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DESENVOLVIMENTO DE GENOSSENSORES PARA O DIAGNÓSTICO DO PAPILOMAVÍRUS HUMANO (HPV)

DANIELLY SANTOS CAMPOS FERREIRA

RECIFE, 2014

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Tese apresentada ao Programa de Pós-graduação em Biologia Aplicada à Saúde do Laboratório de Imunopatologia Keizo Asami – LIKA/UFPE, como requisito para a obtenção do título de Doutor

Orientador: Prof. Dr. José Luiz de Lima Filho

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Aprovada em 28 de julho de 2014, pela comissão examinadora

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Aos grandes amores da minha vida, Augusto e Caroline

Dedico.

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"O pessimista se queixa do vento; O otimista espera que ele mude;
O realista ajusta as velas."
"The pessimist complains about the Wind; The optimist expects it to change;
The realist adjusts the sails."

(William George Ward)

LISTA DE ABREVIATURAS E SIGLAS

AE – Auxiliary electrode

AM – Azul de Metileno

CRPV – Papilomavírus de coelho

CV – Voltametria Cíclica

CYSFILM – L-Cysteine Film

DPV – *Differential Pulse Voltammetry*

dsDNA – Ácido Desoxirribonucléico de fita dupla

EA – Eletrodo auxiliar

ER – Eletrodo de referência

ET – Eletrodo de trabalho

ESD – *Extracted Sample DNA*

FC^+ – Ferroceno

HPV – Papilomavírus Humano

IUPAC – União Internacional de Química Pura e Aplicada

MB – *Methylene Blue*

PCR – *Polymerase Chain Reaction*

PGE – *Pencil Graphite Electrode*

PNA – Ácido Nucléico Peptídico

PV – Papilomavírus

RE – Reference electrode

RSD – *Relative Standard Deviation*

SAM – *Self-Assembled Monolayer*

SELEX – *Systematic Evolution of Ligands by Exponential Enrichment*

SNP – *Single-nucleotide polymorphism*

ss-DNA – Ácido Desoxirribonucléico de fita simples

SUS – Sistema Único de Saúde

SWV – *Square Wave Voltammetry*

WE – Working electrode

WHO – *World Healt Organization*

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RESUMO

Infecções pelo papilomavírus humano (HPV) de alto risco, principalmente o HPV16, podem levar ao desenvolvimento de tumores, como o de câncer cervical. O diagnóstico rápido e preciso associado a uma baixo custo operacional das lesões pré-cancerígenas por HPV é extremamente importante para o sucesso do tratamento. Os tradicionais testes para o diagnóstico desse vírus não preenchem todos os requisitos necessários para um diagnóstico bem sucedido. Dispositivos analíticos, como os biossensores, podem detectar agentes infecciosos de uma maneira mais simples e barata, em comparação com os testes convencionais. Estas características tornam os biossensors uma alternativa promissora para o diagnóstico precoce do HPV. O objetivo do presente trabalho foi o desenvolvimento de genossensores (biossensores de DNA) para o diagnóstico do HPV. O primeiro biossensor foi composto de dois eletrodos, um eletrodo de trabalho (ET) feito de lápis grafite e um eletrodo de referência (ER) feito de Ag/AgCl. O outro modelo de biossensor foi formado por três eletrodos impressos: ET constituído de ouro; ER constituído de Ag/AgCl; e EA (eletrodo auxiliar) constituído de carbono. Nos dois biossensores propostos, um sonda de DNA específica para o gene E6 do HPV16 foi imobilizada sob o eletrodo de trabalho por adsorção e, em seguida, uma sequência alvo foi hibridizada com a sonda imobilizada. No primeiro biossensor, o alvo foi o gene E6 do HPV16 colonado no plasmíedo PGEM-T. Já no segundo biossensor, os alvos foram os oligonucleotídicos sintéticos e o DNA extraído (DE) de amostras de pacientes. Os sinais redox da hibridização, nos dois biossensores, foram analisados pela técnica de voltametria de pulso diferencial. Os resultados mostraram que os biossensores puderam diferenciar a hibridização da não-hibridização. Os dois biossensores propostos apresentaram elevada sensibilidade cujos limites de detecção foram para o primeiro biossensor de 16 pg/μL (7 nM) e o segundo biossensor de 18,13 nM. Além disso, ao contrário dos testes padrão, os dois genossensores foram capazes de detectar a presença do DNA viral sem a necessidade de amplificação do material genético. Isso os tornam mais rápidos e baratos em comparação aos testes convencionais. Os dados obtidos com os biossensores mostraram viabilidade para o diagnóstico de vários tipos de HPVs, permitindo com isso o desenvolvimento de um sistema pioneiro para detecção portátil desse vírus.

Palavras chaves: Papilomavírus humano (HPV), Plasmídeo, HPV16, Biossensor Eletroquímico, Genossensor.

ABSTRACT

Infection by human papillomavirus (HPV) of high risk, particulary the HPV16, may lead to the development of tumors, such as cervical cancer. The rapid and accurate diagnosis associated to low cost of the precancerous lesions by HPV is extremely important for successful treatment. Traditional diagnostics tests for this virus do not all fulfill the necessary requirements for a successful diagnosis. The analytical devices, such as biosensors, can detect infectious agents in a more simple and cheap way as compared to the conventional tests. These characteristics make the biossensors a promising alternative for early diagnosis of HPV. The aim of this work was the development of genosensor (DNA biosensor) for HPV diagnosis. The first biosensor was composed of two electrodes. The pencil graphite was used as a working electrode (WE) and Ag/AgCl was used as a reference electrode (RE). The other model biosensor was composed of three screen-printing electrodes: WE constituted of gold; RE constituted of Ag/AgCl; and AE (auxiliary electrode) constituted of carbon. In both biosensors proposed, a DNA probe specific for E6 gene detection from HPV 16 was immobilized on the working electrode by electrodeposition and then a target sequence was hybridized with the probe immobilized. In the first biosensor, the target was the E6 gene inserted in plasmid pGEM-T. While in the second biosensor, the targets were the synthetic-oligonucleotide and extracted sample DNA (ESD) from patients. The hybridization redox signals were analyzed using differential pulse voltammetry. The results showed that biosensors could differentiate hybridization of non-hybridization. The two biosensors showed high sensitivity which detection limits were in the first biosensor 16 pg/μL (7 nM) and the second biosensor 18.13 nM. Moreover, unlike standard tests, both genossensores were able to detect the presence of viral DNA without the need for amplification of the genetic material. This makes them faster and cheaper than conventional tests. The data obtained with the biosensors showed viability for the diagnosis of various HPV types, thereby allowing the pioneering development of a new portable detection system for viruses.

Keywords: Human papillomavirus (HPV), Plasmid, HPV16, Electrochemical biosensor, Genosensor.

SUMÁRIO

INTRODUÇÃO

As infecção ocasionadas pelos HPVs de alto risco, principalmente a do genótipo 16, estão associadas à cerca de 99% dos casos de câncer cervical e com um número significativo de canceres vaginal, vulvar, peniano, anal e de cânceres de cabeça e de pescoço (1,2).

A citologia da pele e dos tecidos são as principais ferramentas utilizadas para diagnosticar as alterações pré-cancerígenas envolvidas na infecção pelo HPV. A principal alteração citológica para determinação desse vírus é a coilocitose, que se caracteriza por clareamento citoplasmático perinuelear e contorno suave, com reforço periférico e demarcação abrupta. Contudo, outras alterações citológicas, como disqueratose, papilomatose, hiperqueratose, acantose e paraqueratose, também podem ser sugestivas para o HPV (3). O teste citológico é considerado o padrão ouro do diagnóstico do câncer cervical (4). No entanto, essas técnicas são baseadas na análise interpretativa e subjetiva do examinador, o que torna esses métodos pouco sensível (5). Além disso, esses exames não detectam a presença do HPV, mas sim, as alterações citológicas causadas por esse vírus (6).

As técnicas moleculares têm sido utilizadas como ferramentas alternativas para detecção do DNA do vírus. Essas técnicas são extremamente específicas e sensíveis, entretanto, requerem uma complexa infra-estrutura laboratorial, que inclui sofisticados equipamentos, instalações com armazenamento de amostras, insumos de elevado custo e profissionais capacitados (7,8).

O desenvolvimento de um teste que possua todas as qualidades dos moleculares associados à rapidez de resposta e baixo custo operacional, torna este uma alternativa promissora para o diagnóstico precoce desse vírus. Para atender essa demanda, os biossensores podem ser utilizados por possuírem os requisitos necessários para a detecção do HPV.

Os biossensores são ferramentas que combinam biomoléculas imobilizadas (ácidos nucleicos, antígenos, anticorpos, tecidos, células, entre outros) a um transdutor para criar uma superfície que permita a medição qualitativa e/ou quantitativa de um analito específico (9). Essas ferramentas caracterizam-se por possuírem elevada seletividade, sensibilidade, rapidez e linearidade do sinal (10). A sua utilização não

necessita de técnicos especializados, podendo em alguns casos, até mesmo ser utilizado pelo próprio paciente. Portanto, constituem uma alternativa rápida e barata para medidas analíticas aplicadas ao diagnóstico (11).

Deste modo, o desenvolvimento desse dispositivo inovador pode gerar uma economia significativa de recursos, uma vez que sua aplicação dispensaria estrutura laboratorial, podendo alcançar pacientes que residem nas regiões mais remotas, ampliando assim, o acesso ao diagnóstico célere e eficiente. Ademais, o dispositivo pode oferecer outros benefícios como: fácil execução em campo e resultados de simples interpretação.

JUSTIFICATIVA

Vários estudos têm demostrado que diversos tipos de canceres estão fortemente associados às infecções causadas pelo papilomavírus humano (HPV) de alto risco**.** O diagnóstico rápido e preciso do HPV antes que este cause as lesões pré-cancerígenas é extremamente importante para o sucesso do tratamento. O uso de métodos de diagnóstico sensíveis e específicos, de fácil execução e interpretação, que não necessitem de infra estrutura laboratorial e profissionais especializados traz um grande benefício para o diagnóstico eficaz e rápido desse vírus, principalmente nas localidades onde o acesso a exames laboratoriais mais complexos é limitado. Desta maneira, os genossensores são importantes ferramentas que auxiliam no diagnóstico médico, facilitando a detecção precoce de diversos agentes infeciosos.

OBJETIVOS

Objetivo geral

Desenvolver genossensores para o diagnóstico do Papilomavírus humano (HPV).

Objetivos específicos

1- Identificar sequências nucleotídicas consenso e específicas entre os diferentes tipos de HPVs, através de ferramentas de bioinformática para a construção de sondas;

2- Avaliar a especificidade e sensibilidade do genossensor através de testes de hibridização entre as sondas e o DNA alvo, seguindo os critérios de certificação da Anvisa;

3- Avaliar a estabilidade do biossensor frente aos principais parâmetros físico-químicos.

4- Realizar estudo com amostras biológicas para comparação do sistema frente aos métodos utilizados atualmente.

Capítulo I

Revisão da Literatura

1.1 Papilomavírus (PV)

O papilomavírus (PV) é um grupo de vírus pertencente à família *Papillomaviridae,* na qual é constituído por 16 gêneros nomeados por letras gregas (Figura 1) (12,13). Esses vírus possuem forma icosaédrica não envelopada, genoma circular, composto por uma dupla fita de DNA, com aproximadamente 8 000 pares de bases (8Kb) (14–16).

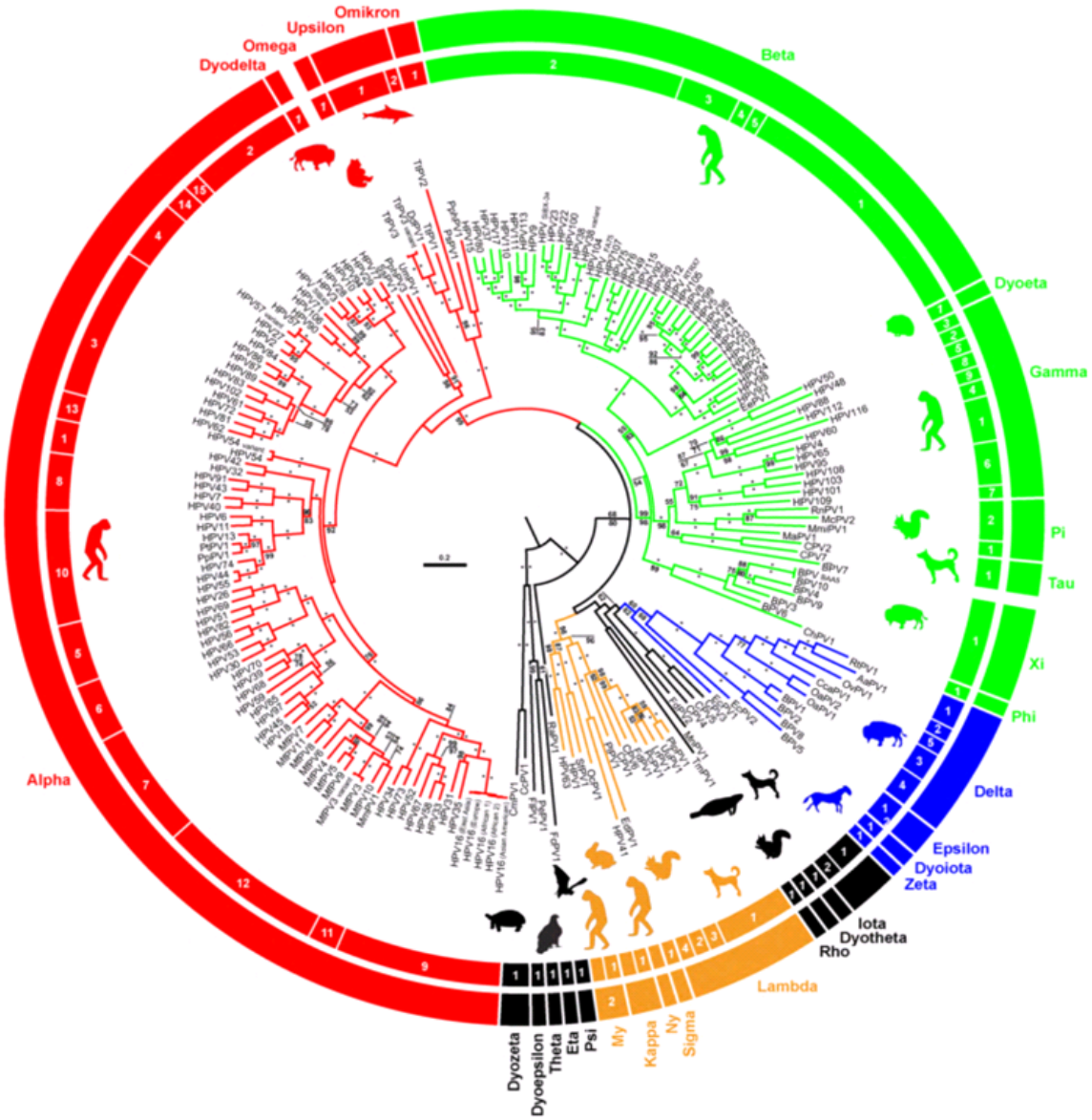

Figura 1. Árvore filogenética dos Papilomavírus. Adaptado de (13).

Os PVs podem ser encontrados em vários vertebrados, incluindo anfíbios, répteis, aves e mamíferos (17). Em geral, cada tipo de PV é específico para cada espécie, tipo de epitélio e localização anatômica (6,17,18).

Na sua grande maioria, os PVs causam tumores benignos (verrugas ou papilomas). No entanto, algumas infecções induzida por genotipos específicos de PVs apresentam um alto risco de progressão maligna, principalmente em humanos (19,20).

1.2 Papilomavirus Humano (HPV)

O HPV é considerado o vírus sexualmente transmissível mais comum do mundo. Cerca de 50 a 80% da população sexualmente ativa irá se contaminar com o vírus em algum momento da vida (4,21). Apesar da transmissão desse vírus dá-se prioritariamente por via sexual, existe relatados de casos de transmissão não sexual, como a transmissão vertical (22,23).

Os HPVs são um grupo heterogêneo de vírus, onde já foram identificados mais de 200 genotipos virais (24). A classificação dentro de uma mesma espécie de HPV não é baseada em sorotipos, e sim, feita com base nas diferenças do próprio genoma. Por isso, os tipos de HPVs são genotipos e não sorotipos (25). Considera-se um novo tipo de HPV quando as sequências de nucleotídeos dos genes L1, E6 e E7 (aproximadamente 30% do genoma viral) diferirem em mais de 10% dos tipos conhecidos. Se esse percentual for menor que 2%, então, o novo vírus isolado é designado como uma variante do mesmo genotipo. Por outro lado, será designado como subtipo viral, quando essas sequências nucleotídicas nessas regiões gênicas diferirem entre 2% e 10% dos tipos já descritos (14,17,26,27).

Além dessa classificação genotipica, os HPVs podem ser classificados epidemiologicamente quanto ao risco de causar câncer cervical, ou seja, em alto risco ou baixo risco oncogênico. Tipos de HPV de baixo risco incluem os tipos 6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81 e 89 (24). Os tipos de HPVs de alto risco incluem os tipos 16, 18, 31, 33, 34, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68, 73 e 82 (4).

1.3 Genoma do HPV

O genoma do HPV é constituído principalmente por 3 regiões (Figura 2): (i) uma região precoce (Early, E) responsável por codificar várias proteínas – E1, E2, E4, E5, E6 e E7 - que regulam a transcrição e a replicação viral e controlam o ciclo celular, conferindo a esses vírus potencial para transformar e imortalizar as células hospedeiras; (ii) uma região tardia (Late, L) - formada pelos genes *L1* e *L2*- responsáveis por codificar as proteínas do capsídeo; e (iii) uma região reguladora LCR (*Long Control Region*) ou URR (*Upstream Regulatory Region)* responsável por estimular ou reprimir a transcrição viral, dependendo do estímulo (28,29).

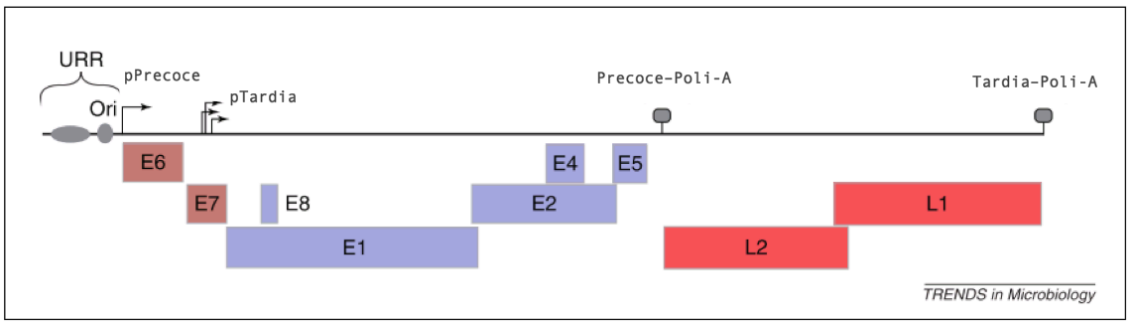

Figura 2. Organização genômica do Papilomavírus. Adaptado de (28).

As proteínas E1 e E2 desempenham importante função na replicação viral e formam um complexo em torno da origem de replicação (ORI) . O gene *E1* é o maior e mais conservado da região precoce do genoma dos HPVs. Esse gene codifica uma fosfoproteína (E1) que desempenha função de helicase e ATPase (6). A E1 se liga cooperativamente à outra fosfoproteína, a E2, formando um complexo multimérico essencial para a replicação dos HPVs (30). A E2 além de poder regular a replicação, também está envolvida na regulação da transcrição, agindo como um fator transcricional. Essa proteína age como forte repressora dos genes *E6* e *E7* (31).

A proteína E4 apresenta três funções: (i) facilitar a liberação do vírus, uma vez que essa proteína pode se ligar a queratina e ao citoesqueleto da célula, desestabilizando os filamentos intermediários das camadas superiores do epitélio e da pele; (ii) parar o cliclo celular do hospedeiro na fase G2, uma vez que se liga aos complexos

CDK/ciclina; e (iii) interagir com a proteína E2, facilitando o seu deslocamento do núcleo para o citoplasma (32,33).

As proteínas E5, E6 e E7 estão envolvidas na transformação celular (16,34). E5 não aparece em todos os tipos de HPV, mas em alguns casos essa proteína pode atuar em conjunto com o fator de crescimento epidermóide (EGF), no sentido de favorecer a proliferação celular, modulando os processos de divisão celular a partir da membrana celular (35). E6 e E7 desempenham importante papel nos processos que culminam com a transformação celular neoplásica. Essas proteínas são capazes de alterar a expressão de proteínas reguladoras da replicação celular, das quais as mais estudadas e conhecidas são a proteína do retinoblastoma (pRB) e a proteína supressora de tumor (p53) (36).

A proteína E6 é conhecida por promover a degradação do p53 e ativar a enzima telomerase, enquanto a proteína E7 se liga e inativa os membros da família do retinoblastoma (37). Quando a proteína E6 do HPV de alto risco se liga à p53, as atividades normais da p53, como (i) parada do ciclo celular em G1, (ii) reparo do DNA e (iii) apoptose, são anuladas (36,38). Com isso, a replicação continua nas células com o DNA danificado, conduzindo à instabilidade do genoma e à acumulação da mutação (39). Por outro lado, quando o produto do gene E7 do HPV se liga à pRb perturba o complexo de ligação entre pRb e o fator de transcrição celular E2F-1, resultando na liberação desse fator, permitindo a transcrição de genes, cujos produtos estimulam a célula a entrar na fase S (fase de síntese) do ciclo celular (2,40,41). A proteína E7 pode também associar-se a outras proteínas, como a ciclina E, que estimulam a síntese de DNA e o ciclo celular, resultando em uma alta proliferação celular (42–44).

Dessa maneira, as proteínas E6 e E7 do HPV de alto risco podem dar origem a uma instabilidade genômica associada a uma alta taxa de proliferação celular. Como consequência, a célula hospedeira acumula danos no DNA que não podem ser reparados, levando-as a transformação em células cancerosas (39).

A E8 ainda não apresenta função determinada em humanos (28). No entanto, estudos realizados por (45) demonstraram um potencial imunogênico desta proteína em papilomavirus de coelhos (CRPV) .

As proteínas L1 e L2 codificam as proteínas principal e secundária do capsídeo, respectivamente. Estudos demonstram que essas proteínas são responsáveis pela imunogenicidade do vírus e carregam determinantes antigênicos gênero-específicos

(2,46). A L1 é a proteína mais conservada dentre todos os HPVs ao contrário da L2 que apresenta a menor conservação (47,48) .

A região reguladora LCR pode variar de 400 a 1000 pb e está localizada entre as regiões *L1* e *E6*. Nessa região, existem além da origem de replicação, sequências estimuladoras e repressoras da transcrição viral responsivas aos hormônios e aos fatores de transcrição celular (12,49,50).

1.4 Interação vírus /hospedeiro

O ciclo biológico dos HPVs tem início quando as partículas virais penetram nas células da camada profunda, que são as células menos diferenciadas do epitélio escamoso, e que ainda têm atividade mitótica (25). Fissuras nesse epitélio possibilitam o acesso do vírus a essas células (51). A partícula viral entra na célula pela interação das proteínas do capsídeo com receptores específicos da superfície celular (32).

Os proteoglicanos, como o heparan sulfato, e a proteína integrina **α**-seis **ß**-quatro têm sido sugeridos como fortes candidatos a receptores iniciais de ligação para o HPV. Esses receptores são frequentemente encontrados na matriz extracelular (ME) e na superfície da maioria das célula. Eles estão envolvidos em várias funções biológicas e por causa de sua localização, são moléculas adequadas para a infecção viral por HPV (18,48,52).

Depois de penetrar na célula, a partícula viral perde seu capsídeo, expondo seu DNA à ação de enzimas nucleares, o que favorece a expressão dos genes virais (53,54). O genoma viral pode ficar dentro do núcleo da célula sob a forma circular, não integrado ao genoma celular (forma episssomal) ou integra-se ao genoma celular do hospedeiro (Figura 3).

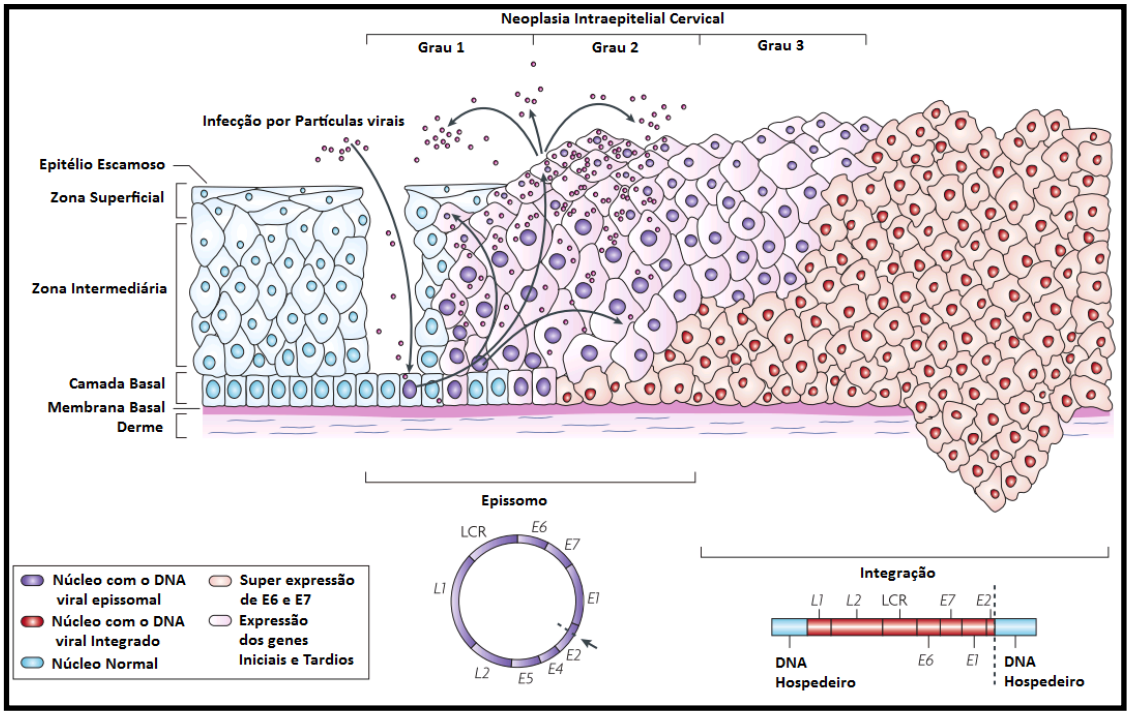

Figura 3. Ciclo de vida do HPV. Adaptado de (53).

Nos estágios precoces da infecção, o DNA epissomal replica-se na camada basal, concomitantemente ao DNA celular (54), Nas infecções por tipos de HPV associados a cânceres é observada, com frequência, a integração do DNA do vírus ao cromossomo do hospedeiro (55), pela ruptura na região de E2 do HPV, responsável pelo controle da transcrição do vírus (53,54,56). A perda do E2 conduz à expressão descontrolada das proteínas E6 e E7, que por sua vez leva a uma perturbação da regulação do ciclo celular do hospedeiro (32,54,56).

1.5 HPV e o Câncer Cervical

O câncer é uma doença causada principalmente por uma série de mutações somáticas em genes específicos que controlam a progressão do ciclo celular e as vias de reparo do DNA, resultando em descontrolada proliferação celular (57).

No geral, estima-se que o HPV está presente em 30% dos casos de cânceres em seres humanos (13). Os tumores malignos e seus precursores estão geralmente associados aos tipos de HPVs de alto risco oncogênicos, enquanto que as lesões

benignas (papilomas) estão associados aos de tipos de baixo risco (58). O DNA dos HPVs de alto risco são identificados em aproximadamente 99% das amostras de cânceres de colo de útero, em 80% das amostras de cânceres de ânus; em 60% das amostras de cânceres de vagina; em 40% das amostras de cânceres de vulva e de pênis; em 25,9% das amostras de cânceres de cabeça e pescoço e em mais de 35% das amostras de tumores da orofaringe (6,56,58,59).

Entre os tipos de câncer atribuível ao elevado risco de infecção por HPV, o câncer cervical tem recebido mais atenção. Com aproximadamente 530 mil casos novos por ano no mundo, o câncer do colo do útero é o segundo tipo de neoplasia maligna mais comum entre as mulheres, sendo responsável pelo óbito de 270 mil mulheres por ano (60).

Segundo o Instituto Nacional de Câncer (INCA), no Brasil, o câncer cervical é o terceiro tipo de câncer mais incidente na população feminina. Para o ano de 2014, no Brasil, são esperados 15.590 novos casos com um risco estimado de 15,33 casos a cada 100 mil mulheres. Sem considerar os tumores de pele não melanoma, estima-se que em 2014, o câncer do colo do útero será o mais incidente na região Norte (23,57/100 mil); o segundo nas Regiões Centro-Oeste (22,19/100 mil) e Nordeste (18,79/100 mil); na região Sudeste (10,15/100 mil), o quarto; e na região Sul (15,87/100 mil), o quinto mais frequente. Na Região Nordeste, os estados que apresentam maiores estimativas são Pernambuco e Sergipe, com 25,40 e 26,21 casos para cada 100 mil mulheres, respectivamente (61).

Estudos epidemiológicos demonstram que os HPVs 16, 18, 31, 33 e 45 são os mais predominantes, sendo responsáveis por mais de 90% dos carcinomas do colo do útero (58,62). O HPV-16 é o mais frequentemente encontrado, respondendo por cerca de metade dos casos de câncer do colo do útero no mundo (63–65). Em segundo lugar, existe uma prevalência do HPV-18, no entanto em alguns países observam-se algumas variações regionais, como no Brasil, onde na Região Centro-Oeste existe uma predominância do HPV-33 em relação ao HPV-18, e na Região Nordeste, onde o segundo em prevalência é o HPV-31 (63).

A infecção persistente por esses tipos de HPVs é considerada causa necessária para o desenvolvimento desse tipo de câncer, mas não suficiente (66). Existem, associado a presença do DNA viral, outros fatores importantes considerados de risco

para o desenvolvimento do câncer de colo de útero, como tabagismo, uso prolongado de contraceptivos orais, multiparidade e agentes infecciosos, como a *Chlamydia trachomatis, Neisseria gonorrhoeae* e o vírus da imunodeficiência humana (HIV) (1,51,62,63).

1.6 Vacinas contra o HPV

A vacinação é um método eficaz para prevenção de doença, especialmente as oriundas de agentes infecciosos, como o HPV (67,68). Existem dois principais grupos de vacinas contra o HPV: as vacinas preventivas, que se baseiam na estimulação da resposta imunológica humoral, e as vacinas terapêuticas, que estimula o desenvolvimento da resposta imune celular, ao sensibilizar células imunocompetentes, para atuar no combate à infecção viral (69).

Comercialmente, apenas se encontra disponível as vacinas preventivas, uma quadrivalente, a Gardasil® (Merck), que protege o indivíduo de quatro tipos de HPVs (6, 11, 16 e 18) e uma bivalente, a Cervarix® (GlaxoSmithKline – GSK) que protege o individuo contra dois tipos de HPVs (16 e 18) (68,70). Contudo, para garantir uma proteção total é necessário a aplicação de três doses da vacina (71), no decorrer de um período de 6 a 12 meses (67,70). Porém, algums pesquisadores sugerem um reforço a cada 5 anos, uma vez que essas vacinas possuem uma eficácia que varia de 5 a 10 anos dependendo do organismo (58,72).

Uma relevante limitação das vacinas preventivas é que essas não proporcionam efeitos terapêuticos contra infecções pré-existentes de HPV (73,74). Como as vacinas preventivas são produzidas a partir de partículas semelhantes à vírus (*virus-like particles* -VLP) oriundas das proteínas da capsídeo viral do HPV (L1 e L2) e as células tumorais associadas ao HPV não expressam níveis detectáveis desses antigénios (L1 e L2), essas vacinas não são eficazes em pacientes com infecção pré-existente (75,76).

Para eliminar as lesões pré-existentes, o ideal seria que as vacinas atingissem antígenos que fossem continuamente expressos em todas as fases da infecção, ou seja, antígenos expressos desde da entrada do vírus até sua integração ao hospedeiro (70,73,75). Pesquisas demostram que, uma alternativa seria a produção de vacinas terapêuticas a partir das proteínas E6 e E7, que possuem a particularidade de serem

constitutivamente expressas em todos os níveis do epitélio das células infectadas pelo HPV (47,73,75,77).

1.7 Diagnóstico do HPV

Um marco histórico importante no conhecimento do câncer de colo uterino foi o estudo realizado por Papanicolaou & Traut, publicado pelo *The American Journal of Obstetrics and Gynecology* em 1941. O teste de Papanicolau, como ficou conhecido esse estudo, é um teste citológico que analisa o esfregaço vaginal com o objetivo de detectar a presença de células anormais na mucosa cervical (4). Esse teste é considerado o padrão ouro do diagnostico do câncer uterino, passando a ser adotado por diversos países para a detecção precoce desse tipo de câncer (4).

No entanto, essa técnica visual possui várias limitações, principalmente por causa da sua baixa sensibilidade (5). Existem várias razões para explicar essa falha, incluindo erros amostrais e de interpretação laboratorial no diagnóstico das lesões (79). Como todo teste citológico, o Papanicolau é baseado em uma interpretação subjetiva das alterações morfológicas celulares e o seu sucesso depende que as amostras sejam coletadas adequadamente (80).

Uma das críticas mais frequentes a esse exame é a alta taxa de falsos negativos, que pode variar de 6 a 68% (81). As principais causas desses resultados falsos negativos são a falta de cuidado na colheita do material, erro na manipulação do esfregaço e ou na interpretação dos achados citológicos (79,81).

Outra falha desse teste é que ele não detecta a presença do HPV, mas sim, as alterações citológicas causadas por esse vírus. Pesquisas demonstram que mulheres com citologia cervical visualmente normal são positivas para o DNA do HPV (6), isso acontece porque as alterações citológicas, causadas por esses vírus, podem progredir lentamente (82,83). A evolução da lesão é um aspecto relevante a ser considerado, visto que ocorre sem sintomas específicos, o que dificulta um diagnóstico precoce (84).

O uso de técnicas tradicionalmente utilizadas para detecção viral, como cultura de células e sorologia não tem apresentado bons resultados para o diagnóstico desse vírus (85). O HPV possui a particularidade de não pode ser cultivado em cultura de células convencionais e os testes sorológicos apresentam precisão limitada (86), isto por

que a infecção gera uma resposta imune humoral contra a proteína L1 do capsídeo e os anticorpos gerados por esta resposta são detectáveis por muitos anos após a primeira infecção (87). Esse fato torna o teste sorológico não adequado para distinguir infecções presentes ou passadas, apresentando assim uma baixa sensibilidade e utilizado apenas para pesquisa (88).

