark transformed tissues and pathological events such as metastasis and shows differential lectin binding patterns that may allow distinguishing between normal, benign, and malignant tumor in various grades. Lectins employed to investigate the glycan profile in transformed tissues constitute useful tools for diagnosis and prognosis of cancer.

7. Lectin-Based Biosensors for Disease Detection

Many known biomarkers established to specific physiological and pathological processes are glycoprotein and glycan detectable in biological fluids and cell surface [163]. Lectin assays developed for glycan analysis attached to circulating glycoproteins or cell surfaces, allowing the detection of diseases and pathogens. Lectin-based biosensors have been developed to detect and quantify glycans [164]. These systems are based on the conversion of lectin-carbohydrate interactions into a measurable signal on a surface, allowing the measurement of biomarkers.

When compared to other lectin techniques such as enzyme-linked lectin assay and lectin microarrays, which use labeled systems that generate color or fluorescence, biosensors can operate in a label-free mode reducing the steps and consumption of reagents. In accordance with type of signal transduction, biosensing methods can be electrochemical, optical, mass, and thermal; however, electrochemical biosensors are more attractive since they are rapid, practical, low cost, and user-friendly assays, available in distinct designs and analytical performance [164, 165]. Electrochemical biosensors have been constructed using electrodes as sensing surfaces, commonly modified with polymers and nanomaterials (carbon nanotubes, gold, magnetic nanoparticles, etc.) to improve the analytical performance and immobilization of biorecognition elements, such as lectins [164]. Techniques such as electrochemical impedance spectroscopy (EIS) and voltammetry are used to measure alterations on the electrode surface and detect interaction ligand-analyte (Figure 6). EIS measurements are based on the detection of changes in charge transfer resistance on the sensor surface after the interactions. Voltammetric techniques such as cyclic voltammetry (CV), differential pulse voltammetry (DPV), and square wave voltammetry (SWV) are based on detection of changes in the current signals generated under the application of a potential on electrode, in the presence of a redox probe [166]. Electrochemical lectin-based biosensors are more attractive as analytical tools of glycans and their application in finding pathogens and diagnosis of diseases reported through detection of biomarkers.

The specific binding affinity of Con A for glucose/mannose was explored in the development of biosensors for distinct applications. An electrochemical biosensor based on Con A was developed for nonenzymatic recognition of glucose [167]. Con A immobilized onto thionine modified electrodes established a sensing surface for specific recognition of glucose. Con A biosensor detected glucose in low concentrations in a linear range from 1.0×10^{-6} to 1.0×10^{-4} M achieved a good limit from detection of 7.5×10^{-7} M. The

biosensor successfully determined glucose in serum samples, being a potential tool for blood glucose analysis monitored in diabetic individuals.

An electrochemical biosensor based on Con A and gold nanoparticles-modified electrode was efficient to recognize serum glycoproteins from patients infected by dengue virus [168]. Another lectin biosensor based on Con A and lipid membranes was constructed for electrochemical detection of abnormal serum glycoproteins from patients contaminated with dengue serotypes I, II, and III (DSI, DSII, and DSIII) [169]. EIS and CV revealed the interactions between Con A and glycoproteins from serum samples, showing more quantitative response to glycoproteins from DSIII. Con A recognizes serum glycoproteins related to dengue infection, being useful for determination of dengue serotypes. Another electrochemical biosensor using Con A showed potential for rapid, sensitive, and selective detection of norovirus (NoV) [170]. The biosensor was composed by a nanostructured gold electrode with Con A immobilized in order to recognize NoV. The results showed a linear relation between current signals and concentration of NoV in the range of 10^2 and 10^6 copies/mL, a great detection limit of 35 copies/mL, and a selectivity of 98% for NoV, being an attractive approach for sensitive and selective quantification of NoV in biological samples.

Sialylation is a common feature observed in glycans and glycoproteins in the occurrence of diseases; electrochemical lectin-based biosensors have been elaborated for determination of sialylated glycoproteins in solutions and biological samples. An ultrasensitive label-free biosensor using the sialic acid-specific lectin *Sambucus nigra* agglutinin type I (SNA-I) immobilized on a self-assembled monolayer (SAM) was developed to quantify the sialylated glycoproteins fetuin and asialofetuin [171]. EIS measurements revealed that SNA biosensor detected both glycoproteins in femtomolar level, showing a promising application for diagnosis of diseases associated with aberrant sialylation. Another SNA-I lectin biosensor using gold nanoparticles detected sialic acid residues in fetuin and asialofetuin down to attomolar level, besides identifying changes in the sialic acid amount [172].

Biosensors, in a larger approach, were constructed based on Con A, SNA-I, and *Ricinus communis* agglutinin (RCA) on SAM-modified gold electrode in order to measure serum glycoproteins in real samples [173]. Glycoproteins were detected in femtomolar level by all three lectin biosensors. Later, they were incubated with human serum samples from healthy individuals and people diagnosed with rheumatoid arthritis (RA) for glycoprofiling of glycan patterns. Con A and RCA biosensors signals for serum from healthy individuals were weaker than signal for serum from RA patients, suggesting a lower exposition of mannose and galactose residues in serum glycans from healthy individuals. SNA biosensor showed higher signals for serum healthy individuals when compared to serum from RA patients, showing that the expression of sialic acid in serum from RA patients is reduced; it is possible to distinguish these samples due to lectin specificities. Another SNA-I biosensor was developed for discrimination of cancer-associated sialyl-Tn (STn) antigen in real samples [174]. SNA-I was immobilized onto screen-printed gold

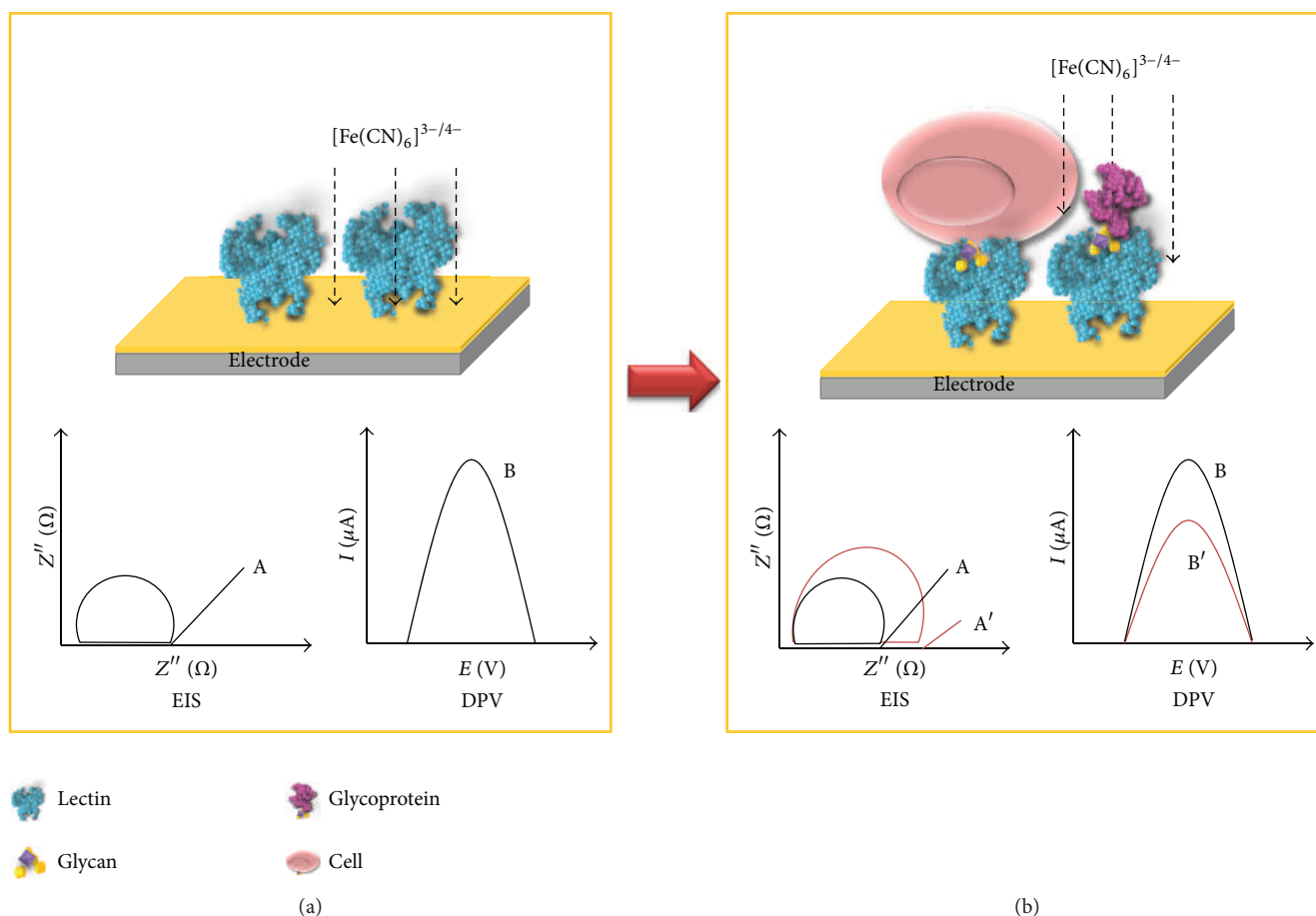


FIGURE 6: Schematic representation of lectin-modified electrode surface before (a) and after binding (b) for measurements of lectin-glycan interactions. In the electrochemical system, measurements are performed in a solution containing a redox probe (e.g., $[\text{Fe}(\text{CN})_6]^{3-/4-}$); reduction or oxidation states generate electrochemical signals (charge transfer resistance for electrochemical impedance spectroscopy, EIS, and current for differential pulse voltammetry, DPV) to monitor electrode surface interactions. Before binding (a), charge transfer resistance or current signals are obtained on lectin-modified electrode surface. After binding (b), the presence of cells or glycoproteins on electrode surface generates a higher blockage for charge transfer and current signals. It is measured as an increase in the charge transfer resistance (represented by semicircle A') for EIS response and a reduction in the current amplitude for DPV response.

electrodes and their surfaces were incubated with serum samples from healthy individuals and patients with malignant tumors to analyze STn-expression in serum glycoproteins. EIS results revealed a differential interaction of SNA-I with STn antigens in serum glycoproteins, distinguishing between healthy and cancer samples.

Evaluation of mannose and sialic acid expression on normal and cancer cells from human lung, liver, and prostate was performed by an electrochemical lectin-based biosensor based on Con A and SNA [175]. Con A and SNA lectins were used as biorecognition element for mannose and sialic acid, respectively. The proposed biosensor could successfully detect the expression levels of specific sugars. Sialic acid was more evident in cancer cells, and mannose showed a high expression in both normal and cancer cells. The biosensor could quantify cancer cells and measure the amount of sialic acid expressed on single cell surface, being a promise approach for profiling glycan expression on cell surfaces providing an early diagnosis and treatment.

Cramoll lectin biosensors have been reported for revelation of glycoproteins in solutions and serum samples by electrochemical finding. A biosensor was developed using gold electrode modified with polyvinyl formal chloroform, Fe_3O_4 nanoparticles, and Cramoll for exposure of fetuin in solutions and glycoproteins from serum patients contaminated with DSI, DSII, and DSIII [176]. EIS and voltammetric measurements showed the interaction of Cramoll with fetuin and serum glycoproteins of DSI, DSII, and DSIII and a higher response to glycoproteins of DSII. Another Cramoll biosensor elaborated with gold nanoparticles, polyaniline, and Cramoll was used to identify abnormal glycoproteins of DSI, DSII, and DSIII, dengue fever, and dengue hemorrhagic fever, present in serum samples [177]. EIS and CV characterizations revealed distinct changes in the charge transfer resistance and current signals after interactions with the serum samples, being able to recognize serum glycoproteins from dengue serotypes. A greater Cramoll binding was observed to DSIII glycoproteins. A label-free Cramoll nanosensor based on

assembled carboxylated carbon nanotubes and poly-L-lysine film showed differential serum glycoproteins from prostate cancer and benign prostatic hyperplasia [9]. DPV responses of nanosensor revealed that Cramoll was able to distinguish between benign and malignant prostate tumor, in addition to showing a significant statistical correlation with the degree of staging prostate cancer. Another Cramoll biosensor contained self-assembled Cramoll lectin on the hybrid cysteine-gold nanoparticles-modified gold electrode and was used as a recognition interface for bacterial lipopolysaccharide (LPS) [10]. CV and EIS results expressed the selective interactions of Cramoll biosensor with LPS from *Escherichia coli*, *Serratia marcescens*, *Salmonella enterica*, and *Klebsiella pneumoniae*. Thus, Cramoll has been able to recognize bacterial LPS and serum glycoproteins, becoming a potential approach for diagnosis of diseases.

The ability of lectins to recognize glycans, in addition to the attractive analytical performance of electrochemical biosensors, has been successfully applied for detection of virus and bacteria as well as glycoprofiling of serum glycoproteins and cell surfaces. Early discovered infections and diseases can help in the diagnosis and appropriate treatment.

8. Lectins as Anticancer Agents

Lectins from several origins exert cytotoxic effects such as inhibition of proliferation and activation of cell death pathways, on different types of cancer cells. In addition, many anticancer lectins usually possess low cytotoxicity to nontransformed cells. This fact is probably associated with the distinct expression of glycans on surface of cancer and normal cells, allowing lectins specifically to recognize malignant cells [11, 12].

The ability of lectins to inhibit the growth of cancer cells in vitro is well documented in the literature and some examples are listed in Table 1 [13–21, 23–25, 27–29, 89, 111, 178–181]. In addition, to reduce cell proliferation, lectins may interact with receptors and other molecules present in cell surface and/or cytosol, activating cell death pathways. MCF-7 (human breast cancer) and HCT-15 (human colorectal adenocarcinoma) cells treated with a lectin isolated from *Morus alba* leaf showed morphological changes and DNA fragmentation that are characteristic of death by apoptosis, which was confirmed by the authors using staining with annexin V and acridine orange/ethidium bromide; in addition, an increased activity of caspase 3 was detected [182]. On the other hand, the *Bauhinia forficata* seed lectin induced necrosis and secondary necrosis in MCF-7 cells, with caspase 9 inhibition [21].

A mannose-binding lectin from *Clematis montana* induced apoptosis in L929 cells (murine fibro sarcoma) with activation of caspases. Authors proposed that there is a correlation between carbohydrate-binding ability and anticancer effect of this lectin since the cytotoxic activity decreased with the assay performed in presence of mannose [23]. In the same sense, Carvalho et al. [20] attributed death of NB4 (leukemia) cells promoted by *Artocarpus heterophyllus* lectin to the recognition of a trimannosyl core of *N*-glycans

containing a β 1,6-*N*-acetylglucosamine branch linked to α -1,6-mannose.

Mechanisms of apoptosis or necrosis induction by lectins have been studied. The treatment of MCF-7 cells with lectin from *Abelmoschus esculentus* led to increasing in expression of proapoptotic genes (caspase 3, caspase 9, and p21) as well as increased Bax/Bcl-2 ratio, being Bax a proapoptotic protein with activity inhibited by Bcl-2 [19]. Wu et al. [181] reported that *Polygonatum odoratum* rhizome lectin induced apoptosis and autophagy in A549 cells (human adenocarcinoma from alveolar basal epithelial cells) and demonstrated that this activity involves the regulation of microRNAs levels. Authors described a downregulation of the microRNA-1290, which leads to amplification of apoptosis and downregulation of Wnt pathway; in addition, the glycogen synthase kinase-3 β was reported as a direct target of this microRNA. On the other hand, authors mentioned upregulation of microRNA-15a-3p, which mediates ROS-p53 44 pathway linked to apoptosis and autophagy. *Bothrops leucurus* venom lectin triggered necrosis in B16-F10 (murine melanoma) cells, with increase in cytosolic calcium concentration and mitochondrial superoxide generation; this lectin activated the opening of mitochondrial permeability transition pore [16]. Yang et al. [183] showed that dimerization and formation of a hydrophobic pocket in the structure of an *Agrocybe aegerita* lectin are essential for its apoptosis-inducing activity.

Lectins may also affect the adhesion ability of cancer cells. Lebecin (a C-type lectin-like protein from *Macrovipera lebetina* venom) inhibited the integrin-mediated attachment of MDA-MB-231 (human breast cancer) cells to fibronectin and fibrinogen [17]; *Bauhinia forficata* seed lectin inhibited adhesion of MCF-7 cells to laminin, collagen I, and fibronectin by decreasing expression of α 1, α 6, and β 1 integrin subunits [21].

As mentioned above, lectins can reach the cytosol and promote several alterations in cell physiology. A remarkable example is the lectin from Northeast China black beans, which was reported to bind HCT116 (colorectal carcinoma) cell membrane and was found in the Golgi apparatus and lysosomes within 3 h after treatment. Authors also described that this lectin caused aggregation of Golgi complex, protein accumulation in the endoplasmic reticulum, mitochondrial malformation, and membrane depolarization [29].

The several reports on the potential of lectins for cancer treatment stimulated studies at in vivo conditions using different models. *Crataeva tapia* bark lectin showed antitumor activity on sarcoma 180 (ascitic tumor) model and its safety for use in future clinical studies was indicated by the low toxicity (LD₅₀ of 2,500 mg/kg) to mice [184]. Lectins isolated from fruiting bodies of *Russula lepida* and *Pleurotus citrinopileatus* also showed antitumor activity in mice, reducing in 80% and 67.3%, respectively, the growth of sarcoma 180 tumor [185, 186].

Cratylia mollis seed lectin encapsulated into liposomes was evaluated for in vivo antitumor activity against sarcoma 180. Shrinkage and 71% inhibition of tumor growth were detected in comparison with untreated group; encapsulation prevented lectin-damaging effects (fibrosis, necrosis, and

TABLE 1: Sources of lectins with in vitro inhibitory effect on growth of different cancer cells.

Source	Affected cells	Reference
	Fungi	
<i>Agrocybe aegerita</i>	HeLa (derived from cervical cancer cells), HL-60 (promyelocytic leukemia), SW480 (lymph node metastasis), SGC-7901, BGC-823, and MGC80-3 (gastric cancer)	[9]
	Animals	
<i>Aristichthys nobilis</i> gills	HeLa	[10]
<i>Bothrops leucurus</i> venom	B16-F10 (murine skin melanoma), HEp-2 (carcinoma), K562 (chronic myelogenous leukemia), NCI-H292 (lung mucoepidermoid carcinoma)	[11, 12]
<i>Macrovipera lebetina</i> venom	MDA-MB-231 (human breast cancer)	[13]
	Plants	
<i>Abelmoschus esculentus</i> seeds	MCF-7 (breast cancer)	[14]
<i>Amaranthus mantegazzianus</i> seeds	URM-106 (rat osteocarcinoma)	[15]
<i>Artocarpus heterophyllus</i> seeds	NB4 (leukemia)	[16]
<i>Bauhinia forficata</i> seeds	MCF-7	[17]
<i>Clematis montana</i> stem	L929 (murine fibrosarcoma), HepG2 (hepatocellular carcinoma), HeLa, MCF-7	[18]
<i>Dioscorea opposita</i> tubers	CNE-2 (nasopharyngeal carcinoma), MCF-7, HepG2	[19]
<i>Glycine max</i> seeds	U373MG (glioblastoma astrocytoma), HeLa, HEp-2, HepG2, MDA-MB-231	[20]
<i>Lotus corniculatus</i>	THP-1 (leukemia), HOP62 (lung adenocarcinoma)	[21]
<i>Microgramma vacciniifolia</i> rhizome	NCI-H292	[22]
<i>Momordica charantia</i> seeds	EAC (Ehrlich ascites carcinoma)	[23]
<i>Morus alba</i> leaves	HCT-15 (colorectal adenocarcinoma), MCF-7	[9]
Northeast China black beans	HCT116 (colorectal carcinoma)	[24]
<i>Phaseolus vulgaris</i> cv. extra long autumn purple bean seeds	HNE-2, CNE-1, CNE-2 (nasopharyngeal carcinoma), MCF-7, HepG2	[25]
<i>P. vulgaris</i> Chinese pinto bean seeds	HONE-1 (nasopharyngeal carcinoma)	[26]
<i>P. vulgaris</i> cv. blue tiger king seeds	HepG2	[27]
<i>Pisum sativum</i> seeds	EAC cells	[28]
<i>Polygonatum odoratum</i>	A549 (alveolar basal epithelial adenocarcinoma)	[29]

lymphocyte infiltration) that were observed in liver and kidney of animals treated with free lectin solution [187].

Pisum sativum and *Momordica charantia* seed lectins showed in vitro and in vivo inhibitory effects on Ehrlich carcinoma (ascitic tumor) in mice. The growth of tumor inhibited in 63% and 75% with *P. sativum* and *M. charantia* lectins, respectively, both administered intraperitoneally at 2.8 mg/kg/day for five consecutive days. The *P. sativum* lectin caused apoptosis involving activation of caspases while the *M. charantia* lectin did not induce this cell death mechanism. The proapoptotic gene Bax was expressed intensively in cells treated with *P. sativum* lectin [28, 180]. The seed lectin from *Glycine max* caused 82.95% inhibition of Dalton's lymphoma in mice that received it through intraperitoneal injection; induction of autophagy and apoptosis, with activation of ROS production, was detected [25].

Finally, the anticancer potential of lectins also includes antimetastatic properties. Leaf lectin from *Viscum album coloratum* showed a preventive effect against lung metastasis caused by B16-BL6 and 26-M3.1 cells in mice that received 20–50 ng of lectin through intravenous administration two days before inoculation of cancer cells. This lectin also inhibited liver and spleen metastasis of L5178Y-ML25 cells when administered one day after tumor inoculation [188].

9. Conclusions

Lectins from diverse sources with distinct carbohydrate recognition events have important roles for many biotechnological applications and disease therapies. In vitro and in vivo uses showed that lectins have protective effects against virus and microorganisms; they are potent modulators of

immune response, mitosis, proliferation, healing, drug delivery therapies, and cancer regression. Altered glycans on cells or tissue surfaces and serum samples can be located using lectin-based techniques, such as histochemistry and biosensors, detecting diseases and infection agents. Thus, this review gathers achievements attributed to lectins with focus in biotechnological/pharmacological and therapeutic applications, being a valuable resource for more studies about biological effects, pathways, and biotechnological potential of lectins. Besides, the effects described for the same or different lectins on biological systems could unravel new interpretations or insights to the field.

Competing Interests

The authors have declared that no competing interests exist.

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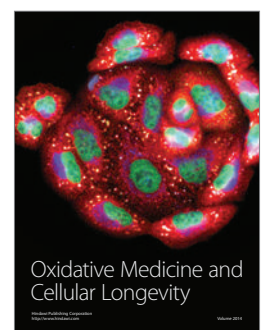
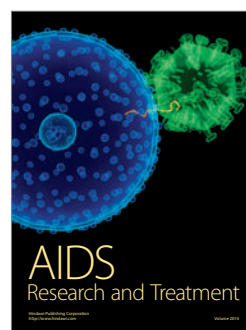
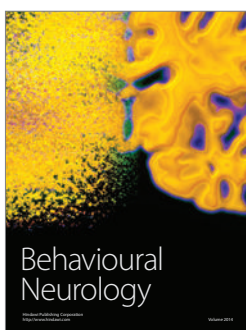
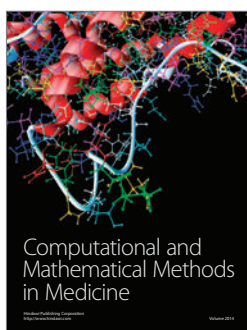
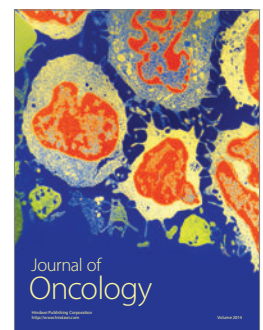
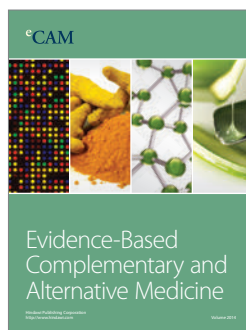
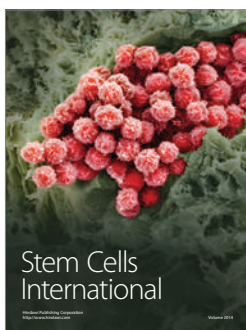
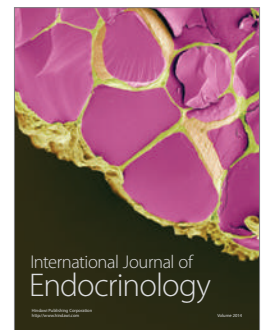
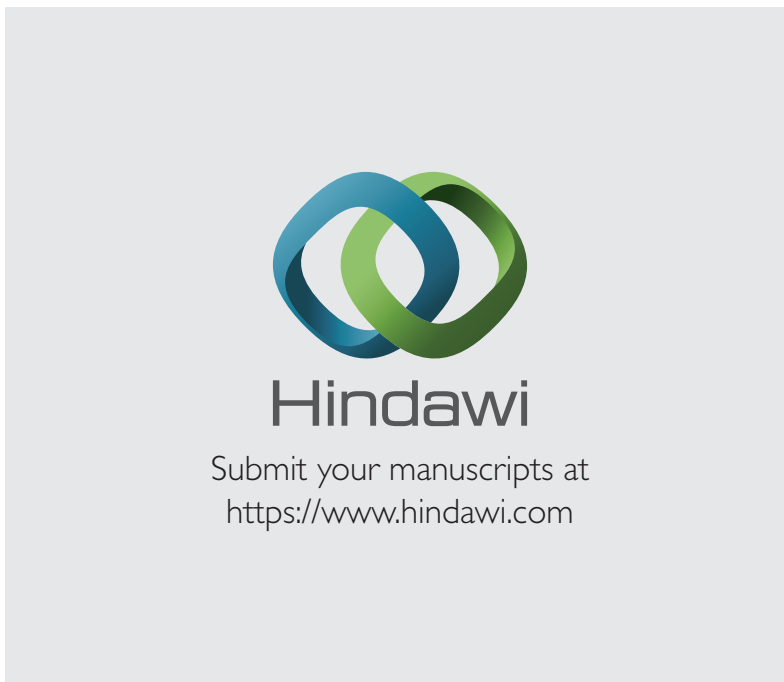
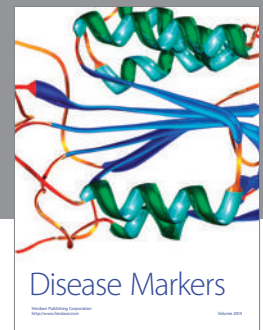
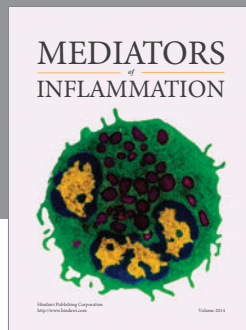
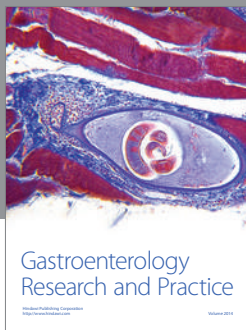
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**APÊNDICE E – ARTIGO PUBLICADO NA REVISTA BIOTECHNOLOGY
JOURNAL INTERNATIONAL**

**Charge transference detection through cyclic voltammograms in the
interaction of *Cratylia mollis* seed lectin-carbohydrate inhibitor**

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Charge Transference Detection through Cyclic Voltammograms in the Interaction of *Cratylia mollis* Seed Lectin-carbohydrate Inhibitor

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Authors' contributions

This work was carried out in collaboration between all authors. Author SRS designed the study, performed protocols, statistical analysis and wrote the first draft of manuscript. Authors SRS, EGS and JEGS managed the analysis of the study. Author PMSS contributed in writing the manuscript. Authors MTSC and LCBBC designed, supervised and managed the whole study. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To evaluate the charge transference and the electrochemical potential of *Cratylia mollis* seed lectin, Cramoll 1,4, adsorbed on Nafion beads after the interaction of glucose ligand.

Study Design: Based on detection of electrochemical currents and potentials of Cramoll 1,4 by electrochemical techniques.

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Place and Duration of Study: Department of Chemistry, Catholic University of Pernambuco, between April 2014 and November 2015.

Methodology: Cyclic Voltammetry (CV) was performed in an electrochemical cell containing three electrodes (a calomel electrode, a platinum wire counter electrode and a platinum electrode), connected to a potentiostat to obtain electrochemical currents related to the charge transference. An electrochemical cell containing a calomel electrode and a platinum electrode coupled to a multimeter was used to register the potentials. A saline solution was used as support to control the charge distribution inside of the cell. Cramoll 1,4-glucose interaction was evaluated in the concentration of 100, 200 and 300 mM of glucose.

Results: CV measurements showed significant charge transference after Cramoll 1,4-glucose interaction. Cathodic and anodic peaks paired near 100 mV were detected in the range 100-300 mM glucose, achieving a maximum current response of 1300 μ A, approximately. Positive electrochemical potentials of Cramoll 1,4 adsorbed to Nafion-beads was achieved showing a linear behaviour with the increase of glucose concentration at 300 mM.

Conclusion: The system is useful for characterization of lectin-carbohydrate interactions and as a glucose sensor to estimate the activity of lectins.

Keywords: *Cratylia mollis*; cyclic voltammograms; Nafion-beads; seed lectin.

1. INTRODUCTION

Lectins are a group of non-immune proteins of ubiquitous distribution in nature that bind carbohydrates with high specificity [1]. Lectin-carbohydrate interaction constitutes an initial biological signal to start physiological responses, such as mitogenic activity and cell-to-cell interactions [2,3]. The measurements of lectin interactions became, therefore, a highly attractive field as a quantitative method to the analysis of many biological phenomena. Lectin-carbohydrate interaction has been broadly studied through physicochemical methods such as nuclear magnetic resonance [4], surface plasmon resonance spectroscopy [5] and X-ray crystallography [6], those required complex/time-consuming manipulations and expensive instruments. In this context, electrochemical methods have already been used to evaluate charge distribution in lectin surfaces and lectin-carbohydrate interactions [7,8]. These methods are of fundamental importance to study macromolecular superficial charge [9] and evaluations of charged protein behaviour in analytical systems [10].

A systematic evaluation based on electrochemical methods uses potentiometric or amperometric signal transducers with small polarizable electrodes, allowing the study of surface-immobilized proteins, such as lectins, and the relationship with their ligands [11,12]. Electrochemical methods can measure directly the lectin-carbohydrate interaction through the detection of charge (electron) transference on the lectin when the interaction occurs. The

electrochemical response can be measured to quantify the interaction and to characterize the behaviour of the lectin in the presence of its specific carbohydrate [7,12].

Monitoring of electrochemical current evaluates lectin-carbohydrate linkage through amperometric methods, such as cyclic voltammetry (CV) [11]. CV is the electrochemical technique frequently used to characterize charge transference processes, based on scanning of the potential from the working electrode using a triangular potential waveform, and measuring the resulting current. The current is plotted versus the potential, generating a cyclic voltammogram [13]. Potentiometric methods are also useful to investigate lectin-carbohydrate interactions and the charge distribution on a lectin surface [7,8]. Measurements of electrochemical potential can be associated to charge transference on lectin submitted to an electric field in the presence of its ligand.

The association of lectin with polymers improves the charge transference, promoting the increase of the electrochemical signal detected [14]. Nafion is a perfluorinated anionic polyelectrolyte belonged to a class of perfluorosulfonated ionomer [15]. X-ray dispersion analysis in Nafion has revealed the presence of metallic patches and sulfonic acid groups as hydrophilic regions and fluorocarbons as hydrophobic regions [16]. The presence of ions in the Nafion structure induces electrostatic intermolecular interactions, which confer attractive physicochemical properties for composition of membranes and catalyst support [17]. Available in different forms,

such as membranes and beads, Nafion has been used for ion-exchange applications and electrocatalyses in electrochemical analyses due to high electrical conductivity, good stability and biocompatibility [17,18]. Additionally, the sulfonic acid groups of Nafion structure promote the stable biomolecule immobilization on solid surface [19].

Cratylia mollis is a native forage from the Caatinga biome of the State of Pernambuco, Northeast of Brazil. A lectin specific to glucose and mannose from *C. mollis* seed named Cramoll has been purified and sequenced [20,21]. Lectin-ligand interactions involving the lectin containing its isoforms 1 and 4 (Cramoll 1,4) have been evaluated by electrochemical techniques [7,8]. Biosensing approaches based in Cramoll 1,4 interactions have been developed for sugar detection in solution [11] and for recognition interface for dengue virus serotypes [22], bacterial lipopolysaccharide [23] and differential diagnostic of prostatic tumour [11]. Proteins adsorbed to polymers should leave available active binding sites, which promote the interaction with selective ligands. In this work, CV measurements and determination of electrochemical potentials were performed to analyse the charge transference signals of Cramoll 1,4 adsorbed to Nafion-beads interacting with different concentrations of glucose, in order to evaluate Cramoll 1,4 activity.

2. MATERIALS AND METHODS

C. mollis seeds were collected in the city of Ibimirim, in the State of Pernambuco, Northeast of Brazil. Glucose and Nafion® NR 50 were purchased from Sigma Aldrich (St. Louis, MO, USA). Other reagents used were of analytical grade. All solutions were prepared with ultrapure water from a Milli-Q system (Billerica, USA).

2.1 Cramoll 1,4 Obtention

Cramoll 1,4 was isolated from *C. mollis* seeds according to Correia and Coelho (1995) [20]. The lectin was extracted from *C. mollis* seed flour in 0.15 M NaCl (10% w/v), overnight at 4°C. The saline extract was submitted to fractionation with ammonium sulfate and the precipitate of the 40-60% fraction was resuspended, dialysed in saline (F40-60) and purified by affinity chromatography on Sephadex G-75. The lectin containing the isoforms 1 and 4 of Cramoll (Cramoll 1,4) was eluted from Sephadex G-75

column with 0.3 M glucose in 0.15 M NaCl [20] and was adsorbed on Nafion-beads surface.

2.2 Preparation of Cramoll 1,4/Nafion-beads

In order to adsorb Cramoll 1,4 on Nafion-beads surface, 2 g of Nafion® NR 50 beads and 3 ml of Cramoll 1,4 in 0.15 M NaCl solution (1 mg/ml) were mixed and kept at rest for 6 h at 4°C. Following, the supernatant was removed and beads were washed with 0.15 M NaCl, to obtain Cramoll 1,4/Nafion-beads.

2.3 Electrochemical Measurements of the Cramoll 1,4-glucose Interactions

The electrochemical evaluation of Cramoll 1,4-glucose interactions was performed in an electrochemical cell containing Cramoll 1,4/Nafion-beads deposited at the bottom, and 0.15 M NaCl solution as reaction media to control the distribution of charges within electrochemical cell. The measurement of electrochemical current by CV technique was carried out using a three-electrode system in the electrochemical cell, containing a platinum electrode ($A = 2 \text{ cm}^2$) as the working electrode, a platinum wire as auxiliary electrode and a calomel saturated electrode as reference electrode (Fig. 1). The system was coupled to an EG&G PAR model 175 potentiostat/ galvanostat (New Jersey, USA) connected and controlled by a microcomputer. CV technique was performed in a potential range of 50 to 150 mV within 0.15 M NaCl solution.

In order to obtain electrochemical potentials, it was used an electrochemical cell containing a platinum electrode (working electrode) and a calomel electrode (reference electrode) coupled to a high impedance multimeter (ICEL IK-1500). The saturated calomel electrode was used since it had a stable potential in relation to temperature (27°C), without variations due to charges in the medium. Electrochemical measurements were made in the absence and presence of glucose after 30 min of incubation. Cramoll 1,4/Nafion-beads (1 g) into the cells were incubated with saline solutions containing glucose at concentrations of 0, 100, 200 and 300 mM were performed (Fig. 1). Each experiment was performed in five repetitions.

