Universidade Federal de Pernambuco

Centro de Ciências Biológicas-CCB

Programa de pós-graduação em ciências biológicas-PPGCB

Desenvolvimento de imunossensor eletroquímico baseado no uso de polímero condutor e nanotubos de carbono para diagnóstico do Infarto agudo do Miocárdio

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Recife, 2016.

Catalogação na Fonte:
Bibliotecário Bruno Márcio Gouveia, CRB-4/1788

Silva, Cybele Emanuele da
Desenvolvimento de imunossesor eletroquímico baseado no uso de polímero condutor e nanotubos e carbono para diagnóstico do infarto agudo do miocárdio / Cybele Emanuele da Silva. – Recife: O Autor, 2016.
60 f.: il.
.
Orientadora: Rosa Amalia Firemam Dutra
Dissertação (mestrado) – Universidade Federal de Pernambuco. Centro de Biociências. Programa de Pós-graduação em Ciências Biológicas, 2016.
Inclui referências e anexos

1. Infarto do miocárdio 2. Coração – Doenças – Diagnóstico I. Dutra, Rosa Amalia Firemam (orient.) II. Título.

616.1237 CDD (22.ed.) UFPE/CCB-2016-339

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Dissertação apresentada ao Programa de Pós-Graduação Stricto sensu de Ciências Biológicas, da Universidade Federal de Pernambuco, como parte dos requisitos exigidos para obtenção do título de Mestre em Ciências Biológicas.

Data da aprovação 24/02/16.

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Recife, 2016.

Dedico este trabalho aos meus pais Roberto e Telma que são essenciais na minha vida, que sempre me apoiam e lutam comigo na realização dos meus sonhos. À minha irmã por todo seu cuidado, amor e atenção dedicados, minha eterna gratidão. Aos meus familiares, por cada dia em que posso compartilhar minha vida com eles. Aos meus verdadeiros amigos que estão ao meu lado em qualquer circunstância.

AGRADECIMENTOS

*Em particular, agradeço a **Jeová Deus** por me presentear com a dádiva da vida e com muitas bênçãos durante todos os anos de minha vida vividos, por todo apoio encontrado mediante orações e alimento espiritual providos.*

*À **Rosa Dutra**, minha orientadora, que pacientemente me ensinou os caminhos da vida científica, e que além da relação profissional a considero como uma amiga que demonstra uma humanidade incrível ao lidar com todos que a rodeiam.*

*Aos meus amigos do **LAPED** por todas as experiências vividas e dias de trabalho compartilhados.*

*Á **Bárbara,** que com toda sua inteligência e competência foi de grande ajuda na minha formação como pesquisadora, sempre me acompanhado de perto no laboratório. Por seus conselhos pertinentes como amiga, minha admiração!*

*Á **Blanca**, com quem dividi muitos bons momentos, risadas e trabalho, e que é um exemplo de companheirismo e disposição para ajudar a todos no que for necessário.*

*Á **Priscila** que sempre é uma grande companheira, me socorrendo no laboratório quando eu mais precisava.*

*Aos meus pais **Roberto e Telma**, que nutro profundo amor, que são e foram essenciais para a minha formação tanto intelectual como pessoal. Sempre estando ao meu lado em tudo que idealizo e me comprometo a fazer, apoiando meus sonhos e fazendo com que cada conquista seja comemorada e às vezes algumas perdas sejam superadas. serei eternamente grata.*

*Á minha irmã **Gabrielle**, que como irmã mais velha é uma mãe para mim, agradeço todos os seus cuidados e preocupações prestados. A amo muitíssimo!*

*Aos meus familiares, em especial, minha **avó Vera**, minhas tias **Socorro** e **Márcia** que alegram todos os meus dias com seu amor, carinho e mimos.*

*Ao meu namorado **Jandiel,** por me proporcionar um amor verdadeiro, estando ao meu lado nos momentos em que mais precisei, sendo meu porto seguro. Obrigada por alegrar e encher meus dias de amor e carinho.*

*À minha amiga do coração **Aline Pitt** pelos longos anos de amizade, companheirismo, avemturas, alegrias e tristezas compartilhadas. Por sempre disponibilizar do seu tempo para me ajudar, apoiar no que for preciso e por todas boas conversas que já tivemos.*

*Á **FACEPE** pelo apoio financeiro prestado, que permitiu a realização do meu mestrado.*

Sou extremamente grata a todos. Meu muito Obrigada!

,

“Que os vossos esforços desafiem as impossibilidades, lembrai-vos de que as grandes coisas do homem foram conquistadas do que parecia impossível”.

Charles Chaplin

RESUMO

A busca por métodos que possibilitem o diagnóstico mais rápido e eficaz do infarto agudo do miocárdio é preponderante atualmente. Como possibilidade analítica mais atrativa, os imunossensores são uma grande tendência na área cardiológica. Entretanto, para o aperfeiçoamento destes dispositivos, buscam-se continuamente algumas estratégias, tais como o uso de nanomateriais e polímeros condutores com objetivo de obter uma plataforma sensora mais sensível, estável e reprodutível. Neste trabalho, um filme nanoestuturado de nanotubos de carbono e polipirrol foi desenvolvido sobre a superfície de um eletrodo de platina para detecção eletroquímica da troponina T cardíaca humana. O filme de pirrol (0,3M) e nanotubos de carbono (0,1 mg/mL) foram eletropolimerizados através da técnica de cronoamperometria (0,8 V) sobre a superfície sensora. Os grupos carboxílicos reativos do filme foram ativados para imobilização covalente dos anticorpos anti-troponina T. Os sítios livres não reativos foram bloqueados com uma solução de glicina (0,01 M). A técnica de voltametria cíclica em sonda redox foi empregada para caracterização do processo de construção da plataforma sensora. Nos resultados obtidos, a eletrossíntese do pirrol em conjunto com os nanotubos apresentou um aumento significativo na transferência de elétrons. O tempo de polimerização do filme sobre a interface do eletrodo foi otimizado quando o ensaio croamperométrico foi programado para 80 s no potencial de 0,8 V. O filme de pirrol e nanotubos apresentou uma boa estabilidade eletroquímica, com coeficientes de variação para os picos redox $\leq$ 5%. Uma vez confirmada à viabilidade do filme, ensaios para otimização da concentração do anticorpo imobilizado, tempo de imunoreação e transferência de carga foram realizados. Uma curva de calibração foi obtida para quantificação livre de marcação da troponina T em amostras limpas, utilizando a técnica de voltametria de pulso diferencial. O imunossensor alcançou uma boa linearidade ($r=0,94$; $p<0,0001$) com erro relativo menor do que 1%, apresentando um limite de detecção de 0,006 ng/mL para cTnT. Com a metodologia proposta foi possível desenvolver um filme nanoestruturado em uma única etapa de síntese para determinação da troponina T humana em níveis de importância clínica.

Palavras-chave: nanotubos de carbono; polipirrol; imunossensor eletroquímico; troponina T cardíaca humana; infarto agudo do miocárdio.

ABSTRACT

The search for methods that allow the fast and effective diagnosis of acute myocardial infarction is currently preponderant. As a more attractive analytical possibility, the immunosensors are a big trend in the field of cardiology. However, for the improvement of these devices, the search for some strategies such as the use of nanomaterials and conducting polymers is continuous in order to obtain a more sensitive, stable and reproducible sensor platform. In this work, a nanostructured film of carbon nanotubes and polypyrrole was developed on the surface of a platinum electrode for electrochemical detection of human cardiac troponin T. The pyrrole film (0.3M) and carbon nanotubes (0.1 mg/mL) were electropolymerized using the chronoamperometry technique (0.8 V) on the sensor surface. The reactive carboxylic groups of the film were activated for the covalent immobilization of anti-troponin T. The non-reactive free sites were blocked with a solution of glycine (0.01 M). The cyclic voltammetry technique in redox probe was used to characterize the process of construction of the sensor platform. In the results, the electrosynthesis of pyrrole in conjunction with the nanotubes had a increase in the electron transfer. The film's polymerization time over the electrode interface was optimized when the chronoamperometric test was set to 80 s in the potential of 0.8 V. The pyrrole and nanotube film exhibited good electrochemical stability, with variation coefficients for the redox peaks ≤ 5%. Once confirmed the viability of the film, testing for optimization of the immobilized antibody concentration, immunoreaction time and charge transfer were performed. A calibration curve was obtained for quantification of the marking free troponin T in clean samples, using the technique of voltammetry differential pulse. The immunosensor achieved a good linearity ($r = 0.94$, $p < 0.0001$) with less than 1% relative errors, presenting a detection limit of 0.006 ng/mL for cTnT. With the proposed methodology has been possible to develop a nanostructured film in a one-step synthesis for determination of human cardiac troponin T at clinical importance levels.