O diagnóstico preciso das infecções por HPV baseia-se na detecção de ácidos nucléicos, como as técnicas de biologia molecular (89). Atualmente, os principais testes disponíveis no mercado, para detecção desse vírus, baseiam-se em 3 categorias: (i) hibridização de ácidos nucléicos; (ii) ensaio de amplificação de sinal; e (iii) amplificação de ácidos nucléicos (Figura 4) (90).

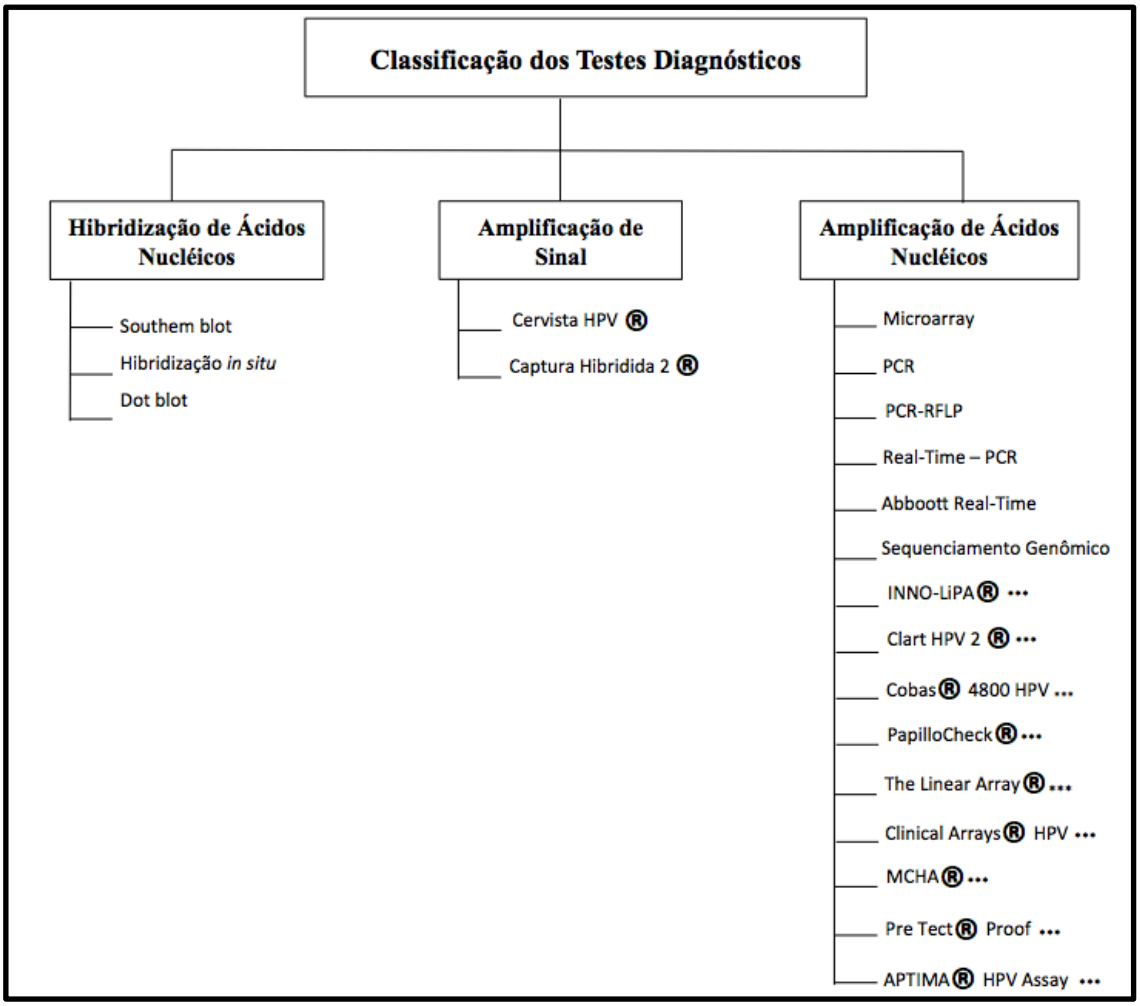

Figura 4. Diagrama esquemático da classificação dos testes diagnósticos do HPV. *** Testes patenteados usados para genotipagem individual do HPV. Adaptado de (90)

De regra geral, as técnicas baseadas na hibridização de ácidos nucléicos utilizam sondas radioativas para detectar o DNA do HPV nas amostras biológicas, não sendo capazes de genotipar o tipo de HPV. As técnicas baseadas na amplificação de sinal utilizam a tecnologia de DNA ramificado (*branched* DNA) ou de captura híbrida para aumentar o sinal do DNA alvo aos níveis detectáveis e apenas permite classificar os tipos de HPV em grupos de risco para o desenvolvimento de câncer. Já , os testes baseados na amplificação de ácidos nucléicos tem como princípio a pré-amplificação em múltiplas cópias do DNA alvo, e alguns métodos são capazes de genotipar individualmente o tipo de HPV (Figura 4) (90).

Essas técnicas moleculares são extremamente específicas e sensíveis, entretanto, requerem uma complexa infra-estrutura laboratorial, que inclui sofisticados equipamentos, instalações com armazenamento de amostras, insumos de elevado custo e profissionais capacitados, o que torna essas técnicas muito onerosas (7,8,85). Deste modo, as técnicas atualmente disponíveis não preenchem todos os requisitos necessários para um diagnóstico bem sucedido do vírus HPV (85,90).

2.1 Biossensores

Sistemas analíticos, como os biossensores, têm sido estudado para o diagnóstico de diversas doenças, incluindo a detecção *in situ* de agentes infeccicosos (91–95). Comparado à instrumentação em um laboratório clássico, os biossensores estão sendo cada vez mais explorados como simples e promissores dispositivos de diagnóstico capazes de diferenciar mais de uma doença numa única amostra (96,97). Alem disso, podem ser uma alternativa rápida e barata para medidas analíticas convencionais, como no monitoramento de substâncias químicas e bioquímicas aplicadas ao diagnóstico clínico (11,98,99).

Os biossensores são ferramentas analíticas que combinam biomoléculas imobilizadas (ácidos nucléicos, antígenos, anticorpos, tecidos, células, entre outros) a um transdutor para criar uma superfície que permita a medição qualitativa e/ou quantitativa de um analito específico (100,101). Estas ferramentas apresentam três componentes distintos (102–104), como ilustra a Figura 5 abaixo:

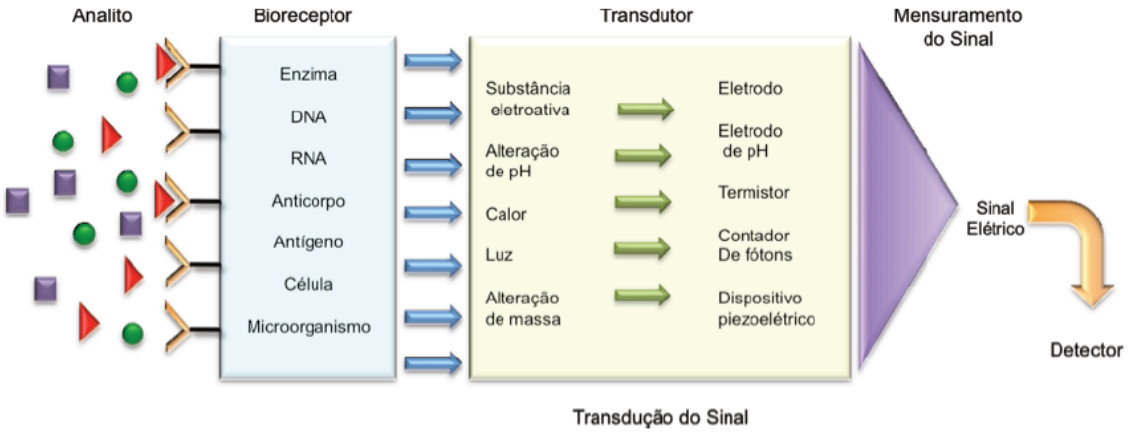

Figura 5. Componentes do biossensor. Adaptado de (102).

a) O bioreceptor: elemento biológico incorporado ao transdutor responsável pela detecção do analito de interesse.

b) O transdutor: componente responsável por converter o sinal biológico num sinal mensurável, geralmente elétrico.

c) O detector: microprocessador onde os sinais oriundos do transdutor são amplificados e analisados.

O princípio de detecção dos biossensores é a "ligação" específica do analito de interesse ao elemento de reconhecimento biológico (bioreceptor) imobilizado a um suporte adequado. A interação específica resulta na alteração de uma ou mais propriedade físico-química (alteração de pH, transferência de elétrons, alteração de massa, transferência de calor, libertação de gases ou íons específicos) que são detectadas pelo transdutor (102). O principal objetivo desses dispositivos é produzir um sinal eletrônico proporcional à concentração do analito específico em tempo real (Luong et al., 2008).

2.2 Classificação dos Biossensores

Os biossensores podem ser classificados de acordo com a origem do elemento de reconhecimento biológico (bioreceptor); com base na natureza do evento de bioreconhecimento; ou de acordo com a energia envolvida na transdução do sinal biológico (transdutor) (105,106).

2.2.1 De acordo com o bioreceptor

A) Biossensor Enzimático: o princípio deste biossensor está baseado na catálise enzimática do substrato (analito) (107). Esse biossensor monitora o desaparecimento de algum reagente ou o aparecimento de algum produto da reação, através de uma de sinal que pode ser uma mudança da concentração de protons; uma liberação ou absorção de gases, como amônia ou oxigênio; uma emissão, absorção ou reflectância de luz; ou uma emissão de calor (108). Muitos fatores exercem influencia sobre o desempenho deste tipo de biossensor, como o uso de pH e temperatura inadequados (109). Dentre os vários tipos de biossensores, os enzimáticos são os mais bem sucedidos comercialmente, representando cerca de 90% do mercado de biossensores (110). O mais popular é o glicosímetro, utilizado para medição de glicose em amostras de sangue (10,111). Este dispositivo utiliza como elemento de reconhecimento a glicose oxidase ou a glicose desidrogenase (111). Contudo, várias outras enzimas são utilizadas na construção de desse tipo de biossensores, tais como a urease, a penicilinase entre outras (109).

B) Imunossensor: é um tipo de biossensor baseado numa reação imunológica específica, onde o antígeno ou anticorpo é imobilizado na superfície do transdutor. Devido ao processo de reconhecimento único e a forte afinidade de interação entre o anticorpo-antígeno, os imunossensors são altamente seletivos e sensíveis e capazes de identificar baixo níveis do analito alvo (112). Entre os analitos que podem ser investigados por esses biossensores estão os hormônios, drogas, marcadores tumorais, vírus, bactérias e poluentes ambientais (pesticidas, por exemplo) (113,114).

C) Biossensor Microbiológico: dispositivo que combina um microrganismo imobilizado a um transdutor (115,116). Esses biossensores tem o principio de operação muito semelhante aos biossensores enzimáticos (107). A vantagem do uso de microrganismos como biocatalisadores reside, principalmente, no fato de que esses têm a capacidade de se adaptarem à condições adversas sem perderam a sensibilidade ao contrário do que ocorre com as enzimas (116,117). Microrganismos, como algas, bactérias e leveduras, oferecem uma alternativa para a fabricação de biossensores, porque eles podem ser produzidos em massa por meio de cultura de célula. Em comparação com as células de outros organismos superiores, como plantas, animais e seres humanos, as células microbianas são mais fáceis de serem manipuladas e têm uma melhor estabilidade e viabilidade *in vitro,* o que pode simplificar o processo de fabricação e melhorar o desempenho dos biossensores (115). Os microrganismos são análogos a uma "fábrica", consistindo de numerosas enzimas e co-fatores/coenzimas, o que os trorna capazes de responder a inúmeros produtos químicos (analitos alvos) (116). Esses biossensores têm sido amplamente aplicados na detecção de vários compostos, como fenol, benzeno, tolueno entre outros (115). A principal limitação para a utilização de desses biossensores é a difusão do substrato e dos produtos através da célula, resultando em uma resposta lenta em comparação aos outros biossensores (118).

D) Genossensor: dispositivo que utiliza uma sonda de ácido nucléico imobilizada como elemento de reconhecimento. Uma das aplicações mais populares desses dispositivos é a detecção de sequências específicas de DNA através do evento de hibridização (110,119). A determinação rápida e confiável da sequência de ácido

nucléico alvo desempenha papel cada vez mais importante no diagnóstico clínico, análises forenses e ambiental, como também no monitoramento da segurança alimentar (120).

2.2.2 De acordo com a natureza do evento de bioreconhecimento

Dentro dessa classificação, o biossensor pode ser dividido em 2 categorias:

A) Dispositivos biocatáliticos: esses biossensores são baseados em uma reação catalisada pela macromolécula (biocepetor) imobilizada ao transdutor. Nesse caso, o elemento biológico converte o analito alvo (substrato) em um produto e as mudanças que se correlacionam a esse evento são registrados pelo transdutor.Três tipos de biocatalisadores (bioceptores) são comumente usados: (i) enzimas; microrganismos; e (iii) fatia de tecido vegetal ou animal (110,121).

B) Dispositivos de bioafinidade: esses biossensores são baseados na ligação seletiva do analito alvo ao bioreceptor levando a formação de um complexo e as alterações físico-químicas provocadas pela formação desse complexo são detectadas pelo transdutor (105). Fazem parte dessa categoria, os biossensores baseados em antígeno, anticorpo, ácidos nucléicos e receptores (121).

2.2.3 De acordo com o transdutor

A) Biossensor Ótico: permite a determinação de analitos utilizando uma ou mais características da radiação ótica, como absorção, índice de refração, fluorescência, fosforescência e comprimento de onda (103,117).

B) Biossensor Piezoelétrico: baseia-se na frequência de ressonância de um material (geralmente cristal). A variação de frequência é proporcional à variação de massa, ou seja, com o aumento da massa, devido à ligação do analito alvo, a frequência de oscilação do cristal é alterada (99,109).

C) Biossensor Térmico: baseia-se na medida da variação de temperatura gerada por reações catalisadoras de enzimas exotérmicas para medir a concentração do analito. A mudança de temperatura é medida através de termistores (103,122).

D) Biossensor Eletroquímico: são os mais frequentemente utilizados. Baseia-se no fato que durante o processo de bio-interação, entre a biomolécula imobilizada e o analito alvo, ocorre reações químicas, que podem produzir ou consumir íons ou elétrons, alterando a propriedade da solução (109).

2.3 Detecção Eletroquímica

Os biossensores eletroquímicos são os mais comuns dos tipos de biossensores em uso, devido à sua portabilidade, custo, eficácia, pequena dimensão, facilidade de utilização e possibilidade de identificar e quantificar compostos específicos em misturas complexas, como em amostras biológicas (10,123). A detecção eletroquímica não é afetada por componentes da amostra, como cromóforos, fluoróforos e partículas, que muitas vezes interferem com a detecção espectrofotométrica. Portanto, as medições eletroquímicas podem ser feitas em amostras coloridas ou turvas, tais como sangue total, sem interferência de glóbulos de gordura, células vermelhas do sangue, hemoglobina e bilirrubina (9).

Os biossensores eletroquímicos podem ser constituídos por dois ou três eletrodos (9,10). Tipicamente uma célula eletroquímica é constituída por um eletrodo de referência, um eletrodo auxiliar e um eletrodo de trabalho. O eletrodo de referência, geralmente composto de prata metálica revestida por uma camada de cloreto de prata (Ag/AgCl), é mantido a uma certa distância do local de reação, a fim de manter um potencial conhecido e estável. O eletrodo auxiliar ou contra-eletrodo tem a função de proteger o eletrodo de referência, pois ele não permite que a corrente, que passa através da solução eletrolítica, passe pelo eletrodo de referência alterando o seu potencial. Já o eletrodo de trabalho serve como elemento transdutor da reação bioquímica (124).

Um sistema de dois eletrodos possui apenas o eletrodo de trabalho e o eletrodo de referência. Esse tipo de sistema é usado quando a densidade da corrente que passa através da solução eletrolítica é baixa o suficiente para não alterar a propriedade do eletrodo de referência. O sistema de dois eletrodos são geralmente preferidos para os

biossensores descartáveis porque a estabilidade a longo prazo do eletrodo de referência não é necessária e o custo é mais baixo (9).

2.3.1 Técnicas eletroquímicas

As técnicas eletroquímicas são geralmente organizadas em quatro principais categorias de medição: corrente (amperometria e voltametria), potencial (potenciometria), condutância (condutimetria) e impedância (impedimetria) (93). Deste modo, os biossensores eletroquímicos podem ser divididos em:

A) Impedimetricos: esses dispositivos têm como função analisar as propriedades de resistividade e capacitividade de materiais em um sistema de excitação provocado por uma pequena tensão de amplitude sinusoidal (frequência) (125). A frequência é variada ao longo de um vasta gama para obter o espectro de impedância e as pequenas alterações na impedância são proporcionais à concentração da espécie analisada (9). As alterações de impedância, ou seja, a capacidade resistiva e capacitiva da transferência de elétrons entre o eletrodo e a solução muda quando o analito se liga ao bioreceptor (126).

B) Condutimétricos: o parâmetro medido quando se utiliza este dispositivo é a condutividade dos eletrólitos produzidos durante a reação bioquímica (109). Este efeito é baseado na mudança do número de cargas carreadas no eletrólito, onde a condutividade é linearmente relacionada a concentração de íons (127).

C) Potenciométricos: esses dispositivos medem a diferença de potencial entre o eletrodo de trabalho e o eletrodo de referência em condições de corrente elétrica constante. Em outras palavras, potenciometria fornece informações sobre a atividade de íons em uma reação eletroquímica (10,128).

D) Amperométricos: são caracterizados pela aplicação de um potencial constante (fixo) na reação eletroquímica, resultando na oxidação ou redução das espécies eletroativas presente na solução (11). Essa reação redox pode ser acompanhada através da medição da corrente na célula eletroquímica (129). A corrente gerada é diretamente relacionada com a espécie eletroquimicamente transformada no eletrodo (124).

E) Voltamétricos: semelhante aos biossensores amperométricos, os voltamétricos também se baseiam na relação corrente-potencial, porém, a corrente é medida durante variações controladas do potencial e não em um potencial fixo. A resposta em pico de corrente é proporcional à concentração do analito. As principais técnicas voltamétricas mais estudadas são a voltametria cíclica e a voltametria de pulso diferencial (130).

2.3.1.1 Principais técnicas voltamétricas

A) Voltametria cíclica: essa técnica é uma das formas mais utilizadas para a obtenção de informação qualitativas sobre o potencial redox de uma reação eletroquímica. Na voltametria cíclica, o eletrodo é analisado por um varrimento de potencial linear, aplicado a uma velocidade constante entre um potencial inicial e um final. Inicia-se a aplicação do potencial em um valor que não altere a reação eletroquímica. Com o aumento do potencial para regiões negativas, ocorre a redução do composto presente na reação, gerando um pico de corrente proporcional à concentração deste composto. Quando o potencial atingir um valor no qual todo o composto estiver sido reduzido, o potencial é varrido no sentido inverso até o valor inicial do procedimento. Nos casos em que o composto presente na reação seja reversível, é possível analisar também a reação de oxidação desse composto, aumentando o potencial de varrimento para regiões positivas. O pico de corrente gerado pela oxidação será simétrico ao pico de redução, como pode ser visualizado no voltamograma da figura 6 (124,130–133).

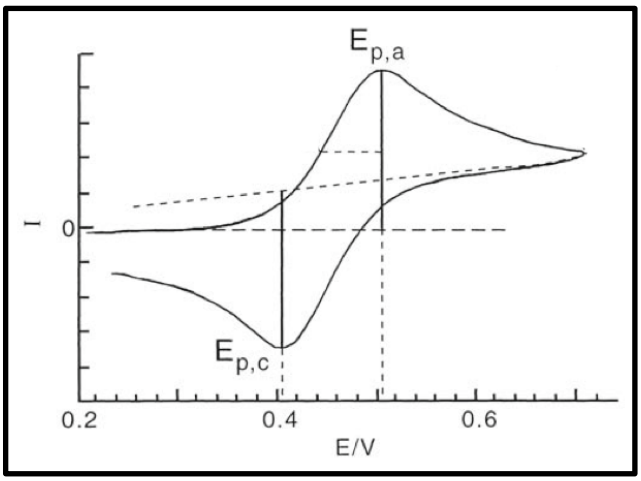

Figura 6. Gráfico da voltametria cíclica (corrente versus potencial). Ep,a é o pico de potencial anódico (pico de oxidação) e Ep,c é o pico de potencial catódico (pico de redução). Adaptado de (132)

B) Voltametria de pulso diferencial: nessa técnica, utiliza-se a aplicação de pulsos de potencial crescente, com amplitude fixa, e em intervalos de tempo curtos no eletrodo de trabalho, gerando um pico de corrente. Nesse modelo, a corrente é lida duas vezes: uma antes da aplicação do pulso (S1) e outra ao final do pulso (S2). O valor final da corrente é a diferença entre a corrente faradaica (S2 – corrente desejável) e a corrente capacitiva (S1- ruído do sistema). O objetivo desse princípio é a obtenção de uma corrente final livre de ruído (Figura 7) (124,130–133)

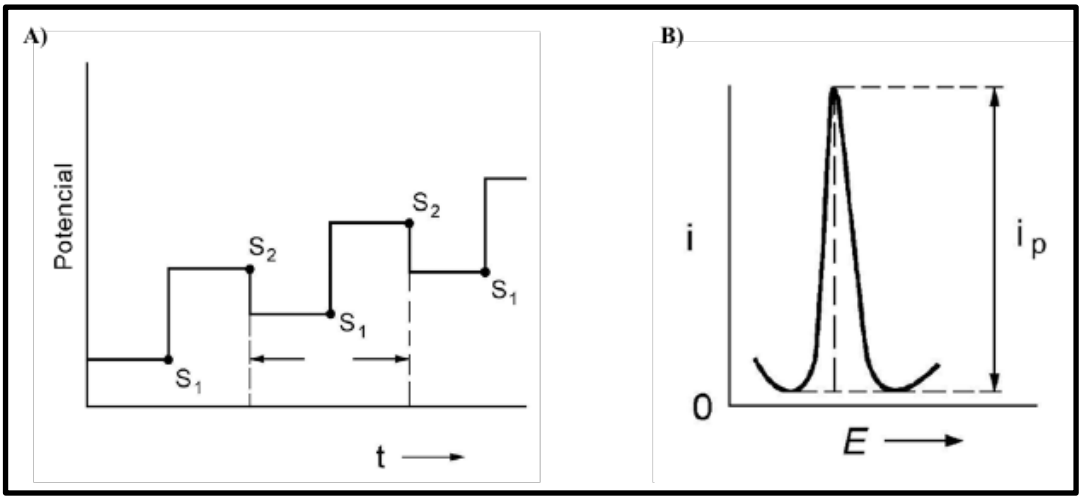

Figura 7. A) Gráfico do potencial aplicado em função do tempo em que S1 determina a corrente capacitiva e S2 determina a corrente faradaica. B) Voltamograma de pulso diferencial (corrente versus potencial) em que o pico de corrente Ip representa a corrente livre de ruído. Adaptado de (131).

3.1 Genossensor

Estes biossensores são dispositivos que possuem, como agente de reconhecimento biológico, um fragmento de ácido nucléico (sonda) conhecido imobilizado a um transdutor (134).

Classicamente, o princípio básico de funcionamento de um genossensor é o reconhecimento da molécula alvo a partir do evento de hibridização, onde a sequência gênica alvo é identificada pela sonda imobilizada, formando um híbrido. Esse reconhecimento é altamente eficiente e específico (135,136). No entanto, ultimamente, muitos genossensores estão utilizando oligonucleotídeos de ácidos nucléicos em um estado conformacional secundário. Nesse caso, o princípio de funcionamento se baseia na capacidade do bioreceptor se ligar com uma elevada afinidade e especificidade a uma molécula alvo, desempenhando um papel de receptor (137).

3.1.1 Elementos de bioreconhecimento dos genossensores

A especificidade do genossenor é essencialmente dependente das propriedades do elemento de bioreconhecimento. Segundo o último relatório da União Internacional de Química Pura e Aplicada – IUPAC (2010), numerosos tipos de ácidos nucléicos estão disponíveis para serem utilizados como elemento de bioreconhecimento nos genossensores, como as moléculas de DNA e RNA, PNA (ácido nucléico peptídico) e aptâmeros (95,134,136).

3.1.1.1 DNA e RNA como elemento de reconhecimento

Entre o DNA e o RNA, o DNA é utilizado com maior frequência devido à sua maior estabilidade frente a uma variedade de condições. O processo de reconhecimento por esses elementos consiste nas interações não covalentes entre as bases da cadeia complementar (alvo) e as bases presente na sonda, seguindo a regra de emparelhamento estabelecida por Watson-Crick (A-T / C-G / U-T) (Figura 8) (136,138).

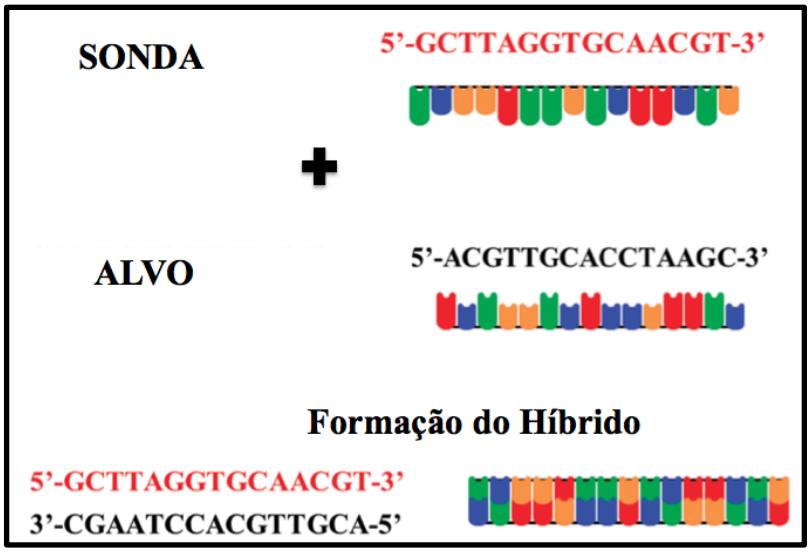

Figura 8. Ilustração do evento de hibridização. Adaptado de (136)**.**

3.1.1.2 PNA como elemento de reconhecimento

O ácido nucléico peptídico (PNA) é um polímero sintetizado artificialmente similar ao DNA ou ao RNA. Esses sintéticos ácidos nucléicos possuem na sua estrutura ligações 2-aminoetilglicina, em vez dos ligantes fosfodiestéricos com cargas negativas presentes nos oligonucleotídeos (Figura 9) (95,134).

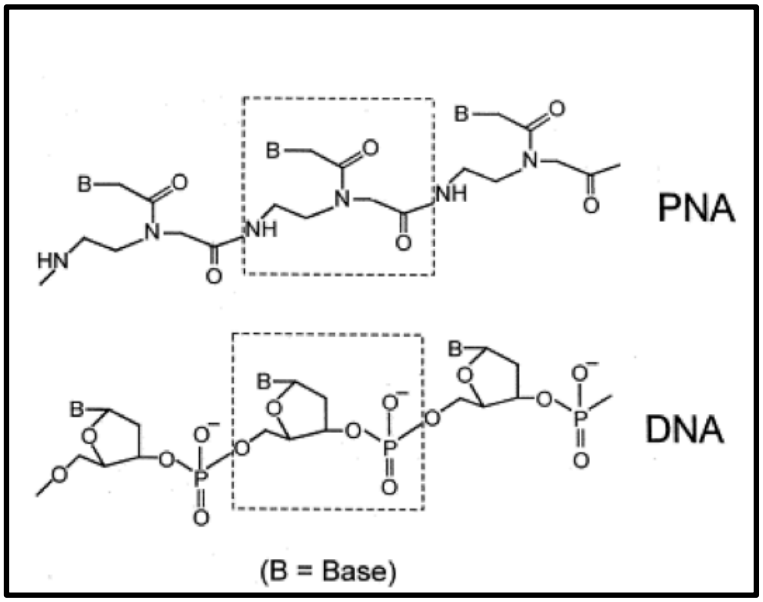

Figura 9. Estrutura química do PNA e DNA, onde B é a nucleobase. Adaptado de (139).

Se comparado com as sondas de oligonucleótidos convencionais, os PNAs são particularmente mais atraente para o desenvolvimento de genossensores. Esses sintéticos ácidos nucléicos além de serem mais estáveis em intervalos amplos de temperatura e pH (140), eles têm maior sensibilidade em detectar mutações de ponto, como polimorfismos de nucleotídeo único (SNPs - *Single-Nucleotide Polymorphisms*), porque a estabilidade do híbrido DNA-PNA é fortemente influenciada por um mal-pareamento de base única (134).

3.1.1.3 Aptâmeros como elemento de reconhecimento

Aptâmeros de ácidos nucléicos são oligonucleotídeos de fita simples (DNA ou RNA), originários de seleção *in vitro* a partir de bibliotecas de sequências aleatórias para obtenção de uma sonda com elevada afinidade de ligação para uma determinada biomolécula alvo (141,142). Esses nucleotídeos são produzidos sinteticamente por um processo conhecido como evolução sistemática de ligantes por enriquecimento exponencial (SELEX – *systematic evolution of ligands by exponential enrichment*), que é um processo repetitivo de ligação, separação e amplificação de ácido nucléicos (141,143).

Os aptâmeros apresentam diversas vantagens que os tornam preferíveis no

desenvolvimento de biossensores (11). Eles possuem estrutura simples; são de fácil síntese e armazenamento; são altamente reprodutíveis; são mais estáveis que o DNA e o RNA por possuírem maior resistência à desnaturação; são quimicamente modificados com maior facilidade; e possuem afinidade e especificidade por ligantes que não são reconhecidos por anticorpos, como íons ou pequenas moléculas (129,144,145).

3.2 Principais métodos de imobilização em genossensores

A forma de imobilização das sondas à superfície do eletrodo depende do tipo e da natureza do transdutor, constituindo um passo fundamental no desenvolvimento do biossensor (134).

Alguns métodos têm sido desenvolvidos para imobilização dos ácidos nucléicos sobre à superfície do eletrodo, os que se destacam são (i) adsorção física, (ii) adsorção química, (iii) ligação por afinidade e (iv) ligação covalente (134).

A) Adsorção física: é o método mais simples de imobilização porque não exige qualquer modificação do ácido nucleico (146). A imobilização por esse método tem sido relatada com base nas interações iônicas que ocorrem entre os grupos carregados negativamente presentes na sonda de ácidos nucléicos e as cargas positivas que cobrem a superfície do eletrodo (109). O método de adsorção física direta consiste na imobilização do elemento de reconhecimento (sonda) diretamente no eletrodo. Esse método se baseia (i) na dissolução do analito modificador (sonda de ácido nucléico) em um solvente apropriado e na exposição do eletrodo a esta solução por imersão, criando filmes finos de ácidos nucléicos, ou por evaporação de pequenos volumes dessa solução, para criar camadas mais grossas de ácidos nucléicos (134); e (ii) na aplicação de um potencial controlado numa solução, contendo a sonda e o eletrodo de trabalho imerso. (134,147,148). Já o método de adsorção física indireta consiste na imobilização do elemento de reconhecimento sobre uma fina camada polimérica formada sobre o eletrodo de trabalho. No caso da imobilização de ácidos nucléicos, um polímero catiónico é usado, o qual forma um complexo estável com os grupos fosfato do ácido nucléico, que são carregados negativamente (134,135,149).

B) Adsorção química: Este método de imobilização se baseia na auto formação de monocamadas (*self-assembled monolayer* - SAM) de um agente, como os alcanos tiolados, sobre a superfície de um suporte, no qual esse agente tenha afinidade (148). Uma das propriedades mais importantes desse método é gerar uma superfície funcional, através das monocamadas, capaz de fixar as moléculas biológicas. Um exemplo clássico são os compostos que apresentam o grupo tiol na sua molécula, como é o caso do aminoácido cisteína (Figura 10) (150–152). Esse grupo apresenta uma alta afinidade pelo ouro (Au), por isso é muito utilizado em biossensores que possuem o eletrodo de trabalho composto por esse metal (134).

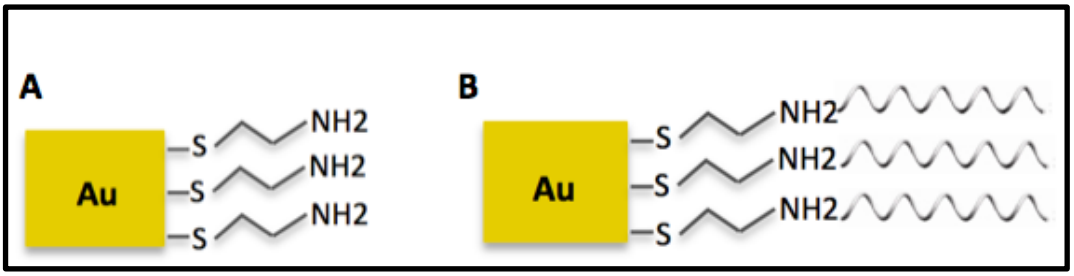

Figura 10. Processo de imobilização de ácidos nucléicos a partir de uma monocamada de grupamento tiol presente no aminoácido cisteína. A) Eletrodo de ouro modificado a partir de uma monocamada de grupamento tiol; B) Eletrodo de ouro modificado a partir de uma monocamada de grupamento tiol imobilizado com uma sonda de DNA.

C) Ligação por afinidade: O complexo avidina (ou estreptavidina)-biotina é o principal exemplo de imobilização por esse método. Esse complexo tem sido amplamente usado na construção de biossensores de ácidos nucléicos. A biotina é uma pequena molécula que se liga com elevada afinidade aos quatros sítios de ligação presentes na avidina ou na estreptavidina. No caso dos genossensores, o ácido nucléico é biotinilado na sua extremidade 5' e a avidina ou a estreptavidina é conectada diretamente ao eletrodo de trabalho (134).

D) Ligação covalente: Neste método, a extremidade final da sonda (elemento de bioreconhecimento) é modificada por grupos funcionais reativos, que apresentam uma alta afinidade pelo tipo eletrodo escolhido. Geralmente, os grupos funcionais utilizados para modificação estão presentes nas cadeias laterais de alguns aminoácidos, como os grupos amino, tiol, carboxílico, entre outros (109). O processo de imobilização por esse método além de ser bastante estável em relação aos demais ele ainda favorece a

formação do híbrido aumentando a sensibilidade de detecção, pois não permite que moléculas inespecíficas se liguem à superfície do eletrodo (109,134).

3.3 Princípios dos métodos de detecção eletroquímica em genossensores

A versatilidade das técnicas eletroquímicas propiciam não apenas a análise estrutural dos ácidos nucléicos, como também sua determinação em baixos níveis de concentração (136). Como citado na seção 3.1, o princípio básico de funcionamento de um biossensor de ácido nucléico é o reconhecimento de uma outra molécula alvo de ácido nucléico a partir do evento da hibridização. A detecção eletroquímica desse evento pode ser feita de duas maneiras (i) uma baseada na medição direta da resposta elétrica dos filamentos de DNA ou (ii) no monitoramento de compostos (indicadores ou intercaladores redox) unidos à rede duplex de DNA (153).