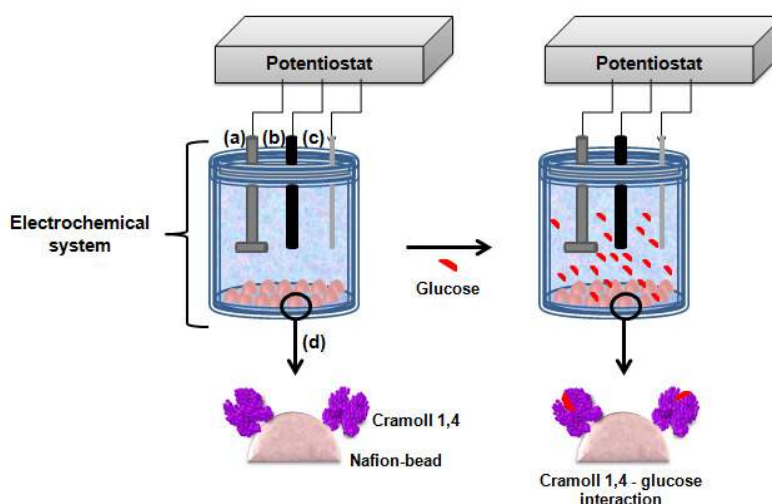


Fig. 1. Schematic representation of the electrochemical system coupled to a potentiostat for voltammetric measurements of Cramoll 1,4/Nafion-beads and glucose interactions, composed by a platinum electrode (a) as a working electrode; a saturated calomel electrode (b) as a reference electrode; an auxiliary electrode (c) and Cramoll 1,4/Nafion-beads (d)

3. RESULTS AND DISCUSSION

Cramoll 1,4 was isolated from *C. mollis* seeds by extraction of powder in 0.15 M NaCl and the precipitate obtained by ammonium sulfate fractionation was chromatographed through Sephadex G-75, according to the procedure described by Correia and Coelho [20]. Cramoll 1,4 elution with 0.3 M glucose generated the chromatogram and electrophoretic pattern in denatured SDS-polyacrylamide gel showed in Fig. 2, in agreement with results reported by the cited authors [20].

Proteins with electroactive sites like the heme group [24] and lectins [7,8] interact with molecules or macromolecules involving charge transference. The interaction measurements of a protein adsorbed to a non-reactive surface and its ligand indicate the capacity of the macromolecule as a redox receptor, with all binding potential directed by the receptor oxidation state [10]. This property of interaction constitutes a basic tool to electrochemical measurements [25]. Firstly, the immobilization of Cramoll 1,4 on the Nafion-beads surface was evaluated through measurements of redox potentials (+E, mV) versus time (from 0 to 80 min) to confirm this immobilization. As showed in Fig. 3, an exponential increasing curve of potentials from Nafion-beads without Cramoll 1,4 was detected, while a decay time was observed for Cramoll 1,4/Nafion-beads, indicating the

immobilization of Cramoll 1,4 on Nafion-beads surface and a reduction in the exposition of superficial charges of Nafion-beads.

In this work, the charge transference from Cramoll 1,4-glucose interaction was detected on the surfaces of Nafion-beads at the bottom of the electrochemical system, but not at the electrode surface (Fig. 1). When the system is submitted to an electric field caused by the working and reference electrodes in the electrolytic medium containing Cramoll 1,4 immobilized on Nafion-beads and glucose, the charge transference of Cramoll 1,4-glucose interaction is detected through electrochemical signals from direct charge exchange of lectin [7,8]. The CV method has been broadly used for charge transference detection in redox systems involving proteins and their interaction with ligands, due to more user-friendly and low cost. The electroactive properties from Cramoll 1,4 binding sites results in the charge transference during the interaction with glucose revealed by induction of redox peaks observed in CV measurements, performed in 0.15 M NaCl solution media. As shown in Fig. 4, the cyclic voltammograms obtained with Cramoll 1,4/Nafion-beads revealed well-defined states of oxi-reduction at 100 mV, represented by two reversible oxidative (anodic) and reductive (cathodic) redox peaks after interaction with glucose, showing that Cramoll 1,4/Nafion-beads - glucose complex can be detected at this potential.

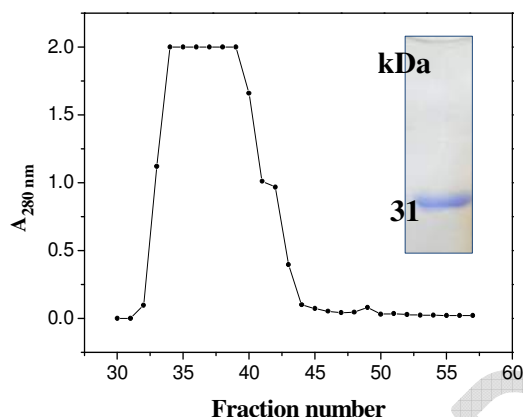


Fig. 2. Purification and partial characterization of Cramoll 1,4. Affinity chromatography of an ammonium sulfate fraction dialysed in 0.15 M NaCl (F40-60) was performed on a Sephadex G-75 column in a flow rate of 30 mL/h and 5 mL fractions were collected. Cramoll 1,4 was eluted with 0.3 M glucose in 0.15 M NaCl (875 mg of protein). The inset shows a denatured SDS-PAGE of Cramoll 1,4 stained with Coomassie Brilliant Blue

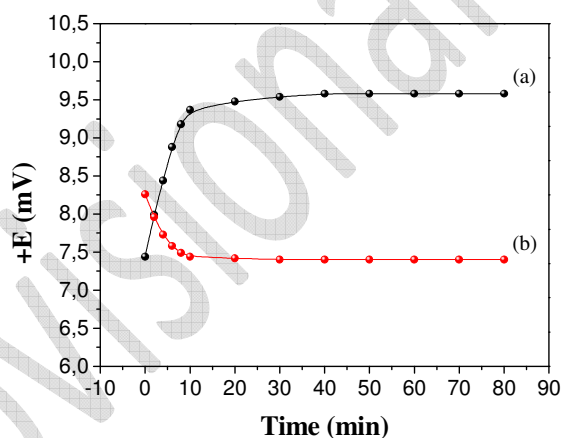


Fig. 3. Measurements of redox potentials versus time for Nafion-beads (a) and Cramoll 1,4/Nafion-beads (b)

The higher cathodic current response was nearly 1300 μ A in the presence of 100-300 mM glucose. Cyclic voltammograms of Cramoll 1,4/Nafion-beads in the absence of glucose did not induce redox peaks. Moreover, the expression of oxi-reduction peaks at 100 mV showed at the CV indicates that Cramoll 1,4 activity was maintained after the adsorption on Nafion-beads. Previous results using potentiometric techniques showed similar electrochemical potential of the interaction between Cramoll 1,4 and glucose in 0.15 M NaCl, of 94 mV [7]. The charge transference of

horseradish peroxidase enzyme immobilized on carbon paste electrode was also monitored by CV, in 10-240 mV within 0.1 M buffer solution to identify hydrogen peroxide, showing double oxidative and revival peaks [24].

In order to analyse linearity of potentiometric response of Cramoll 1,4/Nafion-beads, potentials were measured at different glucose concentrations. Positive electrochemical potentials were registered for Cramoll 1,4/Nafion-beads, as showed in Fig. 5. A potentiometric curve of potential (+E; mV) versus glucose

concentration (mM) was obtained, with linearity from 0 to 300 mM, revealing a linear regression equation $+E(\text{mV}) = 0.036 \cdot [\text{glucose}] + 7.059$ with a correlation coefficient of 0.995 ($p < 0.001$, $n = 4$) in five repetitions. The detection of positive potential for Cramoll 1,4 in the absence of glucose confirms the charge distribution on Cramoll 1,4 surface and its potential redox [7,8]. When a system generates negative

electrochemical potentials it is necessary to use chemical mediators, as signal amplifiers, working in 100 and 200 mV, lowering the action of interferents in biological media [26]. The interaction between Cramoll 1,4/Nafion-beads and carbohydrate can be a model, without mediators, to characterize the lectin-ligand interactions and to determine glucose concentrations.

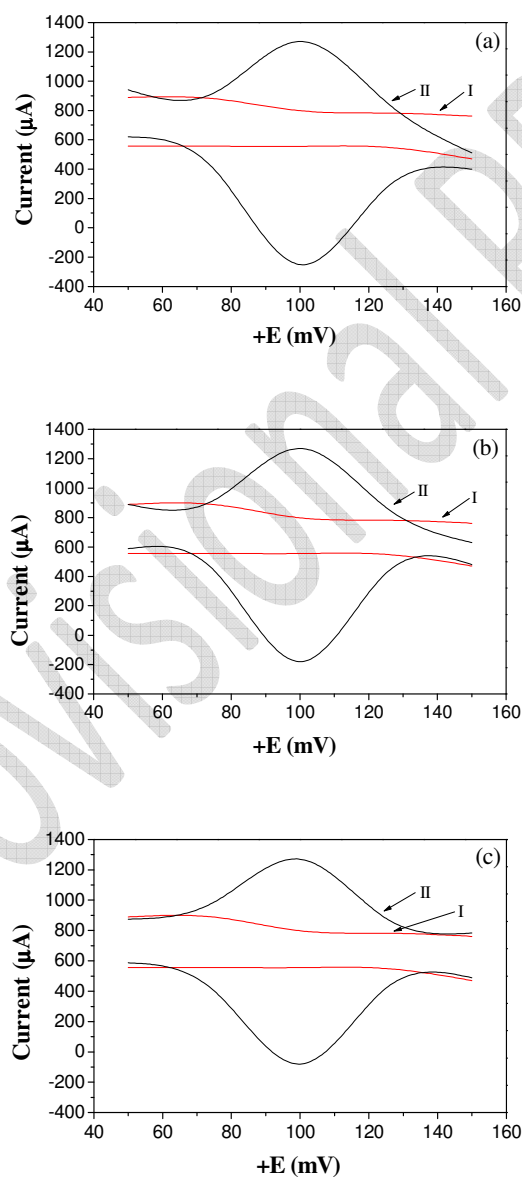


Fig. 4. Cyclic voltammetric measurements of Cramoll 1,4/Nafion-beads before (I) and after (II) interactions with different concentrations of glucose: 100 mM (a); 200 mM (b) and 300 mM (c)

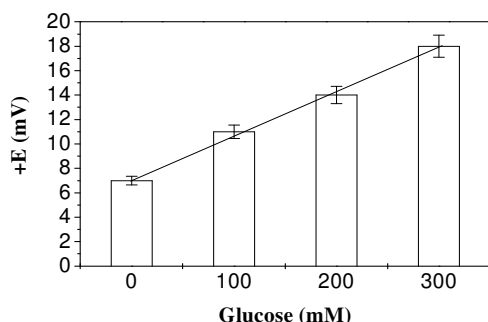


Fig. 5. Electrochemical potentials of Cramoll 1,4/Nafion-beads at different glucose concentrations

4. CONCLUSION

The use of cyclic voltammetry allowed the detection of a redox potential of 100 mV to Cramoll 1,4/Nafion-beads through the interaction with different glucose concentrations and constitutes another approach to determine Cramoll 1,4 activity. Electrochemical potential measurements generate a linear response for glucose concentrations, being an alternative for glucose sensing. Charge transference of lectins is a great phenomenon to evaluate lectin-carbohydrate interactions in biological systems.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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APÊNDICE F – ARTIGO PUBLICADO NA REVISTA JOURNAL OF HOSPITAL INFECTION

***Staphylococcus aureus* and *Staphylococcus epidermidis* infections on implants**

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Review

Staphylococcus aureus and Staphylococcus epidermidis infections on implants

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SUMMARY

Infections are one of the main reasons for removal of implants from patients, and usually need difficult and expensive treatments. *Staphylococcus aureus* and *Staphylococcus epidermidis* are the most frequently detected pathogens. We reviewed the epidemiology and pathogenesis of implant-related infections. Relevant studies were identified by electronic searching of the following databases: PubMed, ScienceDirect, Academic Google, and CAPES Journal Portal. This review reports epidemiological studies of implant infections caused by *S. aureus* and *S. epidermidis*. We discuss some methodologies used in the search for new compounds with antibiofilm activity and the main strategies for biomaterial surface modifications to avoid bacterial plaque formation and consequent infection. *S. aureus* and *S. epidermidis* are frequently involved in infections in catheters and orthopaedic/breast implants. Different methodologies have been used to test the potential antibiofilm properties of compounds; for example, crystal violet dye is widely used for in-vitro biofilm quantification due to its low cost and good reproducibility. Changes in the surface biomaterials are necessary to prevent biofilm formation. Some studies have investigated the immobilization of antibiotics on the surfaces of materials used in implants. Other approaches have been used as a way to avoid the spread of bacterial resistance to antimicrobials, such as the functionalization of these surfaces with silver and natural compounds, as well as the electrical treatment of these substrates.

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Introduction

Biomaterials are natural or synthetic materials, including polymers and metals, used to replace any living tissue that has undergone some accidental damage or destruction due to some pathology or even plastic surgery repair [1]. Researches and

technological advances in the development of biomaterials have shown rapid growth in order to maintain a demand at the population level; the biomaterials can replace or restore the shape and function of a compromised tissue, improving people's quality of life and longevity [2].

Cytocompatibility and preservation of the differentiated phenotype of the cells surrounding the implants are fundamental properties of the biomaterials designed to be integrated to tissues, such as orthopedic implants, whose main objective is the osseointegration [3]. Despite the benefits that implants can offer, they are susceptible to several problems such as lack of integration, inflammatory process, total

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rejection by the receiving individual, and bacterial infection, which is the main cause of implant loss [4]. Bacteria may adhere to form biofilms in foreign bodies placed in patients, such as central and peripheral venous catheters, and in breast/orthopaedic implants, thus establishing infection [5]. In addition, bacteria embedded in biofilms may exhibit greater resistance to environmental conditions as result of the high degree of horizontal gene transfer among them, including antibiotic resistance genes, favouring the infection [6].

Implant infections are most usually caused by staphylococci (about four cases in five). Two species, *Staphylococcus aureus* and *S. epidermidis*, account for around two-thirds of infections [7]. A strategy to eradicate implant infections is prolonged treatment with high doses of antibiotics, often using antimicrobials that act through different mechanisms [8]. However, in clinical practice, infected implants usually require their surgical removal in addition to long-term antibiotic therapy. These problems have stimulated advances in implant surface engineering research, in order to produce implants more resistant to bacterial colonization [9]. One of the most widely adopted strategies is the coating of surfaces with antibiotics; however, this is potentially a hazardous approach due to the risk of selection of drug-resistant micro-organisms, such as methicillin-resistant *S. aureus* (MRSA) [10].

This review aims to clarify mechanisms of *S. aureus* and *S. epidermidis* pathogenicity in implant infections, and to highlight some alternative approaches to preventing infections related to modifications on implant surfaces that could be used in the manufacture of biomaterials.

Methods

Studies were searched in electronic databases according to article titles, abstract contents, and relevance in the field of staphylococcal implant infections. The databases used in this research were PubMed, ScienceDirect, Academic Google and CAPES Journal Portal. The main terms applied were *S. aureus*, *S. epidermidis*, catheter, orthopaedic implant, breast implant, infection, biofilm, antibiofilm activity and antibacterial implant surfaces. Articles were sought that provided new knowledge about the epidemiology of implant infections, the pathogenicity of *S. aureus* and *S. epidermidis* in these infections, and approaches to the prevention of implant-related infections. Each publication identified in the electronic searches was evaluated against these criteria by four authors (W.F.O., P.M.S.S., R.C.S.S. and G.M.M.S.); the selected articles were finally verified and approved by the other authors (G.M., L.C.B.B.C., and M.T.S.C.).

Microbial epidemiology of infections in intravascular catheter and orthopaedic/breast implants

The incidence of local or bloodstream infections associated with intravascular catheters is generally low. However, infections are important, because they are inconvenient to treat, and because serious infectious complications may occur, including sepsis and septic shock, infective endocarditis, and other metastatic infections [11].

Santarpia *et al.* studied 172 patients who had a total of 238 central venous catheters (CVC) used for home parenteral nutrition. Ninety-four of the catheters were associated with

catheter-related bloodstream infection (CRBSI). Coagulase-negative staphylococci (CoNS) were the most frequent causative agents (52.8%); Gram-negative bacteria accounted for 18.6% of infections; 7.1% were caused by fungi, and 15% were by polymicrobial infections [12]. In another study of 85 patients receiving parenteral nutrition, 19% developed CRBSI. Again, *Staphylococcus* spp. (44%) were the most frequent species, followed by *Candida* spp. (25%) [13]. In a recent study by Wu *et al.*, 8% of patients with CVC following gastrointestinal surgery developed CRBSI, and once again CoNS were the most frequent cause of infection [14].

The main micro-organisms that cause infections in orthopaedic implants are Gram-positive bacteria such as *S. aureus*, *S. epidermidis*, and less frequently, *Propionibacterium acnes*; streptococci and enterococci tend to occur in later infections, and Gram-negative bacteria are seen far less frequently [15]. Montanaro *et al.* studied the microbial aetiologies of infections in 242 orthopaedic patients with infections, to investigate their aetiology. Overall, staphylococci accounted for ~75% of all isolates. *S. epidermidis* was the main pathogen in patients with knee and hip arthro-protheses, whereas *S. aureus* was the main pathogen in patients with infections associated with internal and external fixation systems and in patients without implants [16]. A study of 163 patients aged 19–94 years with infected implants in the main joints or long bones of the lower limbs showed a predominance of *S. epidermidis* (51.5%), with 43.6% caused by *S. aureus* (43.6%), and both pathogens isolated from 4.9%. Older patients had a higher mortality rate and higher frequency of infection with methicillin- or multidrug-resistant bacteria [17]. In another study of 115 patients with *S. aureus* orthopaedic implant infections, those who had implants for bone fixation had a lower rate of MRSA infection than those who had arthroplasties. Other risk factors for MRSA were having an open fracture, nursing home residence, renal failure and hospitalization in an intensive care unit. This research raised the possibility of adapting antimicrobial prophylaxis for these higher-risk groups of patients [18].

The majority of isolates from breast-implant infection cases are *Staphylococcus* spp., particularly *S. aureus* and *S. epidermidis* [19,20]. A study with 37 cases of breast implant infection (81% silicone implants and 19% saline implants) showed that the most frequent aetiological agent was *S. aureus* (18 cases) [21]. However, Darragh *et al.* performed two retrospective audits: one with 86 patients undergoing 106 implant-based reconstructions, and another with 89 patients who underwent 105 implant-based reconstructions. In the first audit, bacteria were isolated in three cases, all of which were Gram negative (*Escherichia coli*, two cases; *Pseudomonas aeruginosa*, one case). In the second audit there were five infections, three caused by Gram-negative bacteria and one each caused by *S. aureus* and *S. epidermidis* [22].

Adhesion and biofilm formation: pathogenicity in medical devices

Multidrug-resistant nosocomial pathogens are the most common micro-organisms in medical device infections. They colonize the external and internal region of the catheters and proliferate at a rate of 0.5 cm of surface area per hour, being able to form a thick biofilm in 24 h on the surface of these

plastic devices, from an inoculum with a small number of bacteria [23].

CoNS are important endogenous pathogens of intravascular catheters. Infections tend to be subacute or chronic, whereas infections with *S. aureus* are more likely to be acute due to its ability to stimulate an acute immune response in the host [24]. Figure 1 shows the routes of infection via CVCs, and the mechanism of biofilm formation. The most usual route of infection in short-duration catheters is by migration of micro-organisms from the skin at the insertion site to reach the catheter tip [25]. Catheter hub contamination by contact with contaminated hands, fluids or devices may also lead to an intraluminal colonization of the device [25,26]. More rarely the catheter may be contaminated via the haematogenous route; occasionally, contaminated infusate may introduce micro-organisms into the catheter lumen [26].

Biofilm growth occurs through a series of physical, chemical, and biological processes [27]. The ability of *S. aureus* to adhere to eukaryotic cells and abiotic surfaces through the proteins of its cell wall with subsequent biofilm formation are characterized as important virulence factors in infections associated with implanted biomaterials. The cell-to-cell attachment in the biofilm is known as cohesion [27,28]. Biofilm formation is divided into three steps (Figure 1): initial adhesion to a surface, microcolony formation and biofilm maturation with detachment of bacterial cells. The initial micro-organism adhesion to

surfaces in which the planktonic cells become sessile (Figure 1a) strongly depends on the conditioning layer formed by the adsorption of (macro)molecules on the substrate. The composition of this biofilm favours bacterial adhesion and varies according to the environment to which those surfaces are exposed [29,30]. *S. aureus* and *S. epidermidis* express several microbial surface components that recognize and bind to extracellular matrix molecules, such as fibrinogen and fibronectin proteins, acting in the first stage of biofilm formation in the human body. The matrix proteins are also adsorbed on the surface of medical devices after implantation and may be targets for specific binding to the surface components of staphylococci [31,32]. The initial bacterial adhesion to surfaces is mediated by reversible interactions whose associated physical forces are van der Waals forces and steric–electrostatic interactions [24,27]. Subsequently the bacterial cells adhere irreversibly to the substrates through hydrogen bonds, ionic bonding, and dipole–hydrophobic interactions. Bacterial cell surface structures such as lipopolysaccharides (LPS) and exopolysaccharides also participate in these irreversible interactions [24]. For example, the production of slime exopolysaccharide by *S. epidermidis* is indispensable for its direct adhesion on implants [33].

The secretion of an extracellular polymeric substance (EPS) consisting of extracellular DNA, proteins, lipids and mainly polysaccharides (homo- and heteropolysaccharides) facilitates

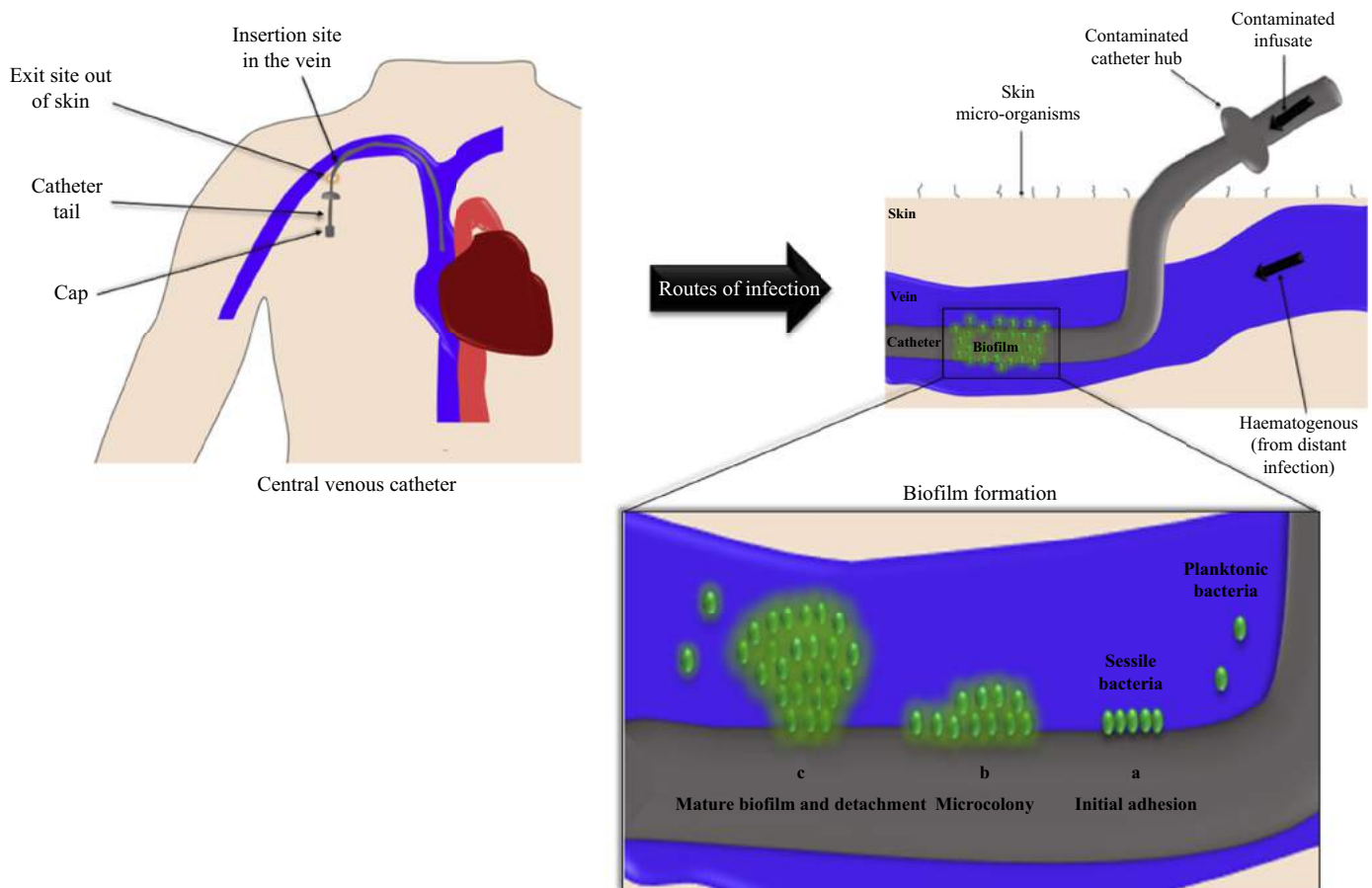


Figure 1. Schematic illustration of central venous catheter infection, showing the main access routes of micro-organisms to cause infection and biofilm formation.

the adhesion between cells and surfaces [24,30]. Bacteria adsorbed on surfaces grow in microcolonies (Figure 1b) and secrete EPS, becoming encapsulated in a hydrogel layer that forms a physical barrier between the microbial community and the extracellular environment [30]. In biofilm development, microcolonies increase progressively and when several layers of cells accumulate on the surface the third stage of formation is reached, indicated by the presence of a mature biofilm (Figure 1c) which is characterized by the presence of macrocolonies surrounded by channels that help to distribute nutrients and signalling molecules. Finally, in order to survive when there is a limitation of nutrients or to spread and colonize other niches, some cells detach from the biofilm individually or in agglomerates [24]. EPS from staphylococci biofilms are composed of extracellular DNA, proteins, amyloid fibrils and polysaccharides such as the polysaccharide intercellular adhesin (PIA) known as poly- β (1–6)-*N*-acetylglucosamine (PNAG), which is the main component responsible by intercellular adhesion in staphylococci. PIA is the primary polysaccharide involved in the formation of *S. aureus* and *S. epidermidis* biofilms, contributing significantly to infections in medical devices and to the evasion of host immune responses [31,34,35]. PIA is synthesized by enzymes encoded by the *icaADBC* locus consisting of four genes, the first and the second being *icaA* and *icaD*, respectively, which together synthesize a transmembrane enzyme with *N*-acetylglucosaminyl transferase activity, since this enzyme is only catalytically active with the junction of the products of these two genes. The *icaC* product appears to translocate PIA to the bacterial surface and the *icaB* product promotes deacetylation of the molecule. Another gene, *icaR*, known as the intercellular adhesin locus regulator, encodes a product that regulates negatively the *icaADBC* locus [35]. The deacetylation of the *N*-acetylglucosamine residues in PIA is of great biological importance, since its free amine group confers positive charge to the molecule. This is then electrostatically attracted to the negative charge on the bacterial cell surface, mainly due to the presence of teichoic acids, contributing to the staphylococci adhesion in biofilms on certain surfaces [31]. The auto-inducing molecule called AI-2, which is a product of the *LuxS* gene and belongs to the *LuxS* quorum sensing system, regulates negatively the expression of the *ica* gene at the transcriptional level in *S. epidermidis* [36]. In *S. aureus* the *Spx* protein (global regulator of stress response genes) induces *icaR* gene expression, which promotes down-regulation of *icaADBC* expression, whereas the protein *Rbf* (protein regulator of biofilm formation) inhibits the expression of *icaR*, leading to an upregulation of *icaADBC* [37]. Bacteria in the biofilm may be 500–5000 times more antimicrobial resistant than planktonic bacteria. Though biofilm initiates the antigenic response in the host by stimulating the production of antibodies, these communities are not yet affected by the host immunogenic response [38,39]. Several mechanisms contribute to biofilm resistance to antimicrobials, such as low penetration of the antimicrobial agent due to biofilm matrix barrier function, presence of persistent dormant cells, and small, highly resistant variant colonies. Reduction of antibiotic susceptibility also occurs: stress-adaptive responses of the bacterial cells in the biofilm may lead to delayed drug penetration or slow cell growth, to changes in the chemical micro-environment within the biofilm, and to upregulation of several biofilm-specific resistance genes [40–42].

Assays applied in the investigation of biofilm formation

Several methods are used to evaluate biofilm formation by bacteria and therefore may be applied to evaluate new compounds with antibiofilm action which may have potential value in implant functionalizations. Among these methods we highlight the tissue culture plate (TCP), tube method (TM), Congo Red agar method (CRA), bioluminescence assay, and fluorescent microscopic examination [43]. Christensen *et al.* were pioneers in investigating the formation of *S. epidermidis* biofilms on smooth surfaces as plastic tubes (by the TM method) and 96-well tissue culture plates (by the TCP method) [44,45]. The TCP method using microtitre plates is one of the most used to evaluate the formation of bacterial biofilms [46]. Knobloch *et al.* carried out a comparative study to evaluate the biofilm formation by *S. aureus* using the TCP, TM, and CRA methods. They verified that among 128 strains analysed, around 57% showed biofilm formation by the TCP method, and the addition of glucose and/or sucrose to the media (brain–heart infusion and trypticase soy broth) strongly influenced biofilm production among the strains. This study also showed that the CRA method is not indicated to evaluate the biofilm formation by *S. aureus*, and the TM method yielded a good correlation with the TCP test, but the classification by TCP test was difficult for biofilm-forming weak strains [47].

Biofilm formation may be quantified by different methods, including the use of dyes such as crystal violet. Dyes are often employed due to the low cost and good reproducibility. Crystal violet binds to negative charges, revealing the total biofilm biomass by the affinity to the bacteria and the EPS [48]. In addition to the total biomass quantification methods, there are assays that quantify the total number of bacterial cells, the number of viable cells, the amount of proteins and polysaccharides, presenting advantages and disadvantages that vary between cost and detection efficiency [48]. It is also possible to analyse the patterns of biofilm formation and to evaluate the activity of bioactive compounds through microscopy. Confocal laser scanning microscopy (CLSM) enables observation of whether the adhered cells forming the biofilm are alive or dead, using dyes such as SYTO-9, which is a fluorescent green dye that binds to nucleic acids and stains live and dead cells. Propidium iodide, which is a fluorescent red dye that penetrates damaged cells, stains dead cells [49].

Trentin *et al.* tested the antibiofilm activity of different plant extracts against *S. epidermidis* at concentrations of 4 and 0.4 mg/mL and evaluated the minimum inhibitory concentration (MIC) that kills 100% of the bacteria [50]. It was observed that the extracts actively inhibited the biofilm formation and that for the *Commiphora leptophloeos* extract the highest concentration tested was also bactericidal, suggesting that the biofilm inhibition at this concentration was due to the death of bacteria [50]. It is important to analyse the effect of the active agent on planktonic bacteria concomitantly to determine whether the compound acts to impede bacterial adhesion and/or biofilm destruction, or whether it kills bacteria, thereby reducing biofilm production. The nematode *Caenorhabditis elegans* is often used as an experimental model for in-vivo assay. Bakkiyaraj and Pandian tested the in-vivo and in-vitro antibiofilm activity of a coral-associated actinobacteria (extract CAA-3) against *S. aureus* [51]. In their study, *C. elegans* was infected

with different strains of *S. aureus* and submitted to the treatment with CAA-3. Intestinal colonization of *C. elegans* was observed by CLSM and measured by Z-stack analysis. The conclusion was that intestinal colonization by *S. aureus* was reduced to ~70% when compared with the control.

The functionalization of nanostructured surfaces with bioactive compounds has attracted considerable interest for several applications. In a study by Qi *et al.*, multiple-wall carbon nanotube surfaces (MWNTs) were covered with a peptide with antimicrobial potential known as nisin, in order to observe *S. aureus* biofilm formation. A decrease of up to 95% in *S. aureus* biofilm formation was observed, compared with a reduction of only 37% for MWNTs alone. In this context, the surface modification used in implants, as functionalization with compounds that have antibiofilm activity, may be an effective alternative in the prevention of post-surgical infections [52].

Strategies for prevention of staphylococcal infections: catheter and implants

Some implant coatings have been used together with antibiotics in preoperative patients to prevent staphylococcal infections [53]. However, studies have also been carried out in order to add antibiotics or other biomolecules to the implants as a preventive action of possible postoperative infections [54]. The surface of implants may be functionalized with compounds having antibiofilm activity to promote a local effect of preventing bacterial adhesion and biofilm formation (Figure 2). This modification of surfaces in biomaterials has been an innovative strategy to be applied in the manufacture of implants.

A film composed of chitosan and gentamicin on titanium (Ti) nanotubes was studied by Feng *et al.* to evaluate the antibiotic effect against *S. aureus* [55]. Antibacterial effect was not observed with the Ti nanotubes without the coating on the bacterial colony. The results indicated that nanotubes coated with gentamicin and chitosan have high resistance to adherence of *S. aureus*, showing an antibacterial activity close to 100%. The coating of chitosan and gentamicin showed excellent antibacterial activity and may be applied to implants [55].

The use of synthetic and natural compounds as an alternative to antibiotics on biomaterial surfaces has also been investigated. Kuehl *et al.* examined the preventive effect on

biofilm production of an implant using a silver-coated titanium–aluminium–niobium metal alloy. The in-vivo tests showed that the compound formed by the Ag-coated alloy was effective in the prevention of postoperative infection by *S. epidermidis*, especially in conjunction with perioperative antibiotic prophylaxis. On the other hand, such silver-coated implants showed only limited effect in the prevention of *S. aureus* infections [9]. Ti alloys are suitable for various requirements of implants [2]. Silver has bactericidal and bacteriostatic properties, and then its controlled release can be obtained with the use of silver nanoparticles. These nanoparticles have been immobilized on medical implant surfaces such as Ti and diamond-like carbon, enabling the prevention of *S. aureus* and *S. epidermidis* biofilm formation, including at the transcriptional level [56,57].

The biomolecules polymethyl methacrylate and polystyrene are widely used in the composition of biomaterials. When these materials were coated with the essential oil from the plant *Ocimum tenuiflorum*, the prevention of bacterial adhesion and *S. aureus* biofilms formation on these substrates was observed [58]. The enzyme deoxyribonuclease I (DNase I), capable of degrading extracellular DNA, was immobilized on Ti surfaces and showed a preventive role for *S. aureus* adhesion and its consequent biofilm formation [59].

Venous catheters can be treated with electrical conduction in order to prevent *S. aureus* biofilm formation, but there are no in-vivo assays due to the lack of patients willing to perform the tests. The applied electrical parameters are safe, avoiding the emergence of arrhythmias. Catheters were submitted to an electric current in units ranging from 4 to 8 μ A and a reduction of 90% of viable bacteria was observed when the current was 4 μ A for a 24 h period. However, the application of a current of 8 μ A did not show a reduction proportional to the current increase [60]. The results appeared encouraging for developing solutions that avoid the contamination of catheters.

Conclusion

Staphylococcus aureus and *S. epidermidis* are frequently involved in infections in catheters and orthopaedic/breast implants. Planktonic bacteria become sessile cells capable of forming microcolonies after adhering to surfaces, secreting biomolecules that make up the EPS where they are embedded.