KEY WORDS: Carbon nanotubes; polypyrrole; electrochemical immunosensor; human cardiac troponin T; acute myocardial infarction.

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Lista de Abreviaturas, Siglas e Símbolos

Ag/AgCl	Prata/cloreto de prata
ACC	*"American College of Cardiology"*
AHA	*"American Heart Association"*
anti-TnT	Anti-troponina cardíaca humana
CA	Cronoamperometria
BRA	Brasil
COOH-CNTs	Nanotubos de Carbono funcionalizados com grupamentos carboxílicos
CK	*"Chreatine kinase"*- Creatinaquinase
cTnT	Troponina T cardíaca humana
DCVs	Doenças cardiovasculares
DPV	Voltametria de Pulso diferencial
DMF	Dimetilformamida
ECG	Eletrocardiograma
CK-MB	*"Chreatine kinase-myocardial band*"– Isoforma cárdica da creatinoquinase
VC	Voltametria Cíclica
CNT	Nanotubos de Carbono
DCV	Deposição de Carbono a Vapor
ESC	*"European Society of Cardiology"*
ECLIA	"*Electrochemiluminescence Immunossay*" - Imunoensaio Eletroquimioluminescente
EDC	*N*-etil-*N´*-(3-dimetilaminopropil) carbodiimida
FT-IR	Infravermelho por transformada de Fourier

E_{pa}	Potencial de pico anódico
E_{pc}	Potencial de pico catódico
EPt	Eletrodo de Platina
SPN	Espanha
USA	Estados Unidos da América
H_2SO_4	Ácido sulfúrico
I	Corrente
IAM	Infarto Agudo do Miocárdio
LDH	Desidrogenase Láctica
I_{pa}	Corrente de pico anódico
I_{pc}	Corrente de pico catódico
$K_3[Fe(CN)_6]$	Ferricianeto de potássio
$K_4[Fe(CN)_6]$	Ferrocianeto de potássio
NACB	*"National Academy of Clinical Biochemistry"*
NHS	*N*-hidroxi succinimida
NTCs	*Nanotubos de Carbono*
OMS	*Organização Mundial de Saúde*
Pi	Pirrol
PPi	Polipirrol
PPY	Polipirrol
Pg	Picogramas
Pt	Platina
PTE	Eletrodo de Platina
TnI	Troponina I
TnT	Troponina T
PBS	Tampão fosfato salino

KCl	Cloreto de Potássio
MWCNTs	Multi-walled Carbon
SWCNTs	Single-walled Carbon Nanotubes
SEM	Microscopia Eletrônica de Varredura

Sumário

1-INTRODUÇÃO

As doenças cardiovasculares (DCVs) são consideradas a maior causa de morte em todo o mundo, de acordo com a Organização Mundial de Saúde (OMS) cerca de 17.5 milhões de pessoas morrem todo ano devido as DCVs , o que corresponde a 31% da taxa de mortalidade mundial. (OMS, 2015). Atrelado aos dados estatísticos, observa-se um grande impacto econômico, visto que, há um aumento na incidência de acometimentos cardiovasculares na população em idade produtiva. Dentre as principais DCVs, o infarto agudo do miocárdio (IAM) destaca-se como seu principal representante relatado em emergências hospitalares (AVEZUM; MAIA; NAKAZONE, 2012).

O IAM caracteriza-se pela ocorrência da isquemia do miocárdio, causada pela interrupção de fluxo sanguíneo nas artérias coronárias. Quando o fornecimento sanguíneo excede um limiar crítico superando os mecanismos de reparação celular os danos causados são irreversíveis resultando na morte do tecido cardíaco (KADIR; TOTHILL, 2010). O retardo no diagnóstico aumenta os riscos de complicações e contribui para maiores gastos nos sistemas públicos de saúde. Dessa forma, o diagnóstico precoce é fundamental para estratificação de riscos e prognóstico do paciente. Na rotina clínica faz-se o diagnóstico do IAM através dos sintomas do paciente e por meio da avaliação das modificações dos traços eletrocardiográficos associados à elevação de biomarcadores cardíacos (SCHULL; STUKEL, 2006). Em particular, os biomarcadores cardíacos são ferramentas que desempenham um papel essencial no diagnóstico do IAM, pois estão presentes em quantidades mínimas durante a fase inicial do processo de isquemia.

As troponinas cardíacas T (cTnT) e I (TnI) são consideradas biomarcadores “padrão ouro” devido a sua cardioespecificidade ao tecido miocárdio. cTnT e TnI fornecem praticamente as mesmas informações a nível clínico, possuindo sensibilidade e especificidade na ordem de 97% comprovadas através dos testes de dosagem empregados na prática laboratorial (BARRETO et al, 2015). A cinética de liberação das cTnT e cTnI permite a mensuração dos níveis séricos a partir 2-4 horas após início dos sintomas, que continuam elevados por um período de 24 horas (JNEID et al., 2013).

Como método para determinação e quantificação da cTnT e TnI utilizam-se atualmente técnicas de imunoensaios enzimáticos, entretanto, tais técnicas demandam mão de obra qualificada, custo elevado e longo tempo de análise (CI, 2012). Uma estratégia para superar as dificuldades existentes é o desenvolvimento de imunossensores.

Os imunossensores eletroquímicos são dispositivos analíticos baseados na interação específica existente entre antígeno-anticorpo. Em uma mistura complexa com soro e plasma essa interação específica torna-se vantajosa, pois permite a detecção e quantificação de biomarcadores em níveis baixos na ordem de picogramas (pg). Comparados com os imunosensaios convencionais, são mais fáceis de utilizar, eficazes, descartáveis e compatíveis com a tecnologia de miniaturização, o que permite a sua portabilidade. A utilização desses dispositivos diminui os custos envolvidos no processo terapêutico, o que faz com que os imunossensores sejam uma ferramenta analítica que vem atraindo atenção nos últimos anos (BURCU; KEMAL, 2015).

No intuito de aprimorar os imunossensores, a nanotecnologia vem sendo empregada no seu desenvolvimento. Os nanomateriais com suas propriedades físicas e químicas únicas com dimensões entre 1-100nm promovem o aumento da sensibilidade e perfomance destes dispositivos (JIANRONG et al., 2004). Dentre os nanomateriais, os nanotubos de carbono (NTCs) são os mais extensivamente utilizados no design dos imunossensores. Os NTCs formam uma superfície nanoestruturada que permite a imobilização de uma maior quantidade de biomoléculas, alcançando alta sensibilidade com baixo limite de detecção (YANG et al., 2015).

Adicionalmente aos nanomateriais, a utilização de polímeros condutores é uma abordagem interessante para a elaboração de imunossensores eletroquímicos (TAM; HIEU, 2011). Combina-se as características dos polímeros condutores, como reprodutibilidade, estabilidade, aderência e homogeneidade na deposição eletroquímica, com aquelas dos NTCs, resultando no aumento do desempenho desses dispositivos. Dentre os polímeros condutores, o polipirrol é uma referência para a elaboração de interfaces sensoras, devido a sua condutividade elétrica, estabilidade e biocompatibilidade (AMOUZADEH; SHAMSIPUR; MOSTAFAIE, 2016).

Neste trabalho, realizou-se a elaboração de uma plataforma sensora nanoestruturada de nanotubos de carbono em uma matriz polimérica de polirrol para

aplicação na detecção eletroquímica livre de marcação da troponina T cardíaca humana (cTnT) no diagnóstico do infarto.

2-OBJETIVOS

2.1- OBJETIVO GERAL

Desenvolver um imunossensor eletroquímico baseado em nanohíbridos de polímeros condutores e NTCs para aplicação no diagnóstico do IAM.