A) Método de detecção direta: No método direto, a formação do duplex é detectada através da medição do sinal eletroquímico, oriundo da oxidação das bases nitrogenadas dos ácidos nucléicos (154). Entre as quatro bases de ácidos nucleicos, a guanina além der ser a base com mais eletroatividade, ela é a mais facilmente oxidada. Devido a essas características, a guanina é a base mais adequada para a detecção de hibridização pelo método de detecção direta (136). Um fator que pode melhorar a detecção do sinal da oxidação da guanina quando se utiliza amostras reais, é a utilização de sondas (elemento de bioreconhecimento) sem a presença de guanina, na qual pode ser trocada pela base inosina (155). Essa base não está naturalmente presente no DNA, mas apresenta o mesmo comportamento da guanina, de ser oxidada e de se ligar a citosina (134,136). A melhoria na detecção do sinal de oxidação das guaninas presentes no DNA da amostra biológica, deve-se a diferença de potencial da oxidação dessas duas bases, o potencial de oxidação da inosina é na faixa de 0,7 V, enquanto que a da guanina é na faixa de 0,9-1,07 V (127,155). Com isso é possível distinguir com eficácia o sinal de oxidação produzido pela sonda e o sinal de oxidação produzido quando o DNA alvo hibridiza com a sonda (Figura 11) (156).

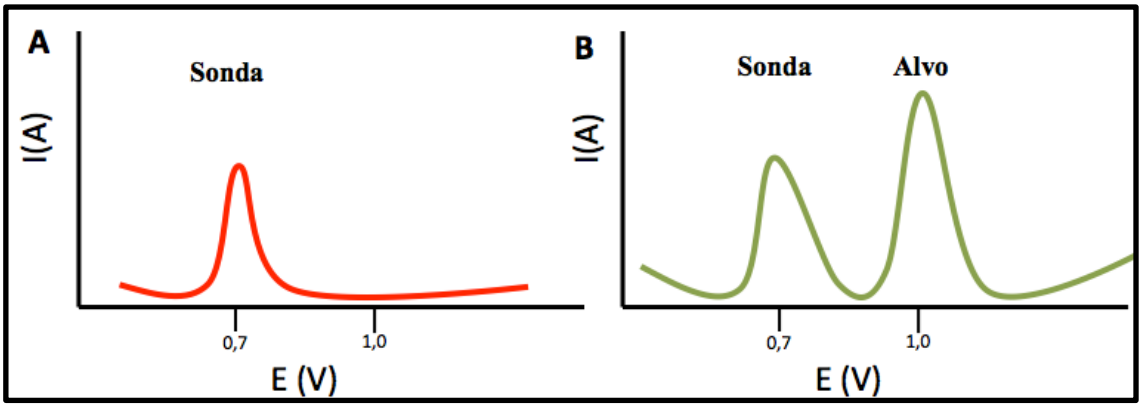

Figura 11. Representação esquemática da faixa de potencial do sinal de oxidação da guanina e da inosina: (A) Faixa de potencial do sinal de oxidação da sonda modificada com inosina; (B) Faixa de potencial do sinal de oxidação do hibridizado (sonda com inosina + alvo com guanina).

B) Método de detecção indireta: Moléculas com propriedades redox têm sido amplamente usadas para monitorar indiretamente a estrutura dos ácidos nucléicos na superfície do eletrodo. Estes compostos podem se ligar ao DNA de fita simples (ss-DNA) e/ou ao DNA de fita dupla (ds-DNA), oferecendo uma resposta eletroquímica capaz de distinguir essas duas estrutura do DNA (154). O azul de metileno (AM) e o ferroceno (FC^+) são composto, com atividade redox sensível à análise eletroquímica e extremamente utilizados nesse método (124). O AM é uma molécula heterocíclica aromática que apresenta alta afinidade pelas bases de guanina (157). A discriminação entre o ss-DNA e o ds-DNA se dá pela diferença do sinal redox do AM quando ligado a essas estruturas de DNA. Esse sinal redox será maior no ss-DNA do que no hibridizado (ds-DNA), pois as guaninas nesse último caso não vão estar facilmente acessíveis, dificultando a ligação do AM ao DNA (Figura 12-A) (158,159). Já o FC^+ e seus derivados são ligantes eletroativos de DNA que interagem com as principais cavidades do ds-DNA, por isso apresentam uma maior afinidade para o hibrido (ds-DNA) que para sonda (ss-DNA) (Figura 12-B) (160).

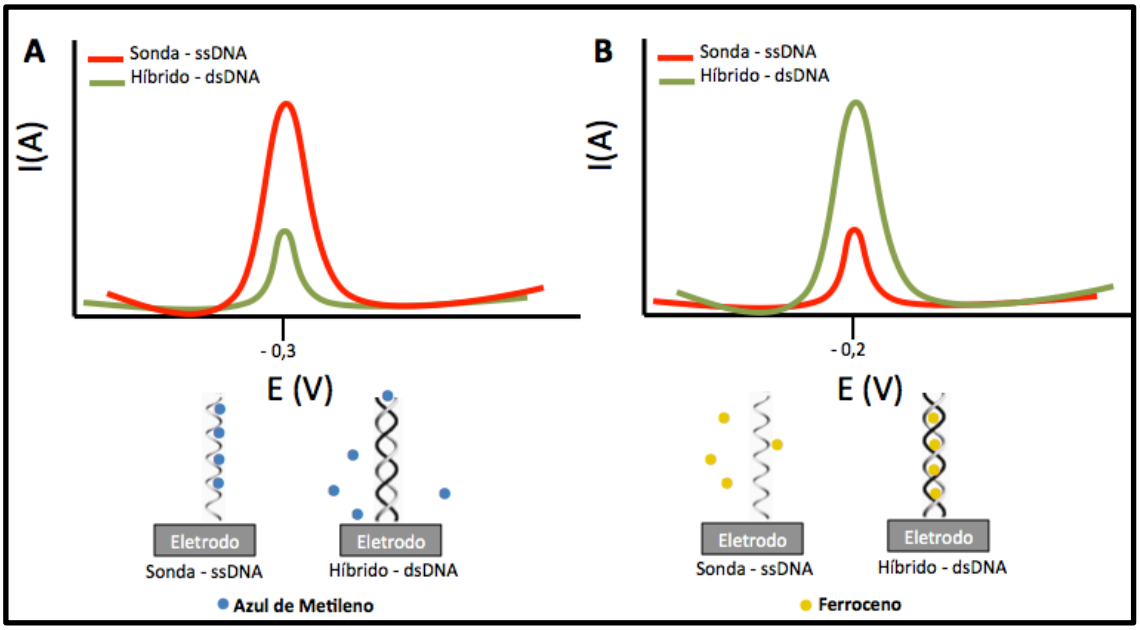

Figura 12. Representação esquemática do sinal de redução do: (A) azul de metileno em ss-DNA e ds-DNA; (B) ferroceno em ss-DNA e ds-DNA.

4.1 Referências

1. Nour NM. Cervical Cancer: A Preventable Death. Rev Obstet Gynecol. 2009;2(4):240–4.

2. Blitzer GC, Smith M a, Harris SL, Kimple RJ. Review of the clinical and biologic aspects of human papillomavirus-positive squamous cell carcinomas of the head and neck. Int J Radiat Oncol Biol Phys. Elsevier Inc.; 2014 Mar 15;88(4):761–70.

3. Miyahara GI, Simonato LE, Mattar NJ, Camilo Jr DJ, Biasoli ER. Correlation between koilocytes and human papillomavirus detection by PCR in oral and oropharynx squamous cell carcinoma biopsies. Mem Inst Oswaldo Cruz. 2011 Mar;106(2):166–9.

4. Echelman D, Feldman S. Management of cervical precancers: a global perspective. Hematol Oncol Clin North Am. Elsevier Inc; 2012 Feb;26(1):31–44.

5. Jin XW, Sikon A, Yen-Lieberman B. Cervical cancer screening: Less testing, smarter testing. Cleve Clin J Med. 2011 Nov;78(11):737–47.

6. Stanley M a. Genital human papillomavirus infections: current and prospective therapies. J Gen Virol. 2012 Apr;93(Pt 4):681–91.

7. Molijn A, Kleter B, Quint W, van Doorn L-J. Molecular diagnosis of human papillomavirus (HPV) infections. J Clin Virol. 2005 Mar;32 Suppl 1:S43–51.

8. Singh R, Sumana G, Verma R, Sood S, Pandey MK, Gupta RK, et al. DNA biosensor for detection of Neisseria gonorrhoeae causing sexually transmitted disease. J Biotechnol. 2010 Nov;150(3):357–65.

9. Ronkainen NJ, Halsall HB, Heineman WR. Electrochemical biosensors. Chem Soc Rev. 2010 May;39(5):1747–63.

10. Pohanka M, Skládal P. Electrochemical biosensors – principles and applications. J Appl Biomed. 2008;6:57–64.

11. Belluzo MS, Ribone MÉ, Lagier CM. Assembling Amperometric Biosensors for Clinical Diagnostics. Sensors (Peterboroug. 2008;8(3):1366–99.

12. De Villiers E-M, Fauquet C, Broker TR, Bernard H-U, zur Hausen H. Classification of papillomaviruses. Virology. 2004 Jun 20;324(1):17–27.

13. Bravo IG, de Sanjosé S, Gottschling M. The clinical importance of understanding the evolution of papillomaviruses. Trends Microbiol. 2010 Oct;18(10):432–8.

14. Bernard H, Burk RD, Chen Z, van Doorslaer K, Hausen H zur, de Villiers E-M. Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. Virology. Elsevier Inc.; 2010 May 25;401(1):70–9.

15. Howley PM. The papillomaviruses. Trans Am Clin Climatol Assoc. 2006;117(617):113–27.

16. Kimple AJ, Torres AD, Yang RZ, Kimple RJ. HPV-associated head and neck cancer: molecular and nano-scale markers for prognosis and therapeutic stratification. Sensors (Basel). 2012 Jan;12(4):5159–69.

17. Burk RD, Harari A, Chen Z. Human papillomavirus genome variants. Virology. Elsevier; 2013 Oct;445(1-2):232–43.

18. Bienkowska-Haba M, Sapp M. The cytoskeleton in papillomavirus infection. Viruses. 2011 Mar;3(3):260–71.

19. Sri JC, Dubina MI, Kao GF, Rady PL, Tyring SK, Gaspari A a. Generalized verrucosis: a review of the associated diseases, evaluation, and treatments. J Am Acad Dermatol. Elsevier Inc; 2012 Feb;66(2):292–311.

20. You J. Papillomavirus interaction with cellular chromatin. Biochim Biophys Acta. 2010;1799:192–9.

21. Tota JE, Chevarie-Davis M, Richardson L a., deVries M, Franco EL. Epidemiology and burden of HPV infection and related diseases: Implications for prevention strategies. Prev Med (Baltim). Elsevier B.V.; 2011 Oct;53:S12–S21.

22. Porterfield SP. Vertical Transmission of Human Papillomavirus From Mother to Fetus: Literature Review. J Nurse Pract. American College of Nurse Practitioners; 2011 Sep;7(8):665–70.

23. Freitas a C, Mariz FC, Silva M a R, Jesus a LS. Human Papillomavirus Vertical Transmission: Review of Current Data. Clin Infect Dis. 2013 Feb 27;1–6.

24. Onon TS. History of human papillomavirus, warts and cancer: what do we know today? Best Pract Res Clin Obstet Gynaecol. Elsevier Ltd; 2011 Oct;25(5):565–74.

25. Camara GNNDL, Cruz MR, Veras VS, Martins CRF. Os papilomavírus humanos – HPV : histórico , morfologia e ciclo biológico. Univ Ciências da Saúde. 2008;01:149–58.

26. Zheng, C.C., Baker Z. Papillomavirus genome structure, expression, and post-transcriptional regulation. Front Biosci. 2006;11:2286–302.

27. Mahdavi A, Monk BJ. Vaccines against human papillomavirus and cervical cancer: promises and challenges. Oncologist. 2005 Aug;10(7):528–38.

28. Bodily J, Laimins L a. Persistence of human papillomavirus infection: keys to malignant progression. Trends Microbiol. Elsevier Ltd; 2011 Jan;19(1):33–9.

29. Amador-Molina A, Hernández-Valencia JF, Lamoyi E, Contreras-Paredes A, Lizano M. Role of innate immunity against human papillomavirus (HPV) infections and effect of adjuvants in promoting specific immune response. Viruses. 2013 Nov;5(11):2624–42.

30. R. Souto, J.P.B. Falhari, A.D. da Cruz. O Papilomavírus Humano: um fator relacionado com a formação de neoplasias. Rev Bras Cancerol. 2005;51(2):155–60.

31. Guzmán-Olea E, Bermúdez-Morales VH, Peralta-Zaragoza O, Torres-Poveda K, Madrid-Marina V. Molecular Mechanism and Potential Targets for Blocking HPV-Induced Lesion Development. J Oncol. 2012 Jan;2012(Il):1–11.

32. Raybould R, Fiander A, Hibbitts S. Human Papillomavirus Integration and its Role in Cervical Malignant Progression. 2011;1–7.

33. Leto MDGP, Santos Júnior GF Dos, Porro AM, Tomimori J. Human papillomavirus infection: etiopathogenesis, molecular biology and clinical manifestations. An Bras Dermatol. 2011;86(2):306–17.

34. Moody C a, Laimins L a. Human papillomavirus oncoproteins: pathways to transformation. Nat Rev Cancer. Nature Publishing Group; 2010 Aug;10(8):550–60.

35. Oh J-M, Kim S-H, Lee Y-I, Seo M, Kim S-Y, Song Y-S, et al. Human papillomavirus E5 protein induces expression of the EP4 subtype of prostaglandin E2 receptor in cyclic AMP response element-dependent pathways in cervical cancer cells. Carcinogenesis. 2009 Jan;30(1):141–9.

36. De Marco F. Oxidative Stress and HPV Carcinogenesis. Viruses. 2013 Jan;5(2):708–31.

37. Boccardo E, Lepique AP, Villa LL. The role of inflammation in HPV carcinogenesis. Carcinogenesis. 2010 Nov;31(11):1905–12.

38. Gómez DT, Santos JL. Human papillomavirus infection and cervical cancer : pathogenesis and epidemiology. Commun Curr Res Educ Top Trends Appl Microbiol. 2007;680–8.

39. Ferraz LDC, Beatriz A, Santos R, Discacciati MG. Ciclo celular , HPV e evolução da neoplasia intraepitelial cervical : seleção de marcadores biológicos. J Heal Sci Ins. 2012;30:107–11.

40. Nevins JR. The Rb/E2F pathway and cancer. Hum Mol Genet. 2001 Apr;10(7):699–703.

41. Mclaughlin-drubin ME, Münger K. The Human Papillomavirus E7 Oncoprotein. Virology. 2009;384(2):335–40.

42. Gómez DT, Santos JL. Human papillomavirus infection and cervical cancer : pathogenesis and epidemiology. 2007;680–8.

43. Mammas IN, Sourvinos G, Giannoudis A, Spandidos D a. Human papilloma virus (HPV) and host cellular interactions. Pathol Oncol Res. 2008 Dec;14(4):345–54.

44. Feller L, Wood NH, Khammissa R a G, Lemmer J. Human papillomavirus-mediated carcinogenesis and HPV-associated oral and oropharyngeal squamous cell carcinoma. Part 1: human papillomavirus-mediated carcinogenesis. Head Face Med. 2010 Jan;6:14–9.

45. Hu J, Cladel NM, Wang Z, Han R, Pickel MD, Christensen ND. GM-CSF enhances protective immunity to cottontail rabbit papillomavirus E8 genetic vaccination in rabbits. Vaccine. 2004 Mar 12;22(9–10):1124–30.

46. Melsheimer P, Kaul S, Ph D, Dobeck S. Immunocytochemical Detection of Human Papillomavirus High Risk Type L1 Capsid Proteins in LSIL and HSIL As Compared with Detection of HPV L1 DNA. Acta Cytol. 2003;47:124–8.

47. Hoory T, Monie A, Gravitt P, Wu T-C. Molecular Epidemiology of Human Papillomavirus. J Formos Med Assoc. 2008 Mar;107(3):198–217.

48. Horvath C a J, Boulet G a V, Renoux VM, Delvenne PO, Bogers J-PJ. Mechanisms of cell entry by human papillomaviruses: an overview. Virol J. 2010 Jan;7:1–7.

49. Stünkel W, Bernard HU. The chromatin structure of the long control region of human papillomavirus type 16 represses viral oncoprotein expression. J Virol. 1999 Mar;73(3):1918–30.

50. Muñoz N, Castellsagué X, de González AB, Gissmann L. Chapter 1: HPV in the etiology of human cancer. Vaccine. 2006 Aug 31;24 Suppl 3:S3/1–10.

51. Adefuye A, Sales K. Regulation of Inflammatory Pathways in Cancer and Infectious Disease of the Cervix. Scientifica (Cairo). 2012;2012.

52. Letian T, Tianyu Z. Cellular receptor binding and entry of human papillomavirus. Virol J. 2010 Jan;7:1–7.

53. Woodman CBJ, Collins SI, Young LS. The natural history of cervical HPV infection: unresolved issues. Nat Rev Cancer. Nature Publishing Group; 2007;7(1):11–22.

54. Saavedra KP, Brebi PM, Roa JCS. Epigenetic alterations in preneoplastic and neoplastic lesions of the cervix. Clin Epigenetics. 2012 Jan;4(1):1–7.

55. Williams GH, Stoeber K. The cell cycle and cancer. J Pathol. 2012 Jan;226(2):352–64.

56. Williams VM, Filippova M, Soto U, Duerksen-Hughes PJ. HPV-DNA integration and carcinogenesis: putative roles for inflammation and oxidative stress. Future Virol. 2011;6(1):45–57.

57. Feller L, Wood NH, Khammissa R a G, Lemmer J. Human papillomavirus-mediated carcinogenesis and HPV-associated oral and oropharyngeal squamous cell carcinoma. Part 1: human papillomavirus-mediated carcinogenesis. Head Face Med. 2010 Jan;6:14.

58. Dochez C, Bogers JJ, Verhelst R, Rees H. HPV vaccines to prevent cervical cancer and genital warts: an update. Vaccine. Elsevier Ltd; 2014 Mar 20;32(14):1595–601.

59. Banura C, Mirembe FM, Katahoire AR, Namujju PB, Mbidde EK. Universal routine HPV vaccination for young girls in Uganda: a review of opportunities and potential obstacles. Infect Agent Cancer. Infectious Agents and Cancer; 2012 Jan;7(1):24.

60. WHO Guidance Note. Comprehensive cervical cancer prevention and control : a healthier future for girls and women. 2013.

61. INCA. Estimativa 2014: Incidência de Câncer no Brasil. Rio de Janeiro; 2014.

62. Stanley M. Pathology and epidemiology of HPV infection in females. Gynecol Oncol. Elsevier B.V.; 2010 May;117(2 Suppl):S5–10.

63. Rosa DD, Bozzeti MC, Silva FR, Silva BR. Papilomavírus humano e neoplasia cervical Human papillomavirus and cervical neoplasia. Cad Saúde Pública. 2009;25(5):953–64.

64. Annunziata C, Buonaguro L, Buonaguro FM, Tornesello ML. Characterization of the Human Papillomavirus (HPV) Integration Sites into Genital Cancers. Pathol Oncol Res. 2012 Mar 27;

65. Machalek D a, Poynten M, Jin F, Fairley CK, Farnsworth A, Garland SM, et al. Anal human papillomavirus infection and associated neoplastic lesions in men who have sex with men: a systematic review and meta-analysis. Lancet Oncol. Elsevier Ltd; 2012 May;13(5):487–500.

66. Tomljenovic L, Shaw CA, Spinosa JP. Human Papillomavirus (HPV) Vaccines as an Option for Preventing Cervical Malignancies : (How) Effective and Safe ? Curr Pharm Des. 2013;19:1–22.

67. Zarchi MK, Behtash N, Chiti Z, Kargar S. MINI-REVIEW Cervical Cancer and HPV Vaccines in Developing Countries. Asian Pacific J Cancer Prev. 2009;10:2001–6.

68. Nieto K, Salvetti A. AAV Vectors Vaccines Against Infectious Diseases. Front Immunol. 2014 Jan;5(January):5.

69. Giraldo PC, José M, Silva PMA, Fedrizzi EN, Gonçalves AKS, Amaral RLG, et al. Prevenção da infecção por hpv e lesões associadas com o uso de vacinas. DST – J bras Doenças Sex Transm. 2008;20(2):132–40.

70. C.F. Hung, B. Ma, A. Monie, S.W. Tsen TCW. Therapeutic human papillomavirus vaccines: current clinical trials and future directions. Expert Opin Biol Ther. 2008;8(4):421–39.

71. S.M. Garland JSS. Human papillomavirus vaccines: current status and future prospects. Drugs. 2010;70:1079–98.

72. Bonanni P, Boccalini S, Bechini A. Efficacy, duration of immunity and cross protection after HPV vaccination: a review of the evidence. Vaccine. 2009 May 29;27 Suppl 1:A46–53.

73. Huang C, Monie A, Weng W, Wu TC. Review Article DNA vaccines for cervical cancer. 2010;2(1):75–87.

74. Nigam A, Saxena P, Acharya AS, Mishra A, Batra S. HPV Vaccination in India: Critical Appraisal. ISRN Obstet Gynecol. 2014;2014:1–5.

75. Ma B, Xu Y, Hung C, Wu T. HPV and Therapeutic Vaccines : Where are We in 2010 ? Curr Cancer Ther Rev. 2010;6(410):81–103.

76. Basu P, Banerjee D, Singh P, Bhattacharya C, Biswas J. Efficacy and safety of human papillomavirus vaccine for primary prevention of cervical cancer: A review of evidence from phase III trials and national programs. South Asian J cancer. 2013 Oct;2(4):187–92.

77. Cho H-J, Oh Y-K, Kim YB. Advances in human papilloma virus vaccines: a patent review. Expert Opin Ther Pat. 2011 Mar;21(3):295–309.

78. Papanicolaou, G.N., H.F. Traut. The Diagnostic Value of Vaginal Smears in Carcinoma of the Uterus. Am J Obstet Gynecol. 1941;42:193–206.

79. Cuzick J, Arbyn M, Sankaranarayanan R, Tsu V, Ronco G, Mayrand M-H, et al. Overview of human papillomavirus-based and other novel options for cervical cancer screening in developed and developing countries. Vaccine. 2008 Aug 19;26 Suppl 1:K29–41.

80. Rocha GA, Melo VH. Biologia molecular no rastreamento das neoplasias cervicais uterinas. Feminina. 2010;38:1–6.

81. Pias ADA, Regina V, Vargas A. Avaliação dos exames citológicos de papanicolaou com células epitelais atípicas e respectivos exames colposcópicos com relação aos exames histopatológicos. Rev Bras Análises Clínicas. 2009;41(2):155–60.

82. Schiffman M, Wentzensen N, Wacholder S, Kinney W, Gage JC, Castle PE. Human papillomavirus testing in the prevention of cervical cancer. J Natl Cancer Inst. 2011 Mar 2;103(5):368–83.

83. Zard E, Arnaud L, Mathian A, Chakhtoura Z, Hie M, Touraine P, et al. Increased risk of high grade cervical squamous intraepithelial lesions in systemic lupus erythematosus: A meta-analysis of the literature. Autoimmun Rev. Elsevier B.V.; 2014 Jul;13(7):730–5.

84. Chow LT, Broker TR, Steinberg BM. The natural history of human papillomavirus infections of the mucosal epithelia. APMIS. 2010 Jun;118(6-7):422–49.

85. Bhatla N, Moda N. The clinical utility of HPV DNA testing in cervical cancer screening strategies. Indian J Med Res. 2009 Sep;130(3):261–5.

86. Burk RD, Chen Z, Van Doorslaer K. Human papillomaviruses: genetic basis of carcinogenicity. Public Health Genomics. 2009 Jan;12(5-6):281–90.

87. Dillner J. The serological response to papillomaviruses. Semin Cancer Biol. 1999;9(6):423–30.

88. Wang K-L. Human papillomavirus and vaccination in cervical cancer. Taiwan J Obstet Gynecol. 2007 Dec;46(4):352–62.

89. Kroupis C, Vourlidis N. Human papilloma virus (HPV) molecular diagnostics. Clin Chem Lab Med. 2011 Nov;49(11):1783–99.

90. Abreu ALP, Souza RP, Gimenes F, Consolaro MEL. A review of methods for detect human Papillomavirus infection. Virol J. 2012 Jan;9:262–71.

91. Nascimento G a, Souza EVM, Campos-Ferreira DS, Arruda MS, Castelletti CHM, Wanderley MSO, et al. Electrochemical DNA biosensor for bovine papillomavirus detection using polymeric film on screen-printed electrode. Biosens Bioelectron. 2012 Oct;38(1):61–6.

92. Campos-Ferreira DS, Nascimento G a, Souza EVM, Souto-Maior M a, Arruda MS, Zanforlin DML, et al. Electrochemical DNA biosensor for human papillomavirus 16 detection in real samples. Anal Chim Acta. Elsevier B.V.; 2013 Dec 4;804:258–63.

93. Sin MLY, Mach KE, Wong PK, Liao JC. Advances and challenges in biosensor-based diagnosis of infectious diseases. Expert Rev Mol Diagn. Informa UK, Ltd.; 2014 Mar;14(2):225–44.

94. Krejcova L, Hynek D, Michalek P, Milosavljevic V, Kopel P. Electrochemical Sensors and Biosensors for Influenza Detection – Literature Survey 2012-2013. 2014;9:3440–8.

95. Bora U, Sett A, Singh D. Nucleic Acid Based Biosensors for Clinical Applications. 2013;1(1):1–8.

96. Johnson BN, Mutharasan R. Biosensor-based microRNA detection: techniques, design, performance, and challenges. Analyst. 2014 Apr 7;139(7):1576–88.

97. Kaur A, Verma M, Singh K. Biosensor and its Clinical Application. Int J Adv Res. 2013;1(3):108–18.

98. Gil EDS, Melo GR De. Electrochemical biosensors in pharmaceutical analysis. Brazilian J Pharm Sci. 2010 Sep;46(3):375–91.

99. Tamayo J, Kosaka PM, Ruz JJ, San Paulo Á, Calleja M. Biosensors based on nanomechanical systems. Chem Soc Rev. 2012 Feb 7;42(3):1287–311.

100. Solanki PR, Kaushik A, Agrawal V V., Malhotra BD. Nanostructured metal oxide-based biosensors. NPG Asia Mater. 2011 Jan;3(1):17–24.

101. Liu S, Guo X. Carbon nanomaterials field-effect-transistor-based biosensors. NPG Asia Mater. Nature Publishing Group; 2012 Aug;4(8):e23.

102. Arya SK, Saha S, Ramirez-vick JE, Gupta V, Bhansali S, Singh SP. Analytica Chimica Acta Recent advances in ZnO nanostructures and thin films for biosensor applications : Review. Anal Chim Acta. 2012;737:1–21.

103. Bohunicky B, Mousa S. Biosensors: the new wave in cancer diagnosis. Nanotechnol Sci Appl. 2011 Dec;4:1–10.

104. Dhull V, Gahlaut A, Dilbaghi N, Hooda V. Acetylcholinesterase biosensors for electrochemical detection of organophosphorus compounds: a review. Biochem Res Int. 2013 Jan;2013:731501.

105. Kirsch J, Siltanen C, Zhou Q, Revzin A, Simonian A. Biosensor technology: recent advances in threat agent detection and medicine. Chem Soc Rev. 2013 Nov 21;42(22):8733–68.

106. Sharma H, Agarwal M, Goswami M, Sharma A, Roy SK, Rai R, et al. Biosensors: tool for food borne pathogen detection. Vet World. 2013 Dec 5;6(12):968–73.

107. Cock LS, Arenas AMZ, Aponte AA. Use of enzymatic biosensors as quality indices : a synopsis of present and future trends in the food industry. Chil J Agric Res. 2009;69(2):270–80.

108. Louzada ES, Luccas PO, Magalhães CS. Construção E Caracterização De Um Biossensor Potenciométrico Para Determinação De Pirogalol. Rev Anal. 2004;2:52–6.

109. Monošík R, Streďanský M, Šturdík E. Biosensors - classification, characterization and new trends. Acta Chim Slovaca. 2012 Apr 1;5(1):109–20.

110. Murugaiyan SB, Ramasamy R, Gopal N, Kuzhandaivelu V. Biosensors in clinical chemistry : An overview. Adv Biomed Res. 2014;2:1–14.

111. Taguchi M, Ptitsyn A, McLamore ES, Claussen JC. Nanomaterial-mediated Biosensors for Monitoring Glucose. J Diabetes Sci Technol. 2014 Mar 2;8(2):403–11.

112. Liu J, Chen C-F, Chang C-W, DeVoe DL. Flow-through immunosensors using antibody-immobilized polymer monoliths. Biosens Bioelectron. 2010 Sep 15;26(1):182–8.

113. Ramírez NB, Salgado AM, Valdman B. The evolution and developments of immunosensors for health and environmental monitoring : problems and perspectives. Brazilian J Chem Eng. 2009;26(02):227–49.

114. Ricci F, Volpe G, Micheli L, Palleschi G. A review on novel developments and applications of immunosensors in food analysis. Anal Chim Acta. 2007 Dec 19;605(2):111–29.

115. Su L, Jia W, Hou C, Lei Y. Microbial biosensors: a review. Biosens Bioelectron. 2011 Jan 15;26(5):1788–99.

116. Lei Y, Chen W, Mulchandani A. Microbial biosensors. Anal Chim Acta. 2006 May 24;568(1-2):200–10.

117. Dai C, Choi S. Technology and Applications of Microbial Biosensor. 2013;2013(August):83–93.

118. D'Souza SF. Microbial biosensors. Biosens Bioelectron. 2001 Aug;16(6):337–53.

119. Hvastkovs EG, Buttry D a. Recent advances in electrochemical DNA hybridization sensors. Analyst. 2010 Aug;135(8):1817–29.

120. Tichoniuk M, Ligaj M, Filipiak M. Application of DNA Hybridization Biosensor as a Screening Method for the Detection of Genetically Modified Food Components. Sensors. 2008 Mar 27;8(4):2118–35.

121. Thévenot DR, Toth K, Durst RA, Wilson G. Electrochemical Biosensors : Recommended Definitions and classification (Technical Report). Pure Appl Chem. 1999;71(12):2333–48.

122. Lee W, Lee J, Koh J. Development and applications of chip calorimeters as novel biosensors. Nanobiosensors Dis Diagnosis. 2012;1:17–29.

123. Pedrero M, Campuzano S, Pingarrón JM. Electrochemical Biosensors for the Determination of Cardiovascular Markers: a Review. Electroanalysis. 2014 Jun 11;26(6):1132–53.

124. Grieshaber D, MacKenzie R, Voros J, Reimhult E. Electrochemical Biosensors - Sensor Principles and Architectures. Sensors. 2008;8:1400–58.

125. Guan J-G, Miao Y-Q, Zhang Q-J. Impedimetric biosensors. J Biosci Bioeng. 2004 Jan;97(4):219–26.

126. Wang Y, Ye Z, Ying Y. New trends in impedimetric biosensors for the detection of foodborne pathogenic bacteria. Sensors (Basel). 2012 Jan;12(3):3449–71.

127. Wang Y, Xu H, Zhang J, Li G. Electrochemical Sensors for Clinic Analysis. Sensors. 2008;8:2043–81.

128. Privett BJ, Shin JH, Schoenfisch MH. Electrochemical sensors. Anal Chem. 2010 Jun 15;82(12):4723–41.

129. Sadik O a, Aluoch AO, Zhou A. Status of biomolecular recognition using electrochemical techniques. Biosens Bioelectron. 2009 May 15;24(9):2749–65.

130. Pacheco WF, Semaan FS, Almeida VGK De, Ritta AGS., Aucélio R. Voltametrias : Uma Breve Revisão Sobre os Conceitos. Rev Virtual Química. 2013;5(4):516–37.

131. Bard AJ, Faulkner LR. Electrochemical methods: Fundamentals and Applications. 2nd ed. New York: John Wiley & Sons, Inc; 2001.

132. Scholz F. Electroanalytical Methods: Guide to Experiments and Applications. Springer; 2010.

133. Manea F. Electrochemical Techniques for Characterization and Detection Application of Nanostructured Carbon Composite. Modern Electrochemical Methods in Nano, Surface and Corrosion Science. InTech; 2014. p. 33–54.

134. Labuda J, Brett AMO, Evtugyn G, Fojta M, Mascini M, Ozsoz M, et al. Electrochemical nucleic acid-based biosensors: Concepts, terms, and methodology (IUPAC Technical Report). Pure Appl Chem. 2010 Apr 20;82(5):1161–87.

135. Teles FRR, Fonseca LP. Trends in DNA biosensors. Talanta. 2008 Dec 15;77(2):606–23.

136. Paleček E, Bartošík M. Electrochemistry of nucleic acids. Chem Rev. 2012 Jun 13;112(6):3427–81.

137. Paniel N, Baudart J, Hayat a, Barthelmebs L. Aptasensor and genosensor methods for detection of microbes in real world samples. Methods. Elsevier Inc.; 2013 Dec 15;64(3):229–40.

138. Pei H, Zuo X, Pan D, Shi J, Huang Q, Fan C. Scaffolded biosensors with designed DNA nanostructures. NPG Asia Mater. Nature Publishing Group; 2013 Jun;5(6):e51.

139. Wang J. DNA biosensors based on Peptide Nucleic Acid (PNA) recognition layers. A review. Biosens Bioelectron. 1998 Oct;13(7–8):757–62.

140. Porcheddu A, Giacomelli G. Peptide nucleic acids (PNAs), a chemical overview. Curr Med Chem. 2005 Jan;12(22):2561–99.

141. Song S, Wang L, Li J, Fan C, Zhao J. Aptamer-based biosensors. TrAC Trends Anal Chem. 2008 Feb;27(2):108–17.

142. Hernandez FJ, Ozalp VC. Graphene and Other Nanomaterial-Based Electrochemical Aptasensors. Biosensors. 2012 Jan 13;2(1):1–14.

143. Santosh B, Yadava PK. Nucleic Acid Aptamers : Research Tools in Disease Diagnostics and Therapeutics. Biomed Res Int. 2014;2014:1–13.

144. Zhou J, Battig MR, Wang Y. Aptamer-based molecular recognition for biosensor development. Anal Bioanal Chem. 2010 Nov;398(6):2471–80.

145. Palchetti I, Mascini M. Electrochemical nanomaterial-based nucleic acid aptasensors. Anal Bioanal Chem. 2012 Apr;402(10):3103–14.

146. Oliveira JE, Consolin-Filho N, Paterno LG, Mattoso LHC, Medeiros ES. Uso de polímeros condutores em sensores. Parte 3: Biossensores. Rev Eletrônica Mater e Process. 2013;1:1–11.