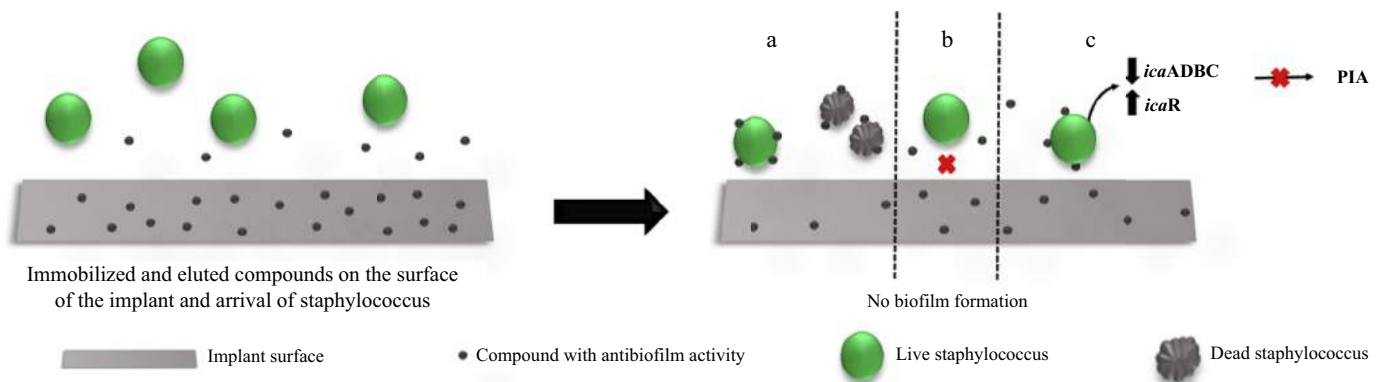


Figure 2. Compounds with antibiofilm activity can be immobilized on the surface of implants. The composites prevent the formation of biofilm, (a) stopping the growth or causing the death of the bacterium; (b) inhibiting the bacterial adhesion on the surface without exerting bacteriostatic/bactericidal effect; (c) and repressing the expression of the genes located in the *icaADBC* locus and stimulating the expression of *icaR* genes which consequently does not promote the production of polysaccharide intercellular adhesin (PIA).

S. aureus and *S. epidermidis* synthesize PIA by the expression of genes located in the *icaADR* locus, and the deacetylation of this adhesin promotes the adhesion of these bacteria to the biomaterial surfaces and the consequent infection. In the mature biofilm, bacteria can establish communication with one another, receive nutrients and water through channels, contributing to their survival on the biomaterial surfaces until they detached and become free-living cells capable of contaminating other locations. Bacteria in biofilm achieve greater resistance to antibiotics and to the immune system of the infected host. Due to the difficulty in treating implant infections, different methodologies have been used to test the potential antibiofilm of compounds; for example, crystal violet dye for in-vitro biofilm quantification offers low cost and good reproducibility. Changes in the surface biomaterials are necessary to prevent biofilm formation. Some studies have investigated the immobilization of antibiotics on the surfaces of materials used in implants. Other approaches have also been used as a way to avoid the spread of bacterial resistance to antimicrobials, such as the functionalization of these surfaces with silver and natural compounds, as well as the electrical treatment of these substrates.

Conflict of interest statement

None declared.

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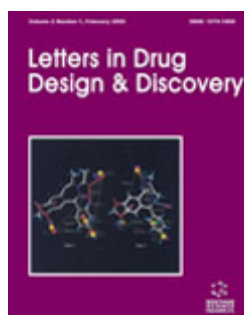
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APÊNDICE G – ARTIGO PUBLICADO NA REVISTA LETTERS IN DRUG DESIGN AND DISCOVERY

Plant antioxidants and mechanisms of action

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REVIEW ARTICLE

Plant Antioxidants and Mechanisms of Action

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Abstract: Background: Free radicals are unstable molecules with one or more unpaired electrons that can associate and oxidize the biological macromolecules causing damage. The human organism is endowed with antioxidant defenses; however, an imbalance between these defenses and the oxidative stress, derived from excess of free radicals, can trigger the development of diseases. The fighting against free radicals can receive the reinforcement of endogenous and exogenous antioxidant molecules. Plants constitute a valuable source of antioxidant compounds, thus, this review explains the activity of the main enzymatic and non-enzymatic antioxidants of plants functioning as exogenous antioxidants for humans.

Methods: Works were searched through electronic databases according to article titles, abstract text, and relevance in the field of plant antioxidant compounds. Articles were analyzed and selected by the authors (WFO and PMSS) and were approved by the other authors (LCBBC and MTSC).

Results: Some pathological conditions can be initiated or exacerbated by free radicals that cause cellular and molecular damages. Plants can produce constitutively antioxidant molecules, with enzymatic or non-enzymatic action; and such synthesis can be increased in certain situations when the plants are exposed to some stressors. These compounds with antioxidant action can be identified in plant extracts and even be purified with elucidation of their chemical structure. Different *in vitro* methodologies can be useful for screening the antioxidant action of these molecules, for example, by evaluating the reducing power of such compounds on commercially available free radicals. Nanotechnology can be used to promote local delivery of antioxidant molecules, for example, by loading such compounds into nanotubes or encapsulating them in nanoemulsions.

Conclusion: Some isolated compounds approached can be used for the design of new drugs. In addition, this article reports new nanotechnological systems that promote the delivery of plant antioxidant molecules, becoming a future perspective for the elaboration of more effective formulations with these compounds.

Keywords: Plant antioxidants, antioxidative mechanisms, free radicals, oxidative stress, nanotubes, nanoemulsions.

1. INTRODUCTION

Reactive oxygen species (ROS) are generated mainly in the electron transport chain (ETC) when some electrons are leaking from the mitochondrial inner membrane and react with oxygen forming chemical species, such as hydroxyl radical ($\text{OH}^\bullet$), superoxide ($\text{O}_2^{\bullet-}$), and hydrogen peroxide (H_2O_2); reactive nitrogen species (RNS) include nitric oxide ($\text{NO}^\bullet$), which is little reactive, and its derivative the peroxynitrite (ONOO^-), a powerful oxidant [1, 2]. Oxidative stress is defined as the impairment of the balance between the generation of ROS/RNS and the ability of cells to neutralize them by the antioxidant defense. The excess of these free radicals has cooperated with the pathogenesis of diseases

by structural alterations and function modulation in proteins, lipids, carbohydrates and nucleic acids [1, 3, 4].

Humans have defense systems to combat free radicals and prevent the consequent triggering of diseases. These systems are classified into two groups: endogenous and exogenous antioxidants. Endogenous antioxidants are the compounds produced by the organism itself and can be enzymatic or non-enzymatic. The major enzymatic nature antioxidants involved in the neutralization of ROS and RNS encompass the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione reductase (GR); endogenous non-enzymatic antioxidants, also known as metabolic antioxidants, include glutathione, lipoic acid, coenzyme Q10, among others [5]. Exogenous antioxidants are not produced by the body and are absorbed in the feeding coming from fruits, vegetables and grains [6]. Components of plants responsible for antioxidant activity, such as phenolic acids, phenolic diterpenes, flavonoids and

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volatile oils, can be a less harmful alternative to synthetic antioxidant products [7, 8].

Therefore, the objective of this review is to provide an understanding of how free radicals participate in the pathogenesis of certain diseases and how plant antioxidants can act as prevention. It is also addressed the use of nanotechnology to promote the delivery of these antioxidant compounds for future inclusion in nutraceuticals and pharmaceuticals formulations.

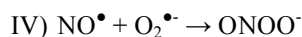
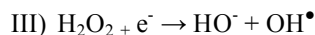
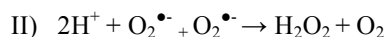
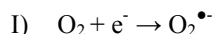
2. FREE RADICALS AND OXIDATIVE STRESS SIGNALS

Radicals are molecules with one or more unpaired electrons normally generated from metabolic pathways, existing in a caged form and in a free-form, known as free radicals, which can interact with cells and tissues. Caged radicals are retained as immobilized molecules, performing functions within their sites of generation and hardly interact with molecules [9]. Free radicals are diffusible molecules, which easily interact with other molecules and unpaired electrons within cells and tissues to achieve a more stable status, being potential oxidizing agents. These radicals are important controllers of regulated redox signaling pathways, but their interaction with cells and tissues components can cause injury, dysfunctions and pathologies [9, 10]. Other terms of more ample meaning have been used to refer to free radicals, such as oxyradicals, ROS, and RNS [11]. These terms include oxidizing species, radical and non-radical, with chemically reactive oxygen-, nitrogen- or also carbon- and sulfur-containing functional groups that can interact with biomolecules and initiate injury in tissues [9]. The generation of ROS and RNS is related to normal cellular reactions, abnormal reactions stimulated by diseases and exposure to dietary xenobiotics and environmental chemicals [12].

$\text{OH}^\bullet$, H_2O_2 , $\text{O}_2^\bullet$, singlet oxygen (O_2) and lipid hydroperoxydes (LOOH) are ROS widely studied and understood. ROS can be generated into a cell through multiple reactions in cell membranes, endoplasmic reticulum, mitochondria and peroxisomes, with the participation of redox and enzymatic systems, as the ETC in the mitochondria and the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) in the cell membrane [11, 13]. The ROS production can also be induced by exogenous factors, such as pollutants, smoke, xenobiotics and radiation. For example, the water radiolysis occurs in the exposition to ionizing radiation, leading the water to lose one electron and generates ROS, mainly $\text{OH}^\bullet$ [14-16]. The reactive species O_2 participates in the aerobic respiration as electron acceptor, and can also result from the decomposition of LOOH, stimulation of neutrophils and macrophages, as well as exposition to UV radiation [14, 17-19]. $\text{O}_2^\bullet$ can be formed during the mitochondrial aerobic respiration, through the reduction of O_2 (Reaction I). It also interacts with transition metals and other radicals, including $\text{O}_2^\bullet$ and $\text{NO}^\bullet$, to form other radicals, as RNS; but hardly react with biological molecules [10, 20, 21]. Another important cellular source of $\text{O}_2^\bullet$ is NADPH oxidase, a membrane-bound enzyme complex present in the extracellular face of the cell membrane that catalyzes the production of $\text{O}_2^\bullet$ from O_2 and NADPH [11]. The H_2O_2 may

be produced through the dismutation of $\text{O}_2^\bullet$ (Reaction II) [22]. It is favorable to penetrate cellular membranes *via* aquaporins [23] and can also interact with transition metals as copper and iron [24] and react with macromolecules such as DNA, inducing lesions as base damage, and single/double-strand breaks [25]. H_2O_2 may be reduced and form $\text{OH}^\bullet$ (Reaction III) [22], which is considered the most reactive ROS, since interacting with organic molecules at diffusion-limited conditions, removing electrons from molecules and inducing the formation of other free radicals [9].

RNS also induce damage in cells and may be generated through the reaction of $\text{NO}^\bullet$ and $\text{O}_2^\bullet$ to produce ONOO^- (Reaction IV) [20]. $\text{NO}^\bullet$ is a highly reactive free radical produced by the enzyme nitric oxide synthase, and acts as an important mediator in the immune response and vascular functions, being also considered a vasodilator and neurotransmitter [11]. ONOO^- is a strong oxidant and nitrating species, highly reactive, which can react with lipids, proteins, nucleic acids, cell membranes and other molecules to produce other RNS, such as nitrogen dioxide (NO_2) and dinitrogen trioxide (N_2O_3), in addition to induction of cell death [26].



Some conditions involving cell component disturbances, including diseases, infections and tissue injury, contribute to the production of high levels of ROS/RNS, which induce and aggravate the initial injury [27, 28]. Many studies have reported the free radicals as oxidizing agents of lipid, protein, carbohydrate, DNA and RNA, as well as of membrane components, causing cellular damage and tissue injury [29]. Free radicals oxidize lipids on cell membranes by a mechanism known as lipid peroxidation, reacting with polyunsaturated fatty acids of cell membranes [30]. In this process, an oxidizing radical oxidizes the polyunsaturated fatty acid and forms a fatty acid radical, which react with an oxygen to form a peroxy radical ($\text{ROO}^\bullet$). $\text{ROO}^\bullet$ can react with other polyunsaturated fatty acid molecules and generates LOOH, which are decomposed to aldehydes and attack other biomolecules. The species $\text{OH}^\bullet$ has been reported as potential inductors of the oxidative damage of proteins, lipids, and mainly DNA, where reacts with base pairs and sugar portions in the oligonucleotides [24]. These reactions generate other radicals and modifications on the DNA bases associated with mutagenesis, cancer and aging. The ROS combined with transition metals as Cu^+ and Fe^{2+} can oxidize proteins and induce oxidative damage to cells and tissues [10]. They also react with different amino acids such as tryptophan, tyrosine, phenylalanine, asparagine, leucine, valine, proline and lysine, producing diverse oxidation products, including hydroxyl residues, carbonyl derivatives and RNS [29]. Moreover, carbohydrates and sugar moieties on proteins and nucleic acid are susceptible to oxidation and can lead to the formation of ketoaldehydes and generate advanced glycation end-products (AGEs), an important factor in the development of degenerative diseases [31].

These markers signal the oxidative stress in diseases and ageing, since cellular components are damaged, and their functions are impaired when free radicals are increased. Oxidative stress has been definite as an imbalance between oxidants and antioxidants in a biological system due to an increase in oxidant levels, impairing the redox control and molecular damage repair [32]. This disturbance in the cell redox status can be generated by increased free radicals. Many dysfunctions have been attributed to free radicals, due to their potential to produce tissue alterations during the injury. Apoptosis is an injury commonly induced by ROS through the mitochondria-mediated or receptor mediated signaling pathways, modulating the activation of caspases, a family of cysteine-aspartate proteases that initiates the apoptosis [9]. As mentioned herein, endogenous antioxidants such as SOD, CAT and GPX are cellular defenses minimizing and neutralizing the oxidative stress, besides the exogenous antioxidants.

Plant-derived foods are rich sources of minerals, dietary fibers and diverse antioxidants such as vitamin A, vitamin C, flavonoids, carotenoids, and phenols. However, these antioxidants may suffer degradation after exposition to oxygen, light, cutting, acid pH, among others, and through the interaction with enzymes as peroxidase and cytochrome oxidase, inducing the decrease of nutrient contents, browning, co-oxidation of biomolecules as fatty acids, and degradation of fruits and vegetables [33, 34]. A study comparing the quality and nutritional content of fresh-cut fruits and whole fruits after specific conditions revealed a decrease in vitamin C and carotenoid content in kiwifruit slices; a decrease of carotenoid in cantaloupe cubes; and a browning in pineapple pieces after light exposure [34].

Oxidative degradation can also be observed in animal-derived food, such as meat and its products. Cooking is an essential step to the processing of meat for food. However, cooking conditions change the composition of meat, due to a reduction in nutritional value, as well as an increase in free radical generations and a decrease in antioxidant protection in meat. The oxidative stress contributes to lipid and protein oxidations, resulting in rancidity, toxic products generation, and deterioration of meat [35-37].

3. FREE RADICALS RELATED PATHOLOGIES

A large number of pathologies have been associated with free radicals, including cancer, diabetes, cardiovascular diseases, inflammatory process, rheumatoid arthritis and neurodegenerative diseases. Cancer is a group of malign diseases whose initiation and progression can be induced by free radicals, from metabolic pathways, or generated through exposure to ionizing and non-ionizing radiation or other exogenous sources. Free radicals may react with DNA, RNA and cellular components, resulting in damage to these molecules, mutagenesis and carcinogenesis induction [29, 38]. Diabetes is a pathological condition characterized by hyperglycemia in non-controlled disease. The elevated blood glucose may induce the formation of ROS as $O_2^{\bullet-}$ and H_2O_2 , through the mitochondrial metabolism and activation of NADPH oxidases. ROS causes oxidative stress and accentuate diabetes complications [39].

Free radicals may also affect the cardiovascular system inducing disease conditions, such as atherosclerosis. ROS can promote the oxidation of low-density lipoprotein (LDL) lipids in plasma, generating oxidized lipids, accumulated by macrophages in arterial walls, leading to foam cell formation, damage on endothelial cells and the development of atherosclerosis [9, 40]. Activated phagocytes, toll-like receptor, NADPH oxidase, smooth muscle cells, and fibroblasts can participate in LDL oxidation through the generation of ROS and RNS, and the release of cytokines and growth factors. High levels of LDL in plasma contribute to the oxidation of lipid process, increasing atherosclerosis risk.

Free radicals are involved in the activation of some genes, signal transduction pathways and cell-to-cell communication, in the context of the immune response, by inducing the release of cytokines [41]. However, an excess of free radicals may accentuate the immune response and initiate chronic inflammatory processes such as rheumatoid arthritis. In this autoimmune disease, antibodies are produced and bind to proteins present in the joint tissue, inducing the activation of neutrophils on site. Activated neutrophils produce ROS, which combined with other inflammatory mediators and proteases, may contribute to injury of tissue, inflammation, and joint degeneration [42].

Several neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, and multiple sclerosis are associated with oxidative stress. The low levels of antioxidant enzymes, high oxygen demand and high lipid content in the central nervous system are factors that contribute to attack of ROS and free radicals on lipids and proteins, causing lipid peroxidation and formation of protein carbonyls, which are observed in these pathologies [29, 43]. The free radicals cause oxidative stress in many pathological conditions; the supplementation with dietary antioxidants, including those from natural plant sources, can help in protection against oxidative stress effects and prevent diseases.

4. PLANT ANTIOXIDANTS

ROS generation in plants occurs through its normal metabolism, such as photosynthesis and respiration; also, by stimulation of different types of environmental stresses, such as high and low temperature, pathogen attack and nutrient deficiency [44]. The main sources of ROS generation in plant cells include photosystems I and II (PS I and PS II) and chlorophyll pigments from chloroplasts; enzymes and complexes I, II and III of mitochondrial ETC; matrix, membrane and metabolic processes in peroxisomes. However, other sites participate in the production of these free radicals, such as cell wall, plasma membrane, endoplasmic reticulum and apoplast [45]. RNS, such as $NO^{\bullet}$ and $ONOO^{\bullet}$, are also generated in some subcellular compartments that include mitochondria, chloroplasts and peroxisomes. The plants have enzymatic and non-enzymatic antioxidant systems to combat toxic effects of free radicals from both stressors and their own aerobic metabolism [46].

4.1. Enzymatic Antioxidants

Plant enzymes with antioxidant activity comprise SOD, CAT, GPX, guaiacol peroxidase (POX), peroxiredoxin (Prx),

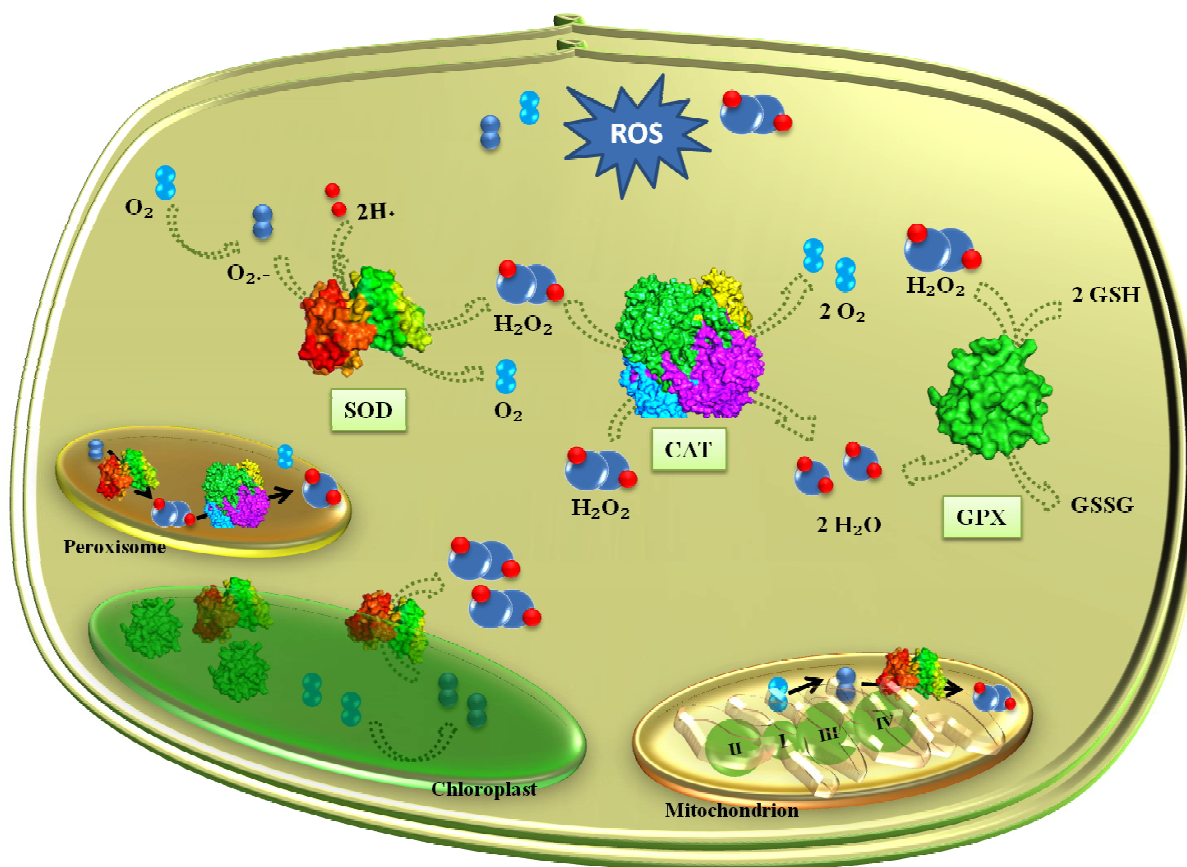


Fig. (1). Schematic representation of a plant cell showing the enzymatic antioxidant mechanisms related to SOD, CAT and GPX.

and enzymes participating in the ascorbate-glutathione (AsA-GSH) cycle. This cycle is the most important antioxidant pathway and involves the enzymes ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and GR [47, 48].

Enzymatic reactions that SOD, CAT and GPX enzymes catalyze within the plant cell are schematized in Fig. (1). SOD enzyme catalyzes the dismutation of $O_2^{\bullet-}$ to O_2 and H_2O_2 ; according to its metal co-factor, SOD can be classified in iron SOD, manganese SOD, and copper-zinc SOD [49, 50]. H_2O_2 can be degraded into H_2O and O_2 by CAT, a tetrameric and heme-containing enzyme found mainly in peroxisomes [51]. H_2O_2 or organic peroxides can be reduced to H_2O by GPX, a non-heme peroxidase that generally uses thioredoxin (Trx) as a reducing agent rather than GSH [52, 53]. POX belong to class III peroxidases, a heme-containing and secretory enzyme found in the extracellular space or in the vacuole, performing two different enzymatic cycles, denominated peroxidative and hydroxylic cycles [54, 55]. Prx is a thiol-dependent enzyme that reduces hydroperoxides and peroxynitrites through its peroxidatic cysteine in the catalytic center (Cys_p) where the thiol of this Cys residue reacts with peroxide; the sulfenic acid is formed and converted to disulfide, and finally, the active thiol is regenerated [53, 56].

Considering the AsA-GSH cycle, APX uses AsA as an electron donor to reduce H_2O_2 to H_2O , whereas AsA is oxidized to monodehydroascorbate (MDHA). This component

is either reduced back to AsA by MDHAR or undergoes non-enzymatic disproportionation to dehydroascorbate (DHA); the resultant DHA is then reduced to AsA in a reaction catalysed by DHAR using GSH as the reductant and undergoes oxidation to glutathione disulfide (GSSG). Finally, GSH is regenerated from GSSG by GR using NADPH [57-59]. Transgenic plants with antioxidant enzymes over-expressed or with its gene silencing has demonstrated that these enzymes, such as APX and MDHAR, protect the plants of the early senescence and abiotic stress, for example, increasing tolerance to salt stress [60-62].

4.2. Nonenzymatic Antioxidants

Nonenzymatic molecules with antioxidant activity synthesized and accumulated in plants participate of their normal physiological functions and/or responses to environmental stress [46]. Phytochemicals belonging to the nonenzymatic antioxidant system include, but are not limited to, ascorbic acid, tocopherols, peptides, carotenoids and phenolic compounds whose chemical structures are represented in Fig. (2).

Ascorbic acid (Fig. 2a), also known as vitamin C, is ubiquitous in plants and its reduced and physiologically active form is AsA whose quantity in different subcellular compartments changes during some abiotic stress conditions that the plant is exposed [63-65]. Ascorbic acid has two ionizable hydroxyl groups and can be found as AsA monoanion, dominant form at physiological pH, or as AsA dianion, gen-

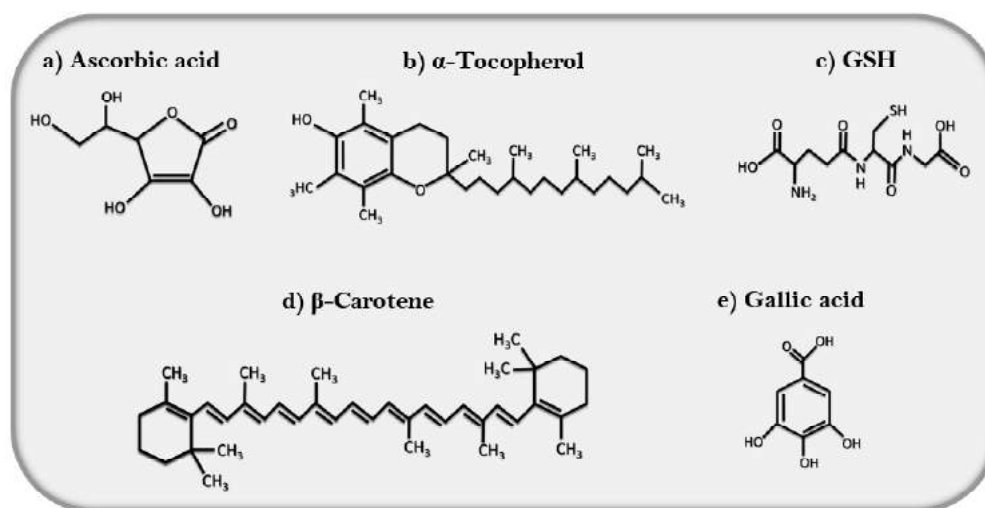


Fig. (2). Chemical structures of representatives belonging to major classes of plant derived nonenzymatic antioxidants. **a)** Ascorbic acid (vitamin C), **b)** α -tocopherol (tocopherols), **c)** glutathione, GSH (peptides), **d)** β -carotene (carotenoids) and **e)** gallic acid (phenolic compounds).

erally present with pH greater than 7. Asa is an excellent reducing agent and undergoes two oxidations generating an ascorbate radical and a dehydroascorbic acid; this acid can receive two electrons from an oxidoreductase and be converted back into AsA [24, 66]. Vitamin C can act directly scavenging $O_2^{\bullet-}$, $OH^{\bullet}$ and O_2 ; also, reducing H_2O_2 to water enzymatically, through APX [67].

Plants synthesize and store four tocopherols (α , β , γ , and δ) in their leaves and seeds; vitamin E deficiency in humans is caused by the lack of α -tocopherol (Fig. 2b), a powerful lipophilic antioxidant [68]. α -Tocopherol, a hydrogen atom donor, is intercepted in the chain-carrying $ROO^{\bullet}$ during lipid oxidation, been capable of protecting polyunsaturated fatty acids, as part of cell membrane phospholipids and LDL [68, 69].

Several plant protein derived peptides have been identified and characterized as potent antioxidants. Generally, these antioxidant peptides have low molecular weight and are obtained through the enzymatic hydrolysis of an extracted protein [70-73]. The high antioxidant capacity of these peptides is often attributed to the presence of hydrophobic and aromatic amino acid residues in their sequences. Hydrophobic residues enable increased peptidic solubility in lipid solution facilitating the interaction of peptides with unstable radicals. Aromatic residues easily donate electrons to free radicals converting them into stable molecules. The histidine residue, for example, is considered as a strong radical scavenger due to its ability to donate photons through its imidazole ring [74-78]. GSH (Fig. 2c) is a low molecular weight free thiol tripeptide that is oxidized by ROS to prevent excessive oxidation of sensitive cellular components; its unique redox and nucleophilic properties are important also in cellular defense against other toxic agents, such as xenobiotics and metal cations [79-81].

Carotenoids, such as β -carotene (Fig. 2d), are natural pigments of the polyene type occurring ubiquitously in all photosynthetic organisms; the molecules are potent scavengers of O_2 and $ROO^{\bullet}$ [82, 83]. Carotenoids can eliminate free

radicals by electron transfer generating a carotenoid radical cation or anion; forming a radical adduct; and by hydrogen atom transfer resulting in a neutral carotenoid radical [83].

Plant secondary metabolites constitute a large group of compounds derived from primary metabolites or their biosynthetic intermediates. These metabolites can be classified through their biosynthetic pathway in three main groups that are terpenes, nitrogen-containing compounds, and phenolic compounds [84, 85]. Aromatic phenolic compounds have a phenyl ring bearing one or more acidic hydroxyl group as represented by gallic acid in Fig. (2e); the major present in plants are anthocyanins, flavonols, flavan-3-ols, hydroxybenzoic acid, hydroxycinnamic acid, and curcumin [85, 86]. Phenolic compounds are extensively studied and commonly used as antioxidants among all plant secondary metabolites; this antioxidant activity is due to its redox properties that make them hydrogen donors [85, 87]. The antioxidant capacity of plants is usually determined through extracts of different plant parts diluted in different chemical reagents. Many researchers isolated the pure compound responsible for this antioxidant activity and chemically characterized such molecules [88-91]. Table 1 shows some isolated molecules of plants with antioxidant potential of studies performed between 2014 and 2017. The purification of these compounds with the elucidation of their chemical structure may be important to stimulate the pharmaceutical industry to make a new design of antioxidant drugs.

5. MECHANISMS OF ANTIOXIDANT ACTION

Exogenous and endogenous antioxidants promote the inactivation of free radicals, preventing the oxidative stress and their consequences. Antioxidants, according to their mechanisms of action, can be classified as primary antioxidants, free radical scavengers, enzymatic antioxidants, chelating agents and mixed antioxidants.

Primary compounds act as donors of hydrogen atoms to free radicals, making them inactive species, blocking the propagation of the chain reaction and the formation of more

Table 1. Isolated compounds of plants with antioxidant activity.

Plants	Plant Parts	Isolated Compounds	Mechanisms of Action	Refs.
<i>Musa</i> AAB var. Nanjanagudu Rasable	Rhizome	4-Epicyclomusalenone; cycloeucalenol acetate and chlorogenic acid.	Scavenging of 1,1-diphenyl-1-picrylhydrazyl (DPPH), $O_2^{\bullet-}$, H_2O_2 , and $NO^{\bullet}$ radicals; β -carotene bleaching inhibition, anti-lipid peroxidation, metal chelating and ferric ion reducing.	[92]
<i>Albizia amara</i> (Roxb.) B. Boivin	Leaves	Budmunchiamine-A.	Scavenging of DPPH radical and β -carotene bleaching inhibition.	[93]
<i>Leucaena leucocephala</i>	Leaves	Quercetin-7-O- α -rhamnopyranosyl-(1 $\rightarrow$ 2 $\rightarrow$)- β -glucopyranoside.	Scavenging of DPPH and 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radicals; metal chelating and ferric ion reducing.	[94]
<i>Dimocarpus longan</i> Lour.	Seeds	2-Hydroxy-3-methoxycaffeic acid 5-O- β -D-glucopyranoside and 3'-O-methyl-4'-O-(4-O-galloyl- α -L-rhamnopyranosyl)ellagic acid.	Scavenging of ABTS radical, oxygen radical absorbance and β -carotene bleaching inhibition.	[95]
<i>Amomum tsaoko</i> Crevost et Lemaire	Fruit	2,3-Dihydro-2-(4'-hydroxyl-phenylethyl)-6-[(3'',4''-dihydroxy-5''-methoxy)phenyl]-4-pyrone and 4-dihydro-2-(4'-hydroxyl-phenylmethyl)-6-[(3'',4''-dihydroxy-5''-methoxyphenyl) methylene]-pyran-3,5-dione.	Scavenging of DPPH radical.	[96]
<i>Astragalus taipashanensis</i>	Roots	7, 2'-Dihydroxy-3'; 4' -dimethoxy-isoflavane; formononetin; isoliquiritigenin; quercetin; kaempferol and ononin.	Scavenging of DPPH and ABTS radicals; ferric ion reducing and lipid peroxidation inhibition.	[97]
<i>Vitex negundo</i> var. <i>heterophylla</i>	Seeds	Vitexnegheteroin A-D; vanilloyl- β -D-(2'-O-4-hydroxybenzoyl)-glucoside; 2-methyl pyromeconic acid 3-O- β -D-glucopyranoside-6'-(O-4''-hydroxybenzoate); breynioside A; 1,6-di-O-4-hydroxybenzoyl- β -D-glucopyranoside, vitexfolin C; salviaplebeiaside and dunnianoside D.	Scavenging of DPPH and ABTS radicals.	[98]
<i>Scadoxus pseudocaulus</i>	Whole plant	5-Hydroxymethyl-2-furancarboxaldehyde; 4-(hydroxymethyl)-5-hydroxy-2H-pyran-2-one and farrerol.	Scavenging of DPPH radical.	[99]
<i>Alpinia officinarum</i>	Rhizome	Galangin and 5-hydroxy-7-(4''-hydroxy-3''-methoxyphenyl)-1-phenyl-3-heptanone.	Scavenging of DPPH, H_2O_2 and $OH^{\bullet}$ radicals.	[100]
<i>Erythrina stricta</i> Roxb.	Stem bark	Erynone; erythrinassinate B and isovanillin.	Scavenging of DPPH radical.	[101]
<i>Cimicifuga dahurica</i> (Turcz.) Maxim	Rhizome	4'-Methoxyl-3'-hydroxy-carboxybenzoyl isoferulic acid anhydride; 2-isoferuloyl piscidic acid; 2-feruloyl piscidic acid and 1-O-feruloyl- β -Dglucopyranoside.	Scavenging of DPPH radical.	[102]
<i>Pappea capensis</i> Eckl. & Zeyh.	Leaves	Quercetin-3-O-rhamnoside and epicatechin	Scavenging of DPPH radical and β -carotene bleaching inhibition.	[103]
<i>Panax ginseng</i> C. A. Meyer (Araliaceae)	Roots	Dammar-12, 24-dien-3 α , 6 β , 15 α -triol-3 α -D-arabinopyranosyl-6 β -L-arabinopyranoside and dammar-24-en-3 α , 6 β , 16 α , 20 β -tetraol-3 α -D-arabinopyranosyl-6 β -D-arabinopyranoside.	Scavenging of DPPH and $NO^{\bullet}$ radicals; ferric ion and phosphomolybdenum reducing.	[104]
<i>Entada abyssinica</i>	Leaves and stem bark	Ursolic acid; quercetin-3-O- α -l-rhamnoside or quercitrin; quercetin-3-O- β -D-glucosyl (1 $\rightarrow$ 4)- α -l-rhamnoside; (8S)-kolavic acid 15-methyl ester; 13,14,15,16-tetranor-3-clerodene-12,18-dioic acid; methyl gallate; entadanin and bis-[(S)-(2,3-dihydroxypropyl)] hexacosanedioate	Scavenging of DPPH and ABTS radicals; ferric ion reducing.	[105]
<i>Achillea fragrantissima</i>	Aerial parts	3,5,4'-trihydroxy-6,7,3'-trimethoxyflavone.	Decreasing intracellular ROS levels in N2a neuroblastoma cells pretreated with amyloid beta.	[106]
<i>Metasequoia glyptostroboides</i>	Cones	Taxoquinone.	Scavenging of DPPH, $NO^{\bullet}$, $O_2^{\bullet-}$ and $OH^{\bullet}$ radicals. Ferric ion reducing.	[107]

(Table 1) contd....

Plants	Plant Parts	Isolated Compounds	Mechanisms of Action	Refs.
<i>Zygophyllum simplex</i> L.	Flowering aerial parts	Myricitrin and luteolin-7- O- β -D-glucoside.	Scavenging of DPPH radical.	[108]
<i>Paulownia catalpifolia</i> T. Gong ex D.Y. Hong	Fruit peels	6-geranyl-4',5',5,7-tetrahydroxy-3'-methoxyflavone (paucatalinone C); 4',5',5,7-tetrahydroxy-3'-methoxy-6-[7-hydroxy-3,7-dimethyl-2(E)-octenyl]flavone (paucatalinone D) and 4',3,5,7-tetrahydroxy-3',5'-dimethoxy-6-[7-hydroxy-3,7-dimethyl-2(E)-octenyl]flavone (paucatalinone E).	Scavenging of DPPH radical.	[109]

reactive radicals [110]. These antioxidants are basically phenolic compounds, including the synthetic polyphenols as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), *tert*-butylhydroquinone (TBHQ) and propyl gallate (PG), as well as natural tocopherols [111].

Free radical scavengers are substances that remove or decrease the oxygen or free radicals in the environment through stable chemical reactions, preventing oxidation and degradation process [112]. Ascorbic acid and its derivatives, hydroquinones, and thiols belong to oxygen and free radical scavenger group.