2.1- OBJETIVOS ESPECÍFICOS

- Caracterizar e otimizar o método de eletropolimerização eletroquímica dos polímeros condutores com NTCs sobre a superfície sensora em uma única etapa de síntese;

- Realizar testes de imobilização dos anticorpos anti-cTnT, para a avaliação da funcionalidade do filme formado;

-Otimizar as condições experimentais do imunoensaio eletroquímico quanto a concentração de anticorpo imobilizado, tempo de imunoreação e transferência de carga na interface sensora;

- Estabelecer a resposta do imunossensor para determinação em tempo real eletroquímica da cTnT, empregando as técnicas de voltametria de pulso diferemcial;

CAPÍTULO 1-REVISÃO BIBLIOGRÁFICA

3- Infarto Agudo do Miocárdio (IAM)

O IAM é definido por um processo isquêmico prolongado e consequente morte celular do músculo miocárdio. A causa mais comum que resulta no IAM é a aterosclesose (KAKOTI; GOSWAMI, 2013), que consiste na formação e ruptura de placas aterosclérosticas (**figura 1**). Hábitos de grande consumo de colesterol, estilo de vida sedentário e predisposição genética contribuem para a deposição de colesterol no endotélio das artérias coronárias. Gradualmente essa deposição torna-se calcificada originando as placas ateroscleróticas. O processo patogênico envolve o desenvolvimento de trombos, devido à agregação plaquetária, após o irrompimento e exposição das placas a cascata de coagulação do organismo. Os trombos danificam o miocárdio pois podem conduzir à oclusão parcial ou total vascular levando a necrose tecidual.(GUYTON; HALL, 2006).

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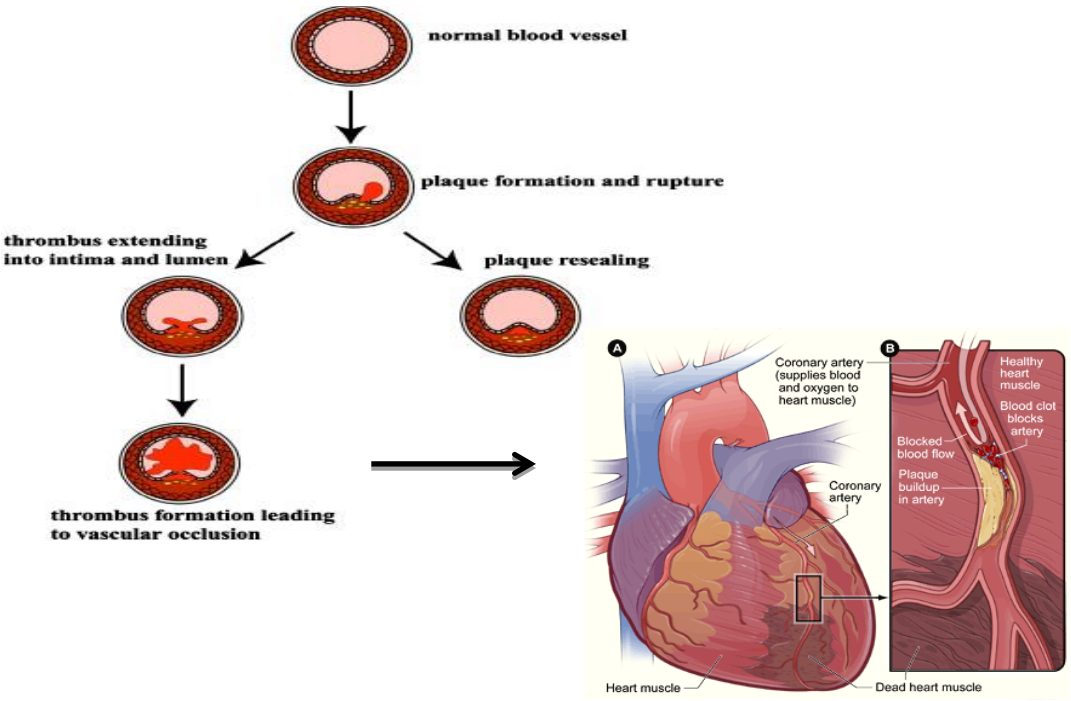
Figura 1: Formação de placas ateroscleróticas com posterior oclusão vascular no infarto agudo do miocárdio.



Fonte: (Goswami et al, 2013).

Estudos da organização mundial de saúde (OMS) definem que o diagnóstico do IAM é realizado através dos sintomas clínicos, anormalidades no eletrocardiograma

(ECG) e níveis alterados de biomarcadores cardíacos (BOERSMA et al., 2003). Algumas técnicas de imagem, como por exemplo, a angiotomografia, também podem ser utilizadas, mas ainda não estão amplamente disponíveis nos sistemas públicos devido ao seu alto custo que dificulta o seu acesso a grande maioria da sociedade (THYGESEN et al., 2007).

Os sintomas isquêmicos incluem vários fatores que são observados em conjunto, como dor no peito que gradualmente aumenta de intensidade e irradia-se para a mandíbula, ombros e braços, suor, naúsea, vômito, tontura, ansiedade e agitação com sensação de morte. Adicionalmente existem sintomas atípicos como dor de cabeça, dor abdominal e palpitações. Esses desconfortos geralmente possuem a duração de > 20 minutos (ABED et al., 2015).

Diante desse quadro clínico de pacientes sintomáticos, realiza-se o ECG que irá apresentar mudanças dinâmicas nos segmentos das ondas ST e Q, que quando presentes, permitem identificar a artéria relacionada com o infarte e estimar o grau de tecido danificado durante o evento do IAM. Entretanto, apenas 57% dos pacientes apresentam alterações eletrocardiográficas precisas para o diagnóstico acurado do infarto (HASANZADEH et al., 2013)

Em relação aos biomarcadores cardíacos, sabe-se que eles desempenham um papel essencial no diagnóstico do IAM, estando presentes em baixos níveis na corrente sanguínea permitindo um diagnóstico durante a fase inicial do evento. Os diversos biomarcadores utilizados são as troponinas (T e I), creatinaquinase (CK, CK-MB), mioglobina dentre outros (KADIR; TOTHILL, 2010).

3.1 Biomarcadores Cardíacos

Biomarcadores cardíacos são definidos como analitos biológicos liberados na corrente sanguínea, com níveis que possam ser mensuráveis, durante ou imediatamente após o dano miocárdio (KAKOTI; GOSWAMI, 2013). São de fundamental importância no diagnóstico do IAM, pois auxiliam no prognóstico do paciente. O processo de liberação dá-se quando as células miocárdicas são irreversivelmente danificas perdendo a integridade de suas membranas e as proteínas são difundidas na corrente sanguínea. A cinética de liberação dos biomarcadores cardíacos depende de diversos fatores como o

tempo de liberação de cada biomarcador, do início do processo isquêmico e do método que será utilizado para a sua detecção (IOANNIDIS et al., 2001).

No passado, para o diagnóstico do IAM mensuravam-se as enzimas Creatinaquinase (CK) total e desidrogenase láctica (LDH), entretanto, com surgimento de outros marcadores bioquímicos específicos para a lesão miocárdica tais enzimas vêm sendo pouco utilizadas. Com isso, a mioglobina, a fração enzimática da creatinaquinase (CK-MB) e as troponinas ganharam espaço na prática (CONTENTS, 2000).

A mioglobina é liberada mais rapidamente no IAM do que as troponinas e a CK-MB. Devido ao seu pequeno tamanho, as moléculas de mioglobina são liberadas no sangue 1 hora após a dor no peito, característica do IAM, chegando a um nível máximo dentro de 2 horas enquanto que as troponinas e a CK-MB levam em torno de 3 e 6 horas respectivamente. Os níveis de mioglobina aumentam na faixa de 90 pg/ml^{-1} até >250 ng/ml^{-1} no período de 90 min, com os níveis de troponinas e CK-MB normais. Possui alta sensibilidade, porém baixa especificidade já que encontra-se tanto no músculo esquelético quanto no cardíaco (EL-SAFTY; FOUAD; EL-, 2016).

A CK-MB (86 kDa) é uma das três formas da isoenzima CK , seus níveis aumentam de 3-12 horas após o início dos sintomas do infarto, atingindo valores de pico em 24 horas, com concentrações variando entre 39–185 ng/mL (MOREIRA et al., 2014). Não podendo ser utilizada como um marcador precoce, a detecção da CK-MB, tradicionalmente, é realizada para a identificação de reinfartos. Sua especificidade é limitada, pois assim como a mioglobina, encontra-se tanto no músculo cardíaco quanto no esquelético. Faz-se necessário seu uso em conjunto com outros biomarcadores (THYGESEN et al., 2007).