147. Pournaghi-Azar MH, Hejazi MS, Alipour E. Developing an electrochemical deoxyribonucleic acid (DNA) biosensor on the basis of human interleukine-2 gene using an electroactive label. Anal Chim Acta. 2006 Jun 16;570(2):144–50.

148. Sassolas A, Leca-Bouvier BD, Blum LJ. DNA biosensors and microarrays. Chem Rev. 2008 Jan;108(1):109–39.

149. Ahuja T, Mir IA, Kumar D, Rajesh. Biomolecular immobilization on conducting polymers for biosensing applications. Biomaterials. 2007 Feb;28(5):791–805.

150. Atta NF, Galal A, El-Ads EH. A novel sensor of cysteine self-assembled monolayers over gold nanoparticles for the selective determination of epinephrine in presence of sodium dodecyl sulfate. Analyst. 2012 Jun 7;137(11):2658–68.

151. Yang W, Gooding JJ, Hibbert DB. Characterisation of gold electrodes modified with self-assembled monolayers of L -cysteine for the adsorptive stripping analysis of copper. J Electroanal Chem. 2001;516:10–6.

152. Wang W. Electrochemical investigation on the film of L-cysteine self-assembled to nanoparticles on a gold electrode. J Biophys Chem. 2012;03(01):39–43.

153. Pedrero M, Campuzano S, Pingarrón JM. Electrochemical genosensors based on PCR strategies for microorganisms detection and quantification. Anal Methods. 2011;3(4):780 –789.

154. Hvastkovs EG, Buttry D a. Recent advances in electrochemical DNA hybridization sensors. Analyst. 2010 Aug;135(8):1817–29.

155. Tosar JP, Brañas G, Laíz J. Electrochemical DNA hybridization sensors applied to real and complex biological samples. Biosens Bioelectron. 2010 Dec 15;26(4):1205–17.

156. Ferapontova E. Electrochemical Indicators for DNA Electroanalysis. Curr Anal Chem. 2011 Jan 1;7(1):51–62.

157. Pan D, Zuo X, Wan Y, Wang L, Zhang J, Song S, et al. Electrochemical Interrogation of Interactions between Surface-Confined DNA and Methylene Blue. Sensors. 2007;7:2671–80.

158. Ortiz M, Fragoso A, Ortiz PJ, O'Sullivan CK. Elucidation of the mechanism of single-stranded DNA interaction with methylene blue: A spectroscopic approach. J Photochem Photobiol A Chem. Elsevier B.V.; 2011 Feb;218(1):26–32.

159. Farjami E, Clima L, Gothelf V, Ferapontova EE. DNA interactions with a Methylene Blue redox indicator depend on the DNA length and are sequence specific †. 2010;1443–8.

160. Teles FRR, França dos Prazeres DM, Lima-Filho JL. Electrochemical Detection of a Dengue-related Oligonucleotide Sequence Using Ferrocenium as a Hybridization Indicator. Sensors. 2007 Oct 30;7(11):2510–8.

Capítulo II

Artigo Científico I

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ORIGINAL ARTICLE

Electrochemical DNA biosensor for the detection of human papillomavirus E6 gene inserted in recombinant plasmid

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KEYWORDS

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Direct DNA detection;
Inosine-probe;
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Electrophoresis standard method

Abstract In the current study, we describe a novel, simple, inexpensive, sensitive, specific, stable and label-free electrochemical DNA biosensor used to identify a target gene cloned into a plasmid. The biosensor was designed with a 23-mer oligonucleotide of guanine-free, which was immobilized on the pencil graphite electrode (PGE) for E6 gene detection from human papillomavirus 16 type (HPV16). The E6 gene was used due to its clinical importance. The optimal probe concentration was obtained in 500 nM. The hybridization detection showed a good linearity in the range of 40–5,000 pg/μL with a detection limit of 16 pg/μL. The electrochemical method showed higher sensitivity and specificity when compared with the agarose gel electrophoresis assay. This technology could be postulated as a new and attractive alternative for cloning analysis in plasmids.

1. Introduction

There are over 160 known types of human papillomavirus (HPV) (Burk et al., 2013), of which 40 can infect the anogenital epithelium and of these 15 are considered oncogenic (Lin et al., 2010). The HPV 16 and 18, classified as high-risk types (HR), are responsible for approximately 60–80% of cervical cancer occurrence worldwide (Carter et al., 2011; Hendry et al., 2013).

Several studies show that HPV encodes two powerful oncogenes, E6 and E7. These oncogenes are constantly expressed in the HR-HPV and are responsible for the malignant

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transformation of cervical cancer (Azam and Shams-ul-Islam, 2010; Boccardo et al., 2010; Carter et al., 2011; Stanley, 2010; Vici et al., 2014). Therefore, the E6 and E7 genes represent the ideal targets for development of therapeutic vaccines, which potentially eliminate pre-existing lesions and malignant tumors by generating cellular immunity against HPV-infected cells (Huang et al., 2010; Kawana et al., 2012; Nieto and Salvetti, 2014).

Progress in the molecular cloning techniques has enabled the relatively quick, easy and cheap manufacture of recombinant vector vaccines (Huang et al., 2010; Hung et al., 2008). Recombinant vector vaccines have many advantages over conventional vaccines and may provide a technological solution for microorganisms that have difficulty growing in cell culture or animal models, like HPV (Ferraro et al., 2011; Lin et al., 2010; Ma et al., 2010).

The most popular technique used for molecular cloning analysis is the electrophoresis method in agarose gels. However, this technique requires well-trained personnel and is time-consuming (Chang et al., 2013; Teles and Fonseca, 2008).

DNA biosensors are commonly employed to detect a specific DNA sequence (Pei et al., 2013; Tosar et al., 2010), but less explored for cloning analysis. In the current study we describe a novel, simple, inexpensive, stable and label-free DNA electrochemical biosensor for the detection of HPV 16 E6 gene cloned in the expression vector.

2. Materials and methods

2.1. Materials

Escherichia coli DH5α (Invitrogen – USA) and pGEM-T Easy (pGEM-T) (Promega – USA) were used as host and cloning vectors, respectively. Bacterial growth was performed in a Luria–Bertani (LB) medium (tryptone 10 g/L, yeast extract 5 g/L and NaCl 10 g/L, pH 7.0), supplemented with 100 mg/L ampicillin, 160 μg/mL X-gal and 0.5 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside). T_4 DNA ligase, DNA size marker and *EcoRI*, *XbaI* and *ApaI* restriction enzymes were purchased from Invitrogen – USA. DNA polymerase and QIAquick PCR Purification Kit were supplied from Clontech Company – USA and QIAgen – Germany, respectively.

Pencil lead (type 4B), commonly composed of natural graphite, a polymeric binder and clay in different percentages, was used as pencil graphite electrode (PGE).

The 23-mer, guanine-free oligonucleotide (5′-ATI CAC CAA AAI AIA ACT ICA AT-3′, purchased from Integrated DNA Technologies – USA), correspondent to the sense strand of the HPV 16 E6 gene, was employed as HPV probe.

The oligonucleotide solutions and dilute solutions of the plasmids were prepared with 0.5 M acetate buffer (pH 5.0) and kept frozen.

2.2. Construction of recombinant bacteria

The pBR322 vector, containing the HPV 16 gene, was used as the backbone for construction of the pGEM-T. E6 amplification was performed using 20 ng DNA; 10 pmol of specific primers: FE6*EcoRI* (5′-GCT GAA TTC ATG CAC CAA-3′) and RE6*XbaI* (5′CGT TCT AGA ATC AGC TGG GT-3′); 2.5 μL Buffer Taq Platinum 10×; 0.5 μL Platinum TaqPol (5 U/μL); 0.5 μL $MgCl_2$ (50 mM); and 0.5 μL dNTP (40 μM).

Amplifications were carried out in Rotor Gene 6.0 (Applied Biosystems – USA) with the following settings: 95 °C for 1 min, followed by 40 cycles at 95 °C for 30 s, 68 °C for 20 s (touchdown each 5 cycles) and 72 °C for 1 min, ending with 72 °C for 1 min. The amplified products were subjected to agarose electrophoresis (Sambrook et al., 1989) and purified with QIAquick PCR Purification Kit.

E6 amplicon was cloned into pGEM-T following the manufacturer's instructions. The vector was transformed in *E. coli* DH5α competent cells by heat shock and incubated at 37 °C for 16 h (Sambrook et al., 1989). Recombinant colonies containing pGEM-T were identified through an ampicillin-resistance medium. The plasmid was extracted from the positive recombinant colonies by alkaline lysis and later analyzed by agarose gel electrophoresis (Sambrook et al., 1989). The *EcoRI* and *XbaI* restriction enzymes were used to liberate the E6 gene from the pGEM-T/E6 and *ApaI* was used to linearize the recombinant vector.

E6 gene sequence was confirmed using DYEnamic™ ET Dye Terminators in an automated DNA sequencing system (MegaBACE 750, GE, Life Science – USA). The electropherogram was analyzed using the base calling algorithm by Sequence Analyzer software (GE, Life Science – USA). The sequence obtained was submitted to BLASTN (NCBI – National Center for Biotechnology Information) for comparison with the E6 gene sequence present in NCBI databank.

2.3. Apparatus

The electrochemical experiments were performed using the AUTOLAB PGSTAT 30 (METROHM AUTOLAB – Netherlands) and the GPES 4.9 software package. Voltammetric signals were measured using a system consisting of two electrodes (Ronkainen et al., 2010; Wang et al., 2008). The pencil graphite was used as a working electrode and Ag/AgCl was used as a reference electrode. The working electrode had a surface area of 28 mm^2 that corresponded to electrochemical area. The working electrode was polished with an emery-impregnated disk to obtain a smooth surface. The reference electrode was produced by screen-printing in silver-ink (ELECTRODAG – Acheson – USA) on a gold wire, which was then dried at 60 °C.

2.4. Activation of PGE

The electrochemical activation of the surface polished PGEs was carried out applying a fixed potential of +1.8 V in 0.5 M acetate buffer solution (pH 5.0), no stirring, for 5 min (Hejazi et al., 2007; Pournaghi-Azar et al., 2006; Souza et al., 2011).

2.5. Immobilization of the probe on the PGE

The probe immobilization was achieved on the activated PGE by applying a fixed potential of +0.5 V, for 5 min in 0.5 M acetate buffer solution (pH 5.0), no stirring (Hejazi et al., 2007; Pournaghi-Azar et al., 2006; Souza et al., 2011).

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2.6. Hybridization

The diluted solutions of the undigested pGEM-T/E6 were denatured at 95 °C, for 5 min, and then immersed in an ice bath for 1 min (Hejazi et al., 2008). The hybridization was performed by immersing the probe-modified PGE into the eppendorf tube, containing 70 μL of the diluted solutions of undigested pGEM-T/E6 at 55 °C, for 5 min. The same protocol was applied for the hybridization of the probe with non-complementary target (undigested pGEM-T/E7), mix target (undigested pGEM-T/E6 and undigested pGEM-T/E7) and in the blank solution, which was composed by 0.5 M buffer acetate (pH 5.0).

2.7. Electrochemical measurements

The electrochemical behavior of the PGE surface was studied using the differential pulse voltammetry (DPV) technique in 20 mM Tris–HCl buffer (pH 7.0) (Hejazi et al., 2007; Pournaghi-Azar et al., 2006; Pournaghi-Azar et al., 2009; Souza et al., 2011). A scanning of the electrode potential was held between +0.3 V and +1.2 V, at a pulse amplitude of 50 mV and a scan rate of 20 mV s^{-1}. The results were treated by means of the GPES software moving average baseline correction, using a "peak width" of 0.01. All the experiments were performed in triplicate at room temperature (23 °C).

2.8. Statistics analysis

Experimental data were analyzed by STATISTICA 8 software. The one-way analysis of variance (ANOVA) was carried out to determine the existence of statistical differences between the samples, then followed by Tukey's post hoc parametric test (Chan, 2003; Montgomery, 2000; Tukey, 1991). A p value of <0.05 was considered significant.

3. Results and discussion

3.1. Construction of recombinant plasmid pGEM-T/E6

The pBR322 vector containing complete genome of HPV16 was used as template DNA for amplification of the E6 gene. The primers used for amplification contained the appropriate endonuclease restriction sites for posterior cloning into the vector (FE6*EcoRI* and RE6*XbaI*). The PCR product (E6 amplicon) was size fractionated using gel electrophoresis. The presence of a 477 bp DNA band confirmed the E6 gene amplification (Fig. S1-A in supplementary information). The E6 amplicon was sequenced to confirm the integrity of the amplification. The nucleotide sequencing was submitted to the BLAST program (NCBI) and showed 100% similarity with the E6 sequence of HPV 16 deposited in the GenBank.

The amplified DNA (E6 amplicon) was ligated to pGEM-T, and the products of the ligation reaction (pGEM-T/E6) were used to transform *E. coli* DH5α competent cells. Plasmid pGEM-T carries genes that provide resistance to ampicillin (Bury-Moné et al., 2009). Thus, the bacteria were plated on solid LB medium supplemented with ampicillin in order to allow for its growth. The bacterial colonies grown indicated that these bacteria incorporated the plasmid. The plasmid extracted from the positive clones was subjected to enzymatic digestion. Digestion of the plasmid with *EcoRI* and *XbaI* confirmed the presence of the DNA inserted (477 bp) in the pGEM-T/E6. According to the gel electrophoresis, the pGEM-T/E6 contained the desired insert (3495 bp), since the E6 gene fragment observed on the gel was of approximately 477 bp, and the pGEM-T vector had a length of approximately 3,018 bp (Fig. S1-B in supplementary information).

3.2. Electrochemical detection of pGEM-T/E6

3.2.1. Influence of the pretreatment on PGE surface

Fig. S2 (supplementary information) shows the DPV voltammograms obtained in 20 mM Tris–HCl buffer (pH 7.0) for non-activated bare PGE, E6 probe immobilized on the non-activated PGE, activated bare PGE, and E6 probe immobilized on the activated PGE.

The electrode surface was pretreated applying a potential of +1.8 V for 5 min in order to obtain a more sensitive and stable analytical signal (Hejazi et al., 2007; Pournaghi-Azar et al., 2006; Souza et al., 2011). Then, the inosine-modified probe (guanine-free) was immobilized on the non-activated and activated PGE. The probe immobilization on the electrode surface occurred by adsorption, which is considered the simplest method to immobilize DNA because it does not require special reagents or nucleic acid modifications (Pividori et al., 2000; Souza et al., 2011).

The inosine oxidation signal was around +0.7 V, well separated from that of the guanine (+0.9 to +1.07 V) (Berti et al., 2009; Palecek, 1960; Tosar et al., 2010; Wang et al., 1998, 2001; Wang and Zhou, 2002). Inosine is a base, which is not naturally present in DNA but can also base-pair with cytosine, as guanine does (Tosar et al., 2010). This fact should be considered when performing detection based on the guanine oxidation signal in real samples (Tosar et al., 2010).

As seen in Fig. S2, no significant difference was observed among the current peaks of the non-activated bare electrode, the activated bare electrode and the probe-modified non-activated electrode. While the signal of probe-modified activated electrode was significantly higher than signals of three other electrodes, which indicate that probe was successfully immobilized on the surface of activated electrode.

These results clearly demonstrate that the application of the potential (+1.8 V) on the PGE surface exerts a positive effect on the adsorption of the oligonucleotides (Souza et al., 2011). The pretreatment of the carbon surface increases its roughness and hydrophilicity, thus facilitating the electrodes' adsorption to DNA (Pividori et al., 2000; Sabzi et al., 2008; Souza et al., 2011; Wang et al., 1996). So, all experiments were performed on activated PGE.

3.2.2. Effect of probe concentration

The effect of the probe concentration is shown in Fig. 1. Different concentrations of the E6 probe (125–2,000 nM) were immobilized on the activated working electrode surface by applying a fixed potential of +0.5 V in acetate buffer (pH 5.0), for 5 min (Hejazi et al., 2007; Pournaghi-Azar et al., 2006; Souza et al., 2011). This potential improves the stability of the immobilized probe onto carbon surface because it increases the electrostatic attraction between the positively charged carbon surface and the negatively charged hydrophilic

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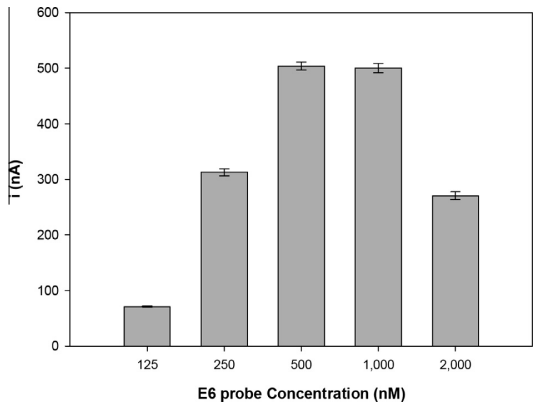

Figure 1 Current peaks of the guanine oxidation signal with different concentrations of the probe analyzed ($n = 3$). Various concentrations of E6 probe modified activated PGE (125, 250, 500, 1,000 and 2,000 nM). The oxidation signal was obtained by differential pulse voltammetry in 20 mM Tris–HCl buffer (pH 7.0).

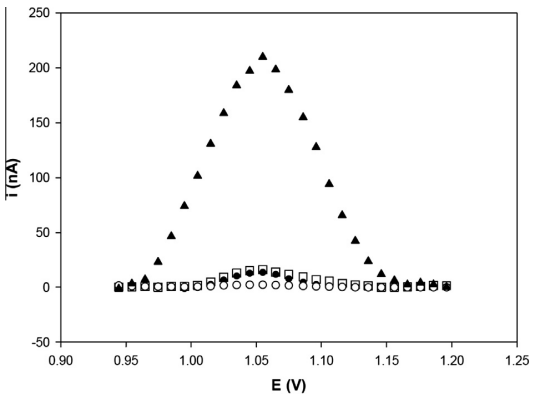

Figure 2 Differential pulse voltammograms of guanine oxidation at activated PGE: before probe immobilization (○), after E6 probe immobilization (●), after hybridization with the undigested plasmid solution (pGEM-T/E6) (▲) and after hybridization with the blank solution (□). Solution concentration: 500 nM solution of E6 probe and 300 pg/μL of target (undigested pGEM-T/E6). The oxidation signal was obtained by differential pulse voltammetry in 20 mM Tris–HCl buffer (pH 7.0).

sugar-phosphate probe backbone (Palecek et al., 1998; Souza et al., 2011).

As seen in Fig. 1, the peak current increased significantly with the increase in the probe concentration. The results showed that the oxidation peak current increased from 125 nM (71 ± 1 nA) to 1,000 nM (500 ± 8 nA). At a concentration of 2,000 nM, there was a decrease of this peak current (271 ± 7 nA). This can be explained by the massive probe accumulation on the graphite electrode, which can lead to the development of overlapping probes (Pournaghi-Azar et al., 2006), resulting in a lower availability of the inosine bases.

The ANOVA parametric test followed by Tukey's test were used to compare the data (significant level $p < 0.05$) (Chan, 2003; Montgomery, 2000; Tukey, 1991). The results showed that there were no statistically significant differences between the concentrations of 500 nM and 1,000 nM ($p = 0.97$). Therefore, the concentration of 500 nM was selected as optimum concentration for immobilization on the activated electrode.

3.2.3. Hybridization detection

Electrochemical hybridization biosensors can potentially be used in DNA detection (Tosar et al., 2010). They can reduce the time and simplify the protocol of several assays, facilitating detection of specific nucleic acid sequence (Campos-Ferreira et al., 2013; Girousi and Kinigopoulou, 2010; Nascimento et al., 2012; Tang et al., 2009). DNA hybridization is based on the ability of ssDNA to recognize its complementary DNA sequence, forming the DNA duplex chain (hybrid) (Campos-Ferreira et al., 2013; Nascimento et al., 2012; Wang et al., 2008).

Fig. 2 shows the electrochemical detection of label-free DNA hybridization by the DPV technique. After probe immobilization on the activated PGE, the modified-electrode was immersed in a microtube containing the denatured target DNA (undigested pGEM-T/E6). The hybridization was directly detected through guanine oxidation signals present in the plasmid sequence. Label-free detection represents an attractive approach for detecting hybridization reaction because it eliminates the indicator addition step, simplifying the sensing protocol (Labuda et al., 2010; Vagin et al., 2003, 2008; Wang et al., 1999).

The voltammogram for probe-modified PGE after hybridization with denatured DNA target showed that there was a significant increase (15-fold) in the oxidation peak current of guanine. This peak current increase represents the hybridization event on the electrode surface.

3.2.4. Analytical performance of the sensor

Firstly, calibration experiments were carried out to evaluate the analytical performance of the sensor. The influence of plasmid concentration on the PGE surface was observed by guanine oxidation signals.

Different concentrations of undigested pGEM-T/E6 (40–15,000 pg/μL) were hybridized on the modified electrode surface and then the guanine oxidation signal was obtained using DPV (Fig. 3).

The peak current increased with the increase in the plasmid concentration and reached a maximum value at concentration 5,000 pg/μL (158 ± 11 nA to 480 ± 28 nA). However, when the concentration reached 15,000 pg/μL, there was a decrease in the oxidation signal (204 ± 21 nA). This decrease can be attributed to the saturation of hybridization surface sites, caused by a steric and electrostatic hindrance in the DNA bases (Erdem et al., 2006; Hejazi et al., 2010; Wang and Kawde, 2001; Wong and Melosh, 2010). Therefore, 5,000 pg/μL of pGEM-T/E6 is the maximum concentration allowed in order to form hybrids on the probe-modified electrode surface.

As seen in Fig. 3, the signal was linear up to 5,000 pg/μL, with a determination coefficient (R^2) of 0.99327 for the complementary target. The regression equation was I (nA) = 0.0616C (pg/μL) + 171.58. The detection limit of the method was 16 pg/μL, estimated by the equation $3\sigma/a$, where

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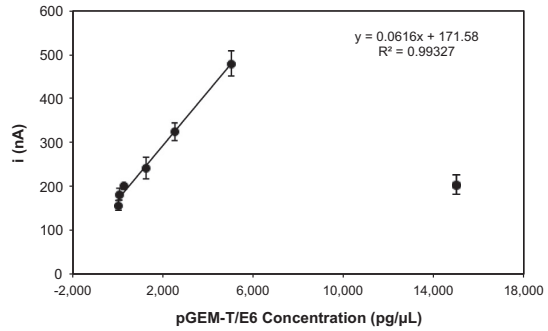

Figure 3 The effect of the different concentrations of the undigested pGEM-T/E6 during hybridization event using DPV response of the guanine oxidation signal (n = 3).The black line represents the linear regression at a concentration range of 40–5,000 pg/μL. The oxidation signal was obtained by differential pulse voltammetry in 20 mM Tris–HCl buffer solution (pH 7.0).

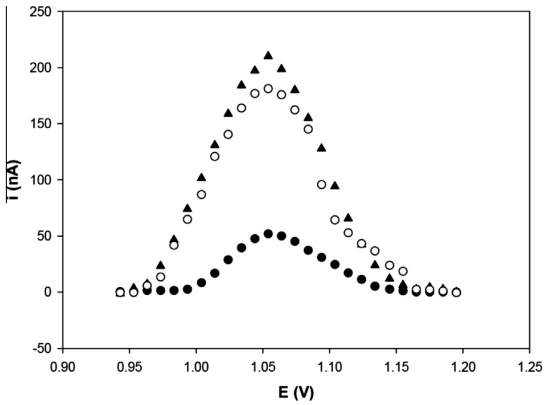

Figure 4 Differential pulse voltammograms of guanine oxidation on the probe-modified PGE: after hybridization with the undigested plasmid solution (pGEM-T/E6) (▲), after hybridization with the non-complementary DNA (undigested pGEM-T/E7) (●) and after hybridization with the undigested mix target (pGEM-T/E6 and pGEM-T/E7) (○). Solution concentration: 300 pg/μL solution of undigested pGEM-T/E6, undigested pGEM-T/E7 and undigested mix target. The oxidation signal was obtained by differential pulse voltammetry in 20 mM Tris–HCl buffer (pH 7.0).

σ was the standard deviation of the blank solution, and a was the slope of the liner regression (Skoog et al., 1998). The detection limit is described as the lowest concentration level of the analyte that produces a detectable response above the noise level of the system (Armbruster and Pry, 2008). Table 1 shows detection limits of some electrochemical DNA sensors (based on label as well as label-free detection). As seen in Table 1, the proposed sensor has a low detection limit, comparable to other DNA sensors.

The relative standard deviation (RSD) over three independently probe-modified electrodes measured at a 300 pg/μL target was 3%, indicating that this concentration allowed for a remarkable reproducibility of the detection method. Therefore, the concentration of 300 pg/μL was selected to realize the selectivity study.

3.2.5. *Selectivity study in optimized conditions*

Control experiments with non-complementary target (undigested pGEM-T/E7) and mixture of complementary and non-complementary targets were carried out to assess whether the suggested DNA sensor responds selectively to the target (Fig. 4).

As seen in Fig. 4, the interaction between non-complementary target and immobilized probe did not lead to a significant increase in the guanine oxidation signal. This result suggests that there was no hybridization of the non-complementary target on the probe immobilized onto the PGE.

On the other hand, the interaction between the mixed target and the immobilized probe lead to a significant increase in the guanine oxidation signal, similar to that of the pGEM-T/E6 (complementary target), indicating that there is hybridization when the complementary target is present in the sample. However guanine signal decreases slightly, probably because of partial hybridizations occurred between target and

Table 1 Comparison of the analytical parameters of different biosensors for DNA detection.

Nucleic acid biosensor	Electrode	Electrochemical technique	Linear range of the hybridization	Detection limit	References
Detection of human interleukine-2 gene	PGE[a]	DPV[b]	10–250 nM	36 pg/μL	Pournaghi-Azar et al. (2007)
Electrochemical detection of human papillomavirus (HPV)	PGE	SWV[c]	–	1.2 ng/μL	Sabzi et al. (2008)
Zirconia based nucleic acid sensor for *Mycobacterium tuberculosis* detection	Gold	DPV	1–150 ng/μL	0.065 ng/μL	Das et al. (2010)
Nanoparticle based DNA biosensor for tuberculosis detection	Carbon	DPV	0.01–10 ng/μL	0.01 ng/μL	Torres-Chavolla and Alocilja (2011)
Electrochemical DNA biosensor for detection of the E6 gene HPV inserted in recombinant plasmid	PGE	DPV	40–5000 pg/μL	16 pg/μL	This work

[a] Pencil graphite electrode.
[b] Differential pulse voltammetry.
[c] Square wave voltammetry.

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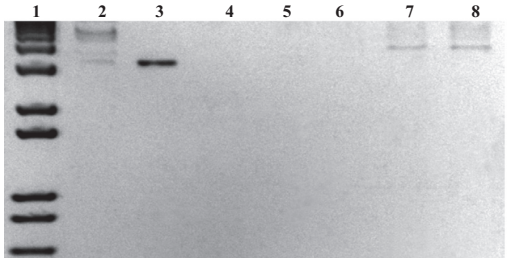

Figure 5 Electrophoresis using a 1% agarose gel stained with ethidium bromide for different concentrations of undigested and linearized plasmid. Lines: (1) 1 kb Plus DNA Ladder (Invitrogen); (2) 120,000 pg/μL of undigested pGEM-T/E6, (3) linearized pGEM-T, (4–8) 40, 2,500, 15,000, 60,000, 120,000 pg/μL of linearized pGEM-T/E6, respectively.

non-complementary target in their mixture solution. These interactions give rise to a slight decrease in availability and hybridization between target and immobilized probe (Pournaghi-Azar et al., 2006; Raoof et al., 2009).

These data demonstrated that this biosensor was able to distinguish between complementary and non-complementary target. The presence of non-complementary samples did not interfere in the specificity of the biosensor.

3.3. Perform biosensor analysis versus electrophoresis for nucleic acid detection

The electrochemical DNA detection performance was compared to the electrophoresis standard method. Samples of different concentrations of the undigested plasmid and linearized plasmid were electrophoresed for 45 min at 80 V on standard 1% agarose gel with ethidium bromide staining (Fig. 5).

As seen in Fig. 5, the electrophoresis method could not detect the presence of the E6 gene in undigested pGEM-T/E6. Thus, the pGEM-T/E6 was linearized with *API* enzyme restriction. However, even with the linearized plasmid, the E6 detection was only possible with a plasmid concentration of at least 60,000 pg/μL. In order to detect the presence of the E6 gene at lower concentrations, it was necessary to submit the samples to amplification by polymerase chain reaction (PCR) (Fig. S3 in supplementary information), which makes the electrophoresis technique more expensive (Singh et al., 2010). On the other hand, the proposed biosensor could detect the presence of the E6 gene inserted in undigested plasmid with a concentration starting from 40 pg/μL, with no need for the PCR amplification assay (Fig. 3).

Agarose gel electrophoresis is the standard method used to separate, identify, analyze, characterize and purify DNA fragments. This technique commonly requires the use of specialized apparatus and toxic and mutagenic reagents, such as ethidium bromide labeling (Kirsanov et al., 2010; Singer et al., 1999; Zhang et al., 2008).

The major limitation of this technique is its low sensitivity. Studies demonstrate that DNA detection by the electrophoresis standard method is only possible at concentrations starting from ng (Carman and Williamson, 1989; Yamauchi et al., 2008). In addition, this method does not provide DNA sequence confirmation (Elenis et al., 2008), reporting but the size of the DNA, which could lead to erroneous results in similar size fragments (Pournaghi-Azar et al., 2008). Another difficulty is the DNA analysis of the undigested plasmid. Due to its conformation, the plasmid must usually be linearized by restriction enzyme before running them on a gel. However, this procedure can increase the cost of the method.

The data demonstrate that the biosensor was more sensitive than the electrophoresis standard method. With this electrochemical device it was possible to detect the presence of DNA at a concentration 1,500-fold lower than the electrophoresis method, without the necessity to employ the restriction enzymes and PCR amplification for analyses. Furthermore, with this biosensor it was possible to confirm the target sequence cloned into plasmid because it makes use of the hybridization event to detect the DNA sequence.

4. Conclusions

This paper reports the development of a label-free electrochemical biosensor for the detection of the HPV E6 gene cloned into plasmid pGEM-T. Under optimum conditions, the electrical signal had a linear relationship with the target DNA concentration ranging from 40 to 5,000 pg/μL. The biosensor's detection limit was 16 pg/μL. Experiments with non-complementary target (E7 gene into pGEM-T) confirmed the selectivity of the biosensor for detecting the E6 gene inserted into pGEM-T.

The electrochemical method showed high sensitivity and specificity when compared with the electrophoresis standard method. The biosensor was able to detect the DNA presence in a 1,500-fold lower concentration than the conventional electrophoresis.

The development of alternative methods, which have high sensitivity and specificity for the DNA detection, is desirable. The proposed method showed a good promise for detecting the presence of a cloned DNA fragment in expression vectors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.arabjc.2014.05.023.

References

Armbruster, D.A., Pry, T., 2008. Limit of Blank, Limit of Detection and Limit of Quantitation. Clin. Biochem. Rev. 29, S49–S52.

Azam, F., Shams-ul-Islam, M., 2010. Prevention of human papilloma virus infection with vaccines. J. Pak. Med. Assoc. 60, 676–681.

Berti, F., Lozzi, L., Palchetti, I., Santucci, S., Marrazza, G., 2009. Aligned carbon nanotube thin films for DNA electrochemical sensing. Electrochim. Acta 54, 5035–5041.

Boccardo, E., Lepique, A.P., Villa, L.L., 2010. The role of inflammation in carcinogenesis. Carcinogenesis 31, 1905–1912.

Burk, R.D., Harari, A., Chen, Z., 2013. Human papillomavirus genome variants. Virology 445, 232–243.

Bury-Moné, S., Nomane, Y., Reymond, N., Barbet, R., Jacquet, E., Imbeaud, S., Jacq, A., Bouloc, P., 2009. Global analysis of extracytoplasmic stress signaling in *Escherichia coli*. PLoS Genet. 5, e1000651.

Campos-Ferreira, D.S., Nascimento, G.A., Souza, E.V.M., Souto-Maior, M.A., Arruda, M.S., Zanforlin, D.M.J., Ekert, M.H.F., Bruneska, D., Lima-Filho, J.L., 2013. Electrochemical DNA biosensor for human papillomavirus 16 detection in real samples. Anal. Chim. Acta 804, 258–263.

Carman, W.F., Williamson, C., 1989. Detection of enzymatically amplified human immunodeficiency virus DNA by oligonucleotide solution hybridization and by incorporation of radiolabeled deoxynucleotides. J. Clin. Microbiol. 27, 2570–2573.

Carter, J.R., Ding, Z., Rose, B.R., 2011. HPV infection and cervical disease: a review. Aust. New Zeal. J. Obstet. Gynaecol. 51, 103–108.

Chan, Y.H., 2003. Biostatistics 102: quantitative data – parametric & non-parametric tests. Singapore Med. J. 44, 391–396.

Chang, W.H., Yang, S.Y., Lin, C.L., Wang, C.H., Li, P.C., Chen, T.Z., Jan, F.J., Lee, G.B., 2013. Detection of viruses directly from the fresh leaves of a *Phalaenopsis orchid* using a microfluidic system. Nanomedicine 9, 1274–1282.

Das, M., Sumana, G., Nagarajan, R., Malhotra, B.D., 2010. Zirconia based nucleic acid sensor for *Mycobacterium tuberculosis* detection. Appl. Phys. Lett. 96, 133703.

Elenis, D.S., Kalogianni, D.K., Glynou, K., Ioannou, P.C., Christopoulos, T.K., 2008. Advances in molecular techniques for the detection and quantification of genetically modified organisms. Anal. Bioanal. Chem. 392, 347–354.

Erdem, A., Papakonstantinou, P., Murphy, H., 2006. Direct DNA hybridization at disposable graphite electrodes modified with carbon nanotubes. Anal. Chem. 78, 6656–6659.

Ferraro, B., Morrow, M.P., Hutnick, N.A., Shin, T.H., Lucke, C.E., Weiner, D.B., 2011. Clinical applications of DNA vaccines: current progress. Vaccines 53, 296–302.

Girousi, S., Kinigopoulou, V., 2010. Detection of short oligonucleotide sequences using an electrochemical DNA hybridization biosensor. Cent. Eur. J. Chem. 8, 732–736.

Hejazi, M.S., Alipour, E., Pournaghi-Azar, M.H., 2007. Immobilization and voltammetric detection of human interleukine-2 gene on the pencil graphite electrode. Talanta 71, 1734–1740.

Hejazi, M.S., Pournaghi-Azar, M.H., Ahour, F., 2010. Electrochemical detection of short sequences of hepatitis C 3a virus using a peptide nucleic acid-assembled gold electrode. Anal. Biochem. 399, 118–124.