Enzymatic antioxidants include enzymes such as SOD, CAT, and GPX, which remove free radicals from a biological system, showing a relevant role in the prevention of oxidative stress [24]. SOD is an enzyme present in the cytosol and mitochondria that converts the radical $O_2^{\bullet}$ into oxygen and H_2O_2 . The H_2O_2 is converted to water and oxygen by the peroxisomal enzyme CAT, and into the water by the enzyme GPX. Peroxyredoxins are a family of peroxidase enzymes that reduces H_2O_2 , as well as organic hydroperoxides and ONOO⁻ [113, 114].

Chelating agents form complex with metallic ions as iron, calcium and copper, which participate in the lipid peroxidation. Ethylenediamine tetraacetic acid (EDTA) is an example of chelating agent that removes these metals and minerals, reducing the lipid peroxidation and the formation of free radicals [115].

Antioxidants and non-antioxidants can act as synergistic substances, improving the antioxidant activity when used in association with antioxidants. Citric acid, tartaric acid, phosphoric acid, lactic acid, sorbitol, phenols, flavonoids and antioxidants of other groups are considered synergistic [116]. There are also antioxidants that act by different mechanisms of action, but their activities complement each other in a biological system. Vitamin E or α -tocopherol acts breaking the chain reaction during lipid peroxidation in cell membranes and lipid particles, through the reaction with lipid peroxyl radicals (ROO[•]) [111]. Ascorbic acid is a free radical scavenger that regenerates vitamin E in cell membrane combined with reducing agents such as GSH [24].

Mixed antioxidants are natural compounds from plants and animals that help to protect cells and tissues from oxidative damage by distinct reaction mechanisms. They include a range of nutrients such as proteins, resveratrol, flavonoids, cinnamic acid derivatives, carotenoids and curcumin, which have been reported as potent antioxidants. Resveratrol is a polyphenol and strong inactivator of ROS and also a free radical scavenger [117]. Flavonoids have been reported as

free radical scavengers and chelating agent of metal ions, preventing the formation of ROS and DNA damage induced by hydroxyl radicals. In this context, the protective effects of flavonoids to DNA also depend on metal ion concentrations, since high concentrations may induce the damage [24, 118]. Quercetin is a flavonol that exhibits a protective effect on DNA against oxidative damage induced by cupric ion at low concentration, resulting from the inhibition of $O_2^{\bullet}$, H_2O_2 and OH[•] on the DNA [24]. Flavonoids as quercetin attack free radicals (Fig. 3) by the interaction with $O_2^{\bullet}$ via a one-electron transfer mechanism; as hydroxyl and peroxyl radicals scavengers, and chelating metal ions [24, 119].

Another class of flavonoids known as anthocyanins prevents the lipid peroxidation via free radical scavenging and metal ion chelating mechanisms [120]. Carotenoids scavenge the ROO[•] generated during lipid peroxidation, inhibiting the chain reaction of lipid peroxidation in membranes and lipoproteins [121]. β -Carotene and lycopene are both carotenoids with potential antioxidant activity to quench O_2 , being the lycopene activity stronger than β -carotene and other natural antioxidants [24, 122]. Cinnamic acids and their derivatives like hydroxycinnamic acids have shown efficient antioxidant action to prevent oxidation of human LDL through the presence of *o*-dihydroxy group in the phenolic ring, as well as the free radical scavenger mechanism similar to flavonoids [123]. Another important natural antioxidant is curcumin, which has shown free radical scavenger and chain breaking antioxidant activities [124].

6. NANOPARTICULATE SYSTEMS: A PROMISING FUTURE FOR ANTIOXIDANT DELIVERY

Nanotechnology has emerged as a new area to manufacture nanosystems capable of promoting the transport and delivery of drugs, increasing the bioavailability of molecules to perform their function in the specific local. Nanoparticles functionalized with antioxidants have been denominated nano antioxidants to use in the prevention and treatment of neurodegenerative diseases [125]. However, other nanosystems can be employed to promote delivery of antioxidants, such as nanotubes and nanoemulsions.

Carbon nanotubes (CNT) are graphene sheets rolled forming a cylinder nanostructure possessing a hexagonal arrangement as shown in Fig. (4). CNT wall can be formed by a single graphene sheet and these nanotubes are denominated single-walled carbon nanotubes (SWCNT); when CNT are constituted of more than one sheet are now called multi-walled carbon nanotubes (MWCNT) [126]. CNT can be absorbed by cells through endocytosis-dependent pathway (receptor mediated or nonreceptor mediated) or by means of

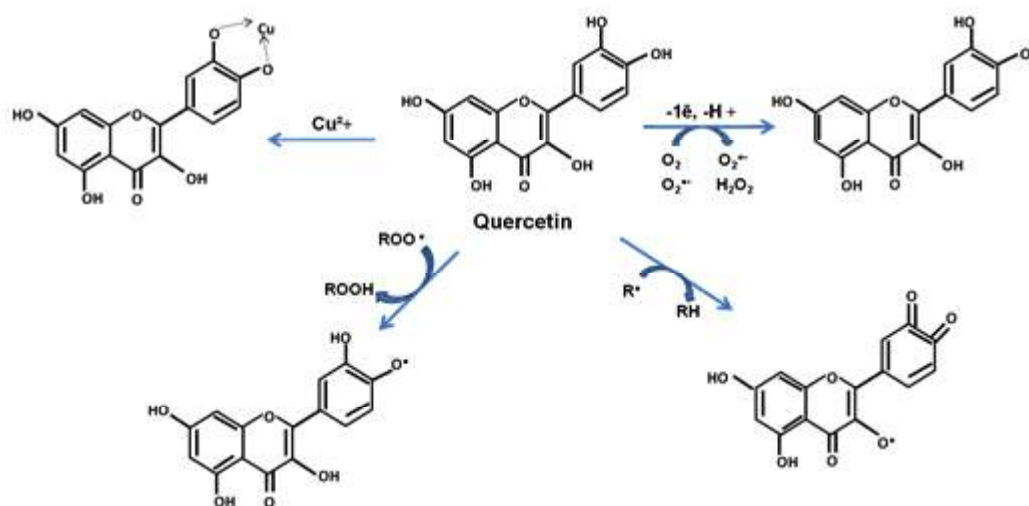


Fig. (3). A simplified representation of radical scavenging and chelating metal ion activities of quercetin, highlighting the superoxide anion, hydroxyl and peroxy radicals, as well as quercetin-Cu complex.

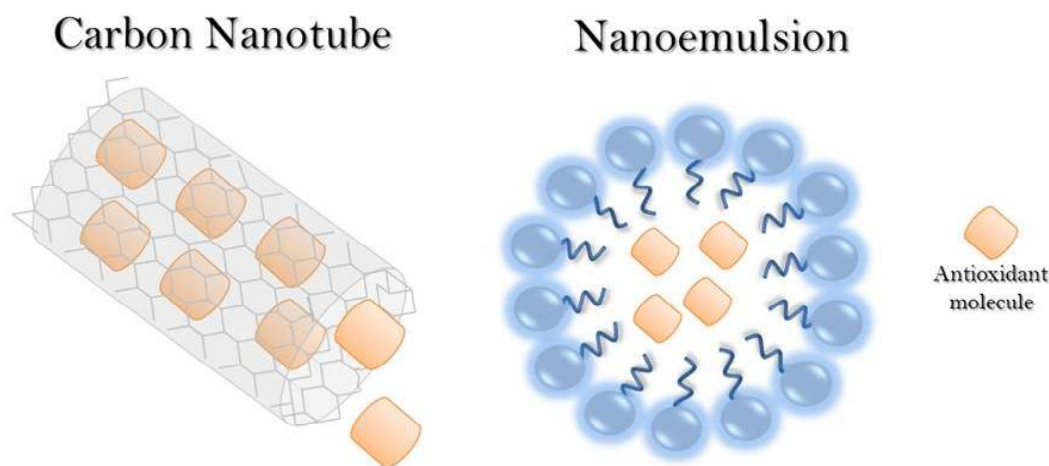


Fig. (4). Carbon nanotube and nanoemulsion as nanoparticulate systems for antioxidant delivery.

endocytosis independent pathway, for example, membrane fusion, direct pore transport and diffusion. CNT have been used as a novel class of nanocarriers for the delivery of therapeutic molecules in a site-specific manner for cancer treatment, for example [127]. CNT functionalized with these molecules can be driven to specific cancer cells; antioxidant compounds loaded in CNT can act to balance the oxidative stress existent in the neoplastic cells [128].

The interaction between a plant bioactive extract with antioxidant activity and CNT was performed through a computational analysis [129]; while the release of CAT enzyme which was loaded onto carbon nanoparticles, including CNT, was evaluated experimentally [130]. Both studies have demonstrated that such systems can be useful in designing new drugs. However, these nanotubes can be toxic to living cells, but when the CNT were functionalized with the phenolic compounds gallic acid and tannic acid there was a considerable reduction of the oxidizing ability of CNT showing a new possibility to improve the biomedical applicability of these nanotubes [131]. In addition, titanium dioxide nanotubes have been used in the manufacture of implants and

their functionalization with quercetin, a flavonoid with antioxidant action, has been performed for the development of drug-releasing implants for localized drug delivery [132].

Nanoemulsions are dispersions of two immiscible liquids, for example, water and oil, stabilized by a surfactant; this nanosystem has attracted attention for use as a vehicle for drug delivery [133]. The surfactant or emulsifying agents are amphiphilic molecules that may reduce interfacial tension of two immiscible liquids. These agents have apolar hydrocarbon tails that interact with non-polar compounds and polar head groups that interact with water-soluble compounds [134]. Therefore, hydrophobic antioxidant molecules may be encapsulated within nanoemulsions as illustrated in Fig. (4). This nanosystem when administered orally, for example, may increase the systemic bioavailability of drug due to an increase in its rate of dissolution [133].

Different vegetable oils, such as olive oil and essential oils, have been efficiently encapsulated in nanoemulsions for the elaboration of formulations promoting efficient antioxidant activity and improving the solubility of liposoluble an-

tioxidant compounds in either aqueous or transdermal application [137-139]. In addition to oils, other compounds such as catechin, quercetin, curcumin and lycopene are being loaded into nanoemulsions and revealing effective potentiation against oxidative stress with promising applications, for example, photoprotection and antifibrotic action [138-143].

Nanoparticle-based therapy is a growing application of nanotechnology in medicine to provide an efficient treatment reducing side effects. Delivery systems based on nanoparticles have been developed in cosmetic industry, for incorporation in the cosmetic formulations containing plant antioxidants, vitamins and coenzymes with anti-ageing, anti-inflammatory and antimicrobial properties [144, 145]. Nanostructures as liposomes, nanoemulsions and lipid nanoparticles have been used to carry antioxidants, acting as a physical barrier to be protected from other formulation components, and improve skin penetration of antioxidant [145, 146]. Nanoparticles modified with polymers and biological ligands have been also developed and tested to improve the stability, selectivity, and controlled release, which is very attractive for local dermatotherapy [145].

A range of skin care products containing antioxidants into nanoparticles is commercially available mainly as approaches for protection against skin damage [147]. For example, Sircuit Addict Firming Antioxidant Serum is an anti-aging product containing grape seed extract, vitamin E and green tea extract carried into carbon nanostructures named fullerenes. Nano-Lipobelle DN CoQ10 oA and Nano-Lipobelle H-AECL are anti-aging with nanoemulsions carrying CoQ10, vitamin E and C, and vitamins A, E and C, respectively. The product Revitalift contains nanosomes carrying pro-retinol A and is used for skin protection.

Delivery systems based on nanoparticles have also been developed and tested in animal models for the treatment of diseases. Polymer-based nanoparticles containing pH responsive nitroxide radical showed a protective effect in renal disease. These nanoparticles disintegrated at pH below 7.0 within the renal acidic lesion and showed high reactive oxygen species scavenging activity in mice, easing the acute kidney injury [148]. Other experiment conducted in rats using chitosan nanoparticles combined with quercetin showed synergistic protective role against oxidative stress and hepatotoxicity induced by carbon tetrachloride, being these nanoparticles a potential candidate as drug delivery in liver treatment [149].

CONCLUSION

Free radicals, mainly ROS and RNS, are generated in biological systems during normal cellular reactions, pathologies and exposure to xenobiotics. An excess of free radicals in a biological system may overcome the potential of endogenous antioxidants, inducing the oxidative stress, injury and diseases. The antioxidants react with free radicals *in vivo* and *in vitro*, removing this oxidant species through distinct mechanisms of action. The dietary supplementation with natural antioxidants from vegetables may help to maintain the balance between free radicals and antioxidants and to prevent oxidative stress and diseases. Substances such as phenols and flavonoids showed antioxidant activity and

therapeutic potentials. Plant antioxidants have been encapsulated in nanotubes and nanoemulsions to improve the bioavailability of these molecules and thus are effective alternatives in preventing damage caused by free radicals. Antioxidant molecules should be purified from plants and the chemical structure of these compounds defined providing a new basis for drug design. Then, more studies are required to evaluate the antioxidant activity *in vivo*, for future applications in therapeutic and preventive medicine.

LIST OF ABBREVIATIONS

ROS	=	Reactive oxygen species
ETC	=	Electron transport chain
OH•	=	Hydroxyl radical
O ₂ •	=	Superoxide
H ₂ O ₂	=	Hydrogen peroxide
RNS	=	Reactive nitrogen species
NO•	=	Nitric oxide
ONOO•	=	Peroxynitrite
SOD	=	Superoxide dismutase
CAT	=	Catalase
GPX	=	Glutathione peroxidase
GR	=	Glutathione reductase
O ₂	=	Singlet oxygen
LOOH	=	Lipid hydroperoxydes
NADPH oxidase	=	Nicotinamide adenine dinucleotide phosphate-oxidase
NO ₂	=	Nitrogen dioxide
N ₂ O ₃	=	Dinitrogen trioxide
ROO•	=	Peroxyl radical
AGEs	=	Advanced glycation end-products
LDL	=	Low-density lipoprotein
PS I	=	Photosystem I
PS II	=	Photosystem II
POX	=	Guaiacol peroxidase
Prx	=	Peroxiredoxin
AsA	=	Ascorbate
GSH	=	Glutathione
APX	=	Ascorbate peroxidase
MDHR	=	Monodehydroascorbate reductase
DHAR	=	Dehydroascorbate reductase
Trx	=	Thioredoxin
GSSG	=	Glutathione disulfide
BHT	=	Butylated hydroxytoluene

BHA	=	Butylated hydroxyanisole
TBHQ	=	Terc-butylhydroquinone
PG	=	Propyl gallate
EDTA	=	Ethylenediamine tetraacetic acid
DPPH	=	1,1-Diphenyl-1-picrylhydrazyl
ABTS	=	2,2'-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid
CNT	=	Carbon nanotubes
SWCNT	=	Single-walled carbon nanotubes
MWCNT	=	Multi-walled carbon nanotubes

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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APÊNDICE H – ARTIGO PUBLICADO NA REVISTA JOURNAL OF APPLIED MICROBIOLOGY

Lectins as antimicrobial agents

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REVIEW ARTICLE

Lectins as antimicrobial agents

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Keywords

antimicrobials, biofilms, lectin-based therapies, lectins, micro-organism infections, quorum sensing.

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Summary

The resistance of micro-organisms to antimicrobial agents has been a challenge to treat animal and human infections, and for environmental control. Lectins are natural proteins and some are potent antimicrobials through binding to carbohydrates on microbial surfaces. Oligomerization state of lectins can influence their biological activity and maximum binding capacity; the association among lectin polypeptide chains can alter the carbohydrate–lectin binding dissociation rate constants. Antimicrobial mechanisms of lectins include the pore formation ability, followed by changes in the cell permeability and latter, indicates interactions with the bacterial cell wall components. In addition, the antifungal activity of lectins is associated with the chitin-binding property, resulting in the disintegration of the cell wall or the arrest of *de novo* synthesis from the cell wall during fungal development or division. Quorum sensing is a cell-to-cell communication process that allows interspecies and interkingdom signalling which coordinate virulence genes; antiquorum-sensing therapies are described for animal and plant lectins. This review article, among other approaches, evaluates lectins as antimicrobials.

Introduction

The extensive use of antimicrobial agents in humans, animals and plants favours the emergence of micro-organisms resistant to these drugs. Despite the large number of broad-spectrum antimicrobial agents commercially available, these micro-organisms frequently develop new resistance mechanisms to escape antimicrobial action and are responsible for increasing nosocomial infections and mortality rate from these diseases.

There is a great interest in isolating antimicrobial substances from natural sources. Proteins and peptides with antimicrobial activity are naturally occurring molecules found in microbes, plants, animals and humans, which act by interfering with the microbial growth by different mechanisms (Nawrot *et al.* 2014; Zhang and Gallo 2016). Lectins are natural carbohydrate-binding proteins that can mediate the identification of micro-organisms through the interaction with complex carbohydrates on microbial surfaces (Procópio *et al.* 2017a) promoting host–pathogen communications, immune defence activation and cell-to-cell

signalling. A growing interest has been developed for the investigation of the lectin role in the interaction between eukaryotic cells and pathogens in infectious disease development and their antimicrobial potential (Dias *et al.* 2015). The antimicrobial roles of lectins include blockade of invasion and infection, inhibition of growth and germination, regulation of microbial cell adhesion and migration.

A variety of laboratory methods can evaluate the efficacy of antimicrobial lectins; the first indication of antimicrobial activity by *in vitro* assays allows discovery of new antimicrobial compounds and their mode of action. Antimicrobial lectins evaluated by *in vivo* assays may promote the preclinical evaluations to approach efficacy, safety and the best dosage (Barton *et al.* 2014).

Frequently, the antimicrobial activity of lectins is related to their carbohydrate-recognition domain (CRD; Procópio *et al.* 2017a). However, this review provides examples of the fact that such specific recognition and binding to microbial surface glycans is not always indicative of this biological activity. In addition, the oligomeric state of these proteins can interfere with their action against micro-organisms.

Several investigations have been carried out worldwide on the antimicrobial activity of natural compounds, including lectins (Silva *et al.* 2016a). Nevertheless, studies that address the mechanism of antibacterial or antifungal action are still in a minority (Ballal and Inamdar 2018; Patin *et al.* 2018; Van Holle and Van Damme 2018). The identification of these mechanisms represents an important step for the effective application of new drugs, since it will be useful to establish strategies of drug delivery, increase effectiveness of possible formulations, as well as will predict mechanisms of future and inevitable microbial resistance (Pichl *et al.* 2016).

Microbial cells use a quorum-sensing (QS) process as an intercellular communication system which enables collectively to adjust behaviour in response to a variety of mechanisms, like density and species diversity (Papenfort and Bassler 2016). Anti-QS mechanisms interfere with gene expression that renders pathogenic microbial avirulent (Silva *et al.* 2016a). Animal and plant lectins have already been tested against a variety of pathogenic microbial cells and showed the capacity to disrupt QS signal transduction and interfering with nonessential functions for cell viability (Klein *et al.* 2015; Jayanthi *et al.* 2017).

Antimicrobial lectins

Lectins contain at least one noncatalytic site for specific and reversible binding to free monosaccharides, oligosaccharides or glycoconjugates. These proteins are widely distributed in nature and have been isolated from microorganisms, plants and animals (Santos *et al.* 2013). The carbohydrate-binding properties of lectins are involved in the interactions with eukaryotic cells and pathogens, playing important roles in the immunological defence against pathogens, blocking of viral infections and inhibition of microbial cell adhesion and migration (Chatterjee *et al.* 2015; Iordache *et al.* 2015).

Antimicrobial effects

Several lectins from natural sources can bind glycans on envelope glycoproteins from viruses (Fig. 1) playing an important role in the prevention of transmission and penetration into host cells (Barton *et al.* 2014; Akkouch *et al.* 2015). Cyanovirin-N (CV-N) is a cyanobacterial lectin from *Nostoc ellipsosporum* considered to be a broad-spectrum virucidal agent against enveloped viruses, such as HIV and ebola virus, through the binding to N-linked high-mannose glycans on the viral envelope (Barrientos and Gronenborn 2005).

Microvirin (MVN) is a mannose-specific lectin from the cyanobacterium *Microcystis aeruginosa* PCC7806 able to inhibit the HIV-1 infection, the syncytium formation

between infected and uninfected CD₄ T cells, and the HIV-1 transmission (Huskens *et al.* 2010). Scytovirin (SVN) is another cyanobacterial lectin from *Scytonema varium* that binds with high affinity to mannose residues on the envelope glycoproteins of viruses and inhibits the virus replication, as observed with the Zaire Ebola virus (Garrison *et al.* 2014). SVN and a red alga-derived lectin called griffithsin (GRFT) also demonstrated *in vitro* activity against genotypes I and II hepatitis C virus through the binding to the envelope glycoproteins E1 and E2, blocking the viral penetration in human hepatocytes (Takebe *et al.* 2013). GRFT is also distinguished by its broad-spectrum antiviral activity against HIV-1, HIV-2 and Ebola virus (Barton *et al.* 2014; Lusvardi and Bewley 2016). Another high mannose-binding lectin (MBL) from the red alga *Eucheuma serra* (ESA-2), that exhibited anti-HIV activity, also showed a potent inhibition on influenza A virus (H1N1) infection with an EC₅₀ of 12.4 nmol l⁻¹ (Sato *et al.* 2015).

A jacalin-related lectin isolated from the fruit of bananas (*Musa acuminata*) named BanLec recognizes high-mannose glycans found on viral envelopes such as HIV-1, resulting in the inhibition of the viral entry into the cell at picomolar concentrations (Swanson *et al.* 2010). Other mannose-binding plant lectins from the rhizomes of *Aspidistra elatior* (AEL) showed significant *in vitro* inhibitory activity against the vesicular stomatitis virus, Coxsackie virus B4 and respiratory syncytial virus (Xu *et al.* 2015).

CvL is a β -galactose-specific lectin isolated from the marine worm *Chaetopterus variopedatus* that demonstrated anti-HIV-1 activity *in vitro* through the inhibition of the production of viral p24 antigen and the cytopathic effect induced by HIV-1, blocking the HIV-1 entry into host cells (Wang *et al.* 2006). Animal lectins binding to viral envelope can also induce the activation of host innate immune response for elimination of the virus. C-type lectins (CTLs), found in animals, are pattern recognition receptors that recognize and bind glycans present in viral envelope and activate host immune responses, including phagocytosis, antigen presentation and T-cell activation (Monteiro and Lepenies 2017).

Lectins have exhibited antibacterial and antifungal activity against Gram-negative, Gram-positive bacteria and fungi, through the interaction with peptidoglycans, polysaccharides, lipopolysaccharides (LPSs), teichoic and teichuronic acids (Fig. 1) on bacterial and fungal cell wall (Iordache *et al.* 2015).

Antimicrobial activities

The cyanobacterial lectin showed antifungal activity against pathogenic cryptococci *Cryptococcus neoformans*

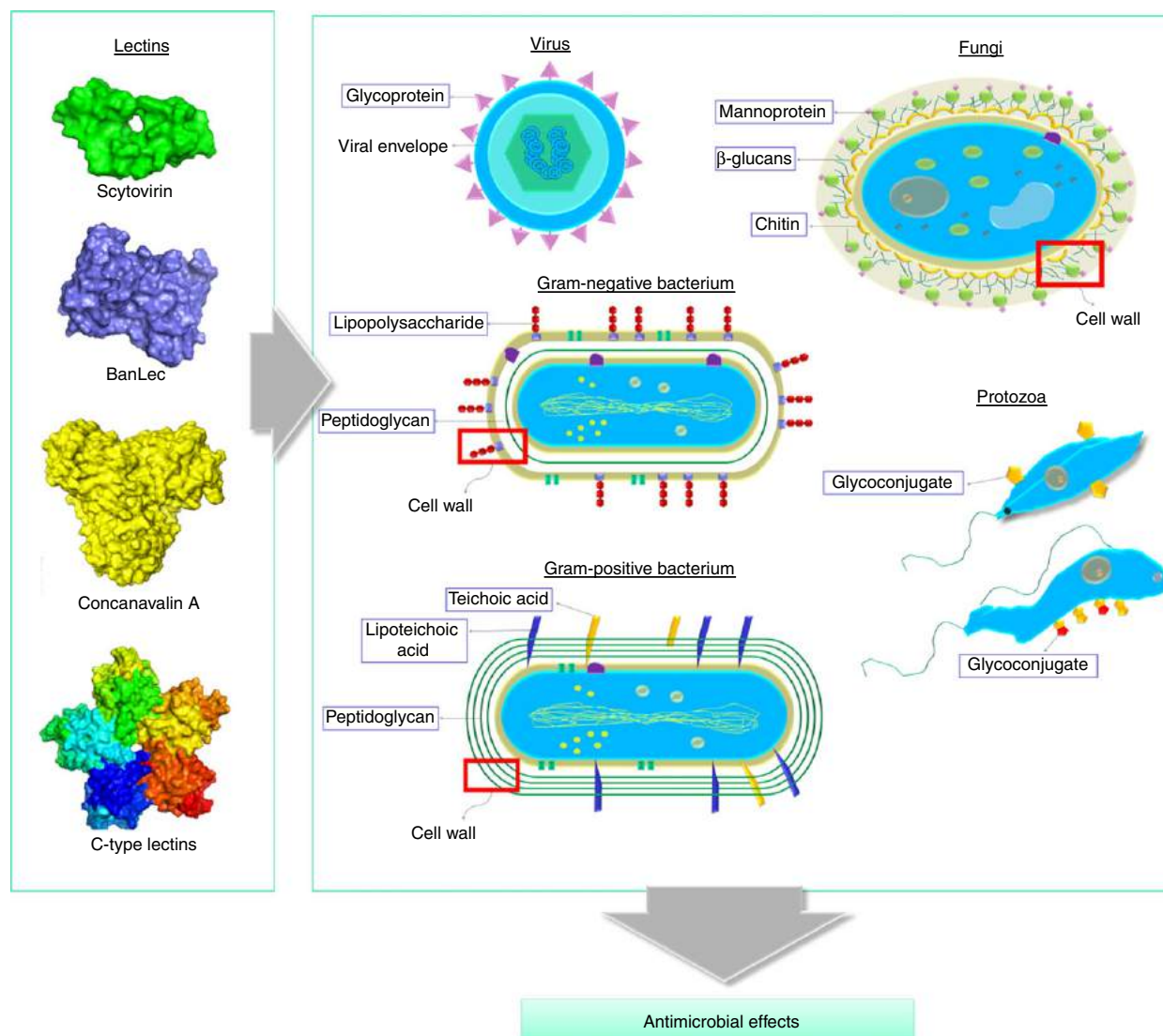


Figure 1 Schematic model of lectin interactions with microbial envelope/cell surface glycan to promote antimicrobial effects. [Colour figure can be viewed at wileyonlinelibrary.com]

var. *neoformans* and *Cryptococcus gattii*, inhibiting cell growth by binding to the cell wall (Jones *et al.* 2017). Lectins present in extracts from the fungal species *Penicillium corylophilum*, *Penicillium expansum* and *Penicillium purpurogenum* exhibited antifungal activity against *Aspergillus niger*, *Candida albicans* and *Saccharomyces cerevisiae*. The lectins also demonstrated antibacterial activity, inhibiting the growth of *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus* (Singh *et al.* 2013).

A fungal lectin from fruiting bodies of the mushroom *Sparassis latifolia* demonstrated antibacterial activity against *E. coli* and resistant strains of *S. aureus* and *Pseudomonas aeruginosa*, and antifungal activity on *Candida* and *Fusarium* species (Chandrasekaran *et al.* 2016).

A chitin-binding lectin isolated from a Bangladeshi cultivar of potato (*Solanum tuberosum* L.) showed antibacterial activity in *Listeria monocytogenes*, *E. coli*, *Salmonella enteritidis* and *Shigella boydii*, and prevented biofilm formation by *P. aeruginosa*. The lectin was also active against fungi *Rhizopus* spp., *Penicillium* spp. and *A. niger* (Hasan *et al.* 2014).

Apuleia leiocarpa seed lectin (ApulSL) demonstrated bacteriostatic effects on the Gram-positive bacteria *S. aureus*, *Streptococcus pyogenes*, *Enterococcus faecalis*, *Micrococcus luteus*, *Bacillus subtilis* and *B. cereus*, and on the Gram-negative bacteria *Xanthomonas campestris*, *Klebsiella pneumoniae*, *E. coli*, *P. aeruginosa* and *S. enteritidis*. ApulSL was also bactericidal against three varieties of *X. campestris* (Carvalho *et al.* 2015). A

galactose-specific lectin from the leguminous *Bauhinia monandra* secondary roots (BmoRoL) showed antifungal activity on phytopathogenic species of *Fusarium*, with highest effect against *Fusarium solani* (Souza *et al.* 2011). Helja is a jacalin-related, mannose-specific lectin obtained from sunflower seedlings that can agglutinate *S. cerevisiae* cells, and inhibited the pathogenic *Candida tropicalis* and *Pichia* genera growth (Regente *et al.* 2014).

A CTL from pacific white shrimp *Litopenaeus vannamei* called LvCTL3 showed an immune activation role in shrimp to bacterial and virus infections against *Vibrio parahaemolyticus* and white spot syndrome virus (Li *et al.* 2014). Other study reports the disruption of staphylococcal biofilms by CTL from *Bothrops jararacussu* venom, as well as the inhibition of biofilm formation of *S. aureus*, *Staphylococcus hyicus*, *Staphylococcus chromogenes*, *Streptococcus agalactiae* and *E. coli* (Klein *et al.* 2015). B-type mannose-specific lectins from the fish *Cynoglossus semilaevis* can reduce the survival rate *in vitro* of *Vibrio harveyi* and reduced bacterial recoveries in the spleen and kidney (Sun *et al.* 2016).

Lectins can recognize cell surface glycoconjugates of protozoa (Fig. 1) and play an important role in the impairing and damaging micro-organism and immune defence activation of the host cell. The mannose-specific lectin from *Cratylia mollis* seeds named Cramoll 1,4 induced the agglutination and inhibition of *Trypanosoma cruzi* epimastigotes *in vitro* (Fernandes *et al.* 2010). In addition, Cramoll 1,4 stimulated plasma membrane permeabilization with Ca^{2+} influx and mitochondrial accumulation, as well as the mitochondrial production of reactive oxygen species (ROS), carrying out a species impairing respiration and epimastigote death by necrosis (Fernandes *et al.* 2010, 2014).

Phaseolus vulgaris (kidney bean) lectin demonstrated a high toxic effect on the trophozoites of the sexually transmitted parasitic protozoa *Trichomonas vaginalis* (Aminou *et al.* 2016). N-acetylglucosamine-specific lectin from *Urtica dioica* (UDA) showed a reducing effect on *Trypanosoma brucei* infectivity (Castillo-Acosta *et al.* 2015). UDA induces changes in the glycan composition on the cell surface, and interacts with these glycans, impairing endocytosis and cytokinesis, promoting the parasite lysis and reducing the infectivity *in vivo* (Castillo-Acosta *et al.* 2015).

C-type lectin from *Bothrops pauloensis* snake venom (BpLec) showed antiparasitic effect on *Toxoplasma gondii*. BpLec reduced the adhesion and replication of *T. gondii* tachyzoite *in vitro* and increased the production of IL-6 and macrophage migration inhibitory factor by infected HeLa cells previously treated with BpLec (Castanheira *et al.* 2015).

***In vitro* and *in vivo* antimicrobial evaluation of lectins**

The antimicrobial activity has been evaluated by *in vitro* and *in vivo* assays and new methodologies have been used to determine the mode of interaction of lectins with micro-organisms and to study their different antimicrobial effects and future applications (Table 1). The first indication of antibacterial and antifungal activity is shown by measuring the inhibition zone in agar medium. Agar disc diffusion provides qualitative results and advantages, such as simplicity, low cost, the ability to test enormous numbers of micro-organisms and antimicrobial agents (Velayutham *et al.* 2017). Moreover, it is impossible to quantify the amount of the antimicrobial agents diffused into the agar medium and distinguish bactericidal and bacteriostatic effects.

The ability of lectins to mediate cross-linking interactions with carbohydrates present on the membrane of micro-organisms is confirmed by agglutination assays (Velayutham *et al.* 2017). The inhibition of this property by free carbohydrates or by a chelant agent specify the mode of this interaction (de Albuquerque *et al.* 2014a). Fungi can also be agglutinated by lectins and this property can be inhibited by carbohydrate (Regente *et al.* 2014). Nowadays, different methodologies are being used to study lectin–micro-organism interaction, such as confocal image, flow cytometry, circular dichroism, colorimetric, fluorescence and western blotting have been also used to study and confirm this interaction (Chikalovets *et al.* 2015; Zhou and Sun 2015; Sun *et al.* 2016; Arasu *et al.* 2017).

In vitro assays can be used to detect and quantify antiviral activity of lectins. In these assays, the cytotoxic ability of virus to a specific cell line is evaluated and compared when the virus is placed in the presence of the lectin. Reduction in viral cytotoxicity by 50% in the lectin presence is used to determine the 50% effective concentration of lectin (EC_{50}). Previously, it was necessary to confirm the absence of cytotoxicity of the lectin to the cell line that is employed in the antiviral test. The interaction between virus and lectin can be confirmed by inhibition with free glycoproteins (Barton *et al.* 2014). The study of antiviral activity of lectins has shown that lectins can affect initial steps of virus entry into the cells, and inhibit the production of viral proteins (Barton *et al.* 2014).

The recognition of membrane glycoconjugates of micro-organisms allows lectins to act as inhibitory agents of biofilm formation and growth. *In vitro* quantification of biofilm formation can be monitored by microtitre plate assay using crystal violet dye and spectrophotometrically measured. Water-soluble *Moringa oleifera* seed

Table 1 *In vitro* and *in vivo* evaluation of antimicrobial lectins

Antimicrobial activity	Method	Lectin	Reference			
Antibacterial	<i>In vitro</i>	Minimum inhibitory and bactericide concentration (MIC and MBC)	Water-soluble <i>Moringa oleifera</i> seed lectin (WSMoL), <i>Punica granatum</i> sarcotesta lectin (PgTeL)	Ferreira <i>et al.</i> (2011); Silva <i>et al.</i> (2016b)		
		Agglutination activity	<i>Oryctes rhinoceros</i> serum lectin	Velayutham <i>et al.</i> (2017)		
		Antibiofilm activity	WSMoL, <i>Calliandra surinamensis</i> leaf (CasuL)	Moura <i>et al.</i> (2015); Procópio <i>et al.</i> (2017b)		
		Bacterial membrane binding	<i>Channa striatus</i> lily type lectin-2 and teleoste C-type (CsLTL-2 and CsCTL1)	Zhou and Sun (2015); Arasu <i>et al.</i> (2017)		
		Disc diffusion	<i>Oryctes rhinoceros</i> serum lectin	Velayutham <i>et al.</i> (2017)		
		Respiration inhibition test	<i>Andrias davidianus</i> lectin (ADL)	Qu <i>et al.</i> (2015)		
		Adhesion inhibition	PgTeL	Silva <i>et al.</i> (2016b)		
		Invasion inhibition	PgTeL	Silva <i>et al.</i> (2016b)		
		Monocyte activation	CsCTL1	Zhou and Sun (2015)		
		Cell permeability	WSMoL, Human lectin (RegIIIa)	Mukherjee <i>et al.</i> (2014); Moura <i>et al.</i> (2015)		
	<i>In vivo</i>	Cumulative mortality and RNAi-mediated silencing	<i>Cynoglossus semilaevis</i> lectins	Sun <i>et al.</i> (2016)		
		Bacterial recovery from the tissue	CsCTL1	Zhou and Sun (2015)		
Antifungal	<i>In vitro</i>	Reduction in fungal growth zone	<i>Microgramma vacciniifolia</i> rhizome lectin (MvRL)	de Albuquerque <i>et al.</i> (2014a)		
		Disc diffusion	<i>Aspergillus panamensis</i> lectin	Singh <i>et al.</i> (2015)		
		Optical microscopy of pathogen, plasma membrane permeabilization assay and reactive oxygen species MIC	CasuL, <i>Helianthus annuus</i> seeds lectin (Helja)	Regente <i>et al.</i> (2014); Procópio <i>et al.</i> (2017b)		
			Helja, <i>Aspergillus panamensis</i> lectin	Regente <i>et al.</i> (2014); Singh <i>et al.</i> (2015)		
		Interaction of lectins with cell wall components	MvRL, Helja	de Albuquerque <i>et al.</i> (2014a); Regente <i>et al.</i> (2014)		
		Agglutination activity	Helja	Regente <i>et al.</i> 2014;		
		Inhibition on germination of spores and hyphal growth	<i>Crenomytilus grayanus</i> lectin (CGL)	Chikalovets <i>et al.</i> (2015)		
		<i>In vivo</i>	Body weight gain and cumulative mortality	Human Mannose-binding Lectin (MBL)	Lillegard <i>et al.</i> (2006)	
		Antiviral	<i>In vitro</i>	50% effective dose (EC50)	Griffithsin (GRFT)	Barton <i>et al.</i> (2014)
				Interaction of Lectins with viral envelope	Scytovirin (SVN)	Garrison <i>et al.</i> (2014)
	Replication inhibition			GRFT	Takebe <i>et al.</i> (2013); Barton <i>et al.</i> (2014)	
	<i>In vivo</i>		Invasion Inhibition	GRFT	Takebe <i>et al.</i> (2013)	
50% infective dose (ID50)			GRFT	Barton <i>et al.</i> (2014)		
Viral recovery from tissues			GRFT, CsCTL1	Takebe <i>et al.</i> (2013); Zhou and Sun (2015)		
	Cumulative mortality	GRFT and SVN	Takebe <i>et al.</i> (2013)			
	Haematology parameters and body weight gain	GRFT	Takebe <i>et al.</i> (2013)			
Antiprotozoal	<i>In vitro</i>	Inhibition of macrophages infection	<i>Synadenium carinatum</i> latex lectin (ScLL)	Afonso-Cardoso <i>et al.</i> (2011)		
		No production	ScLL	Afonso-Cardoso <i>et al.</i> (2011)		
		Expression of cytokines and Inducible nitric oxide synthase	ScLL	Afonso-Cardoso <i>et al.</i> (2011)		
		Agglutinating activity	<i>Cliona varians</i> lectin (CvL)	Moura <i>et al.</i> (2006)		
	<i>In vivo</i>	Transmission electron microscopy of pathogen	<i>Phaseolus vulgaris</i> lectin	Aminou <i>et al.</i> (2016)		
		Stimulation of IFN- γ production and reduction in lesion	ConBr	Barral-Netto <i>et al.</i> (1996)		

lectin (WSMoL) inhibited biofilm formation by *Serratia marcescens* and avoided adherence of *Bacillus* sp. cells on glass (Moura *et al.* 2017).