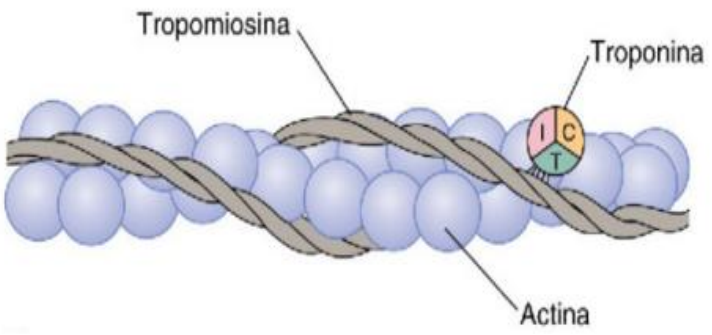
As troponinas cardíacas cTn (T e I) compõem o aparelho contrátil das células do miocárdio, possuindo alta especificidade em comparação com a CK-MB e a mioglobina. Clinicamente as troponinas Te I fornecem informações idênticas, sendo a seleção e escolha do uso de uma ou outra atrelada ao tipo de ensaio laboratorial a ser utilizado. Adicionalmente, as troponinas cardíacas são liberadas rapidamente após o IAM, auxiliando no reconhecimento do tamanho da necrose miocárdica e na existência de IAM em pacientes com angina instável, o que não seria possível utilizando-se outros tipos de marcadores (PLEBANI & ZANINOTTO, 1999).

No ano de 2000 houve uma redefinição dos critérios do IAM proposta pela *European Society of Cardiology* e pelo *American College of Cardiology*, na qual, as troponinas foram recomendadas como marcadores cardíacos padrão-ouro no diagnóstico do IAM. As troponinas cardíacas atendem as necessidades clínicas na avaliação diagnóstica e estratificação de risco no IAM em relação a outros biomarcadores, visto que, sua medida é altamente específica e a detecção no plasma não ocorre na ausência do IAM ("Troponin in ACS 67", 2015).

3.2 Troponinas

As troponinas são proteínas que participam no processo de contração dos músculos esqueléticos e cardíacos. Formam um complexo composto de três proteínas globulares (Troponina C, T e I) localizado nos longos filamentos de tropomiosina (**figura 2**) (COSTANZO et al, 2011). A troponina C (18 kDa) liga-se ao cálcio e regula a ativação dos filamentos finos durante a contração muscular. A troponina T liga-se ao complexo troponina-tropomiosina, sua isoforma cardíaca possui um peso molecular de 37 kDa. A troponina I (23 kDa) possui uma função inibidora, impedindo a contração na ausência de cálcio (TROPONINS, 2002). A troponina C é idêntica tanto no músculo esquelético como cardíaco e, por isso, não é usada clinicamente. Entretanto, os genes codificadores das troponinas I (TnI) e T (cTnT) , cardíaca e esquelética, são diferentes, produzindo proteínas com composição distinta nos dois tecidos, o que permite que anticorpos monoclonais de reatividade cruzada possam ser desenvolvidos (CAMERON et al., 2007).

Figura 2: Complexo troponina composto pelas três subunidades: T, C e I.



Fonte: (Costanzo et al, 2011).

Em termos de especificidade e importância na avaliação dos danos cardíacos diversos autores consideram que as troponinas T e I fornecem informações similares, ambas, apresentam-se com sequências de aminoácidos específicos ao miocárdio. Entretanto, a troponina T permite a detecção de pequenos danos e avaliação do tamanho da lesão do miocárdio (BERTINCHANT et al., 2003). Os valores da troponina T quando mensurados entre 72-92 horas oferecem informações mais fidedignas a esse respeito quando comparada com a troponina I (LICKA et al., 2002);(STEEN et al., 2006).

A troponina T possui quatro isoformas protéicas geradas pelo gene da cTnT (TNNT2). Por serem expressas em diferentes níveis nos corações saudáveis e enfermos, conclui-se que as mesmas participam na função vascular cardíaca (GOMES et al., 2002); (RICHARD et al., 2006).

Diversas organizações científicas internacionais como a *European Society of Cardiology* (ESC), *American College of Cardiology* (ACC), *American Heart Association* (AHA) e a *National Academy of Clinical Biochemistry* (NACB) recomendam o uso desses biomarcadores para realização de diagnósticos estratégicos com prognósticos significativos no IAM (MCDONNELL et al., 2009).

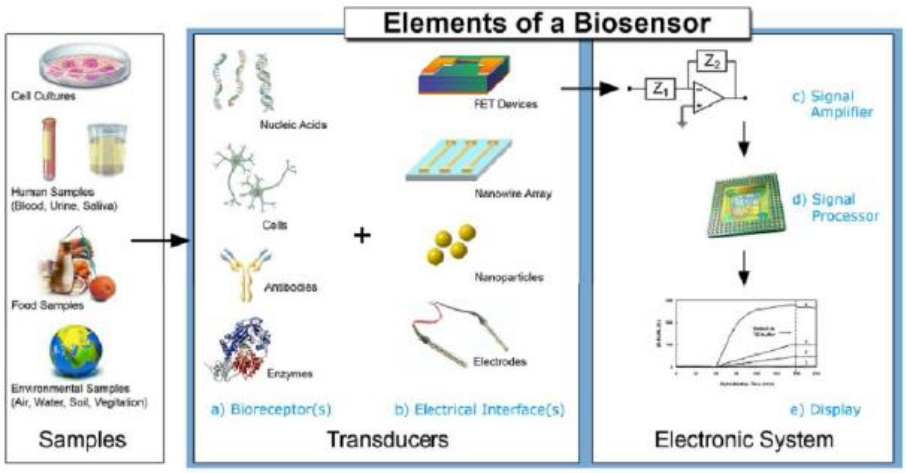
A concentração das troponinas encontra-se elevada no sangue dentro de 3-4 horas após o início da dor precordial, podendo ser detectada durante o evento isquêmico em um período de 3-10 dias. Pode-se analisar os níveis de troponinas em laboratórios através de imunosensaios enzimáticos baseados em reações que envolvem substratos cromogênicos e quimioluminescentes (SILVA et al., 2013). Tais técnicas convencionais baseiam-se em procedimentos com custos elevados, com várias etapas bioquímicas e a utilização de equipamentos caros.

Atualmente, um rápido e sensível método usando biomarcadores cardíacos para confirmação do IAM é desejável. A tecnologia dos testes point-of-care surge para atender tal necessidade, trazendo a possibilidade do diagnóstico nos locais de atendimento ao paciente, como os departamentos de emergências hospitalares. E dentro desse contexto, os biossensores mostram-se promissores como dispositivos que aceleram a tomada de decisão clínica, por diminuir o tempo gasto na solicitação e execução de exames, na coleta de amostras, divulgação e transporte das informações laboratoriais (HUDSON et al., 1999).

4-Imunossensores

Os biossensores baseados na interação antígeno-anticorpo como elementos de reconhecimento são chamados de imunossensores. Estes essencialmente combinam a sensibilidade e especificidade dos imunoensaios com a transdução em um sinal elétrico. Diferentes tipos de transdução podem ser usadas (eletroquímica, piezoelétrica, óptica, etc). Neste caso, antígenos ou anticorpo devem ser imobilizados na superfície do transdutor para a resposta analítica (HOLFORD, DAVIS & HIGSON, 2012; WANG et al., 2008). (**Figura 3**).

Figura 3: Esquema de um biossensor.



Fonte: (Grieshaber, 2008).

Os imunossensores podem ser divididos em marcados e não marcados de acordo com o tipo de método de detecção do sistema transdutor. Os que utilizam o método de marcação, em geral, baseiam-se no fato de que antígenos e anticorpos são espécies inertes e, para a detecção da interação de bioafinidade, há a necessidade de um marcador para o monitoramento da reação. Dessa forma o anticorpo ou o antígeno devem ser marcados com alguma espécie eletroativa ou fluorescente, o que constituiu um conjugado (WANG et al., 2008). Nos imunossensores livres de marcação, a resposta do imunossensor é obtida de maneira direta após o evento de bioreconhecimento. As mudanças físicas resultantes da interação entre antígeno-anticorpo podem ser monitoradas através das alterações: a) no índice de refração

(transdutores ópticos baseados na ressonância de plásmons de superfície); b) na quantidade de massa adsorvida na superfície sensora (transdutores que empregam a microbalança de cristal de quartzo), e c) de corrente elétrica, potenciais (transdutores eletroquímicos) (TANG et al, 2006; WU et al, 2007; GUILBAULT & JORDAN, 2008).