Hejazi, M.S., Pournaghi-Azar, M.H., Alipour, E., Karimi, F., 2008. Construction, electrochemically biosensing and discrimination of recombinant plasmid (pEThIL-2) on the basis of interleukine-2 DNA insert. Biosens. Bioelectron. 23, 1588–1594.

Hendry, M., Lewis, R., Clements, A., Damery, S., Wilkinson, C., 2013. "HPV? Never heard of it!": a systematic review of girls' and parents' information needs, views and preferences about human papillomavirus vaccination. Vaccine 31, 5152–5167.

Huang, C.F., Monie, A., Weng, W.H., Wu, T.C., 2010. DNA vaccines for cervical cancer. Am. J. Transl. Res. 2, 75–87.

Hung, C.F., Ma, B., Monie, A., Tsen, S.W., Wu, T.C., 2008. Therapeutic human papillomavirus vaccines: current clinical trials and future directions. Exp. Opin. Biol. Ther. 8, 421–439.

Kawana, K., Adachi, K., Kojima, S., Kozuma, S., Fujii, T., 2012. Therapeutic human papillomavirus (HPV) vaccines: a novel approach. Open Virol. J. 6 (Suppl. 2: M12), 264–269.

Kirsanov, K.I., Lesovaya, E.A., Yakubovskaya, M.G., Belitsky, G.A., 2010. SYBR gold and SYBR green II are not mutagenic in the Ames test. Mutat. Res. 699, 1–4.

Labuda, J., Brett, A.M.O., Evtugyn, G., Fojta, M., Mascini, M., Ozsoz, M., Palchetti, I., Paleček, E., Wang, J., 2010. Electrochemical nucleic acid-based biosensors: concepts, terms, and methodology (IUPAC Technical Report). Pure Appl. Chem. 82, 1161–1187.

Lin, K., Roosinovich, E., Ma, B., Wu, T.C., 2010. Therapeutic HPV DNA vaccines. Immunol. Res. 47, 86–112.

Ma, B., Xu, Y., Hung, C.F., Wu, T.C., 2010. HPV and therapeutic vaccines: where are we in 2010? Curr. Cancer Ther. Rev. 6, 81–103.

Montgomery, D.C., 2000. Design and Analysis of Experiments. Wiley, New York.

Nascimento, G.A., Souza, E.V.M., Campos-Ferreira, D.S., Arruda, M.S., Castelleti, C.H.M., Wanderley, M.S.O., Ekert, M.H.F., Bruneska, D., Lima-Filho, J.L., 2012. Electrochemical DNA biosensor for bovine papillomavirus detection using polymeric film on screen-printed electrode. Biosens. Bioelectron. 38, 61–66.

Nieto, K., Salvetti, A., 2014. AAV vectors vaccines against infectious diseases. Front. Immunol. 5, 1–9.

Palecek, E., 1960. Oscillographic polarography of highly polymerized deoxyribonucleic acid. Nature 188, 656–657.

Palecek, E., Fojta, M., Tomschik, M., Wang, J., 1998. Electrochemical biosensors for DNA hybridization and DNA damage. Biosens. Bioelectron. 13, 621–628.

Pei, H., Zuo, X., Pan, D., Shi, J., Huang, Q., Fan, C., 2013. Scaffolded biosensors with designed DNA nanostructures. NPG Asia Mater. 5, 1–9.

Pividori, M.I., Merkoçi, A., Alegret, S., 2000. Electrochemical genosensor design: immobilisation of oligonucleotides onto transducer surfaces and detection methods. Biosens. Bioelectron. 15, 291–303.

Pournaghi-Azar, M.H., Alipour, E., Zununi, S., Froohandeh, H., Hejazi, M.S., 2008. Direct and rapid electrochemical biosensing of the human interleukin-2 DNA in unpurified polymerase chain reaction (PCR)-amplified real samples. Biosens. Bioelectron. 24, 524–530.

Pournaghi-Azar, M.H., Ahour, F., Hejazi, M.S., 2009. Differential pulse voltammetric detection of hepatitis C virus 1a oligonucleotide chain by a label-free electrochemical DNA hybridization biosensor using consensus sequence of hepatitis C virus 1a probe on the pencil graphite electrode. Electroanalysis 2, 1822–1828.

Pournaghi-Azar, M.H., Hejazi, M.S., Alipour, E., 2006. Developing an electrochemical deoxyribonucleic acid (DNA) biosensor on the basis of human interleukine-2 gene using an electroactive label. Anal. Chim. Acta 570, 144–150.

Pournaghi-Azar, M.H., Hejazi, M.S., Alipour, E., 2007. Detection of human interleukine-2 gene using a label-free electrochemical DNA hybridization biosensor on the basis of a non-inosine substituted probe. Electroanalysis 19, 466–472.

Raoof, J.B., Hejazi, M.S., Ojani, R., Asl, E.H., 2009. A comparative study of carbon nanotube paste electrode for development of indicator-free DNA sensors using DPV and EIS: human interleukin-2 oligonucleotide as a model. Int. Electrochem. Sci. 4, 1436–1451.

Ronkainen, N.J., Halsall, H.B., Heineman, W.R., 2010. Electrochemical biosensors. Chem. Soc. Rev. 39, 1747–1763.

Sabzi, R.E., Sehatnia, B., Pournaghi-Azar, M.H., Hejazi, M.S., 2008. Electrochemical detection of human papilloma virus (HPV) target DNA using MB on pencil graphite electrode. J. Iran. Chem. Soc. 5, 476–483.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual. Plenum Press, New York.

Singer, V.L., Lawlor, T.E., Yue, S., 1999. Comparison of SYBR Green I nucleic acid gel stain mutagenicity and ethidium bromide mutagenicity in the *Salmonella*/mammalian microsome reverse mutation assay (Ames test). Mutat. Res. 439, 37–47.

Singh, R., Sumana, G., Verma, R., Sood, S., Pandey, M.K., Gupta, R.K., Malhotra, B.D., 2010. DNA biosensor for detection of *Neisseria gonorrhoeae* causing sexually transmitted disease. J. Biotechnol. 150, 357–365.

Please cite this article in press as: Campos-Ferreira, D.S. et al., Electrochemical DNA biosensor for the detection of human papillomavirus E6 gene inserted in recombinant plasmid. Arabian Journal of Chemistry (2014), http://dx.doi.org/10.1016/j.arabjc.2014.05.023

Skoog, D.A., Holler, F.J., Nieman, T.A., 1998. Principles of Instrumental Analysis, 5th ed. Harcourt Brace, Philadelphia.

Souza, E., Nascimento, G., Santana, N., Ferreira, D., Lima, M., Natividade, E., Martins, D., Lima-Filho, J., 2011. Label-free electrochemical detection of the specific oligonucleotide sequence of dengue virus type 1 on pencil graphite electrodes. Sensors 11, 5616–5629.

Stanley, M., 2010. Pathology and epidemiology of HPV infection in females. Gynecol. Oncol. 117, S5–S10.

Tang, L., Zeng, G., Shen, G., Li, Y., Liu, C., Li, Z., Luo, J., Fan, C., Yang, C., 2009. Sensitive detection of lip genes by electrochemical DNA sensor and its application in polymerase chain reaction amplicons from *Phanerochaete chrysosporium*. Biosens. Bioelectron. 24, 1474–1479.

Teles, F.R.R., Fonseca, L.P., 2008. Trends in DNA biosensors. Talanta 77, 606–623.

Torres-Chavolla, E., Alocilja, E.C., 2011. Nanoparticle based DNA biosensor for tuberculosis detection using thermophilic helicase-dependent isothermal amplification. Biosens. Bioelectron. 26, 4614–4618.

Tosar, J.P., Brañas, G., Laíz, J., 2010. Electrochemical DNA hybridization sensors applied to real and complex biological samples. Biosens. Bioelectron. 26, 1205–1217.

Tukey, J.W., 1991. The philosophy of multiple comparisons. Statist. Sci. 6, 100–116.

Vagin, Y.M., Karyakina, E.E., Hianik, T., Karyakin, A.A., 2003. Electrochemical transducers based on surfactant bilayers for the direct detection of affinity interactions. Biosens. Bioelectron. 18, 1031–1037.

Vagin, Y.M., Trashin, S.A., Karyakin, A.A., Mascini, M., 2008. Label-free detection of DNA hybridization at a liquid/liquid interface. Anal. Chem. 80, 1336–1340.

Vici, P., Mariani, L., Pizzuti, L., Sergi, D., Di Lauro, L., Vizza, E., Tomao, F., Tomao, S., Mancini, E., Vincenzoni, C., Barba, M., Maugeri-Saccà, M., Giovinazzo, G., Venuti, A., 2014. Emerging biological treatments for uterine cervical carcinoma. J. Cancer 5, 86–97.

Wang, J., Cai, X., Jonsson, C., Balakrishnan, M., 1996. Adsorptive stripping potentiometry of DNA at electrochemically pretreated carbon paste electrodes. Electroanalysis 8, 20–24.

Wang, J., Jiang, M., Mukherjee, B., 1999. Flow detection of nucleic acids at a conducting polymer-modified electrode. Anal. Chem. 71, 4095–4099.

Wang, J., Kawde, A.N., 2001. Pencil-based renewable biosensor for label-free electrochemical detection of DNA hybridization. Anal. Chim. Acta 431, 219–224.

Wang, J., Kawde, A.N., Erdem, A., Salazar, M., 2001. Magnetic bead-based label-free electrochemical detection of DNA hybridization. Analyst 126, 2020–2024.

Wang, J., Rivas, G., Fernandes, J.R., Paz, J.L.L., Jiang, M., Waymire, R., 1998. Indicator-free electrochemical DNA hybridization biosensor. Anal. Chim. Acta 375, 197–203.

Wang, Y., Xu, H., Zhang, J., Li, G., 2008. Electrochemical sensors for clinic analysis. Sensors 8, 2043–2081.

Wang, J., Zhou, F., 2002. Scanning electrochemical microscopic imaging of surface-confined DNA probes and their hybridization via guanine oxidation. J. Electroanal. Chem. 537, 95–102.

Wong, I.Y., Melosh, N.A., 2010. An electrostatic model for DNA surface hybridization. Biophys. J. 98, 2954–2963.

Yamauchi, E., Watanabe, R., Quikawa, M., Fujimoto, H., Yamada, A., Saito, K., Murakami, M., Hashido, K., Tsuchida, K., Takada, N., Fugo, H., Tu, Z., Maekawa, H., 2008. Application of real time PCR for the quantitative detection of radiation-induced genomic DNA strand breaks. J. Insect Biotechnol. Sericol. 77, 17–24.

Zhang, X., Liu, S., Jiao, K., Gao, H., Shi, Y., 2008. Rapid and cost-effective detection of sequence-specific DNA by monitoring the electrochemical response of 2′-deoxyguanosine 5′-triphosphate in a PCR sample. Analyst 133, 1729–1735.

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Supplementary Information

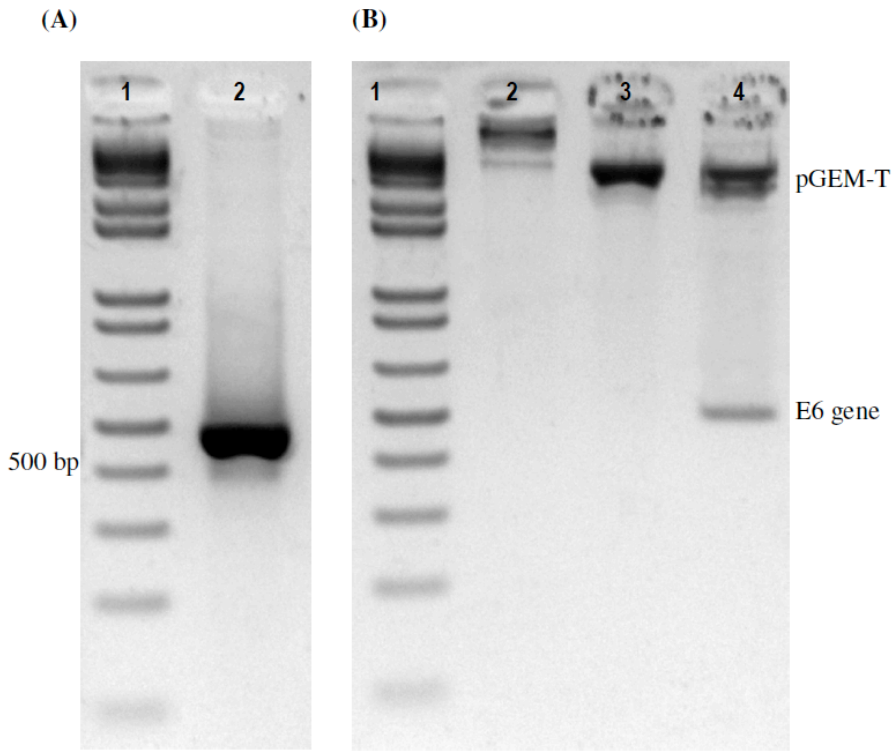

Fig. S1. Amplification of the E6 gene and enzymatic digestion of the pGEM-T/E6 observed in 1% agarose gel. [A] Lines:(1) 1 kbPlus DNA Ladder (Invitrogen), (2) E6 amplification; [B] Lines: (1) 1 kb Plus DNA Ladder (Invitrogen), (2) pGEM-T/E6 extraction, (3) linearized plasmid DNA (pGEM-T/E6) with *ApaI*, (4) digested plasmid (pGEM-T/E6) with *EcoRI* and *XbaI*.

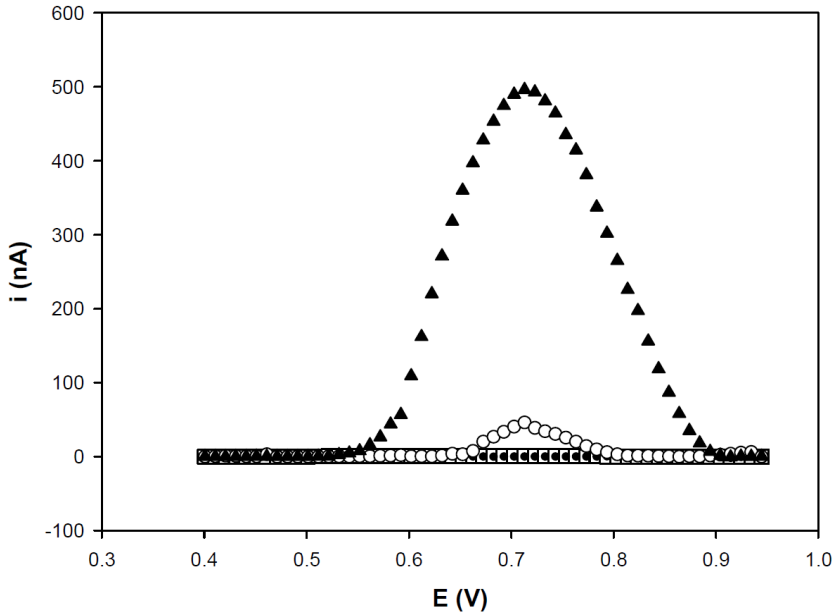

Fig. S2. Differential pulse voltammograms of inosine oxidation signal at non-activated bare PGE (□), E6 probe immobilized on non-activated PGE (○), activated bare PGE (●) and E6 probe immobilized on activated PGE (▲). Solution concentration of the E6 probe was 500 nM. The oxidation signal was obtained by differential pulse voltammetry in 20 mM Tris-HCl buffer (pH 7.0).

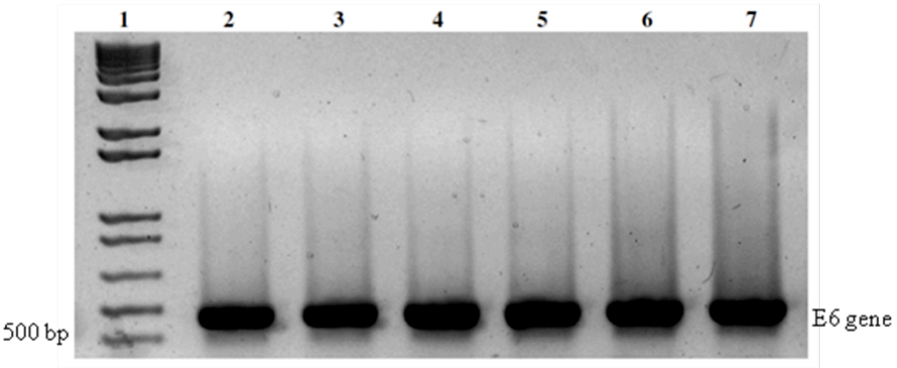

Fig. S3. Electrophoresis using a 1 % agarose gel stained with ethidium bromide for the samples of linearized plasmid DNA (pGEM-T/E6) submitted to PCR amplification. Lines: (1) 1 kb Plus DNA Ladder (Invitrogen), (2 to 7) 40 pg/µL; 70 pg/µL; 1,250 pg/µL; 2,500 pg/µL; 5,000 pg/µL; 15,000 pg/µL of linearized plasmid, respectively.

Capítulo III

Artigo Científico II

Electrochemical DNA biosensor for human papillomavirus (HPV) 16 detection in real samples

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Electrochemical DNA biosensor for human papillomavirus 16 detection in real samples

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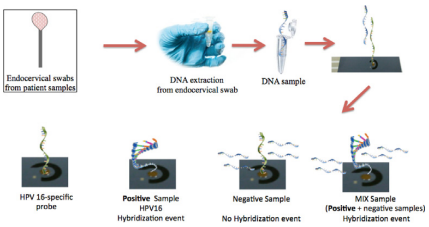
HIGHLIGHTS

- Electrochemical biosensor was used to detecting human papillomavirus (HPV) in real samples from endocervical swabs.
- The detection limit was of 18.13 nM.
- No hybridization with non-complementary sequence showed that the method is selective.
- It can be an excellent approach to detect human papillomavirus (HPV) in real samples.

GRAPHICAL ABSTRACT



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ABSTRACT

An electrochemical DNA biosensor for human papillomavirus (HPV) 16 detection has been developed. For this proposed biosensor, L-cysteine was first electrodeposited on the gold electrode surface to form L-cysteine film (CYSFILM). Subsequently, HPV16-specific probe was immobilized on the electrode surface with CYSFILM. Electrochemistry measurement was studied by differential pulse voltammetry method (DPV). The measurement was based on the reduction signals of methylene blue (MB) before and after hybridization either between probe and synthetic target or extracted DNA from clinical samples. The effect of probe concentration was analyzed and the best results were seen at 1000 nM. The hybridization detection presented high sensitivity and broad linear response to the synthetic-target concentration comprised between 18.75 nM and 250 nM as well as to a detection limit of 18.13 nM. The performance of this biosensor was also investigated by checking probe-modified electrode hybridization with extracted DNA from samples. The results showed that the biosensor was successfully developed and exhibited high sensitivity and satisfactory selectivity to HPV16. These results allow for the possibility of developing a new portable detection system for HPVs and for providing help in making an effective diagnosis in the early stages of infection.

1. Introduction

Epidemiological studies showed that infection with high-risk types (HR) of human papillomavirus (HPV), with large predominance of the genotype 16, is associated with nearly 100% of cases of cervical cancer and with a significant number of vaginal, vulvar, penile, and anal cancers [1,2].

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The oncogenic properties of HR-HPVs reside in the E6 and E7 genes [3]. Expression of these genes can lead to immortalization of keratinocytes, the natural host cells of HPV, suggesting that they significantly contribute to the oncogenic potential of HR-HPVs [4,5].

Currently, HPV diagnosis has been made through cytologic evaluation or visual inspection of the skin and tissues. These techniques have their own limitations, mainly because of poor specificity [6]. However, recent advances in molecular technologies have emerged for HPV detection, as well as the viral types identification. For instance, digene HC2 High-Risk HPV DNA, PapilloCheck®, COBAS® 4800 HPV, The Linear array®, CLART® human papillomavirus 2, INNO-LiPA, Clinical arrays® HPV and others are tests that have shown an excellent sensitivity for the detection of precancerous lesions of the cervix [7,8]. On the other hand, many of these tests do not distinguish individual HPV types, require PCR products, the cost is still relatively high, and require specific apparatus [7,8]. Thus, the development of a miniaturized, rapid, free-PCR and affordable test for HPV DNA makes this a viable alternative compared to current diagnostics for HPV [8–10].

DNA biosensors are analytical devices that are designed to detect a specific sequence of DNA (target) by hybridization with complementary probes immobilized on a solid substrate [11]. Among the different methods that have been used to detect hybridization, the electrochemical methods are more advantageous due to their portability, cost effectiveness, small size, and ease of use [12–14].

Many amino acids polymers have unique properties, which make them excellent platforms for the immobilization of biomolecules on electrodes. They have functional groups that can bind with DNA molecules, which allow for a better performance in the immobilization procedure [15]. The amino acid L-cysteine is a very promising surface modifier. It possesses a thiol group, which can bond strongly to silver or gold electrodes, carboxylic and amino groups, which can interact with several biological molecules, like DNA [16]. Thus, it is ideal to investigate biological systems in an electrochemical environment.

Due to the high rates of prevalence and the difficulties involved in making an effective diagnosis in the early stages of infection, the aim of this study was to develop a platform for the differential diagnosis of HPV16.

2. Materials and methods

2.1. Reagents and materials

All reagents were of analytical purity grade and all solutions were prepared using ultra pure water purchased from Invitrogen (USA). Methylene blue (MB) and L-cysteine were purchased from Sigma–Aldrich (USA). All single-stranded DNA (ssDNA) oligonucleotides were synthesized by Intregrated DNA Technologies (USA). Screen-printed electrodes were purchased from The Gwent Group (UK) for electrochemical detection.

2.2. Aparatus

The electrochemical analysis was carried out with a potentiostat (Autolab PGSTAT) equipped with GPES (4.0.007) software. The voltammetric experiments were performed on a three-electrodes system, consisting of a working, gold electrode (surface area of approximately 3 mm^2), a carbon auxiliary electrode and an Ag/AgCl reference electrode. All electrodes in the above-mentioned system were screen-printed. All hybridization experiments were carried out in hybridization Oven/Shaker (Amersham Pharmacia Biotech).

2.3. Human papillomavirus oligonucleotides

All oligonucleotides (as lyophilized powder) were diluted with ultrapure water and stored as a stock solution in a freezer. The stock solutions of oligonucleotides were diluted in 0.1 M phosphate-buffered saline (PBS) pH 7.4. The following three oligonucleotides sequences were used in this study:

HPV16 probe: 5′-ATG CAC CAA AAG AGA ACT GCA AT-3′
HPV16 target: 5′-AT TGC AGT TCT CTT TTG GTG CAT-3′
Non-complementary DNA: 5′-GTT CCG CCA CGG CAT CAC C-3′.

2.4. DNA Extraction from endocervical swab patient samples, PCR assay and HPV genotyping

Endocervical swabs from 10 patients were collected from Unidade de saúde da família de Jardim Fragoso at Olinda, Brazil. The genomic DNA was extracted from swabs by Wizard Genomic DNA Purification kit (Promega – USA) following the manufacturer's instructions. The genomic DNA extraction was amplified with the primer pair GP5+/GP6+ (forward 5′-TTTGTTACTGTGGTAGATACTAC-3′ and reverse5′-GAAAAATAAACTGTAAATCATATTC-3′). The GP5+/GP6+ primer pair amplifies a fragment of 150 bp from L1 gene.

The PCR was performed using the GoTaq® qPCR Master Mix kit and the following conditions: 1 μL of the extracted DNA, 6.25 μL of GoTaq® qPCR Master Mix, 1 μL of each primer and 3.25 μL of ultra pure water. The amplification was performed in the following conditions: (i) 94 °C for 3 min, (ii) 34 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min and (iii) a final extension at 72 °C for 10 min. The PCR product was visualized by electrophoresis in a 2% agarose gel containing ethidium bromide (1 mg mL^{-1}) and TBE buffer pH 8.4 (89 mM Tris, 89 mM boric acid and 2 mM EDTA) under UV light.

The extracted DNA from endocervical swabs was also used to identify HPV types. HPV genotyping was carried out by PapilloCheck® assay (Greiner Bio-one, Germany) according to manufacturer's recommendations.

2.5. Preparation of the modified electrodes

The screen-printed electrode was washed thoroughly with ultra-pure water. Before modification, the bare electrode was scanned in 0.5 M H_2SO_4 between 0.5 and 1.5 V at 50 mV s^{-1} until a reproducible cyclic voltammogram (CV) was obtained.

The cysteine film was used to allow the immobilization of the DNA probe onto the working electrode. The L-cysteine solution (10 mM) was prepared in 0.1 M PBS pH 7.4. For preparation of the cysteine film, the L-cysteine solution was electrodeposited on the working electrode by cyclic potential scanning from −0.2 to 1.5 V for 15 cycles with a scan rate of 50 mV s^{-1}.

2.6. Probe DNA immobilization

The HPV16 probe was immobilized by adsorption for 30 min at 30 °C on the gold electrode surface that was modified with the cysteine film. Then, the unbound oligonucleotides were washed out from the working electrode with 0.1 M PBS.

2.7. DNA hybridization

The solution containing the HPV16 target was added onto a working electrode with the immobilized HPV16 probe and incubated at 55 °C for 10 min, at a stirring speed of 300 rpm to link the complementary sequence. The Tris–HCl buffer (20 mM, pH 7.0) was used to wash the electrode and remove the non-hybridized

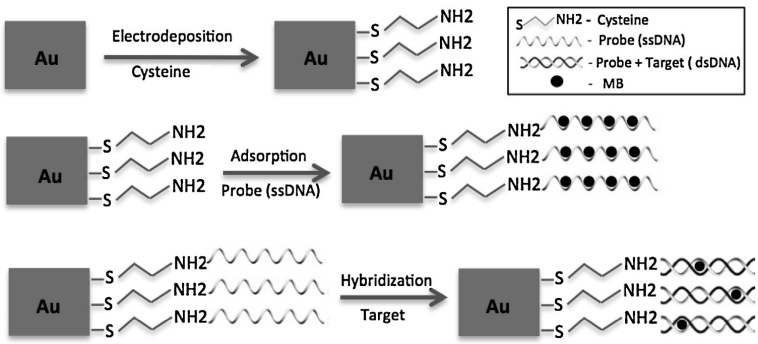

Fig. 1. Schematic drawing of the stepwise DNA biosensor fabrication process.

sequences. The same procedure was applied for interaction of HPV16 probe with non-complementary target.

For the hybridization of the genomic DNA with HPV16 probe on the modified working electrode, the extracted DNA was denatured by heating in a water bath (95 °C) for 5 min and was immediately chilled in ice to obtain denatured ss-DNA. Then, it was added to the modified working electrode surface. The formation of hybridization between the extracted DNA and HPV16 probe was also performed at 55 °C for 10 min at a stirring speed of 300 rpm. The electrode was washed with Tris–HCl buffer (20 mM, pH 7.0) to remove the non-hybridized sequences.

2.8. Electrochemical analysis

The differential pulse voltammetry (DPV) method was used for the electrochemical signal analysis. After the immobilization and hybridization process, 500 μM MB solution in Tris–HCl buffer (20 mM, pH 7.0) was added onto modified working electrode for 5 min and then washed with the Tris–HCl buffer. The DPV measurement of the MB electrochemical reduction was performed in the Tris–HCl buffer, under the following conditions: a potential sweep between −0.6 and 0 V with a scan rate of 20 mV s^{-1}.

3. Results and discussion

3.1. Preliminary investigations

The schematic representation of the fabrication procedure of DNA biosensor is shown in Fig. 1. The electrodeposited L-cysteine film (CYSFILM) was employed as the platforms for the probe immobilization onto gold working electrode surface. The L-cysteine amino acid was chosen because of the special functional end groups, such as —SH, —COOH and —NH_2 [17]. The electrodeposited L-cysteine film was formed by cyclic voltammetry (CV) technique. Electrochemical deposition has been reported as an effective method for immobilization of biomolecules when compared to the traditional process, the self-assembled monolayers method (SAMs) [18].

The ssDNA probe immobilization was achieved by adsorption method. Adsorption is the simplest immobilization method because it does not require special reagents or any nucleic acid modification [19,20]. In this work, the immobilization was based on ionic interactions occurring between the negatively charged phosphate groups of the DNA and the positively charged amine, present in the L-cysteine that covers the surface electrode [20,21].

The essence of the electrochemical DNA detection assay was based either on the electrochemical DPV of ssDNA or double-stranded DNA (dsDNA) using the reduction signals of MB. The MB is covalently attached to DNA and provides a sensitive redox reporter in DNA electrochemistry measurement. This is an aromatic heterocycle molecule that is often employed toward selective discrimination of ssDNA and dsDNA [22].

3.2. Characterization of the probe-modified electrode

The purpose of this experiment was to observe the behavior of probe concentration on the immobilization at the CYSFILM gold electrode through MB electrochemical reduction. The effect of probe concentration is shown in Fig. 2 and was carried out by DPV technique. The results showed that the concentration of 1000 nM had the highest current peaks. The lowest probe concentrations (250 nM and 500 nM) showed lower current signal due to a low amount of immobilized probe on the working electrode surface. The concentration of 2000 nM also showed lower current signal, however, in this case it can be explained by the massive probe accumulation on the electrode that causes probe overlapping [23], resulting in lower availability of the guanine bases and, consequently, a lower availability of MB electrochemical reduction

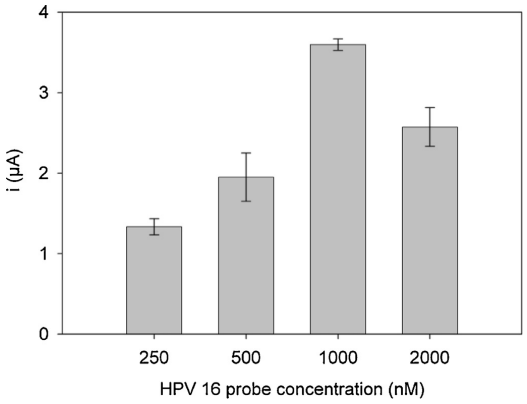

Fig. 2. Histogram of HPV16 probe concentration effect on the MB electrochemical reduction during the immobilization process. The differential pulse voltammetry (DPV) method was used to analyze the current signal in the following conditions: initial potential −0.7 V, end potential 0 V, modulation amplitude 50 mV and scan rate 20 mV s^{-1}. All results plotted were done in triplicates to different HPV16 probe concentrations.

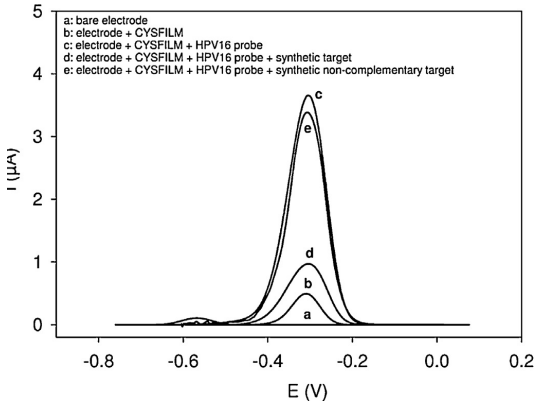

Fig. 3. The differential pulse voltammograms for the MB electrochemical reduction of (a) bare electrode, (b) modified L-cysteine film-gold electrode, (c) 1000 nM HPV16 probe immobilized on modified L-cysteine film-gold electrode before hybridization, (d) 1000 nM HPV16 probe immobilized on modified L-cysteine film-gold electrode after hybridization with 1000 nM HPV synthetic target and (e) 1000 nM HPV16 probe immobilized on modified L-cysteine film-gold electrode after hybridization with 1000 nM synthetic non-complementary target.

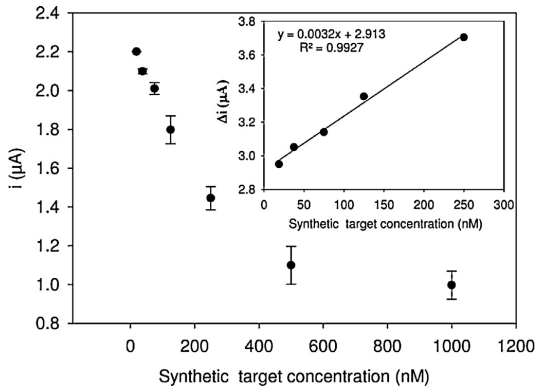

Fig. 4. The effect of the HPV16 target concentration on the MB electrochemical reduction during hybridization. Inset: Linear regression from difference between the DPV signals of MB on the probe modified-electrode in the presence and absence of target (Δi). The differential pulse voltametry (DPV) method was used to analyze the current signal in the following conditions: initial potential −0.7 V, end potential 0 V, modulation amplitude 50 mV and scan rate 20 mV s^{-1}. All results plotted were done in triplicates to different HPV16 target concentration.

[24,25]. Thus, the probe concentration of 1000 nM was selected for electrode modification.

3.3. Detection of hybridization for synthetic oligonucleotides

Detection of target DNA was monitored by means of DPV responses to MB electrochemical reduction on the probe modified-electrode in the absence and presence of target. Fig. 3 shows the DPV voltammograms for MB electrochemical reduction at bare gold electrode (a), CYSFILM electrode (b), probe-modified electrode before hybridization (c), probe-modified electrode after hybridization with synthetic complementary target (d) and probe-modified electrode after hybridization with synthetic non-complementary target (e).

As seen in Fig. 3 it was observed that the probe-modified electrode showed the highest current peak when compared to the other curves. This increase is attributed to strong affinity between MB and free guanine that are present in HPV16 probe. After hybridization of the probe-modified electrode with complementary target, the current signal of MB was decreased. This can be explained due to less MB accumulation on the ds-DNA caused by the inaccessibility of MB to the guanine bases [11,26] or may be due to a steric inhibition of the reducible groups of MB packed between the bulky double helix of the DNA hybrids [15,23,27,28]. Therefore, it is concluded that the decrease in the MB signal represents the extent of the hybridization at the electrode surface. However, the interaction between non-complementary target and probe-modified electrode did not lead to MB signal decrease, due to negligible hybridization. This result indicates that only a complementary target could hybridize on the probe modified-electrode and cause a significant decrease in the accumulation of MB.

3.4. Optimization of hybridization for synthetic oligonucleotides

To identify the conditions for optimal analytical performance of the biosensor proposed, the MB electrochemical reduction was measured after hybridization with synthetic target oligonucleotides at different concentrations (18.75–1000 nM).

Fig. 4 shows that the current signal decreases with the increase of target concentration of up to 250 nM and then it stabilizes at 1000 nM, indicating that all the available probe immobilized on the electrode surface have become involved in hybridization.

As seen on the inset of Fig. 4, the linear regression was obtained from the difference between the DPV signals of MB on the probe modified-electrode in the presence and absence of target (Δi). The calibration curve ($y = 0.0032x + 2.913$) was linear between 18.75 nM and 250 nM, with correlation coefficient of 0.99272. A detection limit of 18.13 nM could be estimated by equation $3\sigma/a$, where σ was the standard deviation of intercept and a was the line slope [15,29]. The relative standard deviation (RSD) over three independently probe modified-electrodes was 0.023% that was measured at 18.75 nM of the HPV16 target, which indicated a remarkable reproducibility of the detection method.