Lectin antibacterial modes of action are investigated by many approaches. Lectins can inhibit the adherence and invasion of bacteria. The lectin from sarcotesta of *Punica granatum* (PgTeL) reduced the adherence and invasion of *Aeromonas* sp., *S. aureus*, *S. marcescens* and *Salmonella enterica* serovar *Enteritidis* to human cells (Silva *et al.* 2016b).

Bacterial cell permeability induced by lectins is visualized by techniques such as fluorescence, quantification of protein leakage, electron microscope and computational modelling studies (Mukherjee *et al.* 2014; Moura *et al.* 2015). Moreover, a new mode of action has been described for antibacterial lectins. The lectin from *Andrias davidianus* (ADL) showed effects on the respiratory chain and energy production of bacterial cells, changing respiratory metabolism and affecting tricarboxylic acid cycle and hexose monophosphate pathway (Qu *et al.* 2015).

Optical and fluorescent microscopies have been increasingly applied to evaluate the mechanism of action of antifungal lectins. By measuring the fluorescence of SYTOX green, a dye that only penetrates cells with a structurally compromised plasma membrane, it was possible to affirm that the lectin from seeds of *Helianthus annuus* (Helja) alters the permeability of *C. tropicalis*, *Pichia membranifasciens* and *C. albicans* (Regente *et al.* 2014). Morphological changes promoted by lectin in fungal cells, such as the formation of pseudohyphae, reduction in hyphae formation and damage to cell walls can also be observed through optical microscopy (Regente *et al.* 2014; Procópio *et al.* 2017b).

Antiparasitic effects of lectins are also evaluated by *in vitro* assays. It can be assessed through reduction in macrophage infection and activation of immune system. Expression of cytokines can be evaluated by RT-PCR using mRNA isolated from macrophages pretreated or not with lectin and infected with protozoa (Afonso-Cardoso *et al.* 2011). The toxic effects of lectin to protozoa have been also demonstrated by transmission electron microscopy of the pathogen after incubation with lectins, such as the lectin from seeds of *P. vulgaris* that causes severe cell damage with cytoplasmic and nuclear destruction (Aminou *et al.* 2016). To study the binding property of lectin to protozoa, the agglutinating activity can be performed (Moura *et al.* 2006).

Some *in vivo* assays are employed to evaluate the antimicrobial effect of lectins and their potential to be used in future clinical trials (Table 1). The mouse model is commonly used to study antimicrobial activity of lectins since it reproduces different aspects of human diseases. Antibacterial lectins have been evaluated by

methods, such as measuring cumulative mortality, genetic assays and bacterial recovery from a tissue of infected animals (Sun *et al.* 2016).

Besides, more rarely, antifungal lectins have been evaluated by *in vivo* tests. By measuring body weight gain and mortality of mice treated with MBL and infected with *C. albicans*, it was possible to confirm that MBL increased resistance of mice to hematogenously disseminated candidiasis with potential to be a therapy to high-risk patients (Lillegard *et al.* 2006).

Antiviral activity of lectin can be evaluated through *in vivo* assays with determination of infective dose (ID₅₀); the sample dilution required to reduce luminescence by 50% in comparison to wells with no sample added. Viral titration from serum of mice infected and indirect parameters can also evidence *in vivo* antiviral activity such as cumulative mortality (Takebe *et al.* 2013; Barton *et al.* 2014).

The intraperitoneal use of a lectin can study its *in vivo* activity against protozoa; the evaluation is performed by histology and cytokine determination. Administration of *Canavalia brasiliensis* lectin (ConBr) did not cure, but reduced lesions of highly susceptible mice infected by *Leishmania amazonenses*, with proliferation of fibroblastic cells in the lesion periphery, a discrete production of conjunctive tissue inducing lymphocytes to produce interferon- γ (Barral-Netto *et al.* 1996).

Molecular characteristics of antimicrobial lectins

Cell surface glycans are intrinsically related with the pathogenicity of some micro-organisms. For example, a variety of glycans make up the cell wall of bacteria which in turn protects these micro-organisms from osmotic lysis; in addition, there are specific sugars expressed only in prokaryotic cells which can be considered important targets for diagnosis and drug action (Tra and Dube 2014).

Lectins that exist as multimers generally undergo further oligomerization after their binding to microbial surface glycans. This oligomeric structure increases functional affinity and favours multivalent binding of the lectin to the micro-organism (Wesener *et al.* 2017). For example, MBL is a human circulating oligomeric protein that acts as a pattern recognition molecule and is capable of recognizing sugars on pathogen surfaces (Ip *et al.* 2009; Carvalho *et al.* 2013). MBL can be found in the serum as trimeric or tetrameric structures; it is known that the tetramer MBL has a higher maximum binding capacity and lower dissociation rate constants for carbohydrates than the trimeric form (Teillet *et al.* 2005; Kjaer *et al.* 2016). This oligomerization of MBL also reflects in its interaction with micro-organisms, once tetrameric

MBL had a higher binding to *S. aureus* than dimeric and trimeric MBL. The structure is composed of four monomers and is also the main oligomeric form present in complexes with MBL-associated serine proteases (Kjaer *et al.* 2016).

Few antimicrobial lectins from other nonhuman sources have had their molecular structure elucidated. A lectin with broad antimicrobial spectrum purified from *Crenomytilus grayanus* mussel was referred as CGL and the three-dimensional structure is depicted in Fig. 2a. The most stable oligomeric form of CGL is a homodimer; each CGL monomer has three galactose-binding sites. Site 1 is composed of sequences Ser22, Ile36, His37 and Glu38; site 2 consists of Ile54, His81, Arg84, His85, Asp86 and Leu89; site 3 contains Asn74, Lys110, Ser113, Ile124, His125, Gly126 and Asp127 (Liao *et al.* 2016). The CRD shape is a double-loop structure stabilized in the base by two highly conserved disulphide bridges (Gasmi *et al.* 2017). Attraction between this lectin and carbohydrate on the microbial surface is represented in Fig. 2b. Lectin can bind to a carbohydrate by attractive forces including hydrogen bonds, hydrophobic and electrostatic interactions (Fernández-Alonso *et al.* 2012). There are also lectins that require ions to interact with their binding sugar, such as CTLs are Ca^{2+} dependent for ligand binding (Vasta *et al.* 2012).

CGL showed anti-HIV activity (Luk'yanov *et al.* 2007); this lectin also has antifungal action, inhibiting the growth of *Pichia pastoris* fungus and antibacterial activity against Gram-negative *E. coli* and Gram-positive *B. subtilis*, *Salinibacterium amurskyense* and *S. aureus*. Interactions between CGL with bacteria and fungi were specifically inhibited by D-galactose (Kovalchuk *et al.*

2013; Chikalovets *et al.* 2015). It was also evidenced that CGL has a high binding activity to the Gram-negative bacteria *Arenibacter troitsensis* and *Chryseobacterium scophthalmum*, but did not promote agglutination and suppression of growth (Kovalchuk *et al.* 2013). Therefore, binding to the microbial surface carbohydrate by lectin is not a predictor of antimicrobial action; however, such a recognition with specificity can be useful for the diagnosis of an infection.

Other antimicrobial lectins that had their molecular structures defined were MCL and BanLec. MCL is a lectin from *Mytilus californianus*, which forms a dimer in solution and each monomer has three carbohydrate-binding sites. MCL has specificity for D-galactose and N-acetyl-D-galactosamine; this lectin was able to agglutinate and inhibit the growth of *Lactobacillus plantarum* and *E. coli* (García-Maldonado *et al.* 2017).

BanLec, isolated from bananas, has been shown to be a lectin with potent anti-HIV action; possibly its ability to bind the mannose structures present on the viral envelope blocks virus entry into the cell (Swanson *et al.* 2010). Previous analyses showed that BanLec was a dimeric protein (Meagher *et al.* 2005). However, a recent study has revealed that this lectin possesses tetrameric stoichiometry in solution, having two carbohydrate-binding sites per monomer considered independent. Unveiling of this configuration consisting of four monomers together with simultaneous binding of their sites to a single and specific high-mannose glycan, results in an enhanced avidity effect with high HIV neutralization (Hopper *et al.* 2017). Therefore, elucidation of the characteristic structure of lectins can stimulate studies of molecular engineering of proteins to improve action against infections in lectin-based therapies.

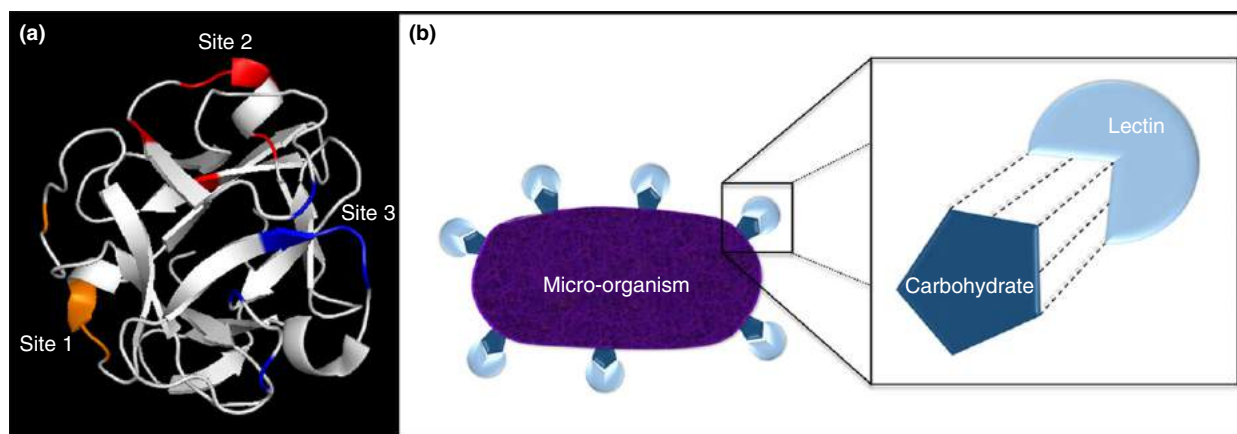


Figure 2 Representation of lectin structure from *Crenomytilus grayanus* mussel (a) and its binding to glycidic moieties on microbial surface (b). Dashed lines show the attractive forces hydrogen bonds, hydrophobic and electrostatic interactions. [Colour figure can be viewed at wileyonlinelibrary.com]

Antimicrobial mechanisms of lectin actions

It is well known that lectins can be involved in the defence of multicellular organisms, displaying an important role in innate immunity. This function is associated with the similar glycan epitopes that occur on the pathogen cell surface, which can constitute target sites for lectin interaction (Wohlschlager *et al.* 2014). The role of lectins in the defence mechanisms of animals or plants may have evolved from their ability to agglutinate and immobilize cells, as well as the toxicity to micro-organisms that can result in death or growth inhibition (Gomes *et al.* 2013; Procópio *et al.* 2017a).

The cell wall of bacteria, in fact, hinders interactions between glycoconjugates on the bacterial membrane and carbohydrate-binding proteins preventing the entry of these proteins into the cell. Therefore, the antibacterial activity against Gram-positive or Gram-negative bacteria depends on the interactions of lectin with bacterial cell wall components such as teichoic and teichuronic acids, peptidoglycans and LPSs through weak linkages such as hydrogen bonds and hydrophobic interactions (Paiva *et al.* 2010).

Some lectins correlate with pore-forming activity, which causes bacterial membrane permeabilization; an interesting example is the lectin named RegIII α , which together with other RegIII CTLs are bactericidal proteins that improve the tolerance to the intestinal microbiota by impairing the interaction of bacteria with human intestinal epithelium. Mukherjee *et al.* (2014), through quantification of uptake of the membrane-impermeant fluorescent dye SYTOX green, demonstrated that this lectin binds with the membrane phospholipids, causing the death of Gram-positive bacteria by inducing the formation of a hexameric membrane-permeabilizing pore. In addition, the authors observed that the lectin induces rapid efflux of the fluorescent dye carboxyfluorescein from liposomes containing acidic phospholipids, indicating that RegIII α interactions with lipid bilayers involve electrostatic interactions between the lectin and acidic lipids in the bacterial membrane. Interestingly, the pore-forming activity of RegIII α was inhibited by LPSs, major constituents of the outer membrane from Gram-negative bacteria, explaining why RegIII α is only bactericidal for Gram-positive bacteria.

The RegIII β lectin, which has its expression upregulated in response to bacterial colonization in murine intestine, is able to bind the lipid A moiety of LPS, the major component of the outer membrane of Gram-negative bacteria (Miki and Hardt 2013). The authors showed that covering the surface-exposed lipid A by an anti-lipid A antibody in the membrane of *Salmonella typhimurium*

results in the inhibition of bactericidal effect of RegIII β , and concluded that the interaction between the lectin and LPS in bacterial surface is essential for antibacterial activity. Also, experiments using the DNA-intercalating fluorescent dye ethidium bromide revealed that RegIII β indeed is able to permeabilize the outer membrane of *Salm. Typhimurium*.

Moura *et al.* (2015) reported the antibacterial activity of the water-soluble lectin from seeds of *M. oleifera* (WSMoL) against corrosive bacteria. The treatment of *S. marcescens* with WSMoL resulted in the loss of wall/membrane integrity and strong leakage of intracellular proteins in a dose-dependent way; this is another example of lectin able to alter the bacterial cell permeability.

The antifungal activity of lectins also has been well reported, and is commonly associated with the ability of these proteins to interact with *N*-acetylglucosamine residues in chitin from fungi cell wall, resulting in inhibitory action on the growth and development of these micro-organisms. The inhibition of fungi growth can result from the binding of lectin to hyphae causing a poor absorption of nutrients, as well as by interference with spore germination process or impairment of synthesis and/or deposition of chitin in the cell wall (Coelho *et al.* 2017).

Corroborating with these reports, the lectin isolated from the leaf pinnulae of *Calliandra surinamensis* (CasuL) was able to inhibit the growth of *Candida krusei*; differential interference contrast microscopy revealed that the lectin induced drastic morphological alterations, including retraction of cytoplasmic content and the presence of ruptured cells or cellular debris (Procópio *et al.* 2017b). In addition, the treatment with CasuL may impair *C. krusei* cell division since the authors observed several cells with incomplete budding/division. When working with the fluorochrome calcofluor, CasuL was able to bind to chitin present in the yeast wall and indicate that CasuL affected the cell wall integrity of *C. krusei*. This effect was attributed to the disintegration of cell wall or to the linkage between the lectin and the chitin, impairing the *de novo* synthesis of cellular wall during yeast development or division.

Antifungal lectins also are able to cause changes in fungal cell permeability. The lectin (Helja) from seedlings of *H. annuus* (sunflower) was able to alter the permeability of the plasma membranes of *C. tropicalis*, *P. membranifasciens* and *C. albicans* yeasts; these results were detected using SYTOX green, through fluorescence microscopy (Regente *et al.* 2014). The treatment of *C. tropicalis* cells with the lectin Helja also resulted in the production of ROS; however, no ROS production was detected in *P. membranifasciens* or *C. albicans* after incubation with the lectin.

Microbial QS, biofilms and lectins

A quorum-sensing process comprises an interspecies or even interkingdom signalling that relies on the production, release, detection and response of secreted and diffusible signalling molecules called autoinducers or QS molecules. The production of QS molecules increases proportionally to population density (Hawver *et al.* 2016).

Quorum-sensing systems have been identified in Gram-negative and Gram-positive bacteria (Hawver *et al.* 2016) and in fungal species (de Albuquerque *et al.* 2014b). Clinically relevant micro-organisms use QS systems to regulate the expression of virulence factors, enzymes, toxins, surfactants, pigments and bioluminescent molecules (Silva *et al.* 2016a).

Bacterial signal molecules comprise several range of chemical classes: Gram-negative bacteria produce small molecules which includes acyl-homoserine lactones (AHLs), alkylquinolones, α -hydroxyketones and diffusible signalling factors (fatty acid-like compounds) (Hawver *et al.* 2016; Silva *et al.* 2016a). Oligopeptides with diverse post-translational modifications comprise QS molecules known as auto-inducer peptides, produced by

Gram-positive bacteria (Hawver *et al.* 2016; Silva *et al.* 2016a). In fungi, alcohols and alcohol derivatives, such as farnesol and tyrosol, AHLs, unsaturated fatty acids and peptides are some identified QS molecules (Dixon and Hall 2015). More details on QS molecules of bacteria and fungi are described in Table 2. Despite differences at molecular level, bacteria and fungi could interact chemically (via QS system) or physically resulting in co-aggregation within a polymicrobial biofilm (Dixon and Hall 2015).

The microbial biofilms are the clearest examples of community behaviour depending on QS systems (Solano *et al.* 2014). This sessile community organization is formed by cells and a self-produced matrix composed of extracellular polymeric substances. The biofilms present significant morphological, physiological and genetic differences from the planktonic lifestyles (Silva *et al.* 2016a). Biofilms are associated with 80% of microbial infections and their high resistance to antibiotics is a significant cause of morbidity and mortality (Brackman and Coenye 2015). Fungal–bacterial biofilms especially occurs in implant or nonbiogenic material such as indwelling catheters or respirators (Wongsuk *et al.* 2016).

Table 2 Some bacterial and fungal QS molecules and their biological roles

Class	Microbial	QS molecule	Biological processes associated (pathogenicity)
Autoinducer peptides (AIP)	<i>Staphylococcus aureus</i>	AgrB, AgrD and AgrC variants	Modulate expression of virulence determinants during the course of a bacterial infection (regulation of toxins, degradative exoenzyme and biofilm formation); relate with antibiotic resistance; interact with the host innate immune system
Acyl-homoserine lactones (HSL)	<i>Vibrio harveyi</i>	AI-1 (3OH-C ₄ -HSL)	Regulate biofilm and protease production, type III secretion (TTS) and bioluminescence expression;
4,5-dihydroxy-2,3-pentanedione (AI-2*)		(S)-THMF-borate (boron-containing AI-2 molecule)	AI-1, AI-2 and CAI-1 are involved in intraspecies, interspecies and intragenera communication respectively
α -hydroxyketones		CAI-1 (cholera autoinducer 1)	
Diffusible signal factor (DSF)	<i>Xanthomonas campestris</i>	Cis-11-methyl-2-dodecanoic acid	Modulate transitions between the planktonic and biofilm-associated lifestyles
Alcohols and alcohol derivatives	<i>Candida albicans</i> , <i>C. dubliniensis</i> and <i>C. tropicalis</i>	Farnesol and farnesoic acid	Control the morphogenesis and biofilm formation; affect the differentiation and cytokine secretion of some immune cells; induce cell death and apoptosis via intracellular reactive oxygen species (ROS) production; modulate drug extrusion; present a role in oxidative stress resistance
	<i>C. albicans</i> , <i>C. dubliniensis</i> and <i>Debaryomyces hansenii</i>	Tyrosol	Promote germ tube formation; increase the adhesion on surfaces; stimulate hypha production during the early stages of biofilm development; induce resistance to oxidative stress
Peptides	<i>Cryptococcus neoformans</i>	Quorum sensing-like peptide (Qsp1)	Induce morphogenesis; promote growth and production of virulence factors

*Universal signal for interspecies communication, existing in both Gram-negative and Gram-positive bacteria, except certain bacteria, such as *P. aeruginosa*. de Albuquerque *et al.* (2014b); Hawver *et al.* (2016); Papenfort and Bassler (2016); Wongsuk *et al.* (2016).

The disruption of QS signalling can interfere with microbial pathogenicity. Nonlethal antimicrobial mechanisms, actually known as antivirulence therapies, comprise a new approach that involves the blockage of QS mechanisms and biofilm development (Dixon and Hall 2015; Silva *et al.* 2016a).

Interference of biofilm development and other anti-QS mechanisms have been described for lectins; the lectin from *B. jararacussu* venom inhibited biofilm development by *S. aureus* and *Staphylococcus epidermidis* without affecting bacterial cell viability (Klein *et al.* 2015). Preformed biofilms of *S. epidermidis* were also significantly disrupted by this lectin. Cavalcante *et al.* (2013) showed that *Streptococcus mutans* cells treated with *Canavalia maritima* lectin (ConM) had reduced expression of genes related with biofilm formation and regulation as well as drug resistance. A lectin from the haemolymph of crab *Portunus pelagicus* was able to reduce biofilm thickness of *Citrobacter amalonatus*, *Proteus vulgaris*, *P. aeruginosa* and *V. parahaemolyticus* in 62, 90, 75 and 88%, respectively, with disruption of biofilm architecture (Jayanthi *et al.* 2017).

Use of lectins in the treatment of micro-organism infections

Delivery strategies are important concerns for the effective use of lectins (and other biological macromolecules) to treat microbial infections. Good delivery systems should prevent or minimize chemical/biological degradation and guarantee specificity regarding the site of action (Fig. 3a,b) as well as avoiding binding to serum proteins, which could lead to undesirable rapid clearance (Nordström and Malmsten 2017).

According to Nordström and Malmsten (2017), macromolecular drugs, such as proteins, can be delivered through nanoparticles composed by inorganic nanomaterials (metals, silica, clays and carbon-based materials) and polymers (polymeric particles, fibres, microgels, multilayers and conjugates). Stabilizing agents attached to nanoparticle surface may prevent chemical and biological degradation; an anchored targeting ligand can help the antimicrobial agent to reach the intended site of action (Fig. 3a,b).

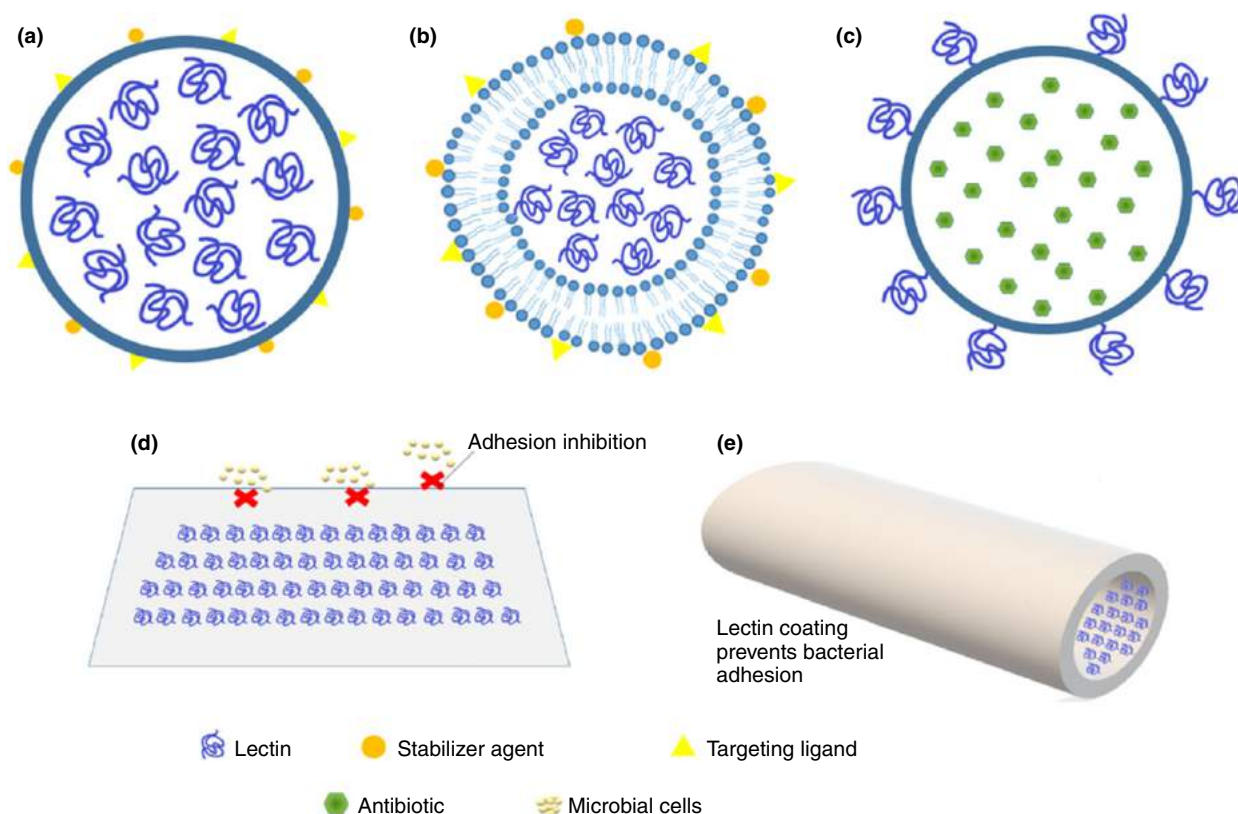


Figure 3 Strategies using lectins for combating microbial infections. Antimicrobial lectins inserted into delivery systems such as nanoparticles composed of metals/polymers (a) and liposomes (b). Lectins as targeting ligands for delivering antibiotic drugs (c); antibiofilm lectins coating medical device surfaces (d) and catheters (e) to prevent microbial attachment and biofilm development. [Colour figure can be viewed at wileyonlinelibrary.com]

In vivo studies have shown that the immunomodulatory potential of some lectins may be useful to combat microbial infections, whether or not the lectin has an antimicrobial action. The treatment of C57BL/6 mice infected with *C. gattii* with the lectin from *C. mollis* seeds, alone or in combination with fluconazole, increased the survival and reduced morbidity as well as decreased pulmonary and cerebral fungal burden; the authors attributed the results to the immunomodulatory effect of the lectin (Jandú *et al.* 2017). In other work, a mannose-binding lectin from *Artocarpus heterophyllus* promoted a Th1 immune response balanced by IL-10 in experimental paracoccidioidomycosis in mice, leading to a reduction in the fungal burden in organs (Ruas *et al.* 2012). Similarly, a MBL from *Artocarpus integrifolia* reduced significantly the number of *Paracoccidioides brasiliensis* colony-forming units in organs of BALB/c mice and induced the release of nitric oxide, INF- α , TNF- α and IL-12 (Coltri *et al.* 2008).

Lectins can be components of systems for the delivery of antimicrobial agents (Fig. 3c). Polymeric nanoparticles conjugated with mannose-specific or fucose-specific lectins were able to selectively adhere to the surface of *Helicobacter pylori* and enhance the release of antimicrobial agents into the bacterial cells (Umamaheshwari and Jain 2003). A lectin from *Arachis hypogaea* was linked to the surface of hepatitis-B surface antigen (HBsAg) nanoparticles enhancing their effectiveness for oral immunization (Gupta *et al.* 2006). Poly(lactide-co-glycolide) nanoparticles coated with wheat germ agglutinin and carrying the antimicrobial agents rifampicin, isoniazid and pyrazinamide were administered to guinea pigs infected with *Mycobacterium tuberculosis* through oral/aerosol route; it was observed that the presence of the drugs in plasma increased in comparison with treatment of uncoated nanoparticles. Furthermore, the number of colony-forming units became undetectable with three doses of nebulization with lectin-coated nanoparticles while 45 doses were necessary to achieve similar result in oral treatment with free drugs (Sharma *et al.* 2004).

Antibiofilm lectins may also be attached to medical device surfaces in order to prevent biofilm establishment (Fig. 3d,e). Moura *et al.* (2017) demonstrated that the coating of glass surface with the water-soluble lectin isolated from *M. oleifera* seeds suppressed the adhesion of *Bacillus* sp. cells. Coating of catheter surface with lectins from human plasma inhibited adhesion of *P. aeruginosa* cells (Gu *et al.* 2001).

Conclusions

Lectins specifically bind to certain carbohydrates, or glycoconjugates, expressed on viruses, bacteria, fungi and

protozoa surfaces. Lectin-carbohydrate interaction can alter permeability of bacterial and fungal cells leading to death and/or inhibition of micro-organism growth and reproduction. Different *in vitro* and *in vivo* methodologies have evaluated the antimicrobial potential of lectins; however, *in vitro* assays are accessible to perform, cost-effective and routinely used to screen antimicrobial lectins. The activity against micro-organisms can change in multimeric lectins according with their state of oligomerization; therefore, elucidating the structure of these proteins is of fundamental importance to potentiate their actions. Lectins can also interfere with biofilm development or mechanism of QS signalling without causing toxicity directly to bacterium or fungus. Then, antimicrobial lectins can be of use as a natural alternative to synthetic and semisynthetic drugs against micro-organisms. In addition, these proteins employed into drug delivery systems, such as encapsulated in nanoparticles, may combat a local infection; or guide delivery of a drug to a specific site.

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Conflict of Interest

The authors have declared that no competing interests exist.

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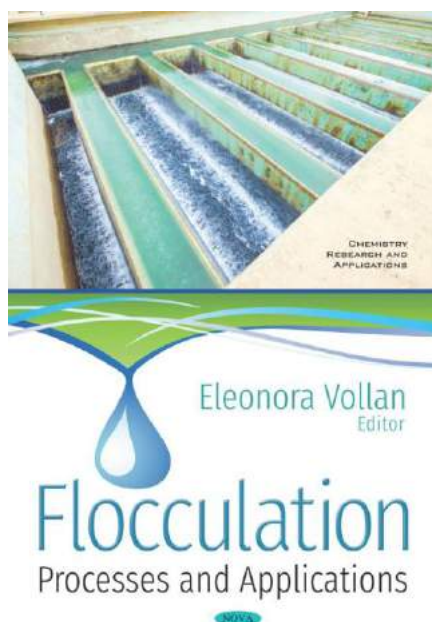
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APÊNDICE I – CAPÍTULO DE LIVRO

Flocculant polysaccharides mainly from plants

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Chapter

FLOCCULANT POLYSACCHARIDES MAINLY FROM PLANTS

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ABSTRACT

Natural polymers are biocompatible, low-cost, and easily available materials of innate origin. These polymers are increasingly preferred over synthetic materials for industrial applications due to their intrinsic properties; they also are alternative sources of raw materials with characteristics of biodegradability, biosafety, and sustainability. Polysaccharides are polymers extracted from plants, algae, animals, fungi or obtained via fermentation, applied on a wide range of uses, from food to biomedical industries. Nowadays, they have been attracting considerable attention as viable alternatives to harmful synthetic flocculating agents for the removal of contaminants from water and wastewater. Then, a great deal of dedicated effort improved the production and performance of natural flocculants based on polysaccharides. The aim of this chapter is to approach current trends in preparation and chemical modification of flocculant polysaccharides derived from plants and their flocculation performance. In addition, aspects including mechanisms of flocculation, chemical modification, the effect of physicochemical factors on flocculating activity, and recent applications of flocculant polysaccharides, also derived from non-plant sources are reviewed.

Keywords: agglomeration, aggregation, coagulation, contamination, flocculation, grafting, polymerization, polysaccharide, wastewater treatment

1. INTRODUCTION

The flocculation phenomenon is related to the clumping of particles with consequent destabilization and coming out of the aggregates from suspension. In a chemical perspective, the flocculation process is essentially physical, occurring due to the contact and adhesion of aggregates by the formation of large-size clusters called flocs, which are excluded of the suspension. Commonly, one can observe flocculation being widely used as synonymous of agglomeration, aggregation, and coagulation (Santos et al., 2014).

Flocculating agents are useful in different industrial fields, such as tapwater and wastewater treatment, dredging, textile, mining, cosmetology, pharmacology, food and fermentation industries and downstream processes (Salehizadeh, Yan and Farnood, 2017). Especially in food and beverage industries, flocculant agents are widely used with the important aim to remove microscopic particles that affect water taste, appearance and texture (Santos et al., 2014).

Flocculants are generally classified into three groups: inorganic flocculants, organic synthetic flocculants, and natural flocculants (or bioflocculants). Although the first two groups are most commonly used due to their effective flocculating performance and low cost, they could be associated to serious environmental and health problems. In order to circumvent the above concerns, natural flocculants have been attracting more attention in utilization (Salehizadeh and Yan, 2014).

Polysaccharides are biopolymers and particularly attractive as natural flocculants due to their inherent properties; they represent one of the most abundant industrial biomaterials usually reported by several studies due to their sustainability, biodegradability and biosafety. Even more, polysaccharides are abundant in nature and commonly found in many higher plants, being frequently produced as a protection mechanism following plant injury (Albuquerque et al., 2017; Rana et al., 2011).

The most common polysaccharide bio-based flocculants are alginate, chitosan, cellulose, and starch; however, microorganisms could also be used as source of raw material (Lin et al., 2014; Teh et al., 2016; Yang et al., 2016). Moreover, polysaccharides may be chemically modified to improve their flocculation performance (Liu et al., 2017b; Lou et al., 2017). Broad industrial application of bioflocculants based on polysaccharides depends on the use of low-cost substrates and the development of more efficient fermentation and recovery processes, as well as the application of cost-effective chemical modification of the extracted polysaccharides for improved performance (Salehizadeh, Yan and Farnood, 2017).

This chapter describes the progress in polysaccharide flocculants with special emphasis for those derived from plants. In particular, aspects

summarized include extraction, purification, modification, characterization, and the broad range of applications in industry.

2. SHORT SUMMARY ON POLYSACCHARIDES

The polysaccharide term gathers collectively quite diverse large carbohydrates composed by monosaccharides, being named homopolysaccharides if they have only one kind of repeating units (for example starch and cellulose), or formed by two or more different monomeric units, named heteropolysaccharides and exemplified as agar, alginate, and carrageenan. Polysaccharides are considered neutral, anionic or cationic depending on its electric charge; in addition, the conformation of the polymer chains is markedly dependent not only on the ionic strength of the medium, but also on the pH, particularly in the case of the polyelectrolytes, and the temperature and the concentration of certain molecules. Several anionic and cationic polysaccharides are widely available in nature and have gained keen interest in food and pharmaceutical fields (Prajapati et al., 2014).