Os sistemas de transdução eletroquímica apresentam propriedades atrativas quando comparados com outros sistemas, pois são facilmente compatíveis para confecção de micro sensores e sistemas portáteis (RODRIGUEZ-MOZAZ *et al*., 2009). Adicionalmente, estes tipos de transdutores oferecem outras vantagens como simplicidade, rapidez na resposta e menor custo. Nos transdutores eletroquímicos, as mudanças das cargas, obtidas através de medidas de capacitância, condutância, potencial e impedância, possibilitam o monitoramento em tempo real da interação antígeno-anticorpo na interface sensora (PIAO et al., 2008). A utilização de nanomateriais aliada a introdução de filmes condutores tem possibilitado o surgimento de transdutores amperométricos, baseados nas medidas de alteração de corrente elétrica através da oxidação e/ou redução de espécies presentes nos eletrólitos em medição. Neste sentido, é possível obter imunossensores diretos sem a necessidade de anticorpos ou antígenos marcados (SILVA et al., 2013).

4.1- Transdutores eletroquímicos

A eletroquímica é uma ferramenta que serve para a realização de diferentes tipos de análises, sendo bastante aplicada em imunossensores. Os métodos eletroanalíticos fazem uso das propriedades elétricas mensuráveis, tais como diferença de potencial, corrente elétrica e acúmulo de cargas na interface, que são derivadas a partir de fenômenos onde uma espécie redox que interage física e quimicamente com os componentes do meio, ou com a interface sensora (BRETT et al., 1996).

Na amperometria eletroquímica, utiliza-se um sistema tri-eletrodico formado pelo eletrodo de trabalho, referencia e auxiliar (contra-eletrodo). A corrente elétrica deve ser medida entre o eletrodo de trabalho e o eletrodo auxiliar, sendo esta mantida constante com relação ao eletrodo de referência. Na experimentação, utiliza-se um

sistema potenciostato/galvanostato acoplado a um microcomputador que permitem o registro de corrente elétrica em tempo real (TURNER, KARUBE & WILSON, 1987)

Os eletrodos de trabalho podem ser de platina, ouro, prata, carbono, grafite ou filmes condutores. Normalmente o tipo de eletrodo de referência utilizado é o prata/cloreto de prata (Ag/AgCl). O eletrodo auxiliar é geralmente confeccionado a partir de condutores inertes como platina e grafite (TURNER, KARUBE & WILSON, 1987)

Nos imunossensores eletroquímicos amperométricos, moléculas biológicas são imobilizadas sobre a superfície do eletrodo de trabalho. Neste caso, a resposta bioquímica da reação antígeno-anticorpo será monitorada quando há a aplicação de um potencial elétrico, sendo estes sensíveis às pequenas perturbações do sistema (WU et al, 2007).

4.2 Técnicas eletroquímicas

Uma grande variedade de técnicas eletroquímicas vem sendo aplicadas a diversas áreas da instrumentação analítica: controle de qualidade de produtos e processos industriais, monitoramento ambiental e nas análises biomédicas. Quando utilizadas para o diagnóstico clínico, estas oferecem opções viáveis para a construção de novos métodos como os biossensores que, além de possuir vantagens em relação à química convencional, não necessitam de grandes quantidades de reagentes e permitem o controle de variáveis (FREIRE et al, 2001)

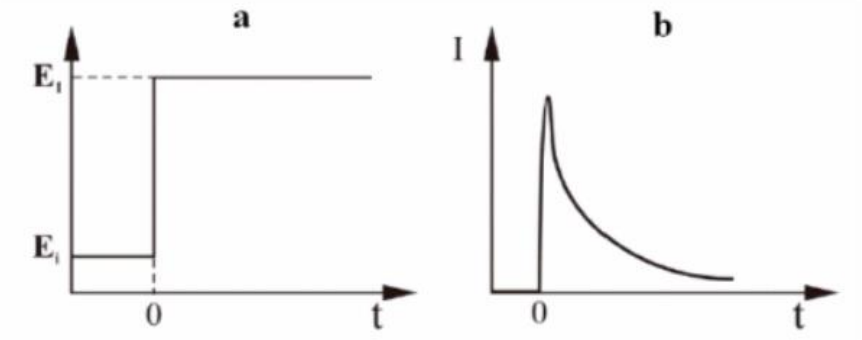
Neste trabalho, as técnicas eletroquímicas de voltametria de pulso diferencial (DPV) e cronoamperometria (CA) foram empregadas para construção do imunossensor.

4.2.1 –Cronoamperometria

Cronoamperometria (CA) é uma técnica eletroquímica em que o eletrodo de trabalho é exposto a uma escala de potencial em um período de tempo especificado. Graficamente a corrente é representada e analisada em função do tempo. O fluxo de corrente correlaciona-se com a concentração das espécies oxidadas ou reduzidas na superfície do eletrodo de trabalho (BARD; FAULKNER, 2001). Essa corrente possui um caráter maior no início do processo e decai rapidamente acompanhando o consumo do analito na interface do eletrodo (KAMAT et al., 2010). Em intervalos de tempo curtos a corrente registrada dominante é capacitiva, já em intervalos longos prevalece a corrente faradaíca (AVCI, 2007).

No início do processo de cronoamperometria, o potencial do eletrodo de trabalho é mantido em E_i (**Figura 4-a)**, no tempo 0 ocorre a alteração no valor de $E_{i,}$ que é rapidamente modificado, obtendo-se a corrente de resposta conforme mostrada na **Figura 4-b.**

Figura 4: Princípio do processo de cronoamperometria a) Potencial aplicado sobre o eletrodo de trabalho; b) Resposta da corrente em função do tempo.



Fonte: (Avci et al, 2007).

Cronoamperometria é utilizada como uma técnica eletroquímica útil na determinação de coeficientes de difusão, em estudos de cinética e seus mecanismos, sendo possível obter essas informações com a realização de poucos experimentos, às

vezes em apenas um (PARAMETERS, [s.d.]). Na execução de vários experimentos, uma análise de caracterização do sistema estudado pode ser obtida através da CA.

Adicionamente, a técnica de CA é bastante explorada para a síntese de polímeros, na qual ocorre a oxidação e redução do monômero e de oligômeros a um potencial fixo. A técnica de CA permite controlar parâmetros importantes durante a eletrodeposição de filmes poliméricos tais como: carga eletrodepositada e consequentemente a espessura do depósito, além da cinética de reação (HOLLER, SKOOG & CROUCH, 2009).

4.3.1 -Voltametria de Pulso Diferencial

Técnicas eletroquímicas voltamétricas baseadas em pulso são mais baratas, seletivas e rápidas, sendo uma intensa área para pesquisa nas últimas duas décadas (GOYAL, 2005). Em especial, a voltametria de pulso diferencial (DPV) é considerada como apropriada na caracterização de sistemas eletroquímicos. Seu princípio baseia-se na aplicação de pulsos duplos sucessivos de potencial com amostragem, no final, do resultado de cada passo do potencial aplicado **(Figura 5)** (MOLINA et al., 2010)**.**

Figura 5: Processo eletroquímico durante a voltametria de pulso diferencial (A-D).