3.5. HPV sample analysis

In order to confirm of the presence of HPV in the extracted DNA from clinical samples, the PCR was carried out using consensus primers, directed at relatively conserved regions of the HPV genome. GP5+/GP6+ primer set, mediated by PCR is the most frequently used amplification system for the detection of HPV-DNA in clinical samples, amplifying DNA fragments in the conserved L1 region of approximately 150 bp [30–32]. In Fig. 5 it is possible to observe two amplification products of 150 bp in the electrophoresis gel that correspond to the HPV L1 gene. These results only confirm the presence of HPV-DNA in the sample.

After HPV detection by PCR, these extracted DNA were used to identify HPV types. The goal of this assay was also to compare the efficiency of the HPV type identification by biosensor proposed. The genotype results obtained by PapilloCheck® showed that the samples ESD1 (extracted sample DNA 1) and ESD2 (extracted sample DNA 2) were positive HPVs for 16 and 45, respectively (data not shown). This is a relatively novel test for the detection of HPV. This assay is a broad-spectrum PCR-based method that uses a consensus primer set that targets the E1 region of HPV DNA and that allows the simultaneous detection and genotyping of 24 different HPV types by DNA chip technology [30,33].

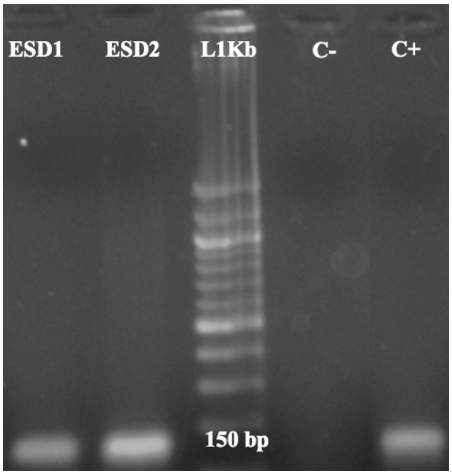

Fig. 5. The amplification of HPV L1 gene with the primer pair GP5+/GP6+ from DNA that was extracted from clinical samples. Lane ESD1: extracted sample DNA 1; Lane ESD2: extracted sample DNA 2; Lane L1Kb: 1 kb Plus DNA ladder (Invitrogen): Lane C−: negative control (ultrapure water) and Lane C+: positive control for the HPVL1 gene in pBR322 – HPV16 plasmid.

3.6. Performance of electrochemical DNA biosensor using real samples of HPV

The performance of this electrochemical DNA biosensor has been investigated by checking probe-modified electrode hybridization with extracted DNA from samples.

In this assay, we used the extracted sample DNA directly on the probe-modified electrode unlike other works that used PCR products [34,35]. Fig. 6 shows a DPV response of biosensor to positive sample of HPV16, positive sample of HPV45 (non-complementary sample) and mix-sample (HPV16 and HPV45). It is possible to observe that in the presence of positive sample of HPV16 the current peaks were decreased, which confirmed the occurrence

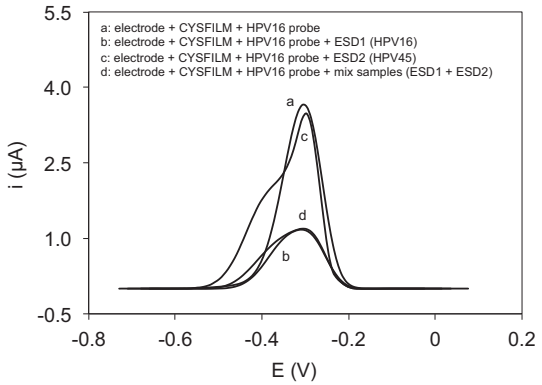

Fig. 6. The differential pulse voltammograms for the MB electrochemical reduction of (a) 1000 nM HPV16 probe immobilized on modified L-cysteine film-gold electrode before hybridization, (b) 1000 nM HPV16 probe immobilized on modified L-cysteine film-gold electrode after hybridization with 1000 nM ESD1 (HPV16); (c) 1000 nM HPV16 probe immobilized on modified L-cysteine film-gold electrode after hybridization with 1000 nM ESD2 (non-complementary target–HPV45) (d) 1000 nM HPV16 probe immobilized on modified L-cysteine film-gold electrode after hybridization with 1000 nM mix samples (ESD1 + ESD2).

of hybridization in the proposed detection system. It was also observed that the biosensor detects the biological samples within the concentration range shown in Fig. 4. Commercial methods, such as PapilloCheck®, also detect low concentrations of DNA, however, using PCR products [8]. Clinically, HPV diagnosis in this concentration range is a technological breakthrough when compared to miniaturized devices like biosensors.

The specificity of the biosensor was studied by the incubation of the non-complementary sample and mix-sample. Fig. 6 shows that the current peak after hybridization of the probe modified-electrode with non-complementary sample increased when compared to the hybrid formed. On the other hand, the interaction between the mix-sample and probe modified-electrode lead to a significant decrease in the current peak, indicating that there is hybridization when the HPV16 is present in the sample.

Another aspect observed in Fig. 6 was the presence of a poorly defined shoulder around −0.4 V, likely due to the chemical degradation of MB. This electrochemical behavior was a sporadic event that occurred only in the hybridization process of the probe modified-electrode with non-complementary sample (curve c). Eventually, several redox compounds, like methylene blue, can undergo irreversible electrochemical degradations and form chemical species that are represented by shoulders in voltammograms [36–41].

These data demonstrated that this electrochemical DNA biosensor able to detect the HPV16 presence in samples and non-complementary samples did not interfere in the specificity of the biosensor.

4. Conclusions

This paper reports the development of an electrochemical DNA biosensor for the detection of one of the most representative high-risk HPV types, the HPV16. The performance of the biosensor was checked using synthetic oligonucleotides and extracted DNA from the cervical swab of a confirmed positive patient. The biosensor was able to detect HPV16 using extracted DNA directly on the modified transducer unlike other works that used PCR products. Thus, results indicate that this electrochemical DNA biosensor is specific to HPV16 and it can be used to distinguish from other HPV types. With these data, it is possible to develop a new portable free-PCR detection system for HPVs, as well as to contribute to an effective diagnosis in the early stages of infection.

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References

[1] C. Annunziata, L. Buonaguro, F.M. Buonaguro, M.L. Tornesello, Pathol. Oncol. Res. 18 (2012) 803–808.
[2] D.A. Machalek, M. Poynten, F. Jin, C.K. Fairley, A. Farnsworth, S.M. Garland, R.J. Hillman, K. Petoumenos, J. Roberts, S.N. Tabrizi, D.J. Templeton, A.E. Grulich, Lancet Oncol. 2045 (2012) 1–14.
[3] E. Tsimplaki, E. Argyri, L. Michala, M. Kouvousi, A. Apostolaki, G. Magiakos, I. Papassideri, E. Panotopoulou, J. Oncol. 2012 (2012) 1–7.
[4] S.S. Tungteakkhun, P.J. Duerksen-Hughes, Arch. Virol. 153 (2008) 397–408.
[5] C.A. Moody, L.A. Laimins, Nat. Rev. Cancer 10 (2010) 550–560.
[6] S.J. Hwang, K.R. Shroyer, J. Oncol. 2012 (2012) 1–9.
[7] M.-N. Didelot, N. Boulle, A. Damay, V. Costes, M. Segondy, J. Med. Virol. 83 (2011) 1377–1382.
[8] A.L.P. Abreu, R.P. Souza, F. Gimenes, M.E.L. Consolaro, J. Virol. 9 (2012) 1–9.
[9] N. Bhatla, N. Moda, Indian J. Med. Res. 130 (2009) 261–265.
[10] E.J. Cho, J.H. Do, Y.S. Kim, S. Bae, W.S. Ahn, J. Med. Microbiol. 60 (2011) 162–171.
[11] J.P. Tosar, G. Brañas, J. Laíz, Biosens. Bioelectron. 26 (2010) 1205–1217.

[12] B. Bohunicky, S.A. Mousa, Nanotechnol. Sci. Appl. 4 (2010) 1–10.
[13] B.J. Privett, J.H. Shin, M.H. Schoenfisch, Anal. Chem. 82 (2010) 4723–4741.
[14] N.J. Ronkainen, H.B. Halsall, W.R. Heineman, Chem. Soc. Rev. 39 (2010) 1747–1763.
[15] G.A. Nascimento, E.V.M. Souza, D.S. Campos-Ferreira, M.S. Arruda, C.H.M. Castelletti, M.S.O. Wanderley, M.H.F. Ekert, D. Bruneska, J.L. Lima-Filho, Biosens. Bioelectron. 38 (2012) 61–66.
[16] L.C. Umuhumuza, X. Sun, Eur. Food Res. Technol. 232 (2010) 425–431.
[17] W. Wang, J. Biophys. Chem. 03 (2012) 39–43.
[18] H. Niu, R. Yuan, Y. Chai, L. Mao, Y. Yuan, Y. Zhuo, S. Yuan, X. Yang, Biosens. Bioelectron. 26 (2011) 3175–3180.
[19] M.I. Pividori, A. Merkoçi, S. Alegret, Biosens. Bioelectron. 15 (2000) 291–303.
[20] A. Sassolas, B.D. Leca-Bouvier, L.J. Blum, Chem. Rev. 108 (2008) 109–139.
[21] R. Beutner, J. Michael, B. Schwenzer, D. Scharnweber, J.R. Soc. Interface 7 (Suppl. 1) (2010) S93–S105.
[22] D. Pan, X. Zuo, Y. Wan, L. Wang, J. Zhang, S. Song, C. Fan, Sensors 7 (2007) 2671–2680.
[23] M.H. Pournaghi-Azar, M.S. Hejazi, E. Alipour, Anal. Chim. Acta 570 (2006) 144–150.
[24] M. Tichoniuk, M. Ligaj, M. Filipiak, Sensors 8 (2008) 2118–2135.
[25] M. Ortiz, A. Fragoso, P.J. Ortiz, C.K. O'Sullivan, Photochem. Photobiol. J. A 218 (2011) 26–32.
[26] A. Erdem, K. Kerman, B. Meric, M. Ozsoz, Electroanalysis 13 (2001) 219–223.
[27] B. Meric, K. Kerman, D. Ozkan, P. Kara, S. Erensoy, U.S. Akarca, M. Mascini, M. Ozsoz, Talanta 56 (2002) 837–846.
[28] L.D. Tran, B.H. Nguyen, N. Van Hieu, H.V. Tran, H. Le Nguyen, P.X. Nguyen, Mater. Sci. Eng. C 31 (2011) 477–485.
[29] M. Gumustas, S. Ozkan, Open Anal. Chem. J. 5 (2011) 1–21.
[30] A.T. Hesselink, D.A.M. Heideman, J. Berkhof, F. Topal, R.P. Pol, C.J.L.M. Meijer, P.J.F. Snijders, J. Clin. Microbiol. 48 (2010) 797–801.
[31] M.G. De Lima Rocha, F.L. Faria, L. Gonçalves, M.D.C.M. Souza, P.Á. Fernandes, A.P. Fernandes, PLoS One 7 (2012) e40988.
[32] A. Asiaf, S.T. Ahmad, M.A. Zargar, S.M. Mufti, S.H. Mir, Genet. Test. Mol. Biomarkers 16 (2012) 904–909.
[33] J. Jones, N.G. Powell, A. Tristram, a N. Fiander, S. Hibbitts, J. Clin. Virol. 45 (2009) 100–104.
[34] H. Gao, X. Qi, Y. Chen, W. Sun, Anal. Chim. Acta 704 (2011) 133–138.
[35] G. Wu, N. Yang, T. Zhang, Z. Wang, X. Lu, J. Kang, Sens. Actuators B. 160 (2011) 598–603.
[36] T. Omata, K. Ono, S.O. Yao-Matsuo, Mater. Trans. 44 (2003) 1620–1623.
[37] M. Melle-Franco, M. Marcaccio, D. Paolucci, F. Paolucci, V. Georgakilas, D.M. Guldi, M. Prato, F. Zerbetto, Am. Chem. Soc. J. 126 (2004) 1646–1647.
[38] M. Panizza, a Barbucci, R. Ricotti, G. Cerisola, Sep. Purif. Technol. 54 (2007) 382–387.
[39] D.H. Evans, Chem. Rev. 108 (2008) 2113–2144.
[40] F. Manea, A. Pop, C. Radovan, P. Malchev, A. Bebeselea, G. Burtica, S. Picken, J. Schoonman, Sensors 8 (2008) 5806–5819.
[41] F. Scholz, Thermodynamics of electrochemical reactions, in: F. Scholz (Ed.), Electroanalytical Methods - Guide to Experiments and Applications, Springer, 2010, pp. 11–32.

Capítulo IV

Considerações Finais e Perspectivas

CONSIDERAÇÕES FINAIS

Este trabalho reporta o desenvolvimento de biossensores de DNA eletroquímico para detecção de um dos mais representativos tipo de HPV de alto risco, o HPV16. A performance dos biossensores foi analisada usando plasmídeo clonados com o gene E6 do HPV16, oligonucletídeos sintéticos e DNA extraídos de amostras humanas.

Os resultados mostraram que esses dispositivos diferenciaram com sucesso as amostras complementares das não-complementares à sonda imobilizada no transdutor, demostrando a sua especificidade. Além disso, apresentaram elevada sensibilidade, cujos limites de detecção foram 16 pg/μL (7 nM) para detecção do gene E6 do HPV16 inserido no plasmídeo e 18,13 nM para o HPV16.

Adicionalmente, os dados demonstraram que, ao contrário das técnicas padrão, os dois biossensores foram capazes de detectar a presença do DNA viral nas amostras sem a necissidade de amplificação do material genético, o que torna esses sistemas mais rápido e mais barato em comparação com os testes tradicionais.

Os dados obtidos indicam que esses sistemas possuem uma grande viabilidade para ser usado no diagnóstico de vários tipos de HPVs, com grandes chances de ser utilizado de forma portátil, permitindo com isso o desenvolvimento de um sistema pioneiro, de fácil execução e interpretação para detecção precoce desse vírus. Além do mais, a mesma metodologia pode ser aplicada para detecção de outros agentes infecciosos.

PERSPECTIVAS

O presente estudo abre a possibilidade para o desenvolvimento de um dispositivo eletroquímico de fácil execução e simples interpretação, que pode ser utilizado para detecção de diversos agentes infecciosos.

Embora nosso sistema não utilize técnicas de amplificação de DNA para as análises, ainda é necessário que o DNA seja extraído das amostras. Por isso, estudos já estão em andamento, com a finialidade de desenvolver um protótipo com um sistema de extração de DNA acoplado ao dispositivo eletroquímico e com isso facilitar a portabilidade do sistema em campo.

Em paralelo, será desenvolvido um dispositivo baseado em aptâmeros para detecção do HPV. O sistema baseado em aptâmero não necessita da extração do DNA das amostras, uma vez que o analito alvo é uma molécula presente na camada externa do vírus. Com isso, o sistema detecta o agente infeccioso diretamente na amostra sem a necessidade de nenhum tratamento prévio.

Por tudo isso, o dispositivo proposto tem grandes chances, em um futuro breve, de ser utilizado no programa de Saúde da Família, nos ambulatórios do Sistema Único de Saúde (SUS), permitindo que pacientes que normalmente se deslocariam para os grandes centros hospitalares para procedimentos de diagnóstico possam ser redirecionado para os centros de saúde locais, evitando grandes aglomerações nos hospitais das grandes cidades. Isso repercutirá não somente na economia para o paciente como para o sistema público.

Anexos

- Artigo colaboração I
- Artigo colaboração II
- Capítulo de livro publicado
- Pedido de Patente I
- Pedido de Patente II
- Pedido de Patente III

Artigo Colaboração I

Souza et al., **2011**. Label-Free Electrochemical Detection of the Specific Oligonucleotide Sequence of Dengue Virus Type 1 on Pencil Graphite Electrodes. Sensors, 11, 5616-5629.

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Article

Label-Free Electrochemical Detection of the Specific Oligonucleotide Sequence of Dengue Virus Type 1 on Pencil Graphite Electrodes

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Abstract: A biosensor that relies on the adsorption immobilization of the 18-mer single-stranded nucleic acid related to dengue virus gene 1 on activated pencil graphite was developed. Hybridization between the probe and its complementary oligonucleotides (the target) was investigated by monitoring guanine oxidation by differential pulse voltammetry (DPV). The pencil graphite electrode was made of ordinary pencil lead (type 4B). The polished surface of the working electrode was activated by applying a potential of 1.8 V for 5 min. Afterward, the dengue oligonucleotides probe was immobilized on the activated electrode by applying 0.5 V to the electrode in 0.5 M acetate buffer (pH 5.0) for 5 min. The hybridization process was carried out by incubating at the annealing temperature of the oligonucleotides. A time of five minutes and concentration of 1 μM were found to be the optimal conditions for probe immobilization. The electrochemical detection of annealing

between the DNA probe (TS-1P) immobilized on the modified electrode, and the target (TS-1T) was achieved. The target could be quantified in a range from 1 to 40 nM with good linearity and a detection limit of 0.92 nM. The specificity of the electrochemical biosensor was tested using non-complementary sequences of dengue virus 2 and 3.

Keywords: dengue virus; nucleic acid biosensor; guanine oxidation

1. Introduction

Dengue is a disease caused by four serologically related viruses called dengue virus (DENV) type-1, -2, -3 and -4. They are members of *Flaviviridae* family and have a single-stranded positive-sense RNA genome of approximately 11 kb. Infection with DENV may cause an acute "influenza-like" febrile disease called classic dengue fever (DF), or progress at the time to dengue hemorrhagic fever (DHF) characterized by bleeding and plasma leakage [1] and dengue shock syndrome (DSS) [2].

The mosquito *Aedes aegypti* (Diptera: Culicidae) is responsible for the spread of diseases such as yellow fever and dengue among humans because of its role as a vector [3]. Yellow fever and dengue are endemic to Central and South America, Asia and Africa [4], and they are important public health problems in the tropic and subtropic areas, which encompass many resource-poor countries. Brazil currently accounts for the majority (80%) of dengue cases reported in Latin America, with co-circulation of three serotypes (DENV 1–3) in most of the country and sporadic epidemic waves in several urban areas [5-7].

Dengue virus is difficult to control due to massive urbanization, overpopulation, continually increasing travel, and failure to maintain effective control programs against the mosquito vector [8]. To date the only option for controlling dengue virus transmission in the human population has been reduction of the population density of the mosquito vectors of dengue [9]. Diagnosis for the detection of dengue infection has been carried out by IgM capture ELISA, virus isolation in mosquito cell lines and live mosquitoes, dengue specific monoclonal antibodies, PCR (polymerase chain reaction) and reverse-transcription polymerase chain reaction (RT-PCR) assays [10], which usually are reportable after 5 or 7 days. Because of the importance of early diagnosis, development of a procedure for detection of viral nucleic acids in a biological sample is highly desirable. The use of a biosensor for the detection of nucleic acid sequences based on nucleic acid hybridization processes can greatly reduce the assay time and simplify the protocol, allowing detection of nucleic acid sequences almost in real time [11-14].

Due to their high sensitivity, small dimensions, low cost, and compatibility with microfabrication technology electrochemical transducers are often used for detecting hybridization events [12,15,16]. Sensitive electrochemical signaling strategies are based on the direct or catalyzed oxidation of nucleic acid nucleotides, as well as the redox reactions of reporter molecules in the indirect detection using chemical indicators [17,18]. The most common indicators for this purpose are heterocyclic dyes (e.g., ethidium bromide and methylene blue) and organometallic complexes (mainly Co, Fe, Os, Pt and Ru) [16].

A technique using direct guanine oxidation signal on carbon paste electrodes in which PCR amplicons of specific genotypes of the factor V Leiden mutation were identified has been previously reported [19]. In this paper, we describe a simple, inexpensive and stable electrode for the possible detection of the dengue virus type 1. It is a nucleic acid-based electrochemical biosensor using the partial sequence of dengue virus type 1 as a probe that allows differentiation of complementary from non-complementary sequences, and utilizing the annealing temperature for the hybridization process.

2. Experimental Section

2.1. Materials and Reagents

Tris base was obtained from Promega (USA), and sodium acetate was obtained from Sigma (USA). UltraPure distilled water was purchased from Invitrogen (USA), and reagents were of analytical grade. All DNA oligonucleotides (probe, target and non-complementary sequences) were synthesized by Integrated DNA Technologies (Brazil). *Probe*: Dengue virus type specific 1 (TS-1P)_ 5'CGTCTCAGTGATCCGGGG 3'; *Target*: Dengue virus type specific 1 (TS-1T)_ 5' CCCCGGATC ACTGAGACG 3'; *Non-complementary*: Dengue virus type specific 2 (TS-2 NC)_ 5' CTGTTCATGG CCCTTGTGG CG 3'; Dengue virus type specific 3 (TS-3 NC)_ 5' GCTCTGTCTCATGATGATGTT A 3'; PolyG-NC_ 5' GGGGGGGGGGGGGGGGGGGG 3'. The oligonucleotide stock solutions and dilute solutions were prepared with 0.5 M acetate buffer (pH 4.8) and kept frozen.

2.2. Bioinformatics

The complete genomes of dengue virus types 1, 2 and 3, corresponding to GenBank_ accession numbers U88536.1, U87411.1 and AY099336.1, respectively, were obtained from the National Center for Biotechnology Information (NCBI) database. The probe specific for dengue type 1 (TS-1P) was constructed based on Duebel [20], and the sequence was aligned using the CLC Combined Workbench v 3.6.1 software. Target sequence (TS-1T) and non-complementary (TS-2 NC and TS-3 NC) sequences were also constructed using this software.

2.3. Apparatus

Electrochemical analysis was carried out with the Autolab PGSTAT apparatus (Metrohm Autolab, The Netherlands). Voltammetric signals were measured using a system consisting of two electrodes [21] Pencil graphite (type 4B) was used as the working electrode, and the reference electrode was screen-printed using Ag/AgCl ink (Electrodag-Acheson, USA) under gold wire and then dried at 60 °C. The experiments were performed in triplicate.

2.4. Preparation of the Graphite Electrode

Pencil lead (type 4B, total length of 3 cm and diameter of 2.5 mm) typically composed of natural graphite was used as the pencil graphite electrode (PGE). The graphite was polished with an emery-impregnated disc to obtain a smooth surface. The body of the pencil was coated well with silicone, resulting in a free area for immobilization of 28.50 mm^2. Afterwards, the graphite electrode

was washed with ultra-pure water to remove possible contaminants on the surface of the electrode. The polished surface of the working electrode was then activated by applying a potential of 1.8 V for 5 min [22]. The working electrode was fixed vertically and immersed in a solution of 0.5 M acetate buffer (pH 4.8) containing the TS-1P probe.

2.5. Immobilization of the DNA Probe

The TS-1P probe was immobilized on the activated electrode by applying 0.5 V [20] to the electrode for 1–10 min in 0.5 M acetate buffer solution (pH 4.8) containing oligonucleotides at different concentrations. The electrode was then rinsed with Tris-HCl (pH 7.0).

2.6. Hybridization of Target Sequence

The working electrode with the immobilized probe was immersed in a solution containing the target oligonucleotides in acetate buffer, pH 4.8, and incubated at 57 °C for 3 min. The electrode was then washed with Tris-HCl (pH 7.0) to remove non-hybridized sequences. The same protocol was applied for the interaction of the probe with non-complementary sequences.

2.7. Electrochemical Analysis

To obtain electrochemical signal data, the biosensor surface with hybridized probe and target nucleic acid was immersed in an electrolytic cell containing Tris-HCl (pH 7.0), and attached to the Autolab potentiostat, equipped with GPES 4.9 software. Differential pulse voltammetry was applied to detect electrochemical signals, whereby a potential sweep was applied between 0.5 and 1.2 V, at a pulse amplitude of 50 mV and scan rate of 20 mV/s. The raw data were treated using the GPES software with a moving average baseline correction using a "peak width" of 0.01.

2.8. Statistical Analysis

Experimental data were analyzed using the Statistica 8 software by non-parametric tests. To evaluate statistical significance, the Mann–Whitney U test was used for the comparison of two unpaired groups. The Kruskal-Wallis test was used for the comparison of multi independent group data. A level of $p < 0.05$ was considered significant.

3. Results and Discussion

3.1. Preliminary Investigation

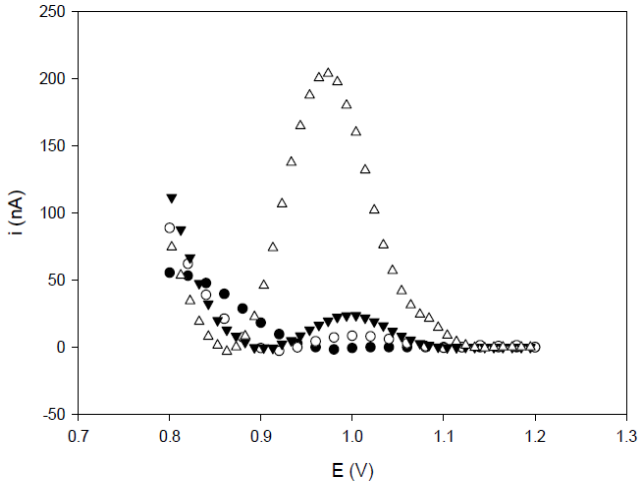
Research has demonstrated the utility of graphite electrodes [23] in the construction of electrochemical biosensor utilizing oligonucleotides [12,15]. The good sensitivity of the carbon electrodes in detecting the oxidation of nucleic acids makes them widely useful in DNA research, especially when inexpensive pencil graphite electrode is used [24]. The immobilization of nucleic acids on the surface of the electrode occurs by adsorption, which is considered the simplest method, and it does not require any special reagents or nucleic acid modifications. The smoothed surface of the pencil graphite was pretreated applying a potential (*vs*. Ag/AgCl reference electrode) of 1.8 V for

5 min. The pretreatment of the carbon surface increases its roughness and hydrophilicity, facilitating the adsorption of the probe on the electrodes [23].

The covalent immobilization of the probe is more stable, but the electrode surface may contain groups capable of reacting with and fragmenting the oligonucleotide molecule, damaging and changing its original structure [25]. The immobilization and hybridization are based on the electrochemical oxidation of guanine and adenine groups, which occurs with different electron transfer reaction rates [26,27]. We detect the oxidation of guanine that it is the most redox active nitrogenous base in DNA strands [12], which has been shown in some works as peaks of +0.93–1.0 V [26,28].

The influence of the pretreatment of the PGE at 1.8 V was investigated. Figure 1 shows no significant difference between the current peaks of activated PGE and TS-1P (1 μM) non-activated PGE. This observation indicates that the adsorption of nucleic acid did not occur. While the signal of the TS-1P (1 μM) on activated PGE were significantly higher than the signals of three other electrodes, due to the immobilization of the oligonucleotides containing guanines. These results demonstrated that applying a potential (1.8 V) on electrodes allows the adsorption of oligonucleotides.

Figure 1. The differential pulse voltammograms of guanine oxidation on (●) non-activated PGE, (○) activated PGE, (▼) TSF-1P (1 μM) immobilized on non-activated PGE and (Δ) TSF-1P (1 μM) immobilized on activated PGE. Voltammetric conditions: scanning potential steps, 20 mV/s; potential amplitude, 50 mV.



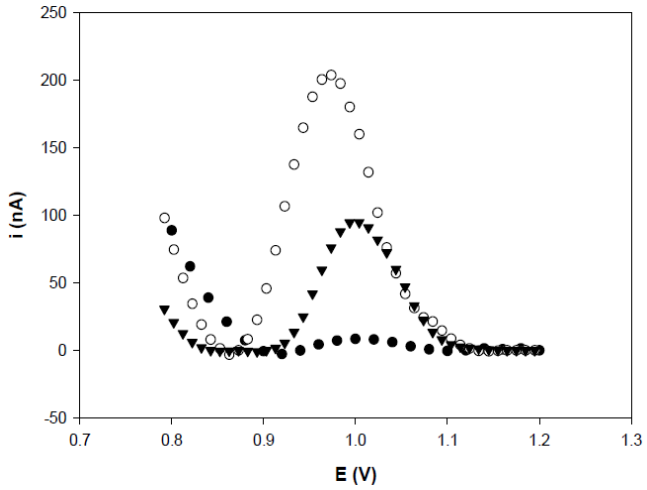
3.2. Detection of Guanine Oxidation via the Differential Pulse Voltammetry

The immobilization of the probe on the activated PGE involved the application of a potential of 0.5 V for 5 min [12,23,29,30]. This potential enhances the stability of the immobilized probe through the electrostatic attraction between the positively charged carbon surface and the negatively charged hydrophilic sugar-phosphate backbone with the bases oriented toward the solution ready to hybridize with the target [27].

The influence of the number of guanine in the probe was investigated immobilizing 1 μM of TS-1P (with seven guanines) and TS-1T (with five guanines) on different working electrodes. Figure 2 shows

that the TS-1P probe (206 ± 4.3 nA) has a current peak two times larger than that of TS-1T (84.7 ± 5.0 nA). These results were similar those obtained by Pournaghi-Azar *et al*. [31] with a biosensor for interleukin-2. They also observed that current peak was higher for the sequence with seven guanines compared to that with one guanine.

Figure 2. The differential pulse voltammograms of guanine oxidation on (●) an activated PGE, (▼) TS-1T (1 µM) immobilized on an activated PGE and (○) TS-1P (1 µM) immobilized on an activated PGE. Voltammetric conditions: Scanning potential steps, 20 mV/s. Potential amplitude, 50 mV.

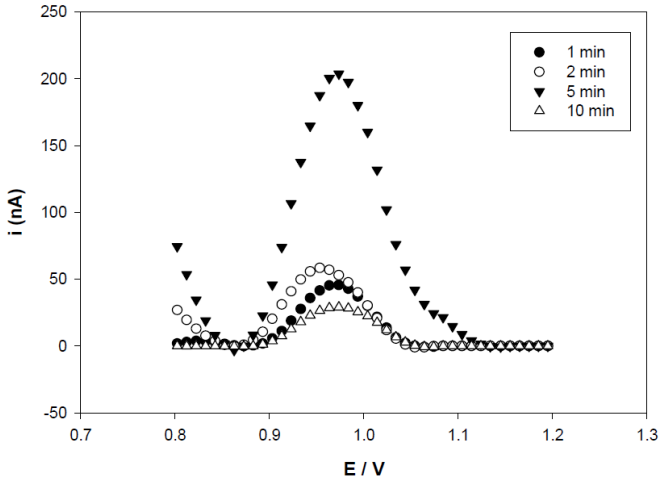


The TS-1T sequence was chosen as a target sequence, because it is present in the dengue virus genome. TS-1P is the sequence complementary to the RNA present in the virus, and thus used as the probe for construction of the biosensor. The data obtained were analyzed using the CLC Combined Workbench v 3.6.1 and NCBI Software.

3.3. Effect of Immobilization Time on the Probe

Probe immobilization is a crucial step for fabricating an electrochemical biosensor [18]. In Figure 3, the results obtained with anodic differential pulse (ADP) show the guanine oxidation signal increasing with time up to 5 min and decreasing significantly after that. This decrease can be attributed to the massive accumulation of the probe [29] on the graphite electrode, leading to an overlapping of the probes and lower availability of guanine bases. These results showed that probe immobilization could be achieved within 1 to 10 min. However, five minutes was suggested as the optimal time for probe immobilization since the electrode achieved a higher peak current. This result also was found in the systems for gene detection of interleukin-2 [29], hepatitis B [15] and electrochemical detection for human papilloma virus [23].

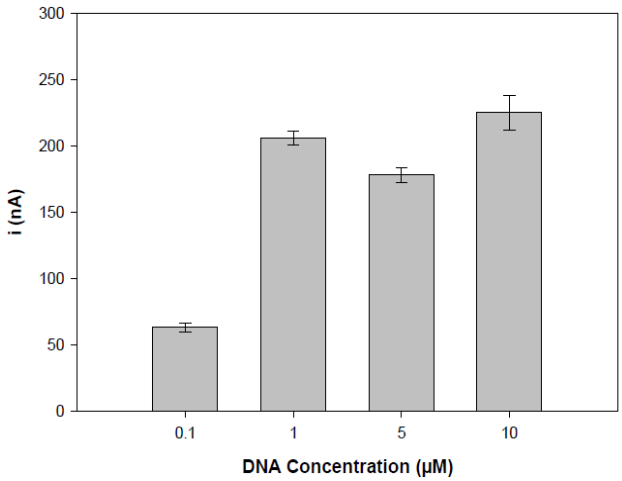
Figure 3. The differential pulse voltammograms of guanine oxidation at TSF-1P (1 µM) immobilized activated for different times (●) 1 min, (○) 2 min, (▼) 5 min and (Δ) 10 min. Voltammetric conditions: scanning potential steps, 20 mV/s; potential amplitude, 50 mV.



3.4. Effect of the Probe Concentration

The effect of probe concentration is shown in Figure 4, it was carried out by differential pulse voltammogram after 5 min of oligonucleotides (TS-1P) immobilization using a potential of 0.5 V in acetate buffer (pH 4.8) containing different concentrations of probe (0.1 to 10 µM).

Figure 4. Current peaks of the guanine oxidation signal with different concentrations of the TS-1P modified activated PGE (0.1 µM, 1 µM, 5 µM and 10 µM). The results were plotted using the means of experiments performed in triplicate.



The results showed that oxidation peak currents increased from 0.1 µM up to 1.0 µM with TS-1P. However, since the 1 µM the signal is stabilized, it was the chosen concentration to carry on further

experiments. The results, analyzed with STATISTICA 8.0 using non-parametric tests (Kruskal-Wallis), showed that 1 μM–10 μM results were statistically identical. Thus, the concentration of the 1 μM and 5 min were considered the best experimental conditions for electrode preparation. Next, the study sought to build a working electrode, with an area completely filled with the oligonucleotide sequence (probe).

3.5. Hybridization Detection

The electrodes modified with nucleic acid (polynucleotide) identify the sequence of complementary bases, through the formation of a double helix. This identification is effective and specific showed in the presence of other non-complementary sequences. When the target oligonucleotide sequence corresponds to the probe (based on the complementarily principle stating that G pairs with C and A with T or U), a hybrid is formed (probe-target).

The development of biosensor DNA or RNA consists of three steps: (a) adsorption of the oligonucleotide probe; (b) hybridization between the probe and its complementary sequence (the target) to form the hybrid; and (c) transduction [27]. The hybridization devices for biological detection of specific oligonucleotides sequences can be fabricated by applying a constant potential [32], incubating at a specific annealing temperature and at room temperature [12,33].