Gums and mucilages are the constituents of polysaccharides, possessing natural source and particular differences: gums readily dissolve in water, while mucilages form viscous masses; in addition, their similarities are related to their broad range of physicochemical properties, which are widely used for applications in cosmetics, paper, pharmacy, textile, adhesive, inks, lithography, paint, explosive, and smoking products (Albuquerque et al., 2017). The preference of polysaccharides when compared to synthetic materials is closely related to their biological and chemical properties, including biocompatibility, biodegradability, polyfunctionality, high chemical reactivity, chirality, chelation and adsorption capacities (Hossain and Mondal, 2014).

Regarding the excellent adsorption behaviour of polysaccharides, it is important to point out characteristics such as high hydrophilicity (due to hydroxyl groups of the monomeric units), presence of a vast number of functional groups (acetamide, primary amino, and/or hydroxyl groups)

with high chemical reactivity, and flexible structure of the polymer chain. In what concerns these advantageous characteristics, the development of new products based on polysaccharides is an attempt to circumvent the issues associated to synthetic polymers and a better way of, rationally, using renewable bio-resources (Hossain and Mondal, 2014; Oladoja et al., 2017).

The global awareness of the society for minimizing the use of synthetic polymers has been growing and gaining special attention in important environmental campaigns because of the increasing environmental damage of this century. In addition, the scientific community try to develop technological alternatives for this issue. The last two decades have been marked by a crescent interest in public and scientific communities about the use and development of biopolymers, which can be obtained from renewable sources, displaying the important characteristic of biodegradability, with the desired physicochemical properties of conventional synthetic materials (Albuquerque and Malafaia, 2017; Rhim, Park and Ha, 2013).

The majority of biopolymers have rather low activity under environmental conditions. The primary task for scientific researches, in what concerns the polysaccharides, is to create a methodology for the synthesis of functional materials derived from them, which have significant properties for the enhancement of their utilization on a practical scale. This is expected to open the possibility of a real knowledge of the nature of intermolecular interactions in aqueous solutions of biopolymers (Oladoja et al., 2017).

The optimal choice of a particular technology for engineering of polysaccharides can be either by chemical, physical or biological modifications. Physical (ultrasonic disruption and microwave exposure) and biological (enzymatic degradation) changes allow modifications of the polysaccharide molecular mass, while the chemical-one could change the substituent types of groups, number and position (Ghimici and Nichifor, 2018).

Polysaccharide bio-based flocculants contain natural polysaccharides that are suspected to exhibit excellent selectivity towards aromatic compounds and metals in the absorption of particles, for example pollutants found in wastewaters (Crini, 2005). However, it is well known that their feasibility is restricted by their physicochemical properties, moderate flocculating activity and short shelf life. In recent years, grafted bioflocculants have been developed and claimed due to their remarkable flocculating ability and biodegradability. They are covalently modified by inclusion of synthetic monomers onto their backbone to synthesise the high molecular weight grafted copolymers that exhibit improved flocculating properties (Crini, 2005; Lee et al., 2014; Lee et al., 2012).

The main methods for polysaccharide modification in aqueous solution can be categorized in: (1) conventional method, (2) microwave initiated grafting method, and (3) microwave assisted grafting method. In the conventional method, a chemical-free radical initiator is added under an inert atmosphere to produce free radical sites on the polymer in order to allow the addition of monomer to form the graft chain. This method is not suitable for an industrial scale of polysaccharide production due to its low reproducibility. The use of microwave irradiation emerges as a promising alternative; in the microwave assisted grafting method, ions are produced by the addition of external redox initiators to the reduction mixture, thus, the free radical initiators facilitates the grafting reaction. In microwave initiated grafting reactions, in turn, no initiators are added and a small amount of hydroquinone is required to inhibit the grafting reactions. It is important to mention that factors including pH, reaction temperature, reaction time, and reactants/initiator/crosslinker dosages and ratios influence the flocculation performance of the modified polysaccharide bio-based flocculants (Kumar, Setia and Mahadevan, 2012; Salehizadeh, Yan and Farnood, 2017; Sen et al., 2009).

3. GENERAL ASPECTS OF THE FLOCCULATION PROCESS

The most commonly used flocculants in industry today are inorganic and synthetic organic flocculants; their effective flocculation activity and low cost are still preferable over the bioflocculants, however, the question of their toxicity to human health and environmental pollution has been a major concern (Okaiyeto et al., 2016).

Inorganic flocculants include alum, polyaluminium chloride (PAC), aluminium chloride, aluminium sulfate, ferric chloride, ferrous sulfates or composites obtained by the mixture of these salts. The flocculation process occurs between the salt of these metals and the negatively charged suspended particles in a solution. This interaction leads to a reduction in surface charge and the formation of microflocs, which in turn aggregates to form larger flocs that can easily settle out of solution (Figure 1). Besides, its application has caused problems of increased metal concentration or residual aluminium in treated water, which may presents human health implications and produces large quantity of sludge whose disposal is another problem (Lee et al., 2014).

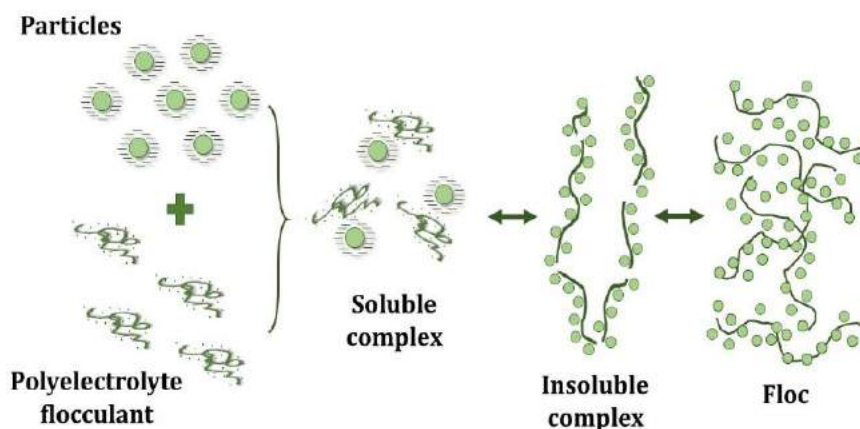


Figure 1. Flocculation mechanism by charge neutralization.

Synthetic organic flocculants include polyacrylamide (PAA), polyethylene amine, and poly(diallyl dimethyl ammonium chloride) (DADMAC). They are commonly derived from oil-based or non-renewable raw materials, usually have a high molecular weight, and present polyelectrolytes in their molecular chain, which enhance their flocculating effectiveness (Lee, Robinson and Chong, 2014; Okaiyeto et al., 2016; Suopajärvi et al., 2013). However, problems associated to Alzheimer's disease (Campbell, 2002) and carcinogenic and neurotoxic risks (Rudén, 2004) to humans were already reported for aluminium and acrylamide salt flocculants, respectively. In addition, these type of flocculants have other limitations related to their relatively high dosage requirement, high pH sensitivity, and poor efficiency for the coagulation of very fine particles (Sharma, Dhuldhoya and Merchant, 2006).

3.1. Mechanisms of Polysaccharide Bio-Based Flocculant

Bio-based flocculants (bioflocculants) are natural organic flocculants, i.e., products based on natural polymers considered of great interest due to their environmentally friendly behaviour and the promise alternative to replace conventional flocculants (Lee, Robinson and Chong, 2014).

Natural polysaccharides from different sources have long been described and widely applied in different fields. In recent decades, there has been an increased interest in the utilization of these biomolecules, particularly the bioactive ones, for various novel applications owing to their inherent properties. In fact, they have high potential to be applied not only in food and fermentation processes, pharmaceutical, cosmetic, downstream processing, but also in water and wastewater treatment (Liu, Willför and Xu, 2015).

The kinetics of flocculation by polymeric flocculants starts with a suitable concentration of the flocculant, which is fed into the suspension; then, the macromolecular flocculant makes contact with the suspended colloids by adsorption through electrostatic interactions, hydrogen bonding, van der Waals forces, etc. This leads to a rearrangement of the

conformation of the adsorbed polymer such that the adsorbed suspended particles aggregate to form large flocs that finally settle down effectively (Bolto and Gregory, 2007; Yang et al., 2016).

The flocculation mechanisms that direct the activities of various polymeric flocculants, including polysaccharides, can be categorized as charge neutralization, charge patching, bridging, and sweeping. Acting as bioflocculants, polysaccharides could destabilise the colloidal particles by increasing the ionic strength and giving some reduction in the zeta potential and thus a decreased thickness of the diffuse part of the electrical double layer. In the other hand, they could specifically adsorb counterions to neutralise the particle charge because they have particular macromolecular structures with a variety of functional groups, such as carboxyl, hydroxyl, amino, and sulphate, which can interact with contaminants. For many years, chitosan, tannins, cellulose, alginate, gums and mucilage have been basing flocculants and attracting wide interest of researchers (Lee, Robinson and Chong, 2014).

It is predicted that some of the active ingredients in the mucilage are responsible for the flocculating property. Therefore, extraction becomes the essential step to isolate the active components that exhibit the flocculating activity from the plants. There are two methods for the production of plant-derived bioflocculants: (1) solvent extraction and precipitation and (2) drying and grinding. Briefly, the solvent extraction and precipitation method starts with the cleaning of the plant materials and then extraction with distilled water overnight, followed by filtration of the mucilaginous extract and precipitation using alcohol. In turn, the drying and grinding method starts with the cleaned materials being dried at high temperature and then grounded and sieved to obtain the bioflocculants. Bioflocculants prepared under solvent extraction and precipitation conditions displayed excellent flocculating ability in the treatment of wastewater with direct flocculation process where no coagulant and pH adjustment are required. Thus, these results suggest that the extraction step is closely related with flocculating efficiency and plays the major role to extract the active constituents with high flocculating activity from the plant materials. In addition, the method used to evaluate the flocculating

efficiency of the plant-derived bioflocculants and optimise the flocculation process is called Jar Test (Lee, Robinson and Chong, 2014; Salehizadeh, Yan and Farnood, 2017).

The most common flocculation mechanisms related for polysaccharide plant-derived bioflocculants are charge neutralization (including electrostatic patch effects) and polymer bridging. These mechanisms are intrinsically dependent on the adsorption of flocculants on particle surfaces; if there is some affinity between polymer segments and a particle surface, the adsorption process occurs (Bolto and Gregory, 2007; Lee, Robinson and Chong, 2014; Salehizadeh, Yan and Farnood, 2017). As already mentioned in section 3, the flocculation mechanism of charge neutralisation is only applicable when the colloid suspended particles and the added flocculants are of opposite charge. In this case, the particle surface charge density is reduced by adsorption of the flocculant polysaccharide; the electrostatic repulsions are reduced to a minimum. This mechanism has been found to be quite effective for low molecular weight polysaccharide flocculants that tend to adsorb and neutralize the opposite charges on the particles (Yang et al., 2016).

Considering the great number of neutral polysaccharides acting as bioflocculants, it is suggested that charge neutralisation is not the mechanism responsible for flocculation. In fact, bridging is the major mechanism in like-charged or neutral polysaccharide flocculants, especially when they extends from the particle's surface into the solution for a distance greater than the distance over which the interparticle repulsion occurs. In this case, segments of the polysaccharide flocculant are adsorbed onto the particle surface resulting in loops and tails extending into solution with the possibility of attachment of dangling polysaccharide segments onto other adjacent particles to form flocs. Bridging could be due to van der Waals force, static, hydrogen bonds or even chemical reaction between some radical groups of the polysaccharide molecule and the particle; in addition, this mechanism is known to be especially effective for high molecular weight polysaccharide flocculants (Salehizadeh, Yan and Farnood, 2017).

Electrostatic patch and sweeping are mechanisms that contribute to the process of flocculation. The first one is caused by polymer flocculants of high charge density interacting with oppositely charged colloidal particles of low charge density. The net residual charge of the polymer patch on one colloidal particle surface can adsorb onto the oppositely charged colloidal particle. The second one forms a bulky precipitate that enmeshed the colloidal particles, which are then either settled out or flocculate together with the precipitate (Salehizadeh, Yan and Farnood, 2017).

Without doubts, flocculation is one of the most important and widely used treatment process of industrial wastewaters. Many authors already highlighted the simplicity and effectiveness of flocculation-coagulation processes (Lee, Robinson and Chong, 2014; Okaiyeto et al., 2016; Salehizadeh, Yan and Farnood, 2017; Teh et al., 2016). In what concerns the use of polysaccharides as bioflocculants, some characteristics could limit their application for certain purposes. For example, chitosan is soluble in acidic media and most of polysaccharides are water-soluble in their native form, therefore they cannot be used as insoluble sorbents (Crini, 2005). In the flocculation process, the polysaccharide biodegradability can lead to the loss of flocs stability and strength (Singh et al., 2000). Also, some polysaccharides have reduced/or no bioactivity in certain conditions (Salehizadeh, Yan and Farnood, 2017). During the time, much attention has been paid to overcome these disadvantages by modifications of polysaccharides.

3.2. The Effect of Physical-Chemical Factors on Flocculating Activity

The chemical composition, physicochemical properties and flocculation activity of flocculants depend on the source of the flocculant and its production method. Characteristics such as charge density and grafting ratio are important in determining the flocculant performance of the substance; even more, earlier studies indicate that flocculation

efficiency could be enhanced by optimizing the grafting ratio (Salehizadeh, Yan and Farnood, 2017).

Besides the flocculant charge density, the flocculation mechanism can be mainly affected by other properties, including dosage, biopolymer molecular weight, the nature of the colloids and aspects such as concentration, pH, ionic strength, composition of culture media, and temperature, especially for production of bioflocculants from bacteria (Chaisorn et al., 2016; Salehizadeh and Yan, 2014).

The effect of pH on flocculating activity is one of the most important external factors affecting bioflocculant production. Several works reported the flocculating activity of different bioflocculants being pH-stable in a wide range of pH values, usually from 3 to 10 (Aljuboori et al., 2013; Huang et al., 2015; Li et al., 2013; Li et al., 2014; Liu et al., 2014b).

The metabolism of microorganisms has direct relationship with cultivating temperature. Maximum enzymatic activation can be obtained at optimal temperatures (Zhang et al., 2007). In this way, temperature is an important parameter for bioflocculant production and cell growth, especially considering bacteria and fungi sources. Thermotolerant microorganisms have an optimum temperature for growth below 45 °C, but ability to grow at higher temperatures; they are also defined as microorganisms able to grow at 60 °C and below 30 °C (Chaisorn et al., 2016).

The flocculant dosage of the polysaccharide is a main aspect for the determination of the flocculating activity of a colloid system. When the polysaccharide dosage is lower than the optimum, the degree of flocculation is insufficient in a colloid system that results in stabilization and charge reversal of the colloidal particles. Thus, there is no bioflocculant enough to adsorb onto the colloidal particle surfaces to bridge between these particles. An overloaded dosage of the polysaccharide flocculants increases the electrostatic repulsion forces between the colloidal particles and increases the distance between the particles to inhibit floc formation and precipitation (Salehizadeh, Yan and Farnood, 2017).

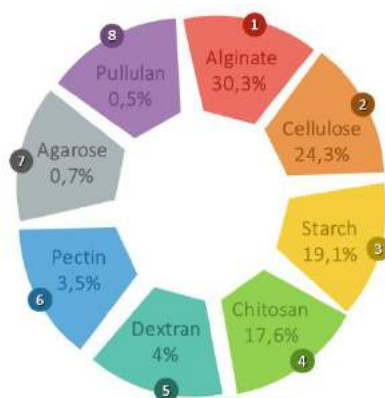


Figure 2. Percentage of publication in the last 10 years about the scientific literature dealing with polysaccharides and flocculants.

4. PLANT FLOCCULANT POLYSACCHARIDES

Polysaccharide flocculants can be classified into three main groups based on their source: marine, microbial, and plant flocculant polysaccharides. Their natural origin could be related to crustacean shell wastes, seaweeds, agricultural/forestry feedstocks, and microorganisms including bacteria, yeast, fungi, and algae. Figure 2 depicts the percentage of published articles reporting polysaccharides and flocculants in the last 10 years.

Considering the main flocculants derived from plant-polysaccharides, we summarized below their properties and the current researches about them.

4.1. Cellulose

Cellulose is a linear polysaccharide consisting of β (1 $\rightarrow$ 4) D-glucose; it represents the most abundant biopolymer on earth, with a global economic importance. The annual production rate of cellulose is estimated at 10^{11} – 10^{12} ton/year, being this polysaccharide the major structural

constituent of the cell wall of plants, but also derived from different origins, such as animals and microorganisms. Cellulose has many advantages, including superior thermal and mechanical properties, in addition to biocompatibility, biodegradability, and cost-effectiveness (Albuquerque et al., 2017; Salehizadeh, Yan and Farnood, 2017).

In what concerns the different pathways to obtain cellulose, it is possible to highlight four: the first one is the most popular and industrially important pathway for isolating cellulose from plants, which includes chemical pulping, separation, and purification processes to remove lignin and hemicelluloses. The second pathway is related to the biosynthesis of cellulose by different types of microorganisms, such as unicellular algae, fungi, and bacteria. The third pathway is the enzymatic *in vitro* synthesis starting from materials such as cellobiosyl fluoride, while the last one is a chemical synthesis, whose production of cellulose occurs through a ring-opening polymerization of the benzylated and pivaloylated derivatives (Chen, Cho and Jin, 2010).

The polymeric structure of cellulose was first demonstrated by Staudinger in 1920. In fact, cellulose is a simple polysaccharide with no branching or substituents in its homogeneous backbone. The morphological hierarchy of this biopolymer is composed by elementary fibrils, packaged into microfibrils, and finally assembled into fibres. Within the cellulose fibrils, there are regions of crystallites, where the cellulose chains are arranged in a highly ordered structure, and amorphous regions of disordering. Five allomorphic forms of cellulose have been known based on the location of hydrogen bonds between and within strands. Natural cellulose, called cellulose I, has two different crystalline structures (I α and I β). Cellulose may be also found with other crystal structures including cellulose II, III and IV, being cellulose II the most stable structure, which can be obtained by alkali treatment of cellulose I (Brinchi et al., 2013; Moon et al., 2011; Roy et al., 2009).

Cellulose obtained from plants are usually composed of hemicellulose, pectin, lignin, and other substances, while bacterial cellulose is completely pure with much higher water content and higher tensile strength (Klemm et al., 2005). The hydroxyl groups are the most targeted reactive groups on

the cellulose main chain, which can fully or partially react with chemical agents to obtain various derivatives with different substitution degrees. The obtained derivatives of cellulose have been applied in food, cosmetic, biomedical, and pharmaceutical industries, however, the application of cellulosic material is limited due to the difficulty in processing. The high crystallinity degree and rigid intra/intermolecular hydrogen bonds result in its insolubility in water and most organic solvents. In addition, cellulose becomes amorphous in water at 320 °C and 25 MPa and can be converted chemically into its monomeric units by reacting with concentrated acids at high temperatures (Deguchi, Tsujii and Horikoshi, 2006; Liu, Willför and Xu, 2014; Zhang, Lin and Yao, 2015).

Problems associated to processing cellulose and its limited solubility are observed for other natural polymers since they present increasingly application in the industrial technology. Most polymers do not present biological activity until modifications are made, so it is important to mention that numerous attempts are being performed to minimize these certain drawbacks with chemical modifications in the polymeric structure (Albuquerque et al., 2017). Cellulose can be considered an efficient alternative to produce environmentally friendly functional materials and chemicals such as bioflocculants due to its inherent physicochemical characteristics, which are improved by chemical modifications (Salehizadeh, Yan and Farnood, 2017).

The most recent researches on bioflocculant production are reporting isolated strains (Lee and Chang, 2018; Shahadat et al., 2017). The unique potential of bioflocculants produced from microorganisms was first investigated in *Levure caseeuse* yeast by Louis Pasteur, and a similar trend in bacterial culture was also observed by Bordet in 1899 (Shahadat et al., 2017). More recently, it was reported that some bacteria can produce bioflocculants by utilizing biomass from raw materials: a lignocellulose-degrading strain *Cellulosimicrobium cellulans* L804 isolated from corn farmland soil presented the ability to produce bioflocculants by the degradation of lignocellulosic biomass (Liu et al., 2015). Liu et al. (2017a) reported an alkaliphilic strain *Bacillus agaradhaerens* C9 bioflocculant producer by using untreated rice bran as carbon source, and Guo et al.

(2018) reported a novel cellulase-free xylanase-producing bacterium G22 with the potential ability to directly convert various biomasses to bioflocculants.

The scientific literature dealing with polysaccharide flocculants derived from plants is still scarce when compared to the works reporting bioflocculants produced from microorganisms, or even those one reporting commercial polysaccharide bioflocculants. However, the importance of these kind of studies rises with the increasingly serious environmental problems especially associated to the discharge of effluents. For example, Liu et al. (2014a) developed an efficient and eco-friendly flocculant from *Phyllostachys heterocycle* bamboo pulp cellulose grafted with polyacrylamide for an effluent from paper mill. After that, Zhu et al. (2016) optimized the method and employed the co-polymer with the metal ions Fe^{3+} , Al^{3+} or Ca^{2+} to treat the effluent from a surfactant manufacturer. Liimatainen et al. (2012) evaluated the effectiveness of a flocculation treatment based on alum and soluble or nanoparticulate anionic derivatives of dialdehyde cellulose derived from the bleached birch chemical wood pulp obtained in dry sheets of *Betula verrucosa* and *B. pendula*, by studying the removal of colloidal material in a model suspension containing kaolin.

4.2. Starch

Starch is a linear, biodegradable, inexpensive polysaccharide synthesized in a granular form by green plants for energy storage over long periods. Starch granules consist of two major components called amylopectin and amylose, which are composed of α -D-glucopyranose residues, forming α -1,4-glucosidic bonds in linear structure of amylose and additional α -1,6-glycosidic branches in amylopectin molecules. Minor constituents such as lipids, proteins, and minerals are present in starch and the levels vary with the origin, whose botanical sources include wheat, rice, corn, barley, sorghum, millet, rye, legumes, banana, mango, potato, cassava and so on. Cassava and maize were known as the major sources of

starch on a commercial scale for a long time (Albuquerque et al., 2017; Ashogbon and Akintayo, 2014; Salehizadeh, Yan and Farnood, 2017).

Starch is soluble in hot water, but insoluble in cold water, alcohol, or other solvents. The differences between amylose and amylopectin structures have indeed significant variance in their properties. Amylose is much more prone to crystallization process, called retrogradation, and can produce tough gels and strong films, while amylopectin retrogrades much slower due to its dispersion in water, which results in soft gels and weak films (Liu, Willför and Xu, 2015; Pérez and Bertoft, 2010).

Starch is an excellent material for biotechnological applications due to its biodegradable, available, low cost, and versatile characteristics; however, its direct applications are limited by its poor processability and intrinsic properties, such as thermal, mechanical, and biological properties. Thus, various chemical, physical, and enzymatic modifications or blending with other materials has supplied solutions to achieve properties that are more desirable (Albuquerque et al., 2017).

Comparing starch and other high-performance natural-polymeric material, it is much cheaper than chitosan (Liu et al., 2017b). Similar to the cellulose, conventional chemical modifications of starch could be performed based on its primary and secondary hydroxyl groups, and can be obtained by esterification, etherification, oxidation, and graft copolymerization (Liu, Willför and Xu, 2015).

Considering that cationic moieties aid flocculation and sterilization through effective charge attractions, various cationic starch-based flocculants have been developed using different modification methods and even considering the lack of commercial synthesis methods and high cost of the cationic monomers. For example, three different starch-based flocculants, all of them containing the strongly cationic quaternary ammonium salt groups but at different positions, have been designed and prepared through etherification, graft copolymerization, or their combination. The effects of chain architectures and charge properties on flocculation of humic acid (HA) and its floc properties have been investigated in terms of several environmental parameters including pH, dose, and initial HA concentration (Wu et al., 2016). Posteriorly, authors

obtained four variations with different cationic contents of two versions of the starch-based flocculants reported in the previous work. The environmental parameters pH and flocculant dose were evaluated with the effects of structural factors, i.e., cationic group contents and distributions, on the flocculation of the hairwork effluents (Du et al., 2017). These starch-based flocculants were also evaluated by the efficient flocculation of real secondary textile dyeing effluents. Starch-graft-poly[(2-methacryloyloxyethyl) trimethyl ammonium chloride] (STC-g-PDMC) and starch-3-chloro-2-hydroxypropyl trimethyl ammonium chloride (STC-CTA) have been systematically investigated and compared with that of the traditional inorganic coagulant polyaluminum chloride, and the obtained results are of significance in guiding the design and selection of a suitable polymeric flocculant in treating target wastewater (Wu et al., 2017a).

Liu et al. (2017b) prepared a series of cationized starch-based flocculants (ST-CTA) containing various quaternary ammonium salt groups on the starch backbone by using a simple etherification reaction. They observed an effective performance for the flocculation of kaolin suspension, two bacterial suspensions (of *Escherichia coli* and *Staphylococcus aureus*), and two contaminant mixtures (kaolin and each bacterium) under most pH conditions. Wang et al. (2013) prepared a cationic grafted starch (ST-g-PDMC) with high flocculation performance for kaolin suspensions and efficient dewatering of anaerobic sludge. More recently, Huang et al. (2017) reported that the St-g-PDMC was efficient for the flocculation of kaolin suspensions and for the inhibition of *E. coli* by introducing new quaternary ammonium groups. A grafted amphoteric starch-based flocculant (carboxymethyl-starch-graft-aminomethylated-polyacrylamide, CMS-g-APAM) was efficiently developed by Huang et al. (2016), in which the cationic groups were randomly distributed on the polyacrylamide branched chains using Mannich reaction. A series of cationic starches were developed by incorporating a cationic moiety (STC-CTA) onto the backbone of starch in presence of NaOH. The flocculation characteristics of these starches were evaluated in silica suspension and compared with various commercially available flocculants by jar test (Pal, Mal and Singh, 2005).

It is important to mention that the majority of researches about starch flocculants deal with the commercial presentation of the polysaccharide. Well-prepared natural flocculants, with or without chemical modifications, owing to their superior performance and environmental friendliness, have wide-ranging uses in wastewater treatment, especially when more effective production techniques are developed and optimized in the near future. Additionally, it is important to highlight the volume of the sludge produced from the treatment associated to them and the considerable reduction in the cost of sludge disposal.

4.3. Pectin

Pectins are ubiquitous plant polysaccharides. They are present in the cell walls located in the middle lamella, and primary and secondary cell wall. The chemical structure of this water-soluble gum is heterogeneous, depending on the origin, location in the plant and extraction method, being composed at least of 65% of galacturonic acid units plus rhamnose, arabinan, galactan and arabinogalactan (Müller-Maatsch et al., 2016; Tamnak et al., 2016). Actually, the uronic acid residues linked through α -1-4-glycosidic bonds make the main structural polysaccharide motif, while there exist three pectin's domains: α -(1-4)-linked linear homogalacturonic backbone (HG) alternating with two types of highly branched rhamnogalacturonans regions called RG-I and RG-II. The first region (RG-I) is substituted with side chains of arabinose and galactose units, while the second one (RG-II) has a highly conserved structure, consisting of the HG backbone branched with eleven different monosaccharides, including some rare sugars such as apiose, aceric acid, 2-O-methylxylose, 2-O-methylfucose, 2-keto-3-deoxy-d- manno-octulosonic acid, and 3-deoxy-d-lyxo-2-heptulosaric acid. In all natural pectins, some of the carboxyl groups exist in the methyl ester form (Albuquerque et al., 2017; Liu, Willför and Xu, 2015).

Pectin can be categorized as anionic polysaccharides mainly derived from the cell wall of plants, and also from food-industrial wastes of fruits, whose main sources are citrus peels (e.g., lemon, lime, orange, and grapefruit), apple pomace, and sugar beet pulp (Babbar et al., 2015; Marić et al., 2018). Moreover, pectin from non-conventional sources has been evaluated, for example, from cocoa husks (Chan and Choo, 2013), mulberry branch bark (Liu, Jiang and Yao, 2011), jackfruit peel (Xu et al., 2018), faba bean hulls (Korish, 2015), sisal waste (Yang et al., 2018), watermelon rind (Maran et al., 2014), pomegranate peels (Pereira et al., 2016), potato pulp (Yang, Mu and Ma, 2018), Ubá mango peel (Oliveira et al., 2018), pistachio green hull (Chaharbaghi, Khodaiyan and Hosseini, 2017), pequi peel (Leão et al., 2018), and banana peel (Oliveira et al., 2016). In the industry, the major pectin component (homogalacturonan) can be obtained using hot water or chemically by acid extraction, but some innovative extraction approaches have been developed to improve extraction process and pectin quality (Salehizadeh, Yan and Farnood, 2017).

Pectin structure determines in a great manner its physicochemical properties and applications; for example, pectin is efficiently used by the food industry due to their ability to form gels under certain circumstances and to increase the viscosity of drinks. It is also widely applied as stabilizers in acid milk products, and some may have other pharmaceutical uses (Müller-Maatsch et al., 2016).

Pectin has many applications because of its low-cost, ready availability, harmless and green nature. However, it may not provide the proper emulsifying activity because of its hydrophilicity. The following modification techniques have been recommended for the improvement of pectin physicochemical and functional properties: chemical modification (such as saponification, distillation, and esterification), enzymatic modification (by pectin methylsterases), and physical modification (by heat treatment and microwave) (Tamnak et al., 2016).

Yokoi et al. (2002) were one of the first authors reporting pectin as an efficient flocculant for various suspensions. They found that pectin had a flocculating activity, however, in that time; there has so far been no report

about flocculating activity of pectin and its applicability as a flocculating agent. Their results demonstrated that pectin had flocculating activity in a kaolin suspension, and this activity was enhanced by the addition of Al^{3+} and Fe^{3+} to the suspension; in relation to organic suspensions such as cellulose and yeast, pectin acted as flocculant when 0.1–0.2 mM Fe^{3+} was present in the suspensions. Other inorganic suspensions of activated carbon and acid clay were flocculated by pectin in the presence of Al^{3+} or Fe^{3+} .

A commercial citrus pectin with 60% esterification and the common organic synthetic flocculant polyacrylamide were characterized and used to optimize the treatment processes of both flocculants in synthetic turbid waste water. The results reported by Ho et al. (2010) were important for the industrial application of pectins since the main concern for industry is to use low flocculant concentrations to achieve maximum results. In this case, the usage of pectin achieved that goal and proved to be effective at a low concentration of 3 mg/L. The influence of four commercial citrus pectins, with different degrees of esterification, and pectin extracted from pomelo (*Citrus maxima*) on the stability of indomethacin suspension was extensively investigated by Piriyaprasarth and Sriamornsak (2011). The results demonstrated that the extracted pectin had comparable activity to the commercial ones. Moreover, the use of low concentration of pectin and ferric ions allowed obtaining indomethacin suspensions with suitable stability and redispersibility. More recently, pectin extracted from Nopal (*Opuntia ficus-indica*) was tested to treat synthetic waste water contaminated with metallic ions (Ibarra-Rodríguez et al., 2017). Authors well-characterized the pectin and then demonstrated its effectiveness in the removal of the heavy metals by coagulation-flocculation treatment.

It is possible to observe that flocculation properties of pectin have been confirmed by the scientific literature. In fact, pectin can be utilized as a harmless bioflocculant, since it is biodegradable, and edible and non-toxic toward humans and the environment; however, the publications are still discreet when compared to the most popular polysaccharide bio-based flocculants.

4.4. Other Polysaccharide Flocculants Derived from Plants

Other polysaccharide bio-based flocculants derived from different and non-traditional plant sources have been reported by the scientific literature (Lee et al., 2014) reported that all of them have mucilaginous texture, due to the main polysaccharide composition, and a neutral pH.

Plantago psyllium seed husk is known as Isabgol husk in India and widely used as laxative. This anionic polysaccharide is extensively studied due to its easy availability and a very economical cost. Mishra et al. (2002) reported for the first time the flocculation efficiency of this polysaccharide with different effluents. Their results demonstrated that this mucilage was found to be a very effective flocculant, capable of removing almost 85 and 95 percentages of suspended solid (SS) from sewage and tannery wastewater samples, respectively. The chemical modification of *P. psyllium* was done by grafting polyacrylamide (PAM), resulting in a graft-copolymer (Psy-g-PAM) and very effective flocculant, capable of removing more than 93% of SS, 72% of total dissolved solids (TDS) and 15.24% of colour from a textile waste water (Mishra et al. 2004a). The same group (Mishra and Bajpai, 2005) assessed *P. psyllium* for the removal of dyes from model textile wastewater containing golden yellow (C.I. Vat Yellow 4) and reactive black (C.I. Reactive Black 5). The mucilage reduced dye concentration by flocculation and settling, suggesting a preference of this bioflocculant for colour removal because of its low capital cost, as well as the lower operating costs when compared to other technologies (Al-Hamadani et al., 2011) evaluating the psyllium husk from seeds of *P. ovata* plant. *Psyllium* was more effective as coagulant aid when used with aluminum chloride for the treatment of landfill leachate.

The mucilage extracted from tamarind (*Tamarindus indica*) seeds was used for the removal of vat and direct dye in aqueous solution (Mishra and Bajpai, 2006). After the promising results obtained in this study, Mishra et al. (2006) used ceric ion used initiated polymerization to obtain a grafted mucilage with acrylamide. The grafted copolymer (Tam-G-PAM) showed better results for dye removal. The optimal flocculant concentration was independent of dye concentration within the concentration range examined. Both the grafted and ungrafted flocculants were more efficient for removal

of azo dyes than for reactive and basic dyes. The application of tamarind seed kernel powder for the sedimentation of clay slurry was examined in association or not with starch. Initial qualitative results indicated that tamarind can be successfully used to reduce the turbidity of clay slurry (Chakrabarti et al., 2008).

Naturally occurring *Cassia angustifolia* (CA) seed gum was evaluated against polyaluminium chloride for its coagulation-flocculation ability to remove colour from synthetic dye solutions. The results demonstrated that the gum was found to be a good working substitute alone or in conjunction with a very low dose of polyaluminium chloride for decolourization of acid and direct, but not for reactive dye solutions (Sanghi, Bhattacharya and Singh, 2002).

Fenugreek, botanically known as *Trigonella foenumgraecum*, is a leguminous plant found in long pods and used for various medicinal purposes. This mucilage was reported for the very first time by (Mishra, Agarwal and Yadav, 2003), proving to be a very effective flocculant for sewage treatment and capable of removing almost 97% of SS and 20% of TDS. The flocculation efficiency of this mucilage was also reported as an efficient flocculant for tannery effluent treatment (Mishra et al., 2004b).

Anastasakis, Kalderis and Diamadopoulos (2009) studied optimal dose, contact time and flocculation efficiency of *Malva sylvestris* (mallow) and *Hibiscus esculentus* (Okra) mucilages to remove turbidity from synthetic wastewater and biologically-treated effluent. In the pharmaceutical field, tablets prepared using the mucilages of *Hibiscus rosasinensis* Linn. and *Abelmoschus esculentus* Linn. (Okra). Authors observed that the higher concentration level of Okra mucilage show a slow and sustained release, and can be considered as an alternative natural excipient in the modified drug delivery systems. They also reported that *Hibiscus* mucilage could be used as a platform for prolonged release if its binder concentrations are increased (Ameena et al., 2010). Fenugreek (from *T. foenumgraecum*) and okra (from *H. esculentus*) were tested as flocculants for treatment of textile wastewater. Results showed that the mucilages were capable of removing more than 90% of SS, 30% of TDS, and 30% of dye using a very low concentration of polysaccharide (Srinivasan and Mishra, 2008).

5. FLOCCULANT POLYSACCHARIDES OF NON-PLANT SOURCED

Despite the considerable number of polysaccharide bio-based flocculants derived from plants, especially cellulose and starch, other sources of polysaccharides could be derived from seaweeds, arthropods, and microorganisms, which might be chemically modified to improve their flocculation performance.

Natural flocculant polysaccharides from microorganisms, microalgae, seaweeds and animals (Table 1) are also attractive substitutes for synthetic flocculants due to their ecofriendly nature, biodegradability, stability, cost-effective production process, and large availability and yield (Salehizadeh, Yan and Farnood 2017). Microorganisms such as bacteria and fungi are able to produce extracellular polymeric substances (EPS) with flocculating activity, including polysaccharides, proteins and glycoproteins.