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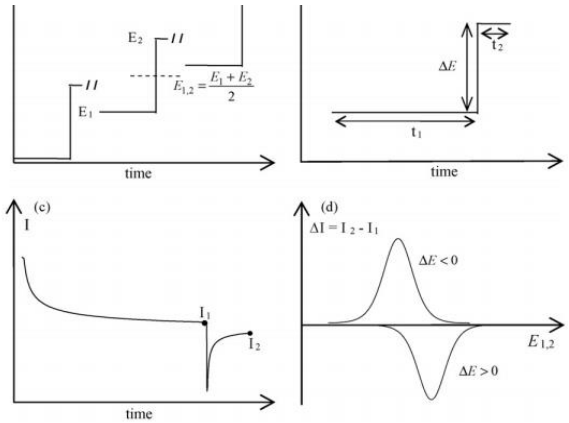


Fonte: (**Molina et al, 2010)**

O uso da voltametria de pulso diferencial tem como principal objetivo a obtenção de melhores valores de corrente que resultem em uma consequente elevação na sensibilidade do sistema a ser analisado, bem como na facilidade da interpretação do sinal de resposta referente aos pulsos aplicados. Para tanto, alguns fatores precisam ser controlados, normalmente medidas de precaução são tomadas em relação a fenômenos de resistência da solução, rugosidade do eletrodo e viscosidade da solução (KANT, 2016).

5- Modificação Eletródica

Na elaboração dos imunossensores, uma das etapas importantes envolve a imobilização de biomoléculas sobre as superfícies sensoras (FREIRE; DURA; KUBOTA, 2001). Neste sentido, com objetivo de desenvolver dispositivos mais sensíveis, reprodutíveis e estáveis modificações na superfície eletródicas são realizadas (WANG; WANG; WU, 2002).

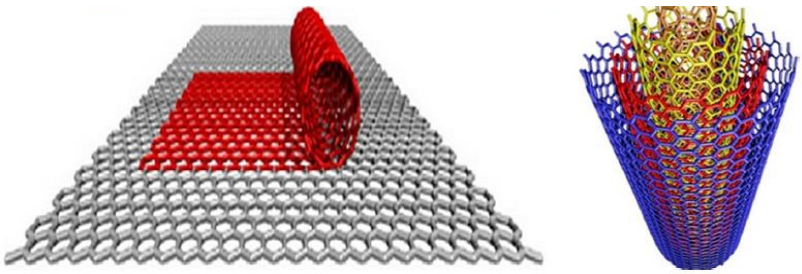
As técnicas de modificação eletródica envolvem o recobrimento dos eletrodos por nanomateriais e filmes poliméricos que permitem a ligação de biomoléculas covalentemente a sítios específicos da superfície do eletrodo. Com isso, altera-se a reatividade do immunosensor,obtendo-se respostas mais acuradas em comparação com uma superfície sem qualquer agente modificado (ELECTRODES; FOR; APPLICATIONS, 2002).

5.1-Nanotubos de Carbono

Os nanotubos de carbono (NTCs) podem ser descritos como um tubo oco compostos de folhas de grafite. Dependendo do número de folhas de grafite os NTCs são divididos em nanotudos de paredes únicas (do inglês: single-walled carbon nanotubes SWCNTs) e nanotubos de múltiplas paredes (do inglês: multi-walled carbon nanotubes MWCNTs). (**Figura 6**) (YANG et al., 2015). A síntese dos NTCs pode ser realizada por três técnicas principais, descargo de arco, ablação a laser e deposição de carbono a vapor (DCV). Com alterações nas variáveis durante a síntese SWCNTs ou

MWCNTs podem ser produzidos. Como exemplo pode-se citar a ablação a laser do grafite em tubo de sílica, que em alta temperatura, resulta normalmente em MWCNTs, porém com o uso de nanopartículas metálicas catalíticas SWCNTs são formados (AQEL et al., 2012). A maioria dos NTCs comercializados são produzidos através de DCV. Após a síntese, os NTCs podem ser funcionalizados através de tratamentos com ácidos fortes que acrescentam grupamentos óxidos, principalmente ácidos carboxílicos para regiões terminais do tubo. Diferentes reações químicas podem ser realizadas, tais como, a funcionalização com grupamentos amidas, tióis e outros (JACOBS; PEAIRS; VENTON, 2010).

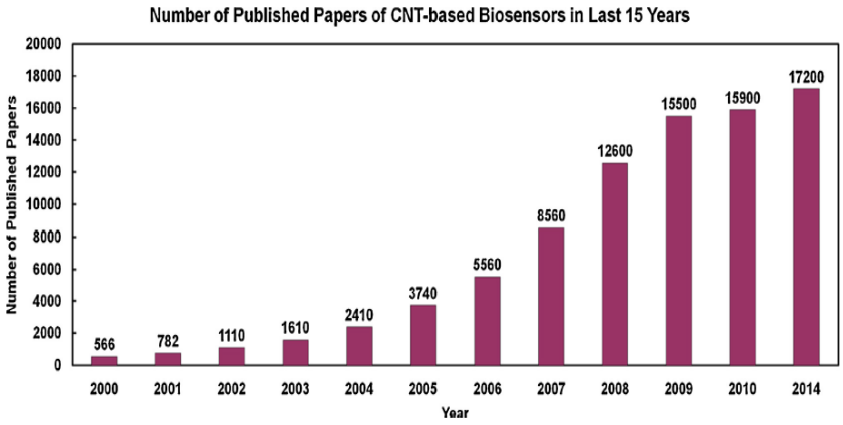
Figura 6: a) SWCNTs; b) MWCNTs.



Fonte: (Yang et al, 2015).

Os NTCs foram descobertos em 1991 por Iijima e rapidamente tornaram-se o objetivo de inúmeras pesquisas científicas, devido a sua alta área superficial e excepcionais propriedades elétricas, mecânicas e eletroquímicas (THOSTENSON; REN; CHOU, 2001). Essas propriedades são sensíveis e podem ser modificadas quando expostas a biomoléculas, o que levou na sua introdução como elementos de detecção em biossensores (YANG et al., 2015). Biossensores baseados em NTCs são relatados como uma nova geração de sistemas de sensoriamento ultra-sensíveis, exibindo vantagens em relação a outros sistemas. O estado da arte da aplicação de NTCs em biossensores é mostrado na **Figura 7,** onde observa-se um aumento rápido e substancial no número de artigos publicados a partir do ano de 2007.

Figura 7: Números de artigos publicados sobre biossensores baseados em NTCs (2007 até 2014).



Fonte: (Yang et al, 2015).

Algumas atribuições dos NTCs aos biossensores envolvem 1) Alta sensibilidade; devido a proporção área/superfície; 2) Permanência da atividade biológica; 3) Rápido tempo de resposta; derivado da cinética de transferência de elétrons 4) Aumento de estabilidade e maior tempo de vida útil (WAN et al., 2013).

Para tanto, modificações eletródicas precisam ser realizadas. A atividade catalítica com o aumento da transferência de elétrons e redução do sobrepotencial em muitos processos eletroquímicos, são propriedades chaves dos eletrodos modificados com NTCs. O processo mais simples de modificação é a dispersão dos NTCs em um pequeno volume de solvente, seguido da sua evaporação, o que permite a implementação de métodos voltamétricos para determinar espécies individuais ou misturas complexas (AGÜÍ; YÁÑEZ-SEDEÑO; PINGARRÓN, 2008). Um exemplo relatado é a detecção de isómeros de nitrofenol, que com o eletrodo de carbono vítreo revestido com NTCs possui um aumento significativo nos picos de corrente (LUO et al., 2008).

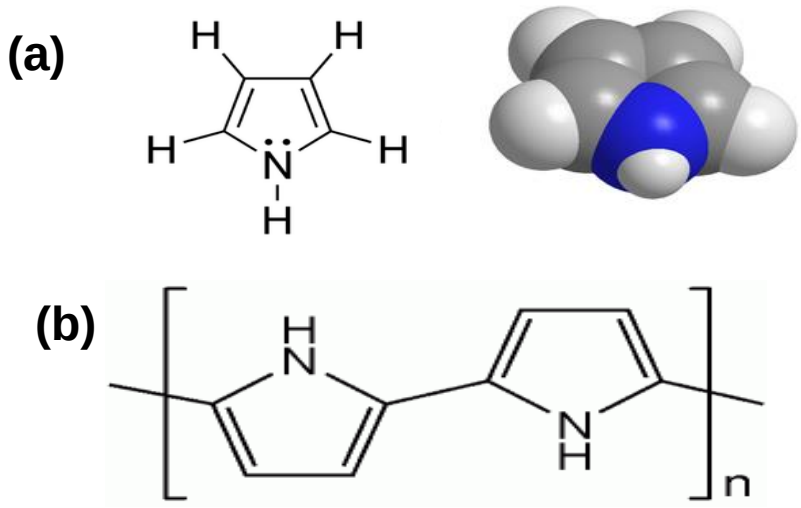
Uma outra estratégia de modificação e revestimento da superfície eletródica envolve o uso de NTCs com polímeros condutores. A combinação das características dos polímeros condutores (boa estabilidade, reprodutibilidade, elevado número de sítios ativos, aderência e homogeneidade na deposição eletroquímica) com aquelas dos NTCs

promove uma complementariedade das propriedades elétricas mecânicas e eletroquímicas, com um efeito sinérgico, resultante em uma melhor performance no desempenho de detecção dos dispositivos sensores (AGÜÍ; YÁÑEZ-SEDEÑO; PINGARRÓN, 2008). Compósitos de nanotubos de carbono e polímeros condutores vem sendo realizados através de polimerização eletroquímica em conjunto com os NTCs (PENG; SUN; CHEN, 2010); (PENG; JIN; CHEN, 2007).