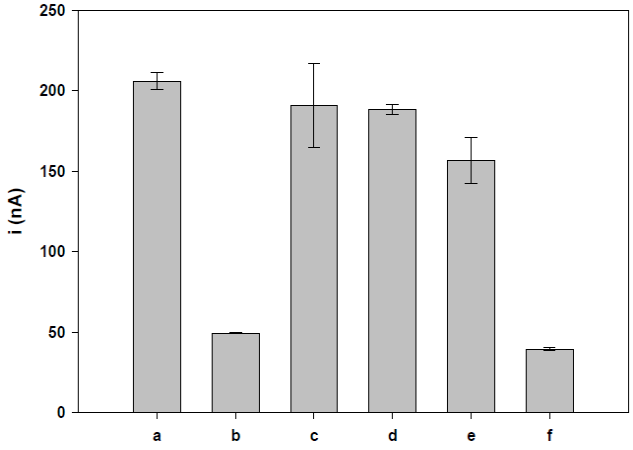
There have been various reports on label-free oligonucleotide biosensors for hybridization detection [12,22]. The oligonucleotide-modified electrode is dipped into a solution of target (DNA or RNA) to test its nucleotide sequence. When hybridization occurs, there is a decrease in electrochemical signals due to the interaction of free guanines of the probe with complementary cytosine bases present in the target sequence [22,27], the principle was used in this work. This is because of less guanine bases are availabile in the hybrid form for oxidation. Another strategy for the detection of guanine oxidation is the use of immobilized inosine-substituted (for guanine) or guanine-free probes [22], where there is a direct detection of oligonucleotide hybridization by the appearance of the oxidation signal due to the presence of guanines in the target sequence [27].

The hybridization experiments were performed in a microtube (0.2 mL), containing the probe-modified PGE, which were immersed in acetate buffer containing a known amount of complementary target oligonucleotides at a specific annealing temperature. Detection of target oligonucleotides was monitored with the formation of a guanine current peak on the probe immobilized on PGE with or without the target sequence.

The results are shown in the voltammogram for guanine oxidation (0.97 V in 20 mM Tris-HCl buffer solution, pH 7) on the probe-modified and activated PGE, before and after hybridization in solutions of complementary and non-complementary oligonucleotides (Figure 5). The current peak generated by guanine oxidation was monitored by the detection of the target oligonucleotides. The probe contains seven guanine bases, and after hybridization with the target sequence it contains only five guanines, thus there is a significant reduction in the guanine oxidation peak.

The current peak of the hybridization at the concentrations 0.5, 1, 10, 20, 30, 40 and 100 nM was statistically different from that observed with the TS-1P sequence at the concentration of 1.0 μM. The results showed that the complementary sequence could produce hybridization, causing a decrease in the guanine oxidation signal.

Figure 5. The differential pulse voltammograms of guanine oxidation at **(a)** TS-1P (1 μM) immobilized on activated PGE before hybridization, **(b)** TS-1P (1 μM) immobilized on activated PGE after hybridization with complementary TS-1T (40 nM), **(c)** with PolyG-NC (40 nM), **(d)** with TS-2 NC (40 nM) **(e)** with TS-3 NC (40 nM) and **(f)** mixture of complementary and non-complementary (PolyG-NC, TS-2 NC and TS-3 NC) (40 nM each). Voltammetric conditions: Scanning potential steps, 20m V/s. Potential amplitude, 50 mV.



3.6. Selectivity Study

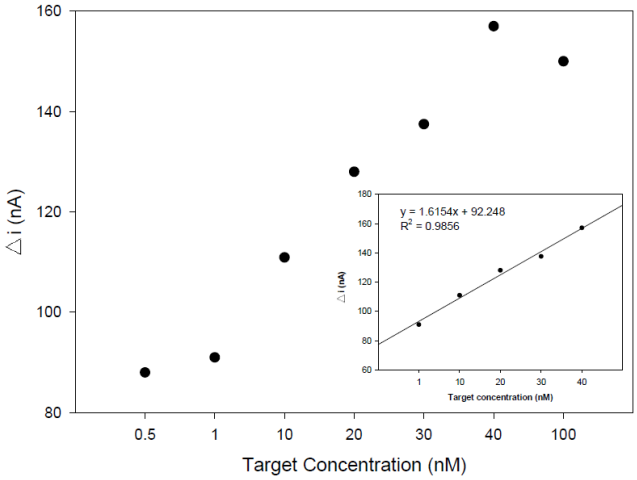
The highest guanine oxidation signal was observed with TS-1P immobilized on PGE, which has seven guanine bases present in the oligonucleotides. Experiments with non-complementary oligonucleotides including TS-2 NC, TS-3 NC and PolyG-NC (Figure 5) were performed to evaluate the selectivity of electrochemical nucleic acid biosensor. The interaction between these non-complementary oligonucleotides and probe did not lead to a significant decrease in the guanine oxidation due to absence of entire hybridization. So, the current peak of hybridized with a non-complementary sequence showed values similar to that observed following TS-1P. The selectivity demonstrates that the sequence (TS-1T) could form a double helix with the complementary oligonucleotides, causing a significant decrease in the guanine oxidation current peak.

These results confirmed that the electrochemical detection in the present study successfully distinguished complementary sequences from non-complementary sequences, using the annealing temperature to perform the hybridization. The selectivity of the biosensor was also examined in a sample containing both complementary and non-complementary sequences in similar proportions. The mixture showed a peak current similar to immobilized TS-1P after hybridization with complementary sequence. This demonstrates that the presence of non-complementary samples did not interfere in the specificity of the biosensor. These results are important for the construction of the device, because the system can be better controlled by regulating temperature than by other means such as the application of a potential.

3.7. Detection Limit

The detection limit was determined using the synthetic target sequence. The difference in the guanine oxidation signal of the probe-modified PGE in the absence and presence of complementary sequence (ΔI) increased when the target concentration increased and leveled off at a concentration of 40 nM. As seen in the inset of Figure 6, the signal was linear between 1 and 40 nM with a correlation coefficient of 0.9856 for complementary target.

Figure 6. Plot of ΔI (difference of guanine oxidation signal of the probe-modified PGE in the absence and presence of the target) *vs*. target concentration. Inset: related calibration graph at concentration range 1–40 nM for complementary target.



The detection limit and quantitation limit values were calculated using the following equations: $y_{LOD} = 3s/m$ and $y_{LOQ} = 10$ s/m, respectively [34], where s is signal of the standard deviation of the blank (0.5 nM), and m is the slope (1.6154) of the related calibration line. The regression equation was $\Delta I = 1.6154C + 92.248$ (C, nM; ΔI, A), thus the detection limit (0.92 nM) and quantitation limit (3.09 nM) were measured, and both values confirmed the sensitivity of the device. The within day and between day reproducibility (R.S.D.%) results of the signal (three independently probes) measured at 40 nM of target were 0.61% and 1.72%, respectively, indicating a remarkable reproducibility of the detection method.

Compared with other nucleic acid biosensors obtained under the same immobilization conditions (adsorption), ours displayed good sensitivity with a hybridization time of 3 minutes. In Table 1, the biosensor proposed in the present work has a low detection limit compared with the others. High-sensitivity nucleic acid detection is essential for clinical diagnosis, pathology, and genetics [35]. The use of an electroactive indicator can provide a slightly lower detection limit when compared with guanine oxidation. However, label-free monitoring based on guanine moiety oxidation signal of target in biosensors are easily manipulated, have fast responses and are inexpensive. Some studies using electroactive molecules showed higher detection limits than our results, such as for the detection of human hepatitis B (7.19 nM) [36] and detection of HPV (3.8 nM) [37].

Table 1. Comparison of nucleic acid biosensors under the same immobilization conditions.

Nucleic acid biosensor	Electrode	Immobilization Method	Electrochemical technique	Probe length (mm)	Linear range of the hybridization	Detection limit	Hybridization time
Hepatitis C virus [38]	Pencil graphite electrode (PGE)	Adsorption	DPV	20	50–750 nM	6.5 nM	15 min
Electrochemical detection of human papilloma virus (HPV) [23]	PGE	Adsorption	SWV	20	—	1.2 ng/μL	3 min
Detection of single nucleotide mutation on p53 [39]	Gold electrode	Adsorption	DPV	15	1–10 nM	0.68 nM	5 min
Oligonucleotide sensors [40]	Carbon past electrode	Adsorption	DPV	20	10–5000 nM	9 nM	5 min
This work	PGE	Adsorption	DPV	18	1–40 nM	0.92 nM	3 min

4. Conclusions

The importance of oligonucleotide biosensors for hybridization reactions via guanine oxidation has been demonstrated. Oligonucleotide biosensors utilizing pencil graphite for the detection of hybridization are useful for identifying specific nucleotide sequences (dengue virus) with selectivity, providing a convenient and rapid electroanalytical method. The hybridization of oligonucleotides on graphite was used in combination with ADP to obtain information about the hybridization reaction using a guanine oxidation signal. The detection limit for this biosensing electrode with complementary DNA target is found to be 0.92 nM within a hybridization time of 3 minutes.

Therefore, we anticipate that modification of the sequences could be used for the diagnosis of other acute infectious diseases and the utility of new electrochemical hybridization utilizing annealing temperature in the detection of hybridization of oligonucleotides using a biosensor for the diagnosis of diseases is promising. The detection of guanine oxidation eliminates the use of some compounds such as methylene blue, metal complexes and toxic substances as hybridization markers in electrochemical DNA biosensors. Breakthroughs in biosensor techniques and prudent design of diagnostics at the molecular level may contribute to future advancements in type-specific dengue diagnosis.

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References

1. Barrett, A.D.T.; Stanberry, L.R. Dengue. In *Vaccines for Biodefense and Emerging and Neglected Diseases*, 1st ed.; Vaughn, D.W., Whitehead, S.S., Durbin, A.P., Eds.; Elsevier. New York, NY, USA, 2009; pp. 288-313.
2. Araujo, J.M.; Nogueira, R.M.; Schatzmayr, H.G.; Zanotto, P.M.; Bello, G. Phylogeography and evolutionary history of dengue virus type 3. *Infect. Genet. Evol.* **2009**, *9*, 716-725.

3. Santos, H.; Tai, M.H. H.; Rocha, L.F.N.; Silva, H.H. G.; Luz, C. Dependence of Metarhizium anisopliae on high humidity for ovicidal activity on *Aedes aegypti* Adelair. *Biol*. *Contr*. **2009**, *50*, 37-42.
4. Ribeiro, K.A.; de Carvalho, C.M.; Molina, M.T.; Lima, E.P.; Lopez-Montero, E.; Reys, J.R.; de Oliveira, M.B.; Pinto, A.V.; Santana, A.E.; Goulart, M.O. Activities of naphthoquinones against Aedes aegypti (Linnaeus, 1762) (Diptera: Culicidae), vector of dengue and Biomphalaria glabrata (Say, 1818), intermediate host of Schistosoma mansoni. *Acta Tropica* **2009**, *111*, 44-50.
5. Siqueira, J.B., Jr.; Martelli, C.M.; Coelho, G.E.; Simplicio, A.C.; Hatch, D.L. Dengue and dengue hemorrhagic fever, Brazil, 1981–2002. *Emerg*. *Infect*. *Dis*. **2005**, *11*, 48-53.
6. Teixeira, M.G; Costa, M.C.; Barreto, N.F.; Barreto, M.L. Dengue: Twenty-five years since reemergence in Brazil. *Caderno de Saúde Publica* **2009**, *25*, 7-18.
7. Braga, C.; Luna, C.F.; Martelli, C.M.; de Souza, W.V.; Cordeiro, M.T.; Alexander, N.; de Albuquerque, M.F.; Junior, J.C.; Marques, E.T. Seroprevalence and risk factors for dengue infection in socio-economically distinct areas of Recife, Brazil. *Acta Tropica* **2010**, *113*, 234-240.
8. Zaytseva, N.V.; Montagna, R.A.; Baeumner, A.J. Microfluidic biosensor for the serotype-specific detection of dengue virus RNA. *Anal*. *Chem*. **2005**, *77*, 7520-7527.
9. Guzman, M.G.; Kouri, G. Dengue: An update. *Lancet Infect*. *Dis*. **2002**, *2*, 33-42.
10. Guzman, M.G.; Kouri, G. Dengue diagnosis, advances and challenges. *Int*. *J*. *Infect*. *Dis*. **2004**, *8*, 69-80.
11. Lee, N.Y.; Jung, Yun K.; Park, H.G. On-chip colorimetric biosensor based on polydiacetylene (PDA) embedded in photopolymerized poly(ethylene glycol) diacrylate (PEG-DA) hydrogel. *Biochem*. *Eng*. *J*. **2006**, *29*, 103-108.
12. Hejazi, M.S.; Pournaghi-Azar, M.H.; Alipour, E.; Karimi, F. Construction, electrochemically biosensing and discrimination of recombinant plasmid (pEThIL-2) on the basis of interleukine-2 DNA insert. *Biosens*.*Bioelectron*. **2008**, *23*, 1588-1594.
13. Karamollaoğlu, H.A.; Oktem, M.M. QCM-based DNA biosensor for detection of genetically modified organisms (GMOs). *Biochem*. *Eng*. *J*. **2009**, *44*, 142-150.
14. Souza, E.V.M.; Nascimento, G.A.; Santana, N.A.; Bruneska, D.; Lima Filho, J.L. Development of electrochemical biosensor for virus dengue diagnostic using graphite electrode. *New Biotechnol*. **2009**, *25*, 378.
15. Meric, B.; Kerman, K.; Ozkan, D.; Kara, P.; Erensoy, S.; Akarca, U.S.; Mascini, M.; Ozsoz, M. Electrochemical DNA biosensor for the detection of TT and Hepatitis B virus from PCR amplified real samples by using methylene blue. *Talanta* **2002**, *56*, 837-846.
16. Teles, F.R.R.; Prazeres, D.M.F.; Lima-Filho, J.L. Electrochemical detection of a dengue-related oligonucleotide sequence using ferrocenium as a hybridization indicator. *Sensors* **2007**, *7*, 2510-2518.
17. Drummond, T.G.; Hill, M.G.; Barton, J.K. Electrochemical DNA sensors. *Nat*. *Biotechnol*. **2003**, *21*, 1192-1199.
18. Marrazza, G.; Chianella, I.; Mascini, M. Disposable DNA electrochemical biosensors for environmental monitoring. *Anal*. *Chim*. *Acta* **1997**, *387*, 297-307.

19. Ozkan, D.; Erdem, A.; Kara, P.; Kerman, K.; Meric, B.; Hassmann, J.; Ozsoz, M. Allele-specific genotype detection of factor V Leiden mutation from polymerase chain reaction amplicons based on label-free electrochemical genosensor. *Anal*. *Chem*. **2002**, *74*, 5931-5936.
20. Deubel, V.; Laille, M.; Hugnot, J.P.; Chungue, E.; Guesdon, J.L.; Drouet, M.T.; Bassot, S.; Chevrier, D. Identification of dengue sequences by genomic amplification: Rapid diagnosis of dengue virus serotypes in peripheral blood. *J*. *Virol*. *Meth*. **1990**, *30*, 41-54.
21. Wang, Y.; Xu, H.; Zhang J.; Li, G. Electrochemical Sensors for Clinic Analysis. *Sensors* **2008**, *8*, 2043-2081.
22. Wang, J.; Rivas, G.; Fernandes, J.R.; Paz, J.L.L.; Jiang, M.; Waymire, R. Indicator-free electrochemical DNA hybridization biosensor. *Anal*. *Chim*. *Acta* **1998**, *375*, 197-203.
23. Sabzi, R.E.; Sehatnia, B.; Pournaghi-Azar, M.H.; Hejazi, M.S. Electrochemical detection of human papilloma vírus (HPV) target DNA using MB on pencial graphite electrode. *J*. *Iran*. *Chem*. *Soc*. **2008**, *5*, 476-483.
24. Dogan-Topal, B.; Uslu, B.; Ozkan, S.A. Voltammetric studies on the HIV-1 inhibitory drug Efavirenz: the interaction between dsDNA and drug using electrochemical DNA biosensor and adsorptive stripping voltammetric determination on disposable pencil graphite electrode. *Biosens*. *Bioelectron*. **2009**, *24*, 2358-2364.
25. Sukhorukov, G.B.; Montrel, M.M.; Petrov, A.I.; Shabarchina, L.I.; Sukhorukov, B.I.; Multilayer films containing immobilized nucleic acids. Their structure and possibilities in biosensor applications. *Biosens*. *Bioelectron*. **1996**, *11*, 913-922.
26. Brett, A.M.O.; Serrano, S.H.P. The electrochemical oxidation of DNA. *J*. *Braz*. *Chem*. *Soc*. **1995**, *6*, 97-100.
27. Palecek, E.; Fojta, M.; Tomschik, M.; Wang, J. Electrochemical biosensors for DNA hybridization and DNA damage. *Biosens*. *Bioelectron*. **1998**, *13*, 621-628.
28. Yardim, Y.; Keskin, E.; Levent, A.; Ozsoz, M.; Senturk, Z. Voltammetric studies on the potent carcinogen, *7*,12-dimethylbenz[a]anthracene: Adsorptive stripping voltammetric determination in bulk aqueous forms and human urine samples and detection of DNA interaction on pencil graphite electrode. *Talanta* **2010**, *80*, 1347-1355.
29. Pournaghi-Azar, M.H.; Hejazi, M.S.; Alipour, E. Developing an electrochemical deoxyribonucleic acid (DNA) biosensor on the basis of human interleukine-2 gene using an electroactive label. *Anal*. *Chim*. *Acta* **2006**, *570*, 144-150.
30. Wang, J.; Cai, X.; Rivas, G.; Shiraishi, H.; Dontha, N. Nucleic-acid immobilization, recognition and detection at chronopotentiometric DNA chips. *Biosens*. *Bioelectron*. **1997**, *12*, 587-599.
31. Pournaghi-Azar, M.H.; Hejazi, M.S.; Alipour, E. Detection of hunan interleukine-2 gene using a label-free electrochemical DNA hybridization biosensor on the basis of non-inosine substitued probe. *Electroanalysis* **2007**, *19*, 466-472.
32. Tosar, J.P.; Kell, K.; Laíz, J. Two independent label-free detection methods in one electrochemical DNA sensor. *Biosens*. *Bioelectron*. **2009**, *24*, 3036-3042.
33. Baeummer, A.J.; Cohen, R.N.; Miksic, V.; Min, J. RNA biosensor for the rapid detection of viable Escherichia coli in drinking water. *Biosens*. *Bioelectron*. **2003**, *18*, 405-413.
34. Gumustas, M.; Ozkzn, S.A. The role of and the place of method validation in drug analysis using electroanalytical techniques. *Open Anal*. *Chem*. *J*. **2011**, *5*, 1-21.

35. Zhu, D.; Tang, Y.; Xing, D.; Chen W.R. PCR-free quantitative detection of genetically modified organism from raw materials. An electrochemiluminescence-based bio bar code method. *Analytical Chemistry* **2008**, *80*, 3566-3571.
36. Zhang, S.; Tan, Q.; Li, F.; Zhang, X. Hybridization biosensor using diaquabis[*N*-(2-pyridinylmethyl)benzamide-$\kappa^2 N,O$]-cadmium(II) dinitrate as a new electroactive indicator for detection of human hepatitis B virus DNA. *Sens*. *Actuat*. *B* **2007**, *124*, 290-296.
37. Nasirizadeh, N.; Zare, H.R.; Pournaghi-Azar, M.H.; Hejazi, M.S. Introduction of hematoxylin as an electroactive label for DNA biosensors and its employment in detection of target DNA sequence and single-base mismatch in human papilloma virus corresponding to oligonucleotide. *Biosens*. *Bioelectron*. **2011**, *26*, 2638-2644.
38. Pournaghi-Azar, M.H.; Ahour, F.; Hejazi, M.S. Differential pulse voltammetric detection of Hepatitis C virus 1a oligonucleotide chain by a label-free electrochemical dna hybridization biosensor using consensus sequence of Hepatitis C virus 1a probe on the pencil graphite electrode. *Electroanalysis* **2009**, *21*, 1822-1828.
39. Raoof, J.B.; Ojani, R.; Golabi, S.M.; Hamidi-Asl, E.; Hejazi, M.S. Preparation of an electrochemical PNA biosensor for detection of target DNA sequence and single nucleotide mutation on p53 tumor suppressor gene corresponding oligonucleotide. *Sens*. *Actuat*. *B* **2011**, in press.
40. Hejazi, M.S.; Raoof, J.; Ojani, R.; Golabi, S.M.; Asl, E.H. Brilliant cresyl blue as electroactive indicator in electrochemical DNA oligonucleotide sensors. *Bioelectrochemistry* **2010**, *78*, 141-146.

Artigo Colaboração II

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Electrochemical DNA biosensor for bovine papillomavirus detection using polymeric film on screen-printed electrode

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ABSTRACT

A new electrochemical DNA biosensor for bovine papillomavirus (BPV) detection that was based on screen-printed electrodes was comprehensively studied by electrochemical methods of cyclic voltammetry (CV) and differential pulse voltammetry (DPV). A BPV probe was immobilised on a working electrode (gold) modified with a polymeric film of poly-L-lysine (PLL) and chitosan. The experimental design was carried out to evaluate the influence of polymers, probe concentration (BPV probe) and immobilisation time on the electrochemical reduction of methylene blue (MB). The polymer poly-L-lysine (PLL), a probe concentration of 1 μM and an immobilisation time of 60 min showed the best result for the BPV probe immobilisation. With the hybridisation of a complementary target sequence (BPV target), the electrochemical signal decreased compared to a BPV probe immobilised on the modified PLL-gold electrode. Viral DNA that was extracted from cattle with papillomatosis also showed a decrease in the MB electrochemical reduction, which suggested that the decreased electrochemical signal corresponded to a bovine papillomavirus infection. The hybridisation specificity experiments further indicated that the biosensor could discriminate the complementary sequence from the non-complementary sequence. Thus, the results showed that the development of analytical devices, such as a biosensor, could assist in the rapid and efficient detection of bovine papillomavirus DNA and help in the prevention and treatment of papillomatosis in cattle.

1. Introduction

Bovine papillomatosis is an infectious disease caused by a double-stranded DNA virus, bovine papillomavirus (BPV) (Campo, 2006; Claus et al., 2008). This virus can induce lesions in the stratified squamous epithelium of the skin and mucous membranes and fibroepithelial tumours (Antonsson and Hansson, 2002; Campo, 2002; Yuan et al., 2010). The BPV belong to the *Papillomaviridae* family, which contains 16 genera (Antonsson and Hansson, 2002; Ogawa et al., 2004). Ten types (BPV1–10) have been well characterised by studies on their biology and genome homology (Claus et al., 2009; Nasir and Campo, 2008). In general, three primers, MY09/MY11, GP5+/GP6+ and FAP59/FAP64, are widely used for papillomavirus identification in humans (first two pairs) and in bovine and other animals (last pair) (Lee et al., 2010; Rai et al., 2011). Research into BPV diagnostics has attracted considerable interest due to problems in the infected animals, such as dermatitis and cancer in different body sites (Borzacchiello and Roperto, 2008; Brandt et al., 2011; Campo, 2002). Consequently, these problems can contribute to economic losses in the agricultural industry. Currently, BPV diagnosis has been made through histological observations of skin and tissues. These models often do not lead to conclusive results and consequently produce a slow diagnosis.

Electrochemical DNA biosensor technologies have been developed due to their several advantages, such as specificity, selectivity, low cost, rapid diagnosis and the possibility of miniaturisation (Siddiquee et al., 2010). A DNA biosensor or genosensor recognises a complementary sequence (target) to a single-stranded DNA (probe) (Labuda et al., 2010; Souza et al., 2011). For a DNA hybridisation sensor, a probe with a defined nucleotide sequence is immobilised on a conductive surface by using immobilisation methods, such as adsorption, cross-linking, encapsulation, an avidin–biotin complex or a covalent attachment (Wang et al., 2008). The key for manufacturing a DNA biosensor is the oligonucleotide immobilisation on different types of transducer surfaces (Wang et al., 2010). Many polymers have been used in

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the development of DNA biosensors (Ghanbari et al., 2008; Jiang et al., 2008; Li et al., 2005) and have the ability to form thin layers on electrodes surfaces. They also have functional groups that can bind with DNA molecules, which allow for better performance in the immobilisation. For instance, poly-L-lysine (PLL) and chitosan are polymers widely used in the construction of DNA biosensors. The PLL can be electrodeposited or adsorbed on several types of electrodes, and it has a functional group that can link with DNA (Stobiecka and Hepel, 2011; Wang et al., 2010). Chitosan is a polysaccharide biopolymer, which is widely used in several industries, biomedicine and biosensors (Wang et al., 2011), that also binds with DNA molecules (Mandong et al., 2007). Thus, the purpose of this present study was to develop a new electrochemical DNA biosensor for bovine papillomavirus detection using polymer films on screen-printed electrodes.

2. Materials and methods

2.1. Reagents and materials

All reagents used were of a high purity. Methylene blue (MB), L-lysine and chitosan were purchased from Sigma-Aldrich (USA). UltraPure™ DNase/RNase-Free distilled water was purchased from Invitrogen (USA). All ssDNA oligonucleotides were synthesised by Integrated DNA Technologies (USA). Screen-printed electrodes were purchased from The Gwent Group (UK) for electrochemical detection.

2.2. Apparatus

A conventional three-electrode system was employed in this work. Gold was used as a working electrode (surface area of approximately 3 mm^2), silver/silver chloride (Ag/AgCl) was used as a reference electrode and carbon was used as a counter electrode. The electrochemical analysis was performed with a potentiostat (Autolab PGSTAT) that was equipped with GPES (4.0.007) software. All hybridisation experiments were carried out in a Hybridisation Oven/Shaker (Amersham Pharmacia Biotech).

2.3. Bovine papillomavirus oligonucleotides

All oligonucleotides were purchased as a lyophilised powder, diluted with ultrapure water and stored as a stock solution in a freezer. The oligonucleotides were diluted from the stock solution in 0.5 M acetate buffer pH 5 for the experiments. The following three oligonucleotides sequences, which were designed by bioinformatics tools, were used in this study:

BPV probe : 5′-TGG AAA TCT TTT TTT GAA AGG CTT TGG-3′
BPV target : 5′-CCA AAG CCT TTC AAA AAA AGA TTT CCA-3′
Non−complementaryDNA : 5′-CCC CGG ATC ACT GAG ACG-3′

2.4. Papilloma samples

15 blood samples were collected from cattle with cutaneous papillomatosis in the Itambé experimental station of Instituto Agronômico de Pernambuco (IPA), which is located in Itambé, Pernambuco, Brazil. Blood samples were kept in EDTA vacutainer tubes and refrigerated at 4 °C until viral DNA extraction.

2.5. Viral DNA extraction and PCR assay

Viral DNA was extracted from the blood samples by using an automated Maxwell® 16 Clinical Instrument System. The Maxwell® 16 Blood DNA Purification Kit was used for DNA purification according to the protocol specifications. After purification, the extracted DNA was amplified with the primer pair MY09 (forward: 5′-CGT CCM ARR GGA ACT GAT C-3′) and MY11 (reverse: 5′-GCM CAG GGC ATA AYA ATG G-3′) to amplify a fragment of 450 bp from the L1 gene. PCR was performed using the GoTaq® qPCR Master Mix kit and the following conditions: 1 μL of the extracted DNA, 6.25 μL of GoTaq® qPCR Master Mix, 1 μL of each primer and 3.25 μL of ultrapure water. The amplification was performed in the following conditions: (i) 94 °C for 3 min, (ii) 34 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min and (iii) a final extension at 72 °C for 10 min. The PCR product was visualised by electrophoresis in a 2% agarose gel containing ethidium bromide (1 mg/mL) and TBE buffer pH 8.4 (89 mM Tris, 89 mM boric acid and 2 mM EDTA) under UV light.

2.6. Preparation of polymer-modified screen-printed electrodes

The polymers chitosan and poly-L-lysine (PLL) were used to allow the immobilisation of the DNA probe onto the working electrode. The chitosan solution (1%) was prepared in an acetic acid solution (1%). The L-lysine solution (0.001 M) was prepared in 0.1 M phosphate-buffered saline (PBS) pH 7.4. For preparation of the chitosan film, 3 μL of the chitosan solution was added onto the working electrode and then incubated until dry at 30 °C for 20 min. For preparation of the PLL film, the L-lysine solution was electropolymerised on the working electrode by cyclic potential scanning from −1.8 to 2.0 V for 10 cycles with a scan rate of 100 mV s^{-1}.

2.7. Experimental design

The influence of the independent variables of the polymer (PLL and chitosan), probe concentration (1 μM and 8 μM of the BPV probe) and immobilisation time (30 min and 60 min) on the dependent variable of the MB electrochemical reduction was evaluated from the results obtained by the 2^3 full factorial design. In this design, a set of 24 experiments was performed in triplicate to allow for the estimation of pure experimental error. The results were analysed by an analysis of variance (ANOVA) at a significance level of $p < 0.05$. All statistical and graphical analyses were carried out with the Statistica 8.0 programme (StatSoft Inc.).

2.8. Probe DNA immobilisation and target DNA hybridisation

The BPV probe in an acetate buffer solution (0.5 M, pH 5) was immobilised by adsorption on a gold electrode surface that was modified with a polymer layer, and the immobilisation time was obtained in the factorial design. Then, the unbound oligonucleotides were removed from the working electrode by washing with PBS.

In the hybridisation process, a solution containing the BPV target in acetate buffer (0.5 M, pH 5) was added onto the working electrode with the immobilised BPV probe and incubated at 55 °C for 10 min with a stirred speed of 300 rpm to link the complementary sequences. This temperature was the best for annealing sequences based on the company's descriptions. A Tris–HCl buffer (20 mM, pH 7.0) was used to wash the electrode and remove the non-hybridised sequences. The same procedure was applied for the interaction of the BPV probe with a non-complementary sequence. For the hybridisation of the papilloma samples with the BPV probe on the modified working electrode, the extracted viral DNA was first heated to 94 °C for 4 min to allow the denaturation of the double helix and then added to the working electrode surface with the immobilised BPV probe. The formation

of a hybrid between the extracted viral DNA and BPV probe was also performed at 55 °C for 10 min with a stirred speed of 300 rpm, and the electrode was then washed with a Tris–HCl buffer (20 mM, pH 7.0) to remove the non-hybridised sequences.

2.9. Electrochemical analysis

For the electrochemical signal analysis, the differential pulse voltammetry (DPV) method was used for the measurement system of the current signals. After the immobilisation and hybridisation process, a 500 μM MB solution in Tris–HCl buffer (20 mM, pH 7.0) was accumulated on the modified working electrode for 5 min, which was then washed with the Tris–HCl buffer. The DPV measurement was performed in the Tris–HCl buffer for the MB electrochemical reduction under the following conditions: a potential sweep between −0.8 and 0 V, modulation amplitude of 50 mV and scan rate of 20 mV s^{-1}.

3. Results and discussion

3.1. Preliminary investigations

In this work, polymers were used to allow for the immobilisation of the probe on the gold working electrode surface. Polymers PLL and chitosan were chosen to be the electropositive compounds, which thus allow for the link with the DNA molecules that are electronegative. With these polymers, it is possible to produce thin films on different supports, such as gold electrodes.

The binding of DNA on PLL or chitosan occurred in a similar way. The immobilisation of the DNA on a PLL-gold surface was primarily due to the electrostatic attraction of the negatively charged phosphate groups from the DNA to the positively charged PLL. It is likely that hydrogen bonding between the NH^{+3} groups of PLL and the oxygens from the phosphate groups in the DNA may contribute to strengthen the attachment of the DNA to PLL (Stobiecka and Hepel, 2011). Chitosan also can form a stable complex with the polyanionic phosphodiester backbones of DNA (Singh et al., 2010). It is susceptible to chemical modifications due to the presence of reactive amino and hydroxyl functional groups and provides a hydrophilic environment for the immobilisation of desired biomolecules (Mandong et al., 2007). From the similarity of the interactions between the DNA and the polymers investigated, an experimental design was carried out to evaluate the efficiency of the polymer layer on screen-printed electrodes during the immobilisation process.

The performance of the biosensor was investigated by differential pulse voltammetry with the redox indicator methylene blue (MB). It is widely known that MB has an affinity to DNA, and several authors have demonstrated two possible mechanisms of interaction. These links can occur by an electrostatic interaction with the negatively charged phosphate groups or by an interaction between the guanine bases, which are mainly in ssDNA (Ortiz et al., 2011; Tran et al., 2011). In this work, the binding of MB occurred by an interaction between the guanine bases in ssDNA (probe) because the phosphate groups were linked to the polymer layer on the gold surface.

3.2. Factorial design analysis in the probe immobilisation

The probe immobilisation efficiency was evaluated from the 2^3 full factorial design, whose independent variables of the polymer, probe concentration (BPV probe) and immobilisation time were investigated. Fig. 1A illustrates the average of the current peak results for the low and high levels of polymer (PLL and chitosan), probe concentration (1 μM and 8 μM) and immobilisation time

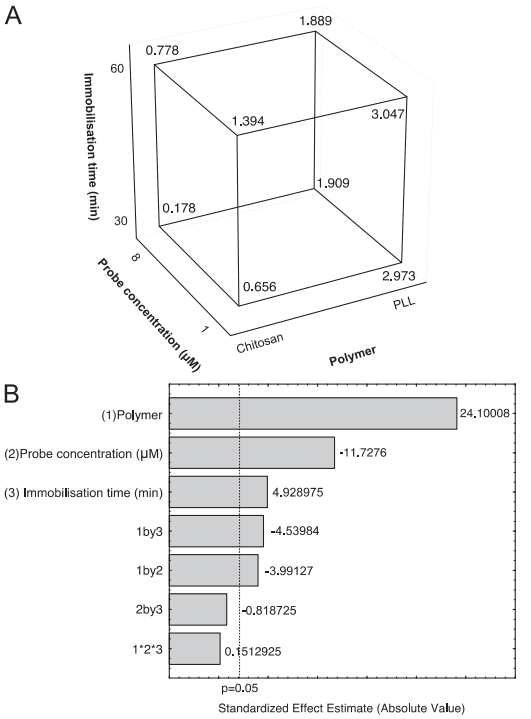

Fig. 1. (A) Graphical representation of the relationships between independent variables polymer, probe concentration and immobilisation time that were analysed by a 2^3 full factorial design. Each independent variable corresponds to an axis, with its respective low and high levels. All results were plotted with the averages of the current peaks (μA) performed in triplicate under the conditions investigated. (B) Pareto bar chart of the standardised effect estimate (absolute value) of the independent variables polymer (1), probe concentration (2) and immobilisation time (3) on MB electrochemical reduction for the 2^3 full factorial design.

(30 min and 60 min). From the 24 experiments that were performed under the conditions investigated, it was observed that the best result for the BPV probe immobilisation process on the MB electrochemical reduction was obtained with the PLL polymer, a probe concentration of 1 μM and an immobilisation time of 60 min (Fig. 1A). The chitosan most likely had no significant results on the MB electrochemical reduction compared to PLL because of its poor electrical conductivity that often results in a low sensitivity for the determination of the analytes (Wang et al. 2011).