Bioflocculants from these sources are also potent alternatives for usage in wastewater treatment, textile, pharmacology and cosmetology industries. This section focuses in recent advances in polysaccharides bioflocculants from marine and microbial origins, considering their features, production, flocculant mechanisms and emerging applications.

5.1. Flocculant Polysaccharides in Bacteria

Bacteria have been reported to produce polymeric bioflocculants such as glycoproteins and exopolysaccharides, as metabolites. Bioflocculants with varied sugar composition have been isolated from bacteria and they have showed strong flocculating activity to kaolin clay, sewage water and heavy metals, being a promise for industrial and biotechnological applications.

Table 1. List of flocculant polysaccharides from bacteria, fungi, marine sources and arthropods

Species	Source	Structure	Reference
Bacteria			
<i>Alcaligenes aquatilis</i> AP4	Palm-oil mill effluent	Glycoprotein	Adebayo-Tayo and Adebami, 2017.
<i>Bacillus cereus</i> SK	Wastewater treatment plant	Glycoprotein	Busi et al., 2016
<i>Bacillus subtilis</i> F9	Wastewater sludge	Polysaccharide and protein	Giri et al., 2015
<i>Bacillus subtilis</i> WD161	Recycled activated sludge of a seafood processing plant	Glycoprotein	Chaisorn et al., 2016
<i>Bacillus thuringiensis</i>	Aquaculture and Nutritional Laboratory, Pearl River Fishery Research Institute, Chinese Academy of Fishery Sciences, Guangzhou, China	Polysaccharide	Wu et al., 2017b
<i>Rhodococcus rhodochrous</i> R-202	Fungal Culture Collection of Lublin	Polysaccharide and protein	Czemierska et al., 2017
<i>Pseudomonas</i> sp. GO2.	Paper mill sludge	Polysaccharide, protein and nucleic acid	Guo et al., 2017
<i>Anabaena</i> sp. BTA992	National repository for cyanobacteria and microgreen algae (freshwater) of the Institute of Bioresources and Sustainable Development, Imphal, Manipur, India	Polysaccharide and protein	Khangembam et al., 2016
<i>Paenibacillus elgii</i> B69	Soil	Polysaccharide	Li et al., 2013
<i>Paenibacillus mucilaginosus</i>	Soil	Polysaccharide	Tang et al., 2015
<i>Klebsiella</i> sp.	Activated sludge of a secondary sedimentation tank in a wastewater treatment plant (WWTP)	Polysaccharide, protein and nucleic acids	Liu et al., 2014b
<i>Streptomyces</i> sp. MBRC-91	Marine sediment	Polysaccharide	Manivasagan et al., 2015
<i>Achromobacter xylosoxidans</i>	Activated sludge of Bongaigaon oil refinery	Glycoprotein	Subudhi et al., 2016

Table 1. (Continued).

Species	Source	Structure	Reference
Fungi			
<i>Curvularia</i> sp. DFH1	Water	Polysaccharide and protein	Abu-Elreesh and Abd-El-Haleem, 2014
<i>Aspergillus flavus</i> S44-1	Microbial Culture Collection Unit (UNiCC), Laboratory of Industrial Biotechnology, Institute of Bioscience, University Putra Malaysia	Polysaccharide and protein	Aljuboori et al., 2013
SGMP ¹ and SGMP ²	White rot	Polysaccharides	Patel et al., 2014
<i>Aspergillus niger</i>	Soil surface	Polysaccharide	Pu et al., 2018
Microalgae/Seaweeds			
<i>Scenedesmus obliquus</i> AS-6-1	Water	Polysaccharide	Guo et al., 2013
Seaweed	Commercial Agar bacteriological	Cationized agarose	Prado et al., 2011
Brown seaweed	Commercial sodium alginate	Polymethyl methacrylate grafted sodium alginate	Rani et al., 2013
Brown seaweed	Commercial sodium alginate	Alginate graft polyacrylonitrile beads Sulfated polysaccharide	Salisu et al., 2016 Yim et al., 2007
<i>Gyrodinium impudicum</i> KG03	Seawater		
Animals			
<i>Brachyura</i>	Commercial chitosan powder from crab shells	Chitosan	Xu et al., 2013
Animal source	Commercial chitosan	3-chloro-2-hydroxypropyl trimethylammonium chloride grafted onto carboxymethyl chitosan	Agbovi and Wilson, 2018
Animal source	Commercial chitosan	Phenylalanine-modified- chitosan	Du et al., 2018

The bacterium *Alcaligenes aquatilis* AP4 produces a flocculant glycoprotein which was isolated by first time from Palm-oil mill effluent (Adebayo-Tayo and Adebami, 2017). The isolated bioflocculant showed a high flocculating activity of approximately 90% and potential for large-scale production.

An exopolysaccharide produced by *Bacillus cereus* SK showed flocculating activity to kaolin of 83.4% at 12 mg, and no considerable toxicity to zebra fish *in vivo*, revealing the potential of this EPS as a bioflocculant in industrial and biotechnological field (Busi et al., 2016). Other study revealed a flocculant glycoprotein produced by *B. subtilis* WD161, composed of mainly protein and sugar, with flocculating activity to precipitate suspended solid in palm-oil mill effluent increased by 35% (Chaisorn et al., 2016). A potent bioflocculant was also purified from *B. subtilis* F9, showing a composition of protein and mainly sugar (88.3%). The bioflocculant was able to flocculate small particles, although its low molecular weight, being considerate an excellent bioflocculant for industrial applications (Giri et al., 2015). A strain of *B. thuringiensis* highly-producer of flocculant was isolated from the biofloc in aquaculture waters. The bioflocculant was a solid substance with white colour and composition mainly of polysaccharides (Wu et al., 2017b).

The flocculation potential of the exopolymer R-202 produced by *Rhodococcus rhodochrous* was investigated (Czemierska et al., 2017). With a composition of polysaccharides and proteins, and negatively charged, the exopolymer R-202 promoted the flocculation of a kaolin suspension with and without the addition of cations, being a promise for applications in flocculation industrial process.

An exopolysaccharide composed of glucose, glucuronic acid, mannose and xylose isolated from bacterium *Paenibacillus elgii* B69 showed high flocculant activity when tested with kaolin clay, dyeing pigment, heavy metal ion and real wastewater (Li et al., 2013). A study about polysaccharide-based flocculants (PSBs) produced by *P. mucilaginosus* GIM1.16 revealed that the production and feasibility of these bioflocculants can be improved with the presence of metal ions in culture

medium. The presence of Ca^{2+} , Mg^{2+} and Fe^{3+} increased the production and flocculating activity of PSBs (Tang et al., 2015).

M-C11 is a bioflocculant isolated from *Klebsiella* sp., composed of sugar (91.2%), protein (4.6%) and nucleic acids (3.9%). This bioflocculant showed high pH and thermal stability in kaolin suspension in the pH range of 4.0 to 8.0 and temperature of 20 to 60°C, and a sludge dewatering performance more efficient than inorganic flocculants (Liu et al., 2014b).

An exopolysaccharide produced by cyanobacterium *Anabaena* sp. BTA992 during its photoautotrophic growth was evaluated and it was observed bioflocculant activity with potential properties for commercial production and industrial applications (Khangembam et al., 2016).

A biomass-degrading bacterium *Pseudomonas* sp. GO2 was able to produce a low-cost bioflocculant from untreated corn stover through directly hydrolyzing biomasses. Biochemical analysis showed a composition of polysaccharides (59%) with uronic acid (34.2%), protein (32.1%) and nucleic acid (6.1%), and a high flocculation potential flocculating to harvest the green microalgae *Chlorella zofingiensis* and *Neochloris oleoabundans* (Guo et al., 2017).

Achromobacter xylosoxidans TERI L1 isolated from oil refinery waste produces an exopolysaccharide bioflocculant encompassed of various functional charged groups with flocculating activity for dispersed kaolin clay particles in suspension and to adsorption of multi metals from environment (Subudhi et al., 2016). Thus, this flocculant is a good candidate for heavy metal removal in contaminated waste-water.

Green synthesis of silver nanoparticles was a method developed based in a polysaccharide flocculant produced by the marine actinobacterium *Streptomyces* sp. MBRC-91. The biosynthesized silver nanoparticles showed strong antibacterial potential in sewage water, being useful for wastewater treatment (Manivasagan et al., 2015). A flocculant exopolysaccharide from *Arthrobacter* sp. B4 was also used for green synthesis of silver nanoparticles, resulting in nanoparticles highly stable, with antimicrobial activity and low phytotoxicity (Yumei et al., 2017). This represents the use of microbial flocculants as a potential tool in many fields, including the medical therapies.

5.2. Flocculant Polysaccharides in Fungi

Conventionally, fungi play valuable roles in environment and are involved in global biotechnological processes, including the production of enzymes, polysaccharides, lipids and pigments. Some fungal species have been reported as bioflocculant polysaccharide producers with high flocculating activities.

An oleaginous fungal *Curvularia* sp. strain DFH1 demonstrated its potential to coproduce lipids and an exopolymer with a powerful flocculant activity of 95% (Abul-Elreesh and Abd-El-Haleem, 2014). The chemical analysis of the exopolymer shows a composition of protein and polysaccharide. An analysis of infrared spectrum indicates the presence of carboxyl and hydroxyl groups, typical in sugar derivatives.

A polysaccharide bioflocculant named IH-7 was produced and isolated from *Aspergillus flavus* fermentation medium (Aljuboori et al., 2013). The bioflocculant consisted basically of protein (28,5%) and sugar (69,7%), including neutral sugar, uronic acid and amino sugar, and showed a good flocculating activity in kaolin suspension without cation addition.

Two bioflocculant exopolysaccharides were isolated from fungal cultures named SGMP1 and SGMP2 found in soil samples collected from New Vallabh Vidyanagar, Gujarat (India). The flocculating activity was 99%, and the fungal isolates could remove the cations Al^{3+} and Fe^{3+} in ideal concentrations for application in bioremediation of heavy metals (Patel et al., 2014).

A bioflocculant (MBFA18) produced by *Aspergillus niger* (A18) isolated from soil sample using potato starch wastewater as nutrients showed strong flocculant efficiency, less dosage, sludge amount and moderate treating condition comparable to the chemical flocculants. A flocculating rate of approximately 90% was achieved for kaolin clay under optimal cultivation condition (Pu et al., 2018).

5.3. Flocculant Polysaccharides in Algae

Exopolysaccharides from marine algae have been explored due to their potential applications, including as bioflocculant agent, considered more efficient than commercial flocculants.

Alginates are polysaccharides firstly isolated from brown seaweeds (*Phaeophyta*), and recently identified as produced also by two genera of soil bacteria, *Pseudomonas* and *Azotobacter* (Hay et al., 2013). This polysaccharide is constituted to over than 200 different types varying in length and array of β -D-mannuronic acid (M) and α -L-guluronic acid (G) monomer units (Albuquerque et al., 2017).

Alginates are biocompatible, biodegradable, and non-immunogenic biopolymer polyelectrolytes (Yang, Xie and He, 2011). The physical properties in aqueous medium for alginates depend not only on the M/G ratio, but also on the distribution of M and G units along the chain. At this moment, it is important to highlight that the main property of alginates is their ability to retain water. Because of their linear structure, and high molecular weight, alginates form strong films and good fibres in the solid state. Their gelling and stabilizing properties are also very important characteristics for alginates; the stiffness of the alginate chains and the complex with counterions could be attributed to the composition (M/G ratio) and distribution of M and G units in the chains. The higher content of G units form stable crosslinked junctions with divalent counter ions (for example, Ca, Ba, and Sr, unless Mg), so the crosslinked network can be considered a gel. In addition, the low pH also forms acidic gels stabilized by hydrogen bonds (Albuquerque et al., 2017; Rinaudo, 2008; Yang, Xie and He, 2011).

As an anionic polymer, alginate forms electrostatic complex when mixed with a cationic polymer. The stability of the formed complex depends on the pH and salt concentration, but even in the best conditions, the complex is insoluble and thus allows the formation of fibres, films, and capsules. They have been applied widely in biomedical, pharmaceutical and biotechnology fields due to its desirable characteristics, versatility and

biocompatibility (Albuquerque et al., 2017; Hay et al., 2013; Salehizadeh, Yan and Farnood, 2017).

Several studies already reported the excellent flocculation and decolourization capabilities of alginates (Dao, Cameron and Saito, 2016; Rani, Mishra and Sen, 2013; Salisu et al., 2016; Yuan and Jia, 2013). Considering the use of alginates for copolymerization, many works described the preparation and characterization of these complexes, also proposing many applications to them, especially for the most investigated polyelectrolyte complex based on alginate and chitosan (Ortiz et al., 2018; Yang et al., 2016).

The sulfated exopolysaccharide p-KG03 produced by the red-tide marine microalga *Gyrodinium impudicum* KG03 showed bioflocculant activity more than 90% in kaolin suspension, and a flocculation rate higher than commercial flocculants as polyacrylamide and zooglan (Yim et al., 2007).

The microalga *Scenedesmus obliquus* AS-6-1 is able to carry out flocculation and self-flocculation due to its cell wall-associated polysaccharides, composed by glucose, galactose, rhamnose and fructose with molar ration of 8:5:3:2:1. Fast flocculating action was observed on suspended cells of *S. obliquus* and *Chlorella vulgaris* in the presence of 0.6 mg/L flocculating agent (Guo et al., 2013).

Agarose is a polysaccharide obtained of certain red seaweed of easy availability. Cationized agaroses were synthesized through the reaction of agarose with 3-chloro-2-hydroxypropyltrimethylammonium chloride in alkaline medium, and showed flocculation activity in assays with colloid kaolin suspensions comparable with commercial cationic polyacrylamides, representing an alternative for water treatment (Prado et al., 2011).

5.4. Flocculant Polysaccharides in Animals

5.4.1. Chitosan

According to Wu et al. (2012), chitin is the second most abundant polymer after cellulose. It is widely synthesized by many living organisms such as fungi, yeasts, algae and squid pen, being found in exoskeletons of crabs, lobsters, shrimp and crab shells, as well as insect cuticles. Depending on its source, chitin occurs mainly as two allomorphs, namely α (the most abundant) and β forms. The chemical structure (poly- β -(1 $\rightarrow$ 4)-N-acetyl-D-glucosamine) of chitin can be partially degraded by acid to obtain a series of oligomers namely oligochitins. Under alkaline conditions, a partial deacetylation of chitin results in one of the most chitin derivative in terms of applications: chitosan, biobased polymer with many bioactivities, including antitumoral, antimicrobial, fungicidal and immunotherapeutic properties, eliciting chitinase, and regulating organism growth (Albuquerque et al., 2017; Salehizadeh, Yan and Farnood, 2017).

Chitosan is a linear, cationic polysaccharide composed of randomly repeating units of β -(1 $\rightarrow$ 4)-linked 2-acetamido-2-deoxy-D-glucopyranose (A-unit; the neutral sugar unit GlcNAc) and 2-amino-2-deoxy-D-glucopyranose (D-unit, the positively charged sugar unit GlcN). The amount of D-units in chitosan is often more than 60%. It shows different degrees of deacetylation (DD) between 40–90% and is available commercially in various molecular weights ranging from 50,000 to 2,000,000 Da. The contents of the A-units in chitosan can vary from 0.7 (70% acetylated) to 0 (0% acetylated, all units charged). Therefore, chitosan can be considered as ampholyte (enriched in A-units) or polyelectrolyte (enriched in D-units). Chitosan is the only commercially available water-soluble cationic polymer, property that emerges as an advantage when compared to the marked insolubility of chitin in all usual solvents. The water solubility of chitosan is related to the positive charges on its primary amino groups, which allows the interaction of this cationic biopolymer with anionic molecules such as glycosaminoglycans (GAG) and proteoglycans (Albuquerque et al., 2017). Moreover, chitosan may be considered one of the most promising natural substitutes for commercial

synthetic polymer flocculants due to its unique flocculation properties, also associated to the presence of these primary amino groups, as well as the potential to employ chemical modifications in this carbohydrate polymer.

Chitosan was used as flocculant agent for harvesting of the microalga *Chlorella sorokiniana*, with flocculation efficiency reaching over 99% at dosage of 10 mg chitosan per gram algal biomass and reducing the volume by 20-50 folds (Xu et al., 2013). Recent studies reported by the scientific literature show the production, characterization, and application of microwaves (Cui et al., 2017), grafted-copolymers (Wang et al., 2016) and other hibrids (Dharani and Balasubramanian, 2015; Jia et al. 2016; Sun et al., 2017) of chitosan and, for example, lignin, ions, antibiotics, acrylamide. Chitosan was functionalized by graft with 3-chloro-2-hydroxypropyl trimethylammonium chloride, generating a new flocculant agent with potential for wastewater treatment (Agbovi and Wilson, 2018). Other chitosan-based flocculant denominated phenylalanine-modified chitosan showed higher removal potential of the antibiotics norfloxacin and tylosin in turbid water than commercial flocculants, being a potent contaminant removal (Du et al., 2018). Great flocculant activity was reported to a terpolymer based on chitosan and lignin (chitosan-acrylamide-lignin), which showed maximum percentage removal of 99.3% and 67.0% for the dyes reactive orange C-3R and methyl orange, respectively (Lou et al., 2018; Lu et al., 2017).

6. POLYSACCHARIDE FLOCCULANTS FOR WASTEWATER APPLICATIONS

Wastewater from different sources can be treated with flocculating polysaccharides, as shown in Figure 3. Contaminant particles dispersed in wastewater, such as dye molecules, can form flocs with these polysaccharides.

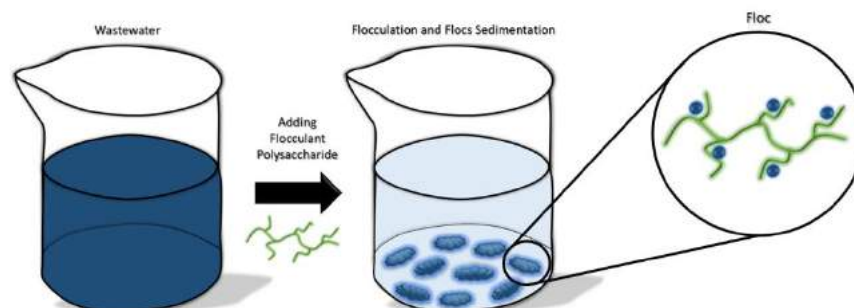


Figure 3. Flocs formation in wastewater by flocculant polysaccharide.

6.1. Dyes Removal

Different types of industries, such as textiles and plastic, use dyes to colouring their products, generating large volumes of colored wastewater. However, dyes are substances capable of absorbing ultraviolet light and consequently decrease this absorption by photosynthetic organisms and reduce dissolved oxygen content of aquatic environment; characterizing, thus, a serious environmental pollutant (Rangabhashiyam et al., 2013; Bello et al., 2017). Coagulation-flocculation has been considered a cost effective technology with excellent color removal ability (Verma et al., 2012).

A polysaccharide with cationic behaviour that is widely used for removal of negatively charged dyes in wastewater is the chitosan; anionic dyes can be adsorbed electrostatically with the protonated amine groups of chitosan (Lee et al., 2014; Vakili et al., 2014). For example, about 99% of the acid blue 92 dye was removed by chitosan, allowing recovery of the biopolymer from formed flocs with the dye using 0.1 M NaOH and with subsequent reuse of chitosan in acetic acid solution (Szygula et al., 2009). Additionally, modifications in chitosan structure as well as its association with other polymers have been increasingly studied to improve flocculant action in dye removal.

A chitosan-based cationic polymer was elaborated through the modification of this polysaccharide by (3-chloro-2-hydroxypropyl)

trimethylammonium chloride; such chitosan modification was useful for dye melanoidin removal from industrial wastewater (Momeni et al., 2018). In an attempt to elaborate amphoteric flocculants, capable of eliminating both anionic and cationic dyes, Yang et al. (2013) prepared grafting flocculants, formed by carboxymethyl chitosan-*graft*-polyacrylamide (CMC-*g*-PAM). These authors have found that the ability to remove methyl orange (anionic dye) and basic bright yellow (cationic dye) initially occurs by charge neutralization between produced flocculant and dyes, but flexibility of PAM graft chains allowed formation of large and insoluble flocs with net-like structure, allowing an easy sedimentation in water treatment (Yang et al., 2013). The synthetic monomer of acrylamide was used to graft chitosan and lignin (CAMCL) forming a ternary graft copolymer, since these two polymers together provide more functional groups to attract dyes, such as orange C-3R, methyl orange and acid black-172. In addition, flocculating mechanism of CAMCL occurred by the charge neutralization, bridging and sweeping effects (Cui et al., 2017; Lou et al., 2018).

Cellulose has a regular structure and matrix formed by hydroxyl groups presenting a high degree of polymerization and susceptible to undergo chemical modifications making it a relevant flocculant (Kono and Kusumoto, 2015; Ferreira et al., 2016). Quaternized celluloses and ampholytes of this polysaccharide in different degree of cationic substitution, for example, had flocculating capacity against anionic dyes, such as acid red 13 and acid blue 92 (Kono and Kusumoto, 2015; Kono, 2017). However, carboxymethyl cellulose undergoing the grafting process with hydrolyzed polyacrylamide, generating CMC-*g*-HPAM, was able to remove the methylene blue cationic dye. The highest removal efficiency occurred under alkaline conditions where the carboxyl groups of CMC-*g*-HPAM were deprotonized, forming -COO^- groups, allowing electrostatic interactions with methylene blue, favoring such flocculation (Cai et al., 2013).

Gum polysaccharides also went through the grafting process, such as the ghatti-crosslinked-polyacrylamide (Gg-cl-PAAM) hydrogel synthesized capable of adsorbing and removing cationic (rhodamine B and

brilliant green) and anionic (methyl orange and congo red) dyes (Mittal et al., 2018). Xylan, a xylose polymer, is another polysaccharide which, when phosphorylated, acted as a flocculant removing more than 95% of cationic ethyl violet dye (Liu et al., 2018). A macromolecule originated using corn ethanol wastewater, named compound biopolymer flocculant (CBF), has in its composition more than 80% polysaccharide; CBF presented methylene blue removal, whose flocculation process occurs primarily by adsorption bridging and charge neutralization by its polar functional groups (Xia et al., 2018). The exopolysaccharide (EPS) produced and purified from *Paenibacillus elgii* B69, was able to promote high rate of decolourization for methylene blue and red X-GRL positive dyes, while less than 50% for anionic and neutral dyes (Li et al., 2013).

6.2. Cleaning of Heavy Metal Ions

Heavy metal ions, such as lead, copper and zinc, can contaminate water due to natural processes, since such elements can be stored in the soil and transported by surface waters. In addition, anthropogenic sources contribute to this type of pollution, such as industrial activities including mining and coal combustion (Kobielska et al., 2018). These ions are extremely toxic and can cause serious human health problems, such as damage to the neurological system, blood disorders, toxicity in different organs and even death (Shtenberg et al., 2015). In front of this serious scenario of contamination with heavy metal ions, to remove these contaminants in wastewater is very important to leave it free or in non-toxic concentrations of these ions; such removal can be carried out through bioflocculants, which include polysaccharides (Salehizadeh and Yan, 2014).

Some polysaccharides, such as xanthan gum and starch, possess many –OH groups and act as metal ion coordination sites; while other polysaccharides, for example, chitosan with –NH₂ groups and sodium alginate apart from –OH groups –COO[–] can bind to the metal ions (Kolya and Tripathy, 2013). Chitosan, for example, promoted simultaneous

adsorption of heavy metal ions (iron, nickel and copper) and salt anion (sulfate). At pH range 5 to 6, chitosan has protonated and unprotonated amine groups, in which the latter group form chelate complexes with salt cations and salt anions are adsorbed through electrostatic interaction on chitosan (Mende et al., 2016). Polysaccharide inulin underwent a carboxymethylation process to synthesize carboxymethyl inulin (MIC), which removed metal ions in a kaolin suspension, reducing the total iron, chromium VI and manganese (II) content. The mechanism proposed for this chelation is the interaction with the cations by oxygen of the ether linkage and by oxygen of the carboxylate group through five membered ring formation (Rahul et al., 2014). A pectin, extracted from *O. ficus-indica* had affinity to the different metallic ions (Ca^{2+} , Cu^{2+} , Zn^{2+} , Cr^{3+} , Ni^{2+} , Pb^{2+} and Cd^{2+}); since pectin is a linear molecule composed of galacturonic acid, whose hydroxyl and carboxylic groups can be responsible for this affinity with the metal ions studied (Ibarra-Rodríguez et al., 2017).

Flocculating power of polysaccharides produced by different bacteria also has been determined. EPS produced by bacteria, such as cyanobacteria, has negative charges and can act as a chelating agent of heavy metal ions with positive charge (Bhunja et al., 2018). The EPS bioflocculant purified from *P. elgii* B69 also had high capacity to adsorb Al^{3+} , followed by Pb^{2+} , Cu^{2+} and Co^{2+} (Li et al.; 2013). A flocculant system was made using EPS from *Rhizobium sp.* and polyethyleneimine (PEI), which was able to remove Cu^{2+} . It was proposed that such process occurred firstly by binding Cu^{2+} to negatively-charged groups, mainly carboxylate, of the EPS followed by link of the EPS- Cu^{2+} complex with PEI resulting in firm and large flocs with easy removal (Escobar et al., 2015). While more than 90% of heavy metals (Zn^{2+} , Cd^{2+} , Pb^{2+} , Cu^{2+} and Ni^{2+}) were adsorbed to the EPS of glycoprotein nature produced by *Achromobacter xylosoxidans* when grown in culture medium supplemented with these ions (Subudhi et al., 2016). A polysaccharide molecule (MSI021) was purified from *Bacillus cereus*; and characterized as a bioflocculant heavy metal remover. A solution containing heavy metal salts (HgCl_2 , ZnCl_2 and CuSO_4) with a bioluminescent bacterium, *Vibrio harveyi*, was treated with

MSI021. The presence of MSI021 in the solution allowed the *V. harveyi* growth, which continued with its luminescent phenotypic expression, differently from the absence of MSI021, in which there was a decrease in the luminescence of this bacterium; such phenomenon was possible due to this biofloculant molecule that mitigated heavy metal toxicity (Sajayan et al., 2017).

6.3. Treatment of Pulp and Paper Industry Effluents

About 42% of global industrial wastewater is produced by pulp and paper industry. The processes in the paper manufacturing involve pulping, bleaching and papermaking; in fact, in all these steps effluents composed of different chemical species are generated (Toczyłowska-Mamińska, 2017). Wastewater from pulp and paper industry have a diversity of organic and inorganic contaminants, such as resin acids, fatty acids, adsorbable organic halide (AOX) and total suspended solids (TSS) besides being able to have high content of biological oxygen demand (BOD) and chemical oxygen demand (COD) (Ashrafi et al., 2015; Kumar et al., 2015). These undiluted untreated effluents are toxic to aquatic organisms and their treatment can be carried through the flocculation physicochemical method, which includes the flocculating polysaccharides (Kumar et al., 2015).

Thus, a copolymer flocculant prepared by grafting (2-methacryloyloxyethyl) trimethyl ammonium chloride onto chitosan (chitosan-*g*-PDMC) has been used to treat pulp mill wastewater. This novel cationic chitosan-based flocculant promoted efficient removal of COD, turbidity and lignin as well as water recovery. Grafting of chitosan showed in favour of the double electric layer compression, charge neutralization and improved the sweep-floc effects (Wang et al., 2009). In another work, raw and undiluted pulp and paper mill effluent was treated with *Cassia obtusifolia* seed gum, which removed COD and TSS an up to 36.2 and 86.9%, respectively; and the flocs formed after the treatment had a morphology highly fibrous-like and aggregate (Subramonian et al., 2014). Polysaccharides from potato starch have been modified by benzylation and

insertion of hydroxypropyl-trimethyl ammonium (HPMA) moieties of different substitution degrees, generating benzyl 2-hydroxypropyl-trimethylammonium starch chloride (BnHPMAS). Flocculation performance was tested for BnHPMAS in a model of paper industry wastewater; these amphiphilic starch derivatives removed contaminants from recycled paper processing, so named stickies, including total organic carbon (TOC) (Genest et al., 2015). A cationic bioflocculant has been produced through polymerizing (2-methacryloyloxyethyl) trimethyl ammonium chloride and xylan, generating xylan-DMC, removed COD, lignin, sugar, BOD and turbidity from pulp mill wastewater; the flocculation of particles occurred by bridging mechanism (Chen et al., 2018).

Natural dicarboxyl cellulose flocculant (DCC) has been synthesized and presented great flocculation activity to the effluent from paper mill; DCC combined with CaCl_2 reduced turbidity, COD and BOD through the process coagulation-flocculation of the effluent suspensions (Zhu et al., 2015). Turbidity reduction from paper mill effluent can allow the recovery of cellulose fibers. Mukherjee et al. (2014) tested flocculant action of guar gum, xanthan gum and locust bean gum polysaccharides and found that guar gum reduced the turbidity and recovered more fibers than the other carbohydrates of a synthetic effluent. In addition, guar gum-based nanocomposite, so called g-GG/SiO₂, has been synthesized by grafting polyacrylamide chains on a guar gum (g-GG) followed by *in situ* nano silica incorporation on g-GG surface. This nanocomposite was able to reduce pollutant contents of paper industry, such as TSS and turbidity, and the flocculation in acidic environment occurs due to electrostatic and patching mechanism; under alkaline conditions the flocculation process happens mainly by bridging (Pal et al., 2015).

6.4. Removal of Agricultural and Food Contaminants

More than 300 million ton of organic waste are produced each year by agricultural and food processing industries in the United States; sustainable

treatment of such waste has become a worldwide challenge (Sheets et al., 2015). These organic substances found in wastewater from such industries comprise TSS, COD, organic colloids, sludge, oil/grease, beyond dissolved inorganics (Teh et al., 2016). In addition, nitrogenous compounds, especially ammonium nitrogen, can be present in large quantities in food industry wastewater (Zhukova et al., 2011). Phosphate is a common constituent of agricultural fertilizers and such chemical specie can contaminate effluents; excess of phosphorus in the aquatic environment can contribute to the process of eutrophication (Adesoye et al., 2014). Therefore, agricultural and food contaminants in wastewater need to be removed, and such process can be performed by flocculant polysaccharides.

Wastewater is generated in potato starch process that is considered one of the most polluted in food industry. Potato starch wastewater (PSW) has been treated with a bioflocculant complex denominated MBF917, produced by *Rhizopus sp.* M9 and M17, such macromolecule is composed of more polysaccharide than protein. MBF917 removed the turbidity and COD from the PSW (Pu et al., 2014). A bioflocculant has been extracted from *Paenibacillus polymyxa*, and such compound showed to be thermostable with indicative of its main backbone being formed by polysaccharide; this bioflocculant was still used to treat PSW by reducing COD and turbidity efficiently (Guo et al., 2015a; Guo et al., 2015b). Another bioflocculant of polysaccharide nature has been produced and isolated in the fermentation liquor of *Rhodococcus erythropolis*. This bioflocculant was able to reduce COD, ammonium and total phosphorus concentrations of the PSW medium (Guo et al., 2018).

Rice starch has been used to perform the treatment of agroindustrial wastewater from palm oil mill; this carbohydrate (at dosage 0.55 g/L) promoted removal of almost 90% TSS from palm oil mill effluent. Flocs produced with this treatment had a compact structure with smoother surface, designating formation of denser and larger flocs by bridging flocculation mechanism (Teh et al., 2014). Removal of orthophosphate (P_i), a very common contaminant in water bodies by agriculture, was obtained using the carboxymethyl chitosan grafted with 3-chloro-2-

hydroxypropyl trimethylammonium chloride, CMC-CTA. The CMC-CTA flocculant efficiently removed P_i and the turbidity under acidic conditions of a simulated wastewater (Agbovi and Wilson, 2018). Therefore, it can be inferred that different polysaccharides are been used for a great versatility in wastewater applications. The composition of EPS molecules produced by microorganisms has been revealed which usually are of glycoprotein nature, and had their flocculating performance well established. While polysaccharides with structure already characterized have undergone chemical changes to improve their flocculant capacity.

CONCLUSION

The purpose of this review was to approach scientific literature dealing with polysaccharide bio-based flocculants with special emphasis for those derived from plants. Moreover, this review emphasized aspects such as extraction, purification, modification, characterization, and the broad range of applications of natural flocculants in industry.

The most current scientific publications demonstrate the industrial concern for the use of new substrates, besides the reduction in the costs of bioflocculants' production and the constant awareness about the substitution of inorganic flocculants for the innovative ones. It was remarkable to note the preference of bioflocculants in comparison with inorganic flocculants due to their low cost, availability, biodegradability and biosafety; in addition, they can be obtained from renewable resources and their application is directly related to the improvement of quality of life.

Several studies investigated the flocculating activity of polysaccharide bio-based flocculants in wastewater treatment; they are technically promising as flocculants with high removal efficiency of suspended solids, total dissolved solids, turbidity, colour and dye. However, its development is limited with variation of flocculating efficiency, short shelf life, and high production cost.

The scientific literature dealing with flocculants polysaccharides derived from plants is still scarce when compared to bioflocculants produced from microorganisms, or even those reporting commercial polysaccharide bioflocculants. Regarding issues such as biosafety and the sake of ecology, more qualitative and quantitative research are necessary for further exploitation and applications of polysaccharide bio-based flocculants in different industries.

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1. <https://www.sciencedirect.com/science/article/pii/S0141813016324394>
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11. <http://www.aimspress.com/article/10.3934/molsci.2016.3.386>

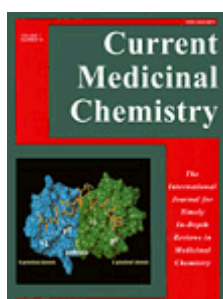
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APÊNDICE J – ARTIGO PUBLICADO NA REVISTA CURRENT MEDICINAL CHEMISTRY

Biomarkers, biosensors and biomedicine

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Biomarkers and Progress in Biomedicine

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Abstract: Discovery of new biomarkers associated with cancer, neurological, cardiovascular and other diseases is necessary. Cancer cells generally express an altered glycosylation profile on their cell surfaces; depending upon the site where carcinogenesis begins, neoplastic cells can synthesize and release specific molecules. Such compounds can be identified thus promoting early and accurate diagnosis of a tumor process. Commonly, some neurological disorders, which can occur physiologically by the process of senescence, are confused with Alzheimer's disease (AD). Therefore, a better diagnosis is vital for determining the prognosis of patients. AD biomarkers have been increasingly studied since this is a very prevalent disease. In addition, cardiovascular diseases are the main cause of death in the world; studies capable of identifying, through molecular probes, the development of an atherosclerotic process in the beginning can lead to an early treatment to avoid an acute myocardial infarction. The aim of this review is to approach biomarkers to be used in diagnoses instead of invasive exams; biosensors for the specific and sensitive detection of these biological markers will also be investigated.

Keywords: Biomarkers; diagnosis; cancer; neurological disease; cardiovascular disease; biosensor.

1 INTRODUCTION

A key indicator of a normal biological condition or pathological process is considered as a biomarker [1]. Therefore, molecular biomarkers, such as proteins, genes and metabolites have been able to differentiate normal, predisease and disease states [2]. Blood is a sample, often obtained without difficulty, whose serum or plasma can be analyzed for identification of biomarkers through different techniques, especially the omics, such as proteomics, genomics and metabolomics; thus, allowing a sensitive and accurate identification of small concentrations of these analytes [3,4].

Cancer is one of the main causes of death in the world and there is still a challenge for its early diagnosis due to tumor heterogeneity, despite the wide knowledge that led to discovery of molecules associated to specific cancers allowing a target therapy [5]. In cancer biology, diagnostic biomarker is fundamental for detection in early stages of tumor development; prognostic biomarker can predict survival of the sick due to ability to indicate tumor aggressiveness; and predictive biomarker indicates the patient's response to therapy [6]. Cancer cells differentiate, for example, in their proliferative capacity, metastatic potential and gene expression and, therefore, biomarkers may be able to indicate specifically the origin of tumor cell [7]. For example, microRNA (miRNA) are small non-coding RNA molecules capable of promoting the epigenetic regulation of various genes, including those involved in tumorigenesis. Detection of miRNA in blood and other body fluids, such as urine and saliva, as well as their concentration and composition may indicate the cell of origin [5,8].