5.2-Polipirrol

O PPi é um polímero condutor bastante promissor devido a boa estabilidade química, facilidade de síntese, reatividade redox e capacidade de apresentar alta condutividade. O monômero de Pi é um composto heterocíclico com caráter aromático, extremamente reativo devido a alta densidade eletrônica no anel. Através da protonação, nos átomos de carbono do anel de Pi, cátions são gerados e estes formam oligômeros que levam à polimerização (**Figura 8**) (GUIMARD, GOMEZ & SCHIMIDT, 2007).

Figura 8: Estrutura química do (a) monômero de Pi e (b) do PPi.

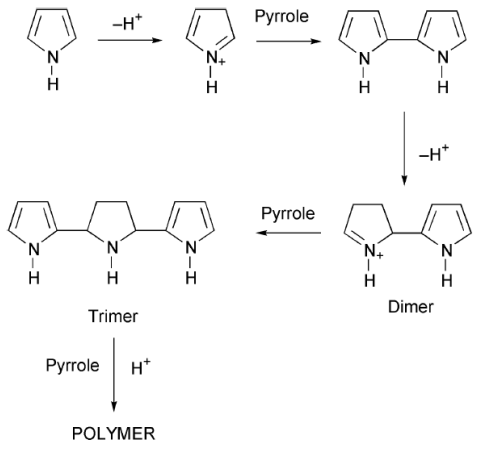


A síntese do PPi pode ser realizada quimicamente e eletroquimicamente. A síntese por via química é baseada na adição de um reagente oxidante a uma solução de monômeros. Estes devem possuir como mesmo potencial de oxidação para ocorrência

da polimerização. A síntese química possui vantagem em relação à síntese eletroquímica, pois se obtém polímeros em grandes quantidades. Porém, na síntese eletroquímica ocorre a formação de um filme com propriedades condutoras aprimoradas passíveis de serem aplicadas em dispositivos eletrônicos e em microssensores. Este método, também denominado de eletropolimerização, permite o controle dos parâmetros de síntese chegando-se a obtenção de um filme polimérico na espessura desejada (RODRÍGUEZ et al 1977).

A eletropolimerização do PPi tem como princípio a passagem de uma corrente anódica através da solução contendo os monômeros, o solvente e o eletrólito, resultando na formação rápida de um filme polimérico condutor diretamente em um substrato, que no caso, são os eletrodos. O processo de eletropolimerização do PPi ocorre via acoplamento das espécies oxidadas, na qual as seguintes etapas são envolvidas: (I) inicialmente o monômero é oxidado e gera um radical cátion; (II) depois há a dimerização do monômero com o acoplamento de dois radicais cátion; (III) o dímero formado é facilmente oxidado, então, novamente, o monômero é re-oxidado e une-se ao trímero (IV) o processo é finalizado quando o radical cátion torna-se pouco reativo ou quando o final da estrutura em cadeia é esterificada (**Figura 8**). Sendo assim, a síntese eletroquímica é um procedimento limpo, rápido e barato, devido a sua realização ser possível em meio aquoso e não aquoso, diferentemente de outros tipos de monômeros (RODRÍGUEZ et al, 1977).

Figura 9: Síntese eletroquímica do PPi.



Fonte: (SADKI et al., 2000).

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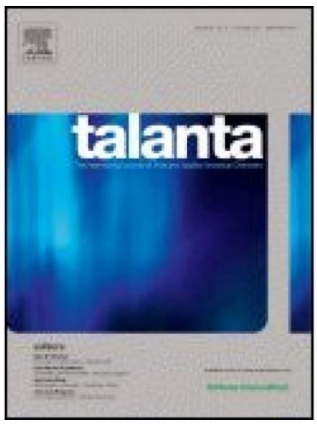
CAPÍTULO 2-Artigo

An ultrasensitive electrochemical immunosensor for cardiac myocardial infarction based on a conducting polymer - carbon nanotube film

Periódico: Talanta



An ultrasensitive electrochemical immunosensor for cardiac myocardial infarction based on a conducting polymer - carbon nanotube film

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Abstract

Immunosensors have emerged as one of the most attractive analytical possibilities for clinical diagnostic due to their simplicity, low cost and practical handling. However, the high level of sensitivity required have been restrictive, as noticed for Troponin T levels, a cardiac marker for acute myocardial infarction, which the cut-off is very low. To overcome these difficulties nanomaterials combined to conductive polymers have shown new perspectives for electrochemical biosensors. In this work, a nanofilm containing carbon nanotubes (CNTs) and polypyrrole (PPy) was formed on the electrode surface in order to detect the cardiac troponin T (cTnT). The nanofilm was obtained by electropolymerization using a three electrode scheme through the chronoamperometry (0.8V). The electrodes were immersed in a mixture containing the dispersed CNT and PPy prepared in H_2SO_4. Anti-cTnT antibodies were covalently immobilized on the CNT-PPy through amide bonds with carboxyl groups of CNTs. The detection of cTnT was performed by voltammetry differential pulse technique using ferrocyanide/ferricyanide as redox probe. The calibration curve obtained achieved a good linearity ($r = 0.944$, $p << 0.0001$) with less than 1% relative errors, and a detection limit of 0.006 ng mL^{-1} for cTnT. The modified electrode showed a good stability, with variation coefficient ≤ 5% after 20 voltammetry cycles. It was possible to develop a nanostructured film in a one-step synthesis for determination of human cardiac troponin T with detection limit in range of clinical relevance for acute myocardial infarction.

Keywords: polypyrrole; carbon nanotube; electrochemical immunosensor; label-free; cardiac troponin T

1. Introduction

Electrochemical immunosensors have been shown an efficient analytical method to apply as point-of-care testing, once these devices associate the high degree of specificity and affinity of antibody-antigen reaction with the simplicity, low cost of the electrochemical systems (AGÜÍ; YÁÑEZ-SEDEÑO; PINGARRÓN, 2008). Recently, the search for more practical and rapid tests in cardiac emergency department have featured the use of immunosensor with a promising tool in the detection of different cardiac biomarkers in the acute myocardial infarction (AMI) diagnosis (BURCU; KEMAL, 2015) . Among the several markers in clinical use, cardiac troponins have been recommended by the Third Universal Definition of Myocardial Infarction as the gold-standard biomarker to detection of the ischemic injury and myocardial necrosis in the AMI (JNEID et al., 2013). The cTnT are released from 2 to 4 h after onset of clinical symptoms of the AMI, reaching to peak between1 and 2 days, and remain in the bloodstream for 10 to 14 days (FATHIL et al., 2015).

Some studies based in the principle of sandwich immunoassays by using the enzymatic reaction of the peroxidase as an indirect marker of antigen-antibody interaction have been described to electrochemical detection of the cTnT [5] [6]. Although selective, these devices have limitations of the sensitivity to detect low concentrations and long time of the sensor response, once an additional reaction is required to detection of the enzymatic reaction. The direct detection of the anti-cTnT and cTnT reaction without the use of labelling had developed by Silva et al. (2013) in printed sensors by measuring the current signal obtained of the pulsed amperometric detection. These methods are able to detect the resistance of the charge in the interface electrode induced by binding of protein. Strategically this technique combined with conductive polymers as matrix of immobilization can be an interesting alternative to monitoring the binding event without labelled immunoreagent and suitable for detection of cTnT in order of the few ng mL^{-1}. In particular, polypyrrole (PPy) is conductive polymers attractive for this application since they have oxidative activity at specify that can be employed to show for the changes in the charge at the sensor interface by using of a standard redox probe as electrolyte electrochemical. Additionally, the PPy have an easy electrochemical synthesis and good biocompatibility (BALINT; CASSIDY; CARTMELL, 2014).

Carbon nanotubes (CNTs) are one of the most studied nanomaterials in the last 20 years and have been shown great advantages when applied in electrochemical immunosensor, such as high sensitivity, because of the large surface area ratio, improving the amount of the immobilized biomolecules and fast response time, due to the ability to mediate fast electron-transfer kinetic (AHMAD et al., 2012)(CASTRO-BELTRAN et al., 2014) . The association of the conductive polymers with the CNTs has been an alternative for development of immunosensor (MESHRAM et al., 2015). Many reports have utilized the layer-by-layer method for deposition of CNT on to polymer as support for attachment on the sensor surface. However, this methodology can result in random aggregation of the CNT in the polymer

composite, favoring 3D structures with low bind capacity for target analyte and low sensibility (PENG; SUN; CHEN, 2010). On the other hand, the one-step synthesis via electropolymerization method has facilitated the nanometer control of the film thickness, resulting robust and reproducible electropolymerized nanofilm. Herein, a nanostructured platform of the CNTs and PPy was developed in a single step synthesis for label-free detection of cTnT. The synergism between PPy and CNT were explored in order to enhance the analytical sensibility to detection of ultra-low cTnT concentrations.

2. Experimental

2.1 Materials and reagents

Carbon nanotubes functionalized with carboxylic groups (COOH-CNTs) (95% pure, average diameter of approximately 10 nm and average length of 1.5 μm) were obtained from Dropsens (Oviedo, SPN). Potassium ferrocyanide ($K_4[Fe(CN)_6]$), potassium ferricyanide ($K_3[Fe(CN)_6]$), N-hidroxysuccinimide (NHS), N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide (EDC), pyrrole (PPy) (98% pure), and glycine were acquired from Sigma Aldrich (St. Louis, USA). The dimethylformamide (DMF) and sulfuric acid (H_2SO_4) were obtained from F.Maia (São Paulo, BRA). The human cTnT and the human monoclonal anti-cardiac troponin T (anti-cTnT) produced in mice were purchased from purchased from Calbiochem (Cambridge, USA). Phosphate-buffered saline (PBS) (0.01 M, pH 7.4) was used in all experiments.

2.2 *Electrochemical and morphological characterization*

Electrochemical measurements were carried out using an IVIUMStat potentiostat (IVIUM Technologies, Eindhoven, NLD) connected to a microcomputer. It was used a conventional electrochemical system, consisting of a platinum electrode (PTE) of 0.5 mm diameter as working electrode, a helical platinum wire electrode as control electrode and an $Ag/AgCl_{(KCl\ sat)}$ electrode as reference electrode that were placed in an electrochemical cell with 10 mL volume. Eletrochemical assays were acquired using cyclic voltammetry (CV) at 0.1 V s^{-1} scan rate performed in 0.005 mol L^{-1} $K_3Fe(CN)_6/K_4Fe(CN)_6$ in the 0.1 mol L^{-1} KCl solution.

Fourier transform infrared (FT-IR) spectroscopy spectra were recorded using a Bruker FT-IR Alpha spectrometer Model IFS-66 (Ettlingen, DEU) in attenuated total reflectance (ATR) mode controlled by OPUS software (version 6.5). The samples were recorded in the wavenumber range of 4000 – 500 cm^{-1} in a room temperature (24 ± 2ºC) with a controlled humidity (~10%).

The morphological characterization of the electrode surface was performed by Scanning Electronic Microscopy (SEM) technique by using a FEI Quanta 200 FEG microscope (Eindhoven, NLD). All SEM analyses were performed using 20 kV acceleration voltages in low-vacuum mode.

2.3 Preparation of the PPy-CNT nanocomposite electrode (PPy-CNT/PTE)

Prior to prepare the nanocomposite on the sensing surface, the electrode was submitted to cleaning and polishing procedures. These steps consisted of manual polishing on a felt wetted with alumina slurry (particle size 0.3 μm) to get a mirror-like surface, followed by immersing in an ultrasonic bath in solution of ethanol and Milli-Q water for 1 min, in order to remove residues of organic and inorganic contaminants.

By finishing the electrode cleaning, the controlled nanocomposite film was obtained by electrochemical method immersing the PTE electrode in the cell containing a mixer of 0.3 mol L^{-1} pyrrole in 0.35 mol L^{-1} H_2SO_4 and 0.1 mg mL^{-1} carboxylated CNTs previously dispersed in DMF (DIAS et al., 2013). The nanocomposite film was achieved by chronoamperometry applying a working potential of 0.8 V vs. Ag/AgCl $_{(KCl\ sat)}$ immersing the Pt electrode in a described mixture.

2.4 Obtaining of the Anti-cTnT/ PPy-CNT/PTE

The carboxylic groups of the carbon nanotubes were employed to the anti-cTnT covalent immobilization by amide bonds. These groups were previously activated with a solution of the 0.002 mol L^{-1} EDC and 0.005 mol L^{-1} NHS prepared in 0.01 mol L^{-1} sodium acetate buffer (pH 5,0) for 2 h. Then, 10 μL of the anti-cTnT (2.5 μg mL^{-1}) in 0.01 mol L^{-1} PBS (pH 7.4) solution were pipetted on the PPy-CNT/PTE for 2 h at room temperature, which was maintained at a moist chamber. In order to avoid the non-specific bindings, the remaining activated carboxylic groups were blocked for 30 min with 0.01 mol L^{-1} glycine in 0.01 mol L^{-1} PBS (pH 7.4). The schematic illustration of the stepwise process of the Anti-cTnT/ PPy-CNT/PTE immunosensor preparation is shown in **Figure 1**.

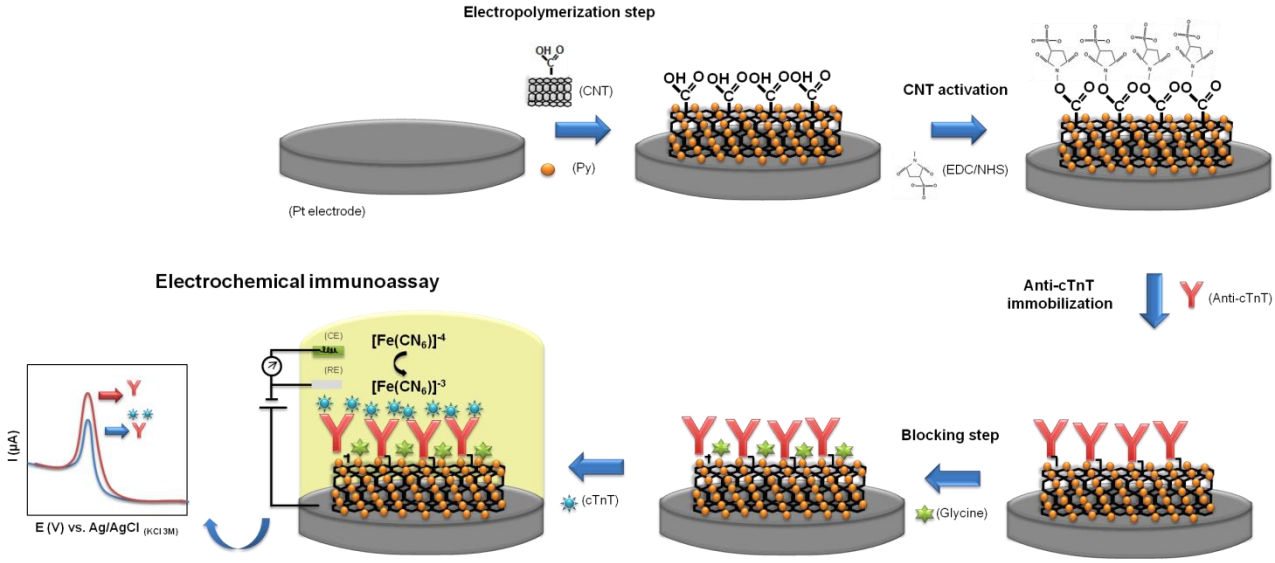


Figure 1. Schematic representation of the immunosensor

2.5 Analytical response and electrochemical characterization of the Anti