The Pareto bar chart (Fig. 1B) represents the estimated effects of the variables and their interactions on the MB electrochemical reduction in decreasing order of magnitude. The length of the bars is proportional to the standardised effect. The vertical line is used to judge which effects are statistically significant. Bars extending beyond this line correspond to the statistically significant effects at a confidence level of 95% (Lima et al., 2009). Statistical analysis showed that the independent variables polymer and immobilisation time had a significant positive effect on the MB electrochemical reduction. Additionally, the independent variable probe concentration and the polymer-immobilisation time and polymer–probe concentration interactions had significant negative effects on the MB current peak. However, the independent variable polymer had the most significant effect, suggesting that PLL can improve the immobilisation process and, consequently, the electrochemical signal. The probe concentration-immobilisation time and polymer–probe

concentration-immobilisation time interactions had no significant effect (Fig. 1B).

The Pareto bar chart (Fig. 1B) also showed that a decrease in the probe concentration could improve the current signal. Therefore, a BPV probe concentration curve was performed using the best conditions observed in the 2^3 full factorial design, which were the modified PPL-gold electrode and an immobilisation time of 60 min. The goal of this experiment was to observe the BPV probe concentration effect on the immobilisation through the MB electrochemical reduction. In Fig. 2, it was observed that the concentrations of 1 μM and 1.5 μM had the highest current peaks; however, the concentration of 1 μM had a better reproducibility compared to 1.5 μM. The other concentrations (0.01 μM, 0.1 μM, 0.25 μM and 0.5 μM) showed lower current signals due to a low amount of immobilised probe on the working electrode surface. Thus, the probe concentration of 1 μM was chosen for the interaction with complementary sequences.

3.3. Optimisation of the hybridisation process

The biosensor recognition array was applied in experiments on the hybridisation reaction between the BPV probe and the BPV target DNA fragment. In this study, the hybridisation was performed with differential potential voltammetry on the MB electrochemical reduction. The goal of this experiment was to determine the optimal hybridisation conditions for the probe and target on the modified PLL-gold electrode while minimising the non-specific interaction. In this regard, the hybridisation was performed with different concentrations of the complementary target sequence. In Fig. 3, it was observed that the current signal increased with the increasing target concentration of up to 100 nM, and then it stabilised at 250 nM. At these concentrations, it was observed that the highest current signal was 0.9 μA (Fig. 3). However, this current signal in the hybridisation decreased when compared to the current signal with the BPV probe (3.4 μA) immobilised on the modified PLL-gold electrode. MB had a strong affinity for the free guanine in the ssDNA. Thus, this difference occurred due to a weak interaction between the dsDNA and MB compared with the ssDNA and MB, which resulted in the decreased intercalation of MB within the dsDNA (Lin et al., 2007; Souza et al., 2009).

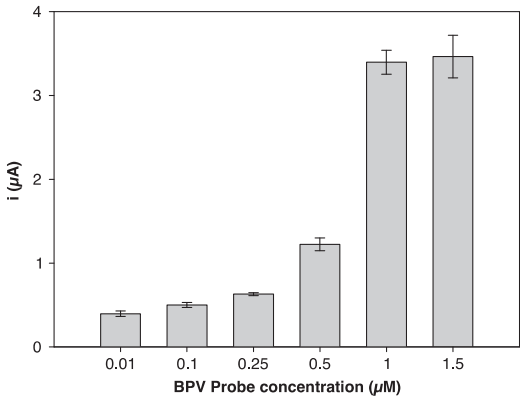

Fig. 2. Histogram of the BPV probe concentration effect on the MB electrochemical reduction during the immobilisation process. The differential pulse voltammetry method was used to analyse the current signals in the following conditions: initial potential −0.8 V, end potential 0 V, modulation amplitude 50 mV and scan rate 20 mV s^{-1}. All results plotted were the averages of triplicates that were performed at different BPV probe concentrations.

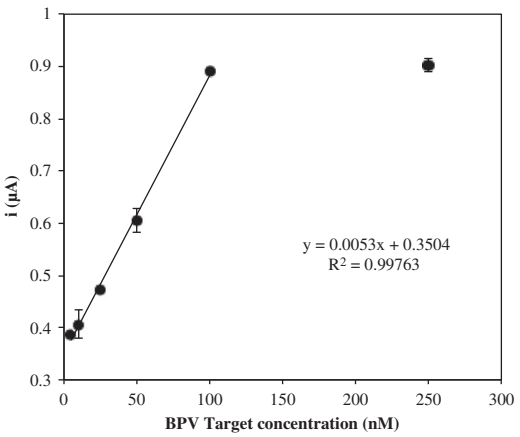

Fig. 3. The effect of the BPV target concentration on the MB electrochemical reduction during hybridisation. The differential pulse voltammetry method was used to analyse the current signals in the following conditions: initial potential −0.8 V, end potential 0 V, modulation amplitude 50 mV and scan rate 20 mV s^{-1}. All results plotted were the averages of experiments that were performed at different concentrations of the BPV target. The black line represents the linear regression at a concentration range of 5–100 nM.

The linear regression obtained from the electrochemical signal regarding the different concentrations of the BPV target is shown in Fig. 3. The calibration curve ($y=0.0053x+0.3504$) is linear between 5 nM and 100 nM with a correlation coefficient of 0.99763 ($p<0.00048$, $n=5$). A detection limit of 4.35 nM could be estimated by equation $3\sigma/a$, where σ is the standard deviation of the intercept and a is the slope of the linear regression (Gumustas and Ozkan, 2011). The relative standard deviation (RSD) was 0.056% over three independent probe-modified electrodes that were measured at 100 nM of the BPV target, which indicated a remarkable reproducibility of the detection method.

3.4. Papilloma sample analysis

In the present paper, the viral DNA extraction from the blood samples was proposed to reduce the steps in the processing of biological samples compared to skin samples (Munday and Knight, 2010). Consequently, the elimination of these steps allowed for the rapid detection of BPV. Two of the fifteen samples were amplified by the primer pair MY09/MY11. This ratio of amplification number to sample number was due to a low concentration of viral DNA in the blood. According to the literature, the identification of BPV was possible in blood samples collected from cows with papillomatosis (Wosiacki et al., 2005). In Fig. 4, it is possible to observe two amplification products of 450 bp in the electrophoresis gel that correspond to the papillomavirus L1 gene. The primer MY is widely used for the amplification and identification of the human papillomavirus L1 gene (Rai et al., 2011). However, many authors have demonstrated the amplification of the BPV L1 gene with primer MY (Ogawa et al., 2004; Silva et al., 2010). Positive and negative controls for the papillomavirus were compared with the PCR products of the bovine samples. After identification of the papillomavirus in the blood samples by PCR, we evaluated the performance of the biosensor with these positive samples. In this assay, we used the extracted viral DNA unlike other studies that used PCR products (Gao et al., 2011; Wu et al., 2011).

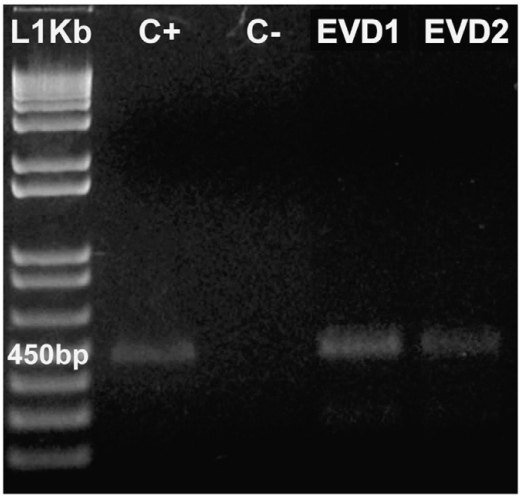

Fig. 4. The amplification of the L1 gene (papillomavirus) with the primer pair MY09/MY11 from viral DNA that was extracted from bovine blood samples. Lane L1(Kb): 1 Kb Plus DNA ladder (Invitrogen); lane C+: positive control for the papillomavirus L1 gene in pBR322.HPV16 plasmid; lane C−: negative control (ultrapure water); lane EVD1: extracted viral DNA 1 and lane EVD2: extracted viral DNA 2.

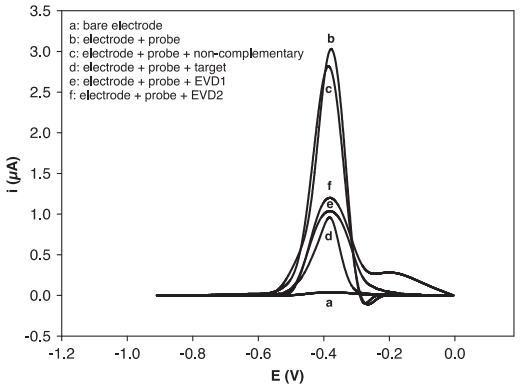

Fig. 5. The differential pulse voltammograms for the MB electrochemical reduction of (a) bare modified PLL-gold electrode; (b) 1 μM BPV probe immobilised on modified PLL-gold electrode before hybridisation; (c) 1 μM BPV probe immobilised on modified PLL-gold electrode after hybridisation with a non-complementary sequence; (d) 1 μM BPV probe immobilised on modified PLL-gold electrode after hybridisation with 100 nM BPV target sequence; (e) 1 μM BPV probe immobilised on modified PLL-gold electrode after hybridisation with 46.90 μg/mL EVD1 and (f) 1 μM BPV probe immobilised on modified PLL-gold electrode after hybridisation with 62.15 μg/mL EVD2.

3.5. Detection of bovine papillomavirus

The performance of the electrochemical DNA biosensor was investigated by checking the hybridisation of the BPV probe, which was immobilised on the modified PLL-gold electrode, with the BPV target sequence, a non-complementary sequence and the viral DNA that was extracted from the papillomatosis samples. Fig. 5 shows a voltammogram with the profiles of current peaks that were generated in different experiments. It is possible to observe that the BPV probe showed the highest current peak when compared to the other curves. This increase occurred due to a strong affinity between MB and the 7 free guanines that are present in the BPV probe. After hybridisation of the BPV probe with the complementary BPV target, the extracted viral DNA 1 (EVD1) and extracted viral DNA 2 (EVD2) current peaks were decreased, which confirmed the occurrence of hybridisation in the proposed detection system. The current peak change could be explained by the steric inhibition of MB packing between the duplex (Tran et al., 2011). The specificity of the biosensor was studied by incubating the non-complementary sequence on the probe electrode. Fig. 5 showed that the electrochemical signal after hybridisation of the BPV probe with non-complementary sequence increased when compared to the hybrid formed. The significant increase in the current signal between the BPV probe and a non-complementary sequence showed that the biosensor was highly selective. However, the experiments with a non-complementary sequence showed a current peak slightly lower than the immobilised BPV probe on the modified PLL-gold electrode, which was most likely due to the non-specific links between non-complementary sequences (Pournaghi-Azar et al., 2007). The bare modified PLL-gold electrode (curve a) did not present an electrochemical signal, which indicated the absence of guanine on the working electrode (Fig. 5). According to the analysed data, the decrease of the current peak represented a positive diagnosis for a bovine papillomavirus infection.

4. Conclusions

In this present work, it was demonstrated a new electrochemical DNA biosensor for detection of the bovine papillomavirus using synthesised oligonucleotides and extracted viral DNA with a high specificity and sensitivity. The modified gold electrode surface can be characterised by a differential pulse voltammetry. PLL presented a better performance than the chitosan during the immobilisation process. In the detection model proposed, the DNA biosensor can detect hybridisation. Therefore, it was possible to identify samples positive for the bovine papillomavirus. The BPV probe after hybridisation resulted in a decrease of the MB electrochemical reduction compared to the BPV probe before hybridisation. This decrease in the MB electrochemical reduction was related to the presence of a BPV infection in the cattle. The data obtained with the biosensor showed viability for the bovine papillomavirus diagnostic, thereby allowing for the development of a new portable detection system for viruses.

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References

Antonsson, A., Hansson, B.G., 2002. Journal of Virology 76 (24), 12537–12542.
Borzacchiello, G., Roperto, F., 2008. Veterinary Research 39 (5), 45.
Brandt, S., Apprich, V., Hackl, V., Tober, R., Danzer, M., Kainzbauer, C., Gabriel, C., Stanek, C., Kofler, J., 2011. Veterinary Microbiology 148 (2–4), 161–167.
Campo, M.S., 2002. Virus Research 89 (2), 249–261.
Campo, M.S., 2006. Bovine papillomavirus: old system, new lessons? In: Campo, M.S. (Ed.), Papillomavirus Research: From Natural History to Vaccines and Beyond. Caister Academic Press, Wymondham, pp. 373–388.
Claus, M.P., Lunardi, M., Alfieri, A.A., Otonel, R.A.A., Sartori, D., Fungaro, M.H.P., Alfieri, A.F., 2009. Brazilian Archives of Biology and Technology 52, 93–98.
Claus, M.P., Lunardi, M., Alfieri, A.F., Ferracin, L.M., Fungaro, M.H., Alfieri, A.A., 2008. Veterinary Microbiology 132 (3–4), 396–401.

Gao, H., Qi, X., Chen, Y., Sun, W., 2011. Analytica Chimica Acta 704 (1-2), 133–138.
Ghanbari, K., Bathaie, S.Z., Mousavi, M.F., 2008. Biosensors and Bioelectronics 23 (12), 1825–1831.
Gumustas, M., Ozkan, S.A., 2011. Open Analytical Chemistry Journal 5, 1–21.
Jiang, C., Yang, T., Jiao, K., Gao, H., 2008. Electrochimica Acta 53 (6), 2917–2924.
Labuda, J., Brett, A.M.O., Evtugyn, G., Fojta, M., Mascini, M., Ozsoz, M., Palchetti, I., Palecek, E., Wang, J., 2010. Pure and Applied Chemistry 82 (5), 1161–1187.
Lee, S.H., Vigliotti, V.S., Pappu, S., 2010. Journal of Clinical Pathology 63 (3), 235–239.
Li, J., Liu, Q., Liu, Y., Liu, S., Yao, S., 2005. Analytical Biochemistry 346 (1), 107–114.
Lima, C.A., Rodrigues, P.M.B., Porto, T.S., Viana, D.A., Lima Filho, J.L., Porto, A.L.F., Cunha, M.G.C., 2009. Biochemical Engineering Journal 43 (3), 315–320.
Lin, X.-H., Wu, P., Chen, W., Zhang, Y.-F., Xia, X.-H., 2007. Talanta 72 (2), 468–471.
Mandong, G., Yanqing, L., Hongxia, G., Xiaoqin, W., Lifang, F., 2007. Bioelectrochemistry 70 (2), 245–249.
Munday, J.S., Knight, C.G., 2010. Veterinary Dermatology 21 (4), 341–344.
Nasir, L., Campo, M.S., 2008. Veterinary Dermatology 19 (5), 243–254.
Ogawa, T., Tomita, Y., Okada, M., Shinozaki, K., Kubonoya, H., Kaiho, I., Shirasawa, H., 2004. The Journal of General Virology 85 (Pt 8), 2191–2197.
Ortiz, M., Fragoso, A., Ortiz, P.J., O'Sullivan, C.K., 2011. Journal of Photochemistry and Photobiology A: Chemistry 218 (1), 26–32.
Pournaghi-Azar, M.H., Hejazi, M.S., Alipour, E., 2007. Electroanalysis 19 (4), 466–472.
Rai, G.K., Saxena, M., Singh, V., Somvanshi, R., Sharma, B., 2011. Veterinary Microbiology 147 (3–4), 416–419.
Siddiquee, S., Yusof, N.A., Salleh, A.B., Abu Bakar, F., Heng, L.Y., 2010. Bioelectrochemistry 79 (1), 31–36.
Silva, M.S., Weiss, M., Brum, M.C., Dos Anjos, B.L., Torres, F.D., Weiblen, R., Flores, E.F., 2010. Journal of Veterinary Diagnostic Investigation 22 (4), 603–606.
Singh, R., Sumana, G., Verma, R., Sood, S., Sood, K.N., Gupta, R.K., Malhotra, B.D., 2010. Thin Solid Films 519 (3), 1135–1140.
Souza, E., Nascimento, G., Santana, N., Ferreira, D., Lima, M., Natividade, E., Martins, D., Lima, J., 2011. Sensors 11 (6), 5616–5629.
Souza, E.M., Nascimento, G.A., Santana, N.A., Bruneska, D., Lima Filho, J.L., 2009. New Biotechnology 25 (Suppl. 0), S378.
Stobiecka, M., Hepel, M., 2011. Biomaterials 32 (12), 3312–3321.
Tran, L.D., Nguyen, B.H., Van Hieu, N., Tran, H.V., Nguyen, H.L., Nguyen, P.X., 2011. Materials Science and Engineering C 31 (2), 477–485.
Wang, J., Zhang, S., Zhang, Y., 2010. Analytical Biochemistry 396 (2), 304–309.
Wang, Q., Zhang, B., Lin, X., Weng, W., 2011. Sensors and Actuators B: Chemical 156 (2), 599–605.
Wang, Y., Xu, H., Zhang, J., Li, G., 2008. Sensors 8 (4), 2043–2081.
Wosiacki, S.R., Barreiro, M.A.n.B., Alfieri, A.F., Alfieri, A.A., 2005. Journal of Virological Methods 126 (1-2), 215–219.
Wu, G., Yang, N., Zhang, T., Wang, Z., Lu, X., Kang, J., 2011. Sensors & Actuators B: Chemical 160 (1), 598–603.
Yuan, Z.Q., Bennett, L., Campo, M.S., Nasir, L., 2010. Virus Research 149 (1), 124–127.

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3.1 BIOSSENSOR ESPECÍFICO PARA SEQUÊNCIA DO VÍRUS DA DENGUE SOROTIPO 1[1]

Elaine Virgínia Martins de Souza
Gustavo Alves do Nascimento
Danielly Santos Campos Ferreira
Mariana Souza de Arruda
José Luiz de Lima Filho

Introdução

A dengue é uma doença que afeta mais de 100 milhões de pessoas no mundo (BRASIL, 2011), e ocorre principalmente em países tropicais, onde as condições são mais favoráveis à proliferação do mosquito transmissor (*Aedes aegypti*). A dengue pode ser causada por quatro sorotipos do vírus, chamados dengue (DENV) -1, -2, -3 e -4. O vírus da dengue (DENV) pertence à família *Flaviviridae*, e o seu genoma é composto por uma fita simples de RNA com aproximadamente 11 kb. A infecção com DENV pode causar dengue clássica (DF) que se caracteriza por um estado febril, ou pode progredir para uma dengue hemorrágica (DHF) caracterizada por manifestações como a síndrome hemorrágica de choque na dengue (DSS) (ARAÚJO et al., 2009).

Os principais sinais e sintomas da dengue clássica podem ser febre, geralmente alta (39 ºC a 40 ºC) e de início abrupto, associada à cefaléia, prostração, mialgia, artralgia, dor retroorbitária, exantema maculopapular acompanhado ou não de prurido. Anorexia, náuseas, vômitos e diarréia podem ser observados. No final do período febril, podem surgir manifestações hemorrágicas como epistaxe, petéquias, gengivorragia, metrorragia e outros.

[1] Agradecemos o apoio financeiro do Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) - Doenças negligenciadas, e ao Laboratório de Imunopatologia Keizo Asami (LIKA).

Pedido de Patente I

Biossensor Eletroquímico de Ácido Nucléico. Depositada em 03/06/2011.

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1.3 CNPJ/CPF: 216382414-49
1.4 Endereço Completo: Av. Professor Moraes Rego S/N, Cidade Universitária,
1.5 CEP: 50761-901 1.6 Telefone 08121268484 1.7 Fax: 08121268485
1.8 E-mail: joseluiz60@mac.com

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☐ Código de controle alfanumérico no formato de código de barras: fl.

☐ Listagem de sequências em formato impresso: fls.

☐ Declaração de acordo com o artigo da Resolução INPI nº 228/09: fls.

11. Documentos anexados (assinale e indique também o número de folhas):
(Deverá ser indicado o nº total de somente uma das vias de cada documento)

☒	11.1 Guia de Recolhimento	1 fls.	☒	11.5 Relatório descritivo	6 fls.
☐	11.2 Procuração	fls.	☒	11.6 Reivindicações	2 fls.
☐	11.3 Documentos de Prioridade	fls.	☒	11.7 Desenhos	5 fls.
☐	11.4 Doc. de contrato de trabalho	fls.	☒	11.8 Resumo	1 fls.
☒	11.9 Outros que não aqueles definidos no campo 11 (especificar) Lista de Inventores				1 fls.

12. Total de folhas anexadas (referentes aos campos 10 e 11): 16 fls.

13. Declaro, sob penas da Lei, que todas as informações acima prestadas são completas e verdadeiras.

Recife 03 fevereiro 2011
Local e Data

Assinatura e Carimbo

Inventores (72):

6.1 Nome: Gustavo Alves do Nascimento

6.2 Qualificação: Biólogo 6.3 CPF: 030.061.254-01

6.4 Endereço completo: Rua Guedes Pereira n 100 Ap 502 Casa Amarela Recife-PE

6.5 CEP: 52060-150 6.6 Telefone: (+55) (81) 32657583 / (81) 99685801

6.7 E-Mail: galvesn23@gmail.com

6.1 Nome: Mariana Souza de Arruda

6.2 Qualificação: Bióloga 6.3 CPF: 053.804.204-43

6.4 Endereço completo: Avenida Inácio Monteiro, nº 1012, Cordeiro, Recife-PE

6.5 CEP: 50721-320 6.6 Telefone: (81) 32279321/ (81) 99549724

6.7 E-Mail: mariana.s.arruda@gmail.com

6.1 Nome: Danielly Santos Campos Ferreira

6.2 Qualificação: Biomédica 6.3 CPF: 036.776.764-36

6.4 Endereço completo: Rua Setúbal, 984, apt 102, Boa Viagem, Recife-PE

6.5 CEP: 51030-010 6.6 Telefone: (+55) (81) 3341-5393 / 8863-1888

6.7 E-Mail: daniellylsantos@hotmail.com

6.1 Nome: Elaine Virgínia Martins de Souza

6.2 Qualificação: Biomédica 6.3 CPF: 041.392.544-75

6.4 Endereço completo: Rua Francisco Leopoldino nº 405 Apt 101 A

6.5 CEP: 50980-060 6.6 Telefone: 81 88743625

6.7 E-Mail: elainevms@yahoo.com.br

6.1 Nome: Danyelly Bruneska Gondin Martins

6.2 Qualificação: Bióloga 6.3 CPF:

6.4 Endereço completo:

6.5 CEP: 6.6 Telefone:

6.7 E-Mail: bruneska@gmail.com

Pedido de Patente II

Biossensor Eletroquímico para Detecção Específica de Papilomavírus Humano. Depositada em 17/11/2011.

INPI INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL PROTOCOLO GERAL

Uso exclusivo do INPI

Eletrônica 019110000305
17/11/2011 11:47 REPE
0000221112122103
Espaço reservado ao protocolo

PI1106062 - 0
Espaço para etiqueta

DEPÓSITO DE PEDIDO DE PATENTE OU DE CERTIFICADO DE ADIÇÃO

Ao Instituto Nacional da Propriedade Industrial:
O requerente solicita a concessão de um privilégio na natureza e nas condições abaixo indicadas

1. Depositante (71):

1.1 Nome: José Luiz de Lima Filho

1.2 Qualificação Médico

1.3 CNPJ/CPF: 216382414-49

1.4 Endereço Completo Av. Prof. Moraes Rego S/N, Cidade Universitária

1.5 CEP: 50761-901 1.6 Telefone (81)21268484 1.7 Fax: (81)21268485

1.8 E-mail: joseluiz60@mac.com

☐ continua em folha anexa

2. Natureza: ◉ Invenção ○ Modelo de Utilidade ○ Certificado de Adição

Escreva, obrigatoriamente, e por extenso, a Natureza desejada: Invenção

3. Título da Invenção ou Modelo de Utilidade ou Certificado de Adição(54):
BIOSSENSOR ELETROQUÍMICO PARA DETECÇÃO ESPECÍFICA DE PAILOMAVÍRUS HUMANO

☐ continua em folha anexa

4. Pedido de Divisão: do pedido Nº Data de Depósito:

5. Prioridade: ☐ interna ☐ unionista

O depositante reivindica a(s) seguinte(s):

País ou organização de origem	Número de depósito	Data do depósito

6. Inventor (72):

☐ Assinale aqui se o(s) mesmo(s) requer(em) a não divulgação de seu(s) nome(s)

6.1 Nome: Deborah Maria Landim Zanforlin

6.2 Qualificação Estudante 6.3 CPF: 066.369.614-30

6.4 Endereço completo Av. Prof. Moraes Rego S/N, Cidade Universitária

6.5 CEP: 50761-901 6.6 Telefone: (81)21268484 6.7 Fax: (81)21268485

6.8 E-Mail: deborahzanforlin@hotmail.com

☒ continua em folha anexa

7. Declaração na forma do item 3.2 do Ato Normativo nº 127/97:

☐ 7.1 Declaro que os dados fornecidos no presente formulário são idênticos ao da certidão de depósito ou documento equivalente do pedido cuja prioridade está sendo reivindicada.

☐ em anexo

8. Declaração de divulgação anterior não prejudicial: (Período de Graça):
(art. 12 da LPI e item 2 do AN nº 127/97) ☐ em anexo

9. Procurador (74)

9.1 Nome:

9.2 CNPJ/CPF: 9.3 API/OAB:

9.4 Endereço completo

9.5 CEP: 9.6 Telefone: 9.7 Fax:

9.8 E-Mail:

10. Listagem de sequências Biológicas (documentos anexados) (se houver):

☐ Listagem de sequências em arquivo eletrônico: nº de CDs ou DVDs (original e cópia).

☐ Código de controle alfanumérico no formato de código de barras: fl.

☐ Listagem de sequências em formato impresso: fls.

☐ Declaração de acordo com o artigo da Resolução INPI nº 228/09: fls.

11. Documentos anexados (assinale e indique também o número de folhas):
(Deverá ser indicado o nº total de somente uma das vias de cada documento)

☒	11.1 Guia de Recolhimento	1 fls.	☒	11.5 Relatório descritivo	7 fls.
☐	11.2 Procuração	fls.	☒	11.6 Reivindicações	3 fls.
☐	11.3 Documentos de Prioridade	fls.	☒	11.7 Desenhos	7 fls.
☐	11.4 Doc. de contrato de trabalho	fls.	☒	11.8 Resumo	1 fls.
☒	11.9 Outros que não aqueles definidos no campo 11 (especificar) Lista de inventores				1 fls.

12. Total de folhas anexadas (referentes aos campos 10 e 11): 20 fls.

13. Declaro, sob penas da Lei, que todas as informações acima prestadas são completas e verdadeiras.

Recife, 17 de novembro de 2011
Local e Data

Assinatura e Carimbo

Nome: Danielly Santos Campos Ferreira
CPF: 036.776.764-30
Qualificação: Biomédica
Endereço completo: Av. Prof. Moraes Rego S/N, Cidade Universitária
CEP: 50761-901
Telefone: (81)21268484
FAX: (81)21268485
Email: daniellylsantos@hotmail.com

Nome: Gustavo Alves do Nascimento
CPF: 030.061.254-01
Qualificação: Biólogo
Endereço completo: Av. Prof. Moraes Rego S/N, Cidade Universitária
CEP: 50761-901
Telefone: (81)21268484
FAX: (81)21268485
Email: galvesn23@gmail.com

Pedido de Patente III

Plataforma Transdutora Composta de 3,4-Etilenodioxitiofeno Funcionalizado com Ácido Aminobezosulfônico Humano. Depositada em 23/07/2014.

< Uso exclusivo do INPI >

INPI eletrônica
23/07/2014 01914000018º 15:12 REPE
BR 10 2014 018116 4

Espaço reservado para o protocolo | Espaço reservado para a etiqueta | Espaço reservado para o código QR

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
Sistema de Gestão da Qualidade
Diretoria de Patentes

DIRPA	Tipo de Documento: Formulário	DIRPA	Página: 1/3
Título do Documento: Depósito de Pedido de Patente		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

Ao Instituto Nacional da Propriedade Industrial:
O requerente solicita a concessão de um privilégio na natureza e nas condições abaixo indicadas:

1. Depositante (71):

1.1 Nome: José Luiz de Lima Filho

1.2 Qualificação: Médico

1.3 CNPJ/CPF: 216382414-49

1.4 Endereço Completo: Avenida Professor Moraes Rêgo, 1235

1.5 CEP: 50670-901

1.6 Telefone: (81)2126-8080 1.7 Fax:

1.8 E-mail: joseluiz60@mac.com

☐ continua em folha anexa

2. Natureza: ☒ Invenção ☐ Modelo de Utilidade ☐ Certificado de Adição

3. Título da Invenção ou Modelo de Utilidade (54):

PLATAFORMA TRANSDUTORA COMPOSTA DE 3,4-ETILENODIOXITIOFENO FUNCIONALIZADO COM ÁCIDO AMINOBENZENOSULFÔNICO

☐ continua em folha anexa

4. Pedido de Divisão: do pedido Nº **Data de Depósito:**

5. Prioridade: ☐ Interna (66) ☐ Unionista (30)

O depositante reivindica a(s) seguinte(s):

País ou Organização do depósito	Número do depósito (se disponível)	Data de depósito

☐ continua em folha anexa

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
Sistema de Gestão da Qualidade
Diretoria de Patentes

DIRPA	Tipo de Documento: Formulário	DIRPA	Página: 2/3
Título do Documento: Depósito de Pedido de Patente		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

6. Inventor (72):

☐ Assinale aqui se o(s) mesmo(s) requer(em) a não divulgação de seus nome(s), neste caso não preencher os campos abaixo.

6.1 Nome: José Luiz de Lima Filho

6.2 Qualificação: médico

6.3 CPF: 216382414-49

6.4 Endereço Completo: Av. Professor Moraes Rêgo - 1235, Cidade Universitária

6.5 CEP: 50670-901

6.6 Telefone: (81) 2126-8080 6.7 FAX:

6.8 E-mail: joseluiz60@mac.com

☒ continua em folha anexa

7. Declaração de divulgação anterior não prejudicial. ☐
Artigo 12 da LPI – período de graça.
Informe no item 11.13 os documentos anexados, se houver.

8. Declaração na forma do item 3.2 da Instrução Normativa PR nº 17/2013:

☐ Declaro que os dados fornecidos no presente formulário são idênticos ao da certidão de depósito ou documento equivalente do pedido cuja prioridade está sendo reivindicada.

9. Procurador (74):

9.1 Nome:

9.2 CNPJ/CPF: 9.3 API/OAB:

9.4 Endereço Completo:

9.5 CEP:

9.6 Telefone: 9.7 FAX:

9.8 E-mail:

☐ continua em folha anexa

10. Listagem de sequências biológicas. ☐
Informe nos itens 11.9 ao 11.12 os documentos anexados, se houver.

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
Sistema de Gestão da Qualidade
Diretoria de Patentes

DIRPA	Tipo de Documento: Formulário	DIRPA	Página: 3/3
Título do Documento: Depósito de Pedido de Patente		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

11. Documentos Anexados:

(Assinale e indique também o número de folhas):
(Deverá ser indicado o número total de somente uma das vias de cada documento).

		Documentos Anexados	folhas
☒	11.1	Guia de Recolhimento da União (GRU).	1
☐	11.2	Procuração.	
☐	11.3	Documentos de Prioridade.	
☐	11.4	Documento de contrato de trabalho.	
☒	11.5	Relatório descritivo.	7
☒	11.6	Reivindicações.	1
☒	11.7	Desenho(s) (se houver). Sugestão de figura a ser publicada com o resumo: nº, _____ por melhor representar a invenção (sujeito à avaliação do INPI).	5
☒	11.8	Resumo.	1
☐	11.9	Listagem de sequências em arquivo eletrônico: _____nº de CDs ou DVDs (original e cópia).	
☐	11.10	Código de controle alfanumérico no formato de código de barras referente às listagem de sequências.	
☐	11.11	Listagem de sequências em formato impresso.	
☐	11.12	Declaração relativa à Listagem de sequências.	
☒	11.13	Outros (especificar) Lista de autores	2

12. Total de folhas anexadas: 17 **fls.**

13. Declaro, sob as penas da Lei que todas as informações acima prestadas são completas e verdadeiras.

Recife, 23 de julho de 2014
Local e Data

Assinatura e Carimbo

6. Inventores (72)

6.1 Maria Amélia Carlos Souto Maior Borba

6.2 Qualificação: Estudante 6.3 CPF: 089.615.204-90

6.4. Endereço: Avenida Professor Moraes Rêgo, 1235 Cidade Universitária

6.5 CEP: 50670-901 6.6 Telefone: (81) 9821-7535

6.7 Email: mariameliaborba@gmail.com

6.1 Danielly Santos Campos Ferreira

6.2 Qualificação: Biomédica 6.3 CPF: 036.776.764-36

6.4 Endereço: Avenida Professor Moraes Rêgo, 1235 Cidade Universitária

6.5 CEP: 50670-901 6.6 Telefone: (81) 8863-1888

6.7 Email: 8771-0112

6.1 Deborah Maria Landim Zanforlin

6.2 Qualificação: Biomédica 6.3 CPF: 066.369.614-30

6.4 Endereço: Avenida Professor Moraes Rêgo, 1235 Cidade Universitária

6.5 CEP: 50670-901 6.6 Telefone: (81) 9516-6364

6.7 Email: deborahzanforlin@gmail.com

6.1 Gustavo Alves do Nascimento

6.2 Qualificação: Biólogo 6.3 030.061.254-01CPF:

6.4 Endereço: Avenida Professor Moraes Rêgo, 1235 Cidade Universitária

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6.1 Natália Cybelle Lima Oliveira

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6.1 Mirella Monteiro Silva

6.1 Qualificação: Estudante 6.3 CPF: 093644924-12

6.4 Endereço: Avenida Professor Moraes Rêgo, 1235 Cidade Universitária

6.5 CEP: 50670-901 6.6 Telefone: (81) 9900-5740

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6.1 Renato Pessoa e Melo Neto

6.2 Qualificação: Estudante 6.3 CPF: 015.094.064-50

6.4 Endereço: Avenida Professor Moraes Rêgo, 1235 Cidade Universitária

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6.1 Sérgio Luiz Rocha Gomes Filho

6.2 Qualificação: Biólogo 6.3 CPF: 048.710.584-25

6.4 Endereço: Avenida Professor Moraes Rêgo, 1235 Cidade Universitária

6.5 CEP: 50670-901 6.6 Telefone: (81) 9163-1636

6.7 Email: rochagomesfilho@gmail.com

6.1 Wessulla Suzana Bezerra Ribeiro

6.2 Qualificação: Educadora Física 6.3 CPF: 029.491.634-24

6.4 Endereço: Avenida Professor Moraes Rêgo, 1235 Cidade Universitária

6.5 CEP: 50670-901 6.6 Telefone: (81) 8771-0112

6.7 Email: wessullas@yahoo.com.br

6.1 Mariana Souza Arruda de Santana

6.2 Qualificação: Bióloga 6.3 CPF: 053.804.204-43

6.4 Endereço: Avenida Professor Moraes Rêgo, 1235 Cidade Universitária

6.5 CEP: 50670-901 6.6 Telefone: (81) 3031-3710

6.7 Email: mariana.s.arruda@gmail.com