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In addition, neurological disorders present a peculiar feature that is the neurodegeneration, which can affect motor ability as well as cognition and memory [9]. Treatment of these diseases, such as Alzheimer and Parkinson's diseases, can be inefficient since there are delay in diagnosis, thus the search for biomarkers capable to diagnose quickly and promote patient monitoring is critical [9-11]. Moreover, cardiovascular diseases leading cause of death worldwide and their risk factors can be identified with the clinical profile of patient, such as hypertension, diabetes mellitus and hypercholesterolemia; however, cardiovascular risk evaluation based on biomarker analysis is essential to stratify the risk and promote better treatment for the patient [12,13].

These molecular signatures may be able to identify early a pathological process, however few biomarkers have been used in clinical practice, since there is deficit of methodologies to discover such molecules and lack molecular devices to use in routine assays [14]. In order to detect more sensitively these specific biomarkers, with the possibility of real-time measurements, new technologies to manufacture biosensors have been investigated to develop simple and portable platforms capable of evaluating complex samples [15,16].

In front of this scenario, this review approaches recent biomarkers discovered related to cancer, neurological and cardiovascular diseases with emphasis on findings in non-invasive pathways. In addition, new technologies to make

biosensors are also discussed to stimulate the development of devices capable of identifying such analytes more efficiently.

2 MOLECULES THAT PREDICT CANCER

A good candidate to be a biomarker useful in cancer diagnosis should have a higher level in patients with this disease and be able to predict tumor size and phenotype. In addition, it should decrease with tumor extermination and increase again in the circulation in case of tumor relapse, as well as having easy monitoring [17]. In order for the biomarkers to be applied clinically, it is first necessary to discover them in basic research, to undergo analytical validation and then promote clinical implementation [18]. In this review, the approach in relation to the neoplasm is focused on molecules that have been discovered and specifically associated with breast cancer (BC) and prostate cancer (PC), which most affect women and men, respectively; and the lung cancer (LC) that is the type that kills most in the world [19].

2.1 Breast Cancer

Some tumors in the breast cannot be identified through clinical and radiological examinations. Then, the search for new diagnostic methods non-invasive and cost effective becomes critical [20]. Besides to tumor cells that can be found in the circulation, cell-free tumor DNA (ctDNA) molecules, containing genetic and epigenetic modifications, can be released by these cells into the blood and measurement of ctDNA can help to diagnose precociously and at the genomic level to induce a targeted therapy [7,20]. Figure 1 illustrates circulating ctDNA in peripheral blood, which can be detected and measured in breast, prostate and lung cancers. For example, methylated ctDNA (met-ctDNA) from frequently methylated genes, such as RASSF1A in BC, was considered a more sensitive marker than carcinoembryonic antigen (CEA) and cancer-associated antigen (CA) 15-3, considered classic markers of BC; moreover, met-ctDNA proved to be a good candidate for monitoring the tumor response to neoadjuvant chemotherapy [21].

In addition, salivary biomarkers have been explored to correlate and aid in the diagnosis of BC [22]. Human epidermal growth factor receptor 2 (HER2) as well as CA 15-3, which is used to assess the overexpression of MUC1 gene, are overexpressed in BC. Autoantibodies IgG and IgM anti-HER2 and anti-MUC1 were significantly higher in the saliva of patients with this type of cancer than in healthy women [23]. Another study using chromatographic methods to analyze saliva samples of BC patients showed that such patients had 18 endogenous metabolites, such as specific types of lysophosphatidylcholine and monoacylglycerol, whose salivary levels were significantly different from those of healthy patients [24].

Human endogenous retroviruses (HERV) were able to integrate into the human genome; these HERV are nonfunctional, however many studies have correlated the development of certain types of tumors with HERV-encoded protein which may be potentially oncogenic [25]. For example, expression of HERV type K (HERV K) envelope gene (*env*) protein has increased expression in BC patients with invasive ductal carcinoma and this protein participates in the process of tumorigenesis and metastasis of BC cells [26,27].

2.2 Prostate Cancer

Prostate-specific antigen (PSA), whose production is regulated by androgens, is generally elevated in PC, although there are patients with low PSA and high-grade disease [28]. In addition, elevated PSA can still be found in other urological disorders, such as urinary tract infection, benign prostatic hyperplasia (BPH) and prostatitis [29]. An alternative that may be useful to differentiate PC and BPH is measuring the urinary levels of furan and p-xylene, volatile organic compounds that show up with significant differences between the two pathologies before and after prostate massage [30]. In addition, lipid composition of urinary exosomes, vesicles released by tumor cells containing molecules, has been analyzed and the lipids phosphatidylserine (18:1/18:1) and lactosylceramide (d18:1/16:0) have highest significance between healthy controls and PC patients [31].

Four miRNA molecules (miR-4289, miR-326, miR-152-3p and miR-98-5p) have been reported as candidates to promote early diagnosis of PC, since levels of these miRNAs were altered in relation to healthy patients and such molecules act on target genes that regulate PC pathogenesis [32]. Moreover, another study showed that the score of three miRNA (miRNA-223, miRNA-24 and miRNA-375) in serum samples could predict the reclassification of PC patients under active surveillance along with PSA levels [33]. It has been verified that in PC the plasma levels of cell-free DNA (cfDNA), using ALU sequence with 115 and 247 base pairs, ALU 115 and ALU 247, respectively, are higher than in BPH [34].

2.3 Lung Cancer

Based on histology, LC can be divided into two subtypes: small cell lung cancer (SCLC) and non-small cell lung carcinoma (NSCLC) that is subdivided into adenocarcinoma, squamous type and large cell type [35]. The paradigm of LC diagnosis has been reconsidered due to the use of biomarkers capable of detecting such tumors that may respond to personalized treatments [36].

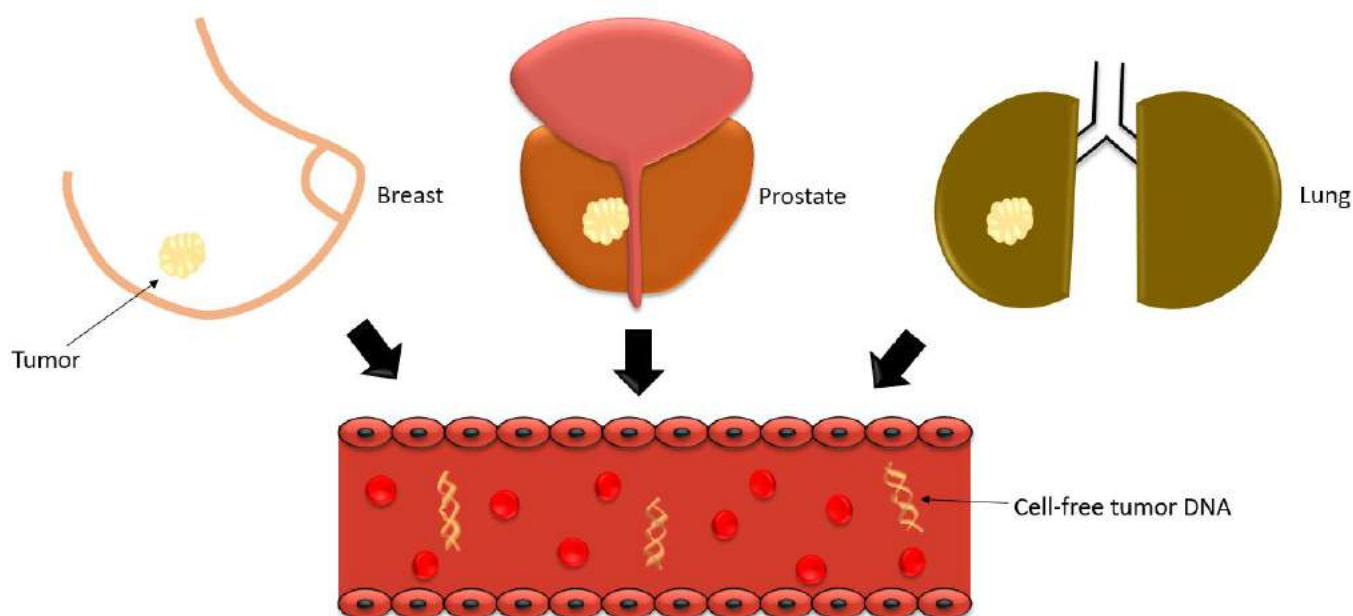


Figure 1. Representation of cell-free tumor DNA (ctDNA) released by tumor cells of breast, prostate and lung neoplasms in bloodstream that can be valuable biomarkers for diagnosis and/or monitoring of these cancers using blood samples.

In spontaneous sputum samples, it was identified metabolites, involved in metabolism of amino acids and lipids, only in individuals with LC; healthy controls did not have [37]. Other metabolites that have been characterized as specific and sensitive biomarkers to determine diagnosis and prognosis of LC were glycerophospho-N-arachidonoyl ethanolamine and sphingosine, since sphingolipid metabolism pathway in this type of cancer is altered [38].

Expression of three HERV *env* genes (HERV P, HERV H, and HERV K) was identified in blood of LC patients having significant difference in levels of their transcripts that are higher in adenocarcinoma than in SCLC and squamous cell carcinoma [39]. It has been reported that a long noncoding RNA (lncRNA), a non-protein encoding RNA class containing more than 200 nucleotides, named lncRNA XLOC_009167, may be useful for the diagnosis of LC, since this lncRNA was most expressed in the whole blood of LC patients than in healthy or in patients with pneumonia [40]. 5-Methylcytosine is a regulator of gene expression and the generated product of its oxidation, 5-hydroxymethylcytosine (5hmC), signatures in cfDNA and may serve as a biomarker for SCLC in blood samples [41]. In another study that also evaluated cfDNA, high levels of ALU 115 and ALU 247 were observed in NSCLC patients whose increase in these levels was proportional to more advanced stages of the disease and in cases of metastasis [42].

3 BIOLOGICAL MARKERS OF NEUROLOGICAL DISORDERS

3.1 Alzheimer's Disease

The most common cause of dementia is Alzheimer's disease (AD) which is characterized by damage or destruction of neurons that lead the person becoming unable to perform everyday functions, such as walking [43]. Main neuropathological findings of this disease include: amyloid plaques, formed by the aggregation of products from the proteolytic cleavage of β -amyloid protein ($A\beta$), $A\beta_{1-40}$ and $A\beta_{1-42}$, causing neuronal death and loss of synapses; intracellular neurofibrillary tangles (NFTs), formed by the hyper-phosphorylated tau proteins that are deposited within neurons in certain brain sites [44]. Biomarkers for AD should be sensitive and specific enough to promote differentiation from other types of dementia and the normal senescence process [45]. These biomarkers include neuroimaging techniques and specific proteins in cerebrospinal fluid (CSF), however these compounds capable of diagnosing AD can be studied from other less invasive sources, such as plasma and urine-based biomarkers [45,46].

$A\beta_{1-40}$ and $A\beta_{1-42}$ were detected in peripheral blood of patients with AD at lower concentrations than in amnesic mild cognitive impairment (MCI) and nonamnesic MCI. In addition, plasma $A\beta_{1-42}$ correlated with certain patient factors, such as age and Mini-Mental State Examination as well as with CSF biomarkers [47]. The salivary tau protein has been associated to the diagnosis of AD, since the values of the phosphorylated tau (p-tau)/total tau (t-tau) ratio were significantly higher in AD patients than normal elderly control [48].

Oxidative stress has important participation in the pathogenesis of AD since the brain is rich in fatty acids that are sensitive to peroxidation; there is no powerful antioxidant system and as this organ uses a lot of oxygen there is an accumulation of its reactive species [49]. Thus, an early diagnosis for AD has been reported using as plasma analytes six compounds derived from lipid peroxidation, isoprostanoids, since such molecules had higher levels in AD patients than healthy group [50]. Other compounds originated from lipid peroxidation were also detected in the urine of AD patients and could serve as potential biomarkers of this neurological disease [51].

3.2 Parkinson's Disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder, being behind only of AD, and individuals affected with this disease have typical motor dysfunctions, such as postural instability, bradykinesia and rest tremor [52]. Pathogenesis of PD is characterized by loss of dopaminergic neurons in the substantia nigra and deposition of Lewy bodies, which are composed mainly of aggregates of the presynaptic intracellular protein named α -synuclein (α -syn) [53,54]. Scientific community has been hardworking to identify biomarkers related to cognitive dysfunction in PD, among which it aims to identify the degree of neurodegeneration, neurotransmitter deficiency, proteinopathy and abnormalities of brain function and connectivity [55]. However, we will highlight some biomarkers that can be found in non-invasive sources.

Most sanguine α -syn is found in red blood cells (RBC), therefore, levels of α -syn oligomers were determined in erythrocytes of PD patients; values of α -syn oligomer/total RBC protein ratio were higher in these patients than in controls [56]. Levels of dimer and dimer/monomer α -syn ratio in RBC were higher in PD patients with mutation in the α -syn gene and PD patients with mutation in the glucocerebrosidase gene (*GBA* gene, which leads to α -syn accumulation) in relation to individuals with this disease without mutation [57]. An abnormal metabolic profile was found in plasma of PD patients; main classes of these metabolites were glycerol phospholipids, fatty acids, bile acids, phenylalanine and tyrosine amino acids, which may act as potential biomarkers for PD [58].

Low systemic antioxidant capacity has been related to early PD, since a decreasing order was verified in values of blood reduced glutathione (Br-GSH) in, respectively, healthy controls, REM sleep behavior disorders (PD prodromal patients) and people with PD diagnosed; therefore, values of Br-GSH, together with olfactory loss and cardiovascular dysautonomia, may help to diagnose precociously PD [59]. Leucine rich repeat kinase 2 (LRRK2) and DJ-1 proteins, whose mutated genes can participate in the pathogenesis of PD, were detected in urinary exosomes with no significant difference among PD patient groups and non-PD controls;

however, the value of DJ-1 was significantly different between PD and non-PD males [60]. Mutation in the LRRK2 gene is related to idiopathic PD leading to an increase in autophosphorylation of this protein, mainly in the Ser-1292 residue; thus, elevated Ser(P)-1292 LRRK2 levels in urinary exosomes were correlated with idiopathic PD and to complications in cognitive impairment of PD patients [61].

4 NEW COMPOUNDS TO DIAGNOSE CARDIOVASCULAR DISEASES

Cardiovascular diseases (CVDs) are a group of disorders affecting the heart and blood vessels, of clinical interest, including myocardial infarction, arrhythmia, atherosclerosis and other conditions, representing the most prevalent causes of death globally, from infancy to old age [62]. Obesity, unhealthy diet, tobacco use, diabetes and hyperlipidaemia are some risk factors for CVDs. The earlier identification of subjects at increased risk for cardiovascular events is of great importance in the medicine to improve prevention, detection and management of these diseases. For example, myocardial infarction is an acute CVD that requires immediate detection and treatment, due to the necrosis of myocardium and ischemia, causing irreversible damages. In this context, the measurement of cardiac specific biomarkers in the blood can reveals an elevated concentration, confirming the heart disease and indicates the times of incidence of the attack and the location of damaged cells.

Numerous biomolecules have been related as efficient specific biomarkers for different CVDs diagnosis, which alone or in combination, provide information about the prediction of risks, gravity of condition, location of damage, and initial time of progression from the clinical condition. More often, cardiovascular biomarkers are found in the patient's blood, serum and plasma, and their levels can be measured in order to monitor the cardiovascular state. Troponin I (cTnI) is a cardiac protein considered the gold standard biomarker for diagnosis of myocardial infarction, due to its elevation related to damage of myocardium [63]. Myoglobin also has diagnosis value as biomarker of myocardial infarction since the rise in blood occurs in a short time after the disease onset (1-3 h); however, this protein can be elevated in skeletal and cardiac muscular damage [64]. As traditional cardiac biomarkers of CVDs, there are also the cardiac troponin T (cTnT), the creatine-kinase-MB (CK-MB) mass and the heart fatty acid binding protein (h-FABP), all considered biomarkers mainly for myocardial infarction; also, the C reactive protein, is an indicator of CVD related with inflammation. Evaluation of CVDs is complemented by physiological biomarkers, such as glucose, serum ferritin, lipid profile including total cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol and triglycerides, as well as apolipoproteins. In addition, some hormones as anti-diuretic hormone (ADH) and the brain natriuretic peptide (BNP) are

secreted in response to cardiac stress and can be useful as biomarkers for diagnostic and prognostic of CVDs.

Currently, identification and quantification of sugar, proteins, lipids and small metabolites including micro-RNAs represents a novel group of plasma-derived biomarkers of much interest in cardiovascular field, to improve CVDs detection and prediction [65-67].

Protein glycosylation is the most common post-translational modification of proteins and consists in the addition of a glycan portion (carbohydrate) to the protein by a specific enzyme. The participation of glycosylated proteins in biological process as cell adhesion, signal transduction, cell homeostasis and inflammation have been reported, being considered early markers of diseases. A research application named GlycA has been related as potential biomarker of incident cardiovascular events related to inflammation [65]. GlycA is measured by nuclear magnetic resonance (NMR) spectroscopy based on signals from N-acetyl methyl groups of the N-acetylglucosamine residues located on specific glycan moieties of inflammatory glycoproteins as α 1-acid glycoprotein, haptoglobin, α 1-antitrypsin, α 1-antichymotrypsin and transferrin. A significant positive relation between baseline levels of N-acetyl methyl groups from specific glycans and CVD events, being an approach for CVD prevention and diagnostic.

Levels of glycated hemoglobin (HbA_{1c}) have been also associated with CVDs incidence [68]. HbA_{1c} is the form of hemoglobin linked to glucose, naturally found within erythrocytes, and its level can be measured in a blood sample to determinate long-term high blood glucose levels over the previous 90-120 days, being recommended for screening and monitoring people for diabetes mellitus.

Fatty acid-binding protein 4 (FABP4) belongs to a family of 14 – 15 kDa proteins and is mainly found within adipocytes and macrophages. In addition, called adipocytes FABP (A- FABP) or aP2, are released from adipocytes and acts in the regulation of the transport of lipids, glucose and lipid metabolism related to inflammatory and metabolic responses, lipolysis during fasting and hepatic glucose production, also playing an important role in insulin resistance and cardiovascular events. Elevation of FABP4 levels in the blood is frequently associated with insulin resistance, diabetes mellitus, obesity, atherosclerosis and

other cardiovascular dysfunctions, being useful as biomarker of CVDs [69].

Growth differentiation factor 15 (GDF – 15) is an injury and inflammatory responsive cytokine, also considered a biomarker for CVDs [70,71]. It is highly expressed in cells as cardiomyocytes and vascular smooth muscle cells, being its levels associated with cardiovascular risk. Higher concentrations of GDF-15 can be measured in serum/ plasma samples and indicates increased risk to develop CVDs.

More than fifty circulating microRNAs (miRNAs) have been identified as significant biomarkers for diagnosis and progression stages of CVDs in plasma and serum, especially the miR-1, -133a/b, -145, -208a/b and -499 [72,73]. The miRNAs are small non coding RNAs that modulate gene expression, regulating vital functions in heart and blood vessels, as angiogenesis, cell differentiation and apoptosis, showing changes in their expression during CVDs [74]. A study evaluated the miRNAs miR-1, miR-133a, miR-499 and miR-208a in healthy and acute myocardial infarction (AMI) patients in order to identify miRNA biomarkers specific for AMI (Wang et al., 2010). miRNA microarray and real-time polymerase chain reaction (PCR) revealed a very low concentration of miR-1, miR-133a and miR-499 and absence of miR-208a in healthy human plasma, while the four miRNAs showed levels significantly higher in plasma from AMI patients when compared with healthy people, patients with non-AMI disease and patients with other CVDs. With attention for miR-208a, that showed undetectable in non-AMI patients and detectable in 90.9% AMI patients and in 100%, within 4h from symptoms start [75]. Thus, miR-208 levels were considered a specific and sensitive biomarker for early detection of AMI. More recently, miR-499 was also suggested as a sensitive biomarker of AMI [76]. This study was conducted using plasma samples of 227 patients with chest pain and 100 healthy people from departments of emergency and cardiology of Wuxi Second People's Hospital. The miR-499 levels measured by reverse transcriptase PCR were significantly higher in 142 AMI patients against 85 non-AMI patients and 100 healthy individuals. The plasma levels of miRNA-499 were detectable after 1h onset the symptoms and increased gradually within 9h, also showing a positive correlation with serum creatine-kinase MB (CK-MB) and cTnI, sensitivity of 80% and specificity of 80.28%.

Epigenetic factors such as DNA methylation have been considered as biomarkers of CVDs, including mitochondrial DNA (mtDNA) methylation levels. The mitochondria in platelets are important source of energy for their functions, and methylation in mtDNA may results in platelet dysfunction, carrying out cardiovascular pathogenesis, as atherosclerosis and evolution of thrombotic events. The methylation of mtDNA can results in an epigenetic regulation of platelet activity, compromising its function in the cardiovascular context and contributing to CVD development. This conclusion start through the observation of mtDNA methylation levels, which was significantly higher in four genes in CVD patients when compared with healthy individuals [77].

5 BIOSENSORY PLATFORMS FOR DETECTION OF BIOMARKERS

Currently, an increasing number of research groups have developed techniques based on biosensor technology for noninvasive and early detection of biomarkers in biological fluids and cell surface associated to disease diagnostics, including CVDs, cancer, diabetes and neurological disorders [78]. Biosensors are analytical devices composed by a recognition bioreceptor integrated with a physiochemical transducer, developed for detection of a specific biomarker at the low level of concentration in early stages of the disease (Figure 2). Biomolecules such as antibodies, lectins, enzymes and nucleic acids, or artificial molecular imprinting polymers and aptamers have been used for biomarker recognition. Transducers are devices that converts the biological recognition event into electrical signals, such as electrochemical, optical, piezoelectric and magnetic [79,80].

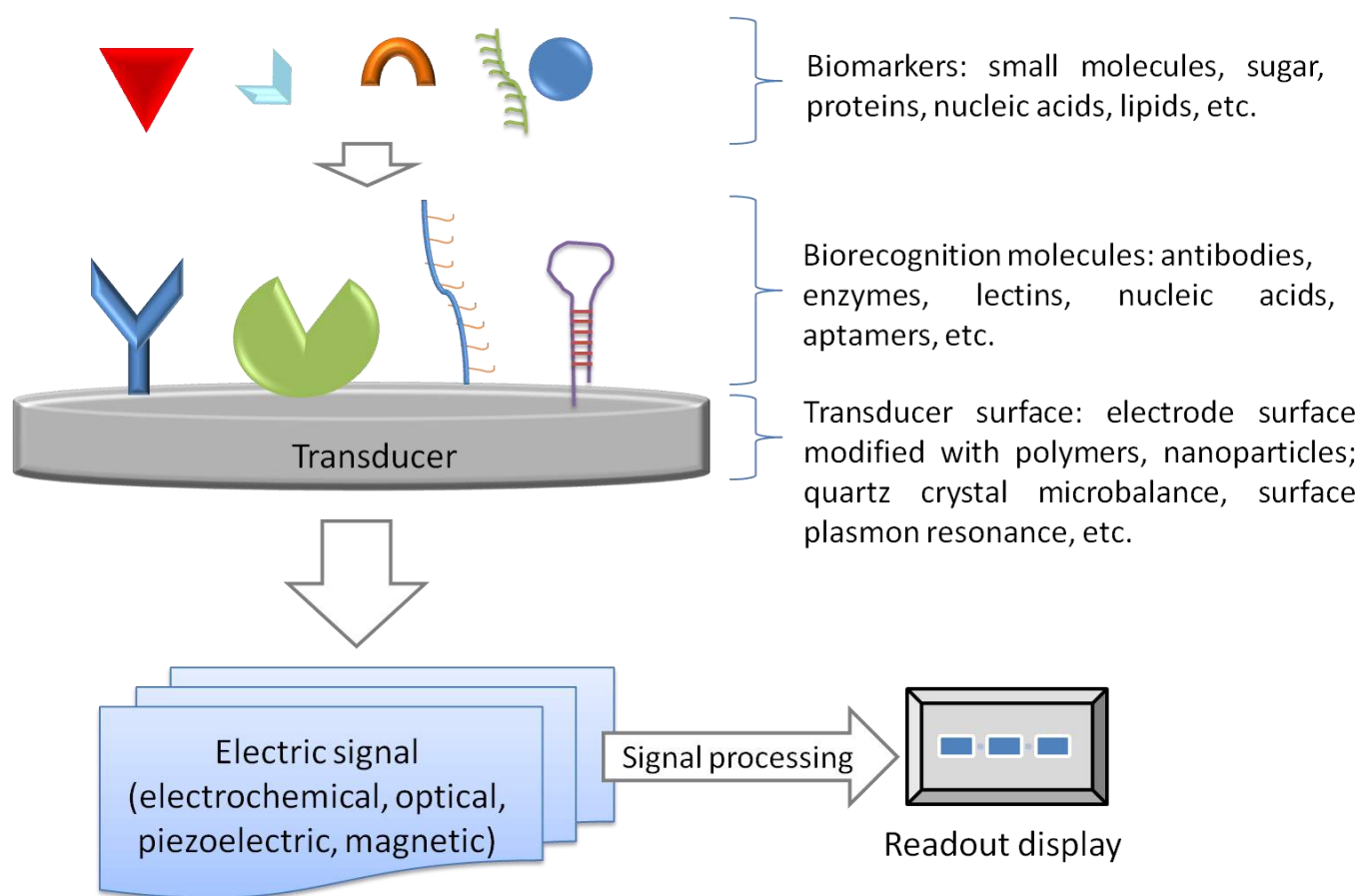


Figure 2. Schematic representation of a biosensor system.

Biosensors are advantageous tools when compared to conventional methods, since provide rapid measurement, real time monitoring of analytes, high specificity and sensitivity, ability to measure a range of molecules, ease of use, high level of precision, miniaturization, simplicity that facilitates

their use in remote areas worldwide, and require low volume reagents and samples [81].

Numerous biosensing platforms have been successfully constructed and demonstrated immense potential in sensing wide range of biomolecules in medical diagnostics. The

scientific advances in medicinal and technological fields along the years have improved sensor production quality, stability, quickness, sensitivity, specificity and reproducibility of biosensors [78]. Nanotechnology represents an advance improving biosensor development; more sensitive and rapid detections are obtained with nanomaterials such as carbon nanotubes, magnetic nanoparticles, gold nanoparticles and quantum dots for probing or signal amplification [82]. Affinity ligands can be attached to nanoparticles generating bioconjugates; these are also important tools for specific profiling of biomarkers and detection of cancer cells, as well as therapy on the site of disease, since selectively bind to receptors on abnormal cells [83]. In addition, bioinformatics and omics technologies provide rich database useful for discovery of new biomarkers [84]. These resources stimulate an increasing demand for construction of diverse sensing approaches, based on optical, electrochemical, piezoelectric and colorimetric detection of proteins, nucleic acids, sugars, lipids and secondary metabolites as disease biomarkers. Here, we focus in biosensors for diagnosis of cancer, neurologic disorders and cardiovascular diseases.

A sensitive optical biosensor based on Au/ZnO thin film surface plasmon resonance (SPR) system was developed for measurement of the breast cancer marker carbohydrate antigen CA 15-3 in human saliva [85]. The biosensor showed a linear detection range of CA 15-3 in human saliva of 2.5-20 U/mL, allowing lower levels than the cut-off point in cancer patients (4 U/mL), being useful to measure levels of CA 15-3 in saliva and as diagnostic tool for breast cancer. An ultrasensitive electrochemical biosensor with a simple design was developed for detection of attomolar levels of miRNA-155, a breast cancer biomarker [86]. Anti-miRNA-155 was immobilized onto Au-screen printed electrode and used for biomarker recognition. The final biosensor shows ability to hybridize miRNA-155 and a low detection limit of 5.7 aM of mi-RNA-155 in real human serum samples, as well as a good selectivity for breast cancer antigen CA15-3 and bovine serum albumin. Thus, this simple, sensitive and selective device can quantify mi-RNA and provide diagnosis of breast cancer and other diseases.

Prostate cancer diagnosis is a challenge because prostate benign diseases also increase serum levels of the prostate serum antigen (PSA), the biomarker of prostate cancer. In this context, novel biomarkers have been revealed through the changes of glycosylation patterns of proteins associated to cancer [87,88]. Changes of glycosylation can be detected by the use of lectins, those are carbohydrate recognizer proteins. A label-free electrochemical biosensor of glassy carbon electrode based on *Cratylia mollis* lectin immobilized onto assembled carboxylated carbon nanotubes and poly-L-lysine film was evaluated for differential diagnostic of prostate cancer and benign prostatic hyperplasia interacting with glycoproteins expressed on serum samples of patients

[89]. The lectin biosensor showed distinct response signals related to degree of staging prostate cancer and benign hyperplasia, being able to distinguish between the two conditions and a new alternative for prostate cancer diagnosis.

An ultrasensitive and specific biosensor based on SPR was created to measure levels of circulating miRNA-21 and -10b associated to pancreatic cancer [90]. It was developed a design regenerative of SPR based on gold nanoprisms, which improved the miRNAs hybridization signals. Detection in the subfemtomolar levels was obtained in measurements of patient plasma, founding nearly 4-fold higher levels of miRNA-21 and -10b in plasma of pancreatic cancer patients when compared with other techniques of RNA quantification, such as RT-PCR. This is one more selective and sensitive strategy based on biosensors for assaying miRNAs in plasma useful for diagnosis of many diseases.

A microfluidic immunosensor was designed for the sensitive detection of an epithelial cancer biomarker, EpCAM (epithelial cell adhesion molecule) using silver nanoparticles covered with chitosan. Anti-EpCAM monoclonal antibodies were used to recognize and capture the biomarker in the peripheral blood sample. The immunosensor achieved detection limit of 2.7 pg mL⁻¹, and a short-time response of 34 min, against a detection limit of 13.9 pg mL⁻¹ and a time response of 270 min of the commercial ELISA, without to prejudice the specificity and reproducibility [91].

In the field of neurological disorders, a growing interest by diagnosis biosensors based has been evidenced.

An immunomagnetic biosensor for the Alzheimer's disease biomarker amyloid-beta peptide 1-42 is a promise for non-invasive and early diagnosis of the disease [92]. Here, a gold electrode was modified using magnetic nitrogen-doped graphene for immobilization of antibodies of A β 1-28 for specific biorecognition of amyloid-beta peptide 1-42. The biosensor showed a low limit of detection into picomolar range, covering the cut-off level of the analyte. Furthermore, the biosensor was reproducible, stable, with a fast response time and low cost. Another biosensor proposed for Alzheimer's disease diagnosis was based on electrochemical detection of amyloid-beta oligomers in blood or cerebrospinal fluid [93]. The biosensor was constructed using graphene oxide/gold nanoparticles hydrogel electrode, and thiolated cellular prion protein (PrPc) peptide was immobilized onto this surface as probe for specific recognition of amyloid-beta oligomers. The analysis were executed by electrochemical impedance spectroscopy; an increase in the electron-transfer resistance was registered after the binding amyloid-beta oligomers and PrPc, also showing high specificity, sensitivity and low detection in picomolar range.

CVD's biomarkers have also been explored as sensitive targets for biosensor approaches, mainly traditional biomarkers as myoglobin and cardiac troponin. Electrochemical and label-free detection of myoglobin (Mb) was reported by Kumar et al. (2016), using a nanostructured aptamer-functionalized black phosphorus nanosheets on electrodes [94]. The nanosheets were functionalized with poly-L-lysine to immobilize the anti-Mb DNA aptamers on electrodes. Low detection limit in picomolar range and good sensitivity was achieved for Mb in serum samples, showing a potential alternative for diagnosis of CVDs.

Other aptamer-based biosensor was recently developed for electrochemical detection of cardiac troponin I [95]. Screen-printed carbon electrodes were modified with gold nanoparticles and conductive polymers to immobilize the aptamers. The biosensor showed great analytical performance in a buffer and serum-added solution, with a dynamic range of 1 – 100 pM and a detection limit of 1.0 pM, lower than cut-off levels of 40 – 700 pg/mL. This is a highly sensitive, simple, portable and innovative approach for cardiovascular diagnosis.

Some biosensors have been developed based on a multiplex detection, the known multiplexed biosensors. These platforms integrate the measurement of different biomolecules in a same device, providing to identify panels of biomarker for diseases [96, 97]. Clinical diagnostic based on individual biomarker is not reliable, because has a possibility of false positive or false negative. Thus, determinations of multiple biomarkers have showed to improve the accuracy for conclusive diagnosis, monitoring and prognostic than a single biomarker determination. It is vitally important for early diagnose of diseases as cancer, some cardiovascular diseases and neurological disorders in patients [98, 99].

A multiplexed biosensor was developed for fluorescent and visual monitoring of cancer cells, using graphene oxide-based aptameric nanosensor in microfluidic paper device, and quantum dots coated mesoporous silica nanoparticles as fluorescent probe. This nanosensor showed highly satisfactory sensitivity for multiple cancer cells monitoring for medical diagnostic [100]. Simultaneous measurement of the biomarkers β -amyloid, acetylcholine and glutamate for Alzheimer's disease detection is possible using an optical biosensor [101]. The fluorescent dye carboxy SNARF-1-dextran was co-immobilized with the enzymes glutamate dehydrogenase and acetylcholinesterase into the biosensor surface for sensing of glutamate and acetylcholine, respectively, and the fluorescent dyes Amplex red and FITC-dextran were immobilized with the enzyme horseradish peroxidase for β -amyloid and hydrogen peroxide detection. The biosensors showed an excellent analytical performance for multianalysis in human serum samples, and limits of detection achieving the nanomolar range in the presence of biomarkers for Alzheimer's disease.

In this context, biosensors have also been developed in microarrays format in order to improve the multiplexing capabilities, providing the detection of genes, oligonucleotides, sequences, and pathogens [96, 102]. A grating-coupled surface plasmon resonance imaging based on a gold-coated sensor chip was produced in microarray model, with capacity to measure simultaneously over 1000 discrete regions of interest by utilizing a microarray of antibodies or other capture biomolecule immobilized on the chip [96]. The system simultaneously detected toxins, pathogens, and specific antibodies to environmental agents.

CONCLUSIONS

There is an incessant search for new biomarkers identified in non-invasive sites, such as blood, urine and saliva, able to determine a specific diagnosis for patients. In different types of cancers, tumor cells can release DNA molecules for circulation, which can be determined in blood samples. In neurological disorders whose diagnosis sometimes requires invasive procedures, such as the use of cerebrospinal fluid, biomarkers have been detected, for example, in the urine, favoring early detection of these diseases, assessing their severity. Discover of biomarkers for CVDs is a progress in the early diagnosis challenger, prevention and adequate treatment, as well as non-invasive and rapid detection with day-to-day clinical practice incorporation. Biosensors have been introduced in search and point-of-care detection of biomarkers for signal-amplification, high sensitivity and specificity, quick time response, low cost, simplicity and multianalytical assays.

CONFLICT OF INTEREST

The authors declare that no competing interests exist.

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[1] J. van der Geer, J.A.J. Hanraads, R.A. Lupton, The art of writing a scientific article, J. Sci. Commun. 163 (2010) 51–59.

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[2] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000.

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[3] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304.

Reference to a website:

[4] Cancer Research UK, Cancer statistics reports for the UK. <http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/>, 2003 (accessed 13 March 2003).

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ANEXO B – NORMAS PARA SUBMISSÃO À REVISTA ELECTROANALYSIS

Author Guidelines

1 Aims and Scope

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[1] P. T. Kissinger, *Anal. Chem.* **1986**, 59, 1287; b) H. Fujimori, K. Osada, *Chem. Lett.* **1994**, 456.

Books:

[2] E.J. Bentz, *Synthetic Fuels Technology Overview with Health and Environmental Impacts*, Ann Arbor Scientific Press, Ann Arbor, MI **1981**.

[3] M. Hite, in *Laboratory Safety, Theory and Practice* (Eds: A. A. Fuscaldo, B. J. Erlick, B. Hindman), Academic Press, New York **1983**, pp. 29-57.

[4] E.B. Sansone, *Ph.D. Thesis*, University of Michigan, Ann Arbor, MI **1967**.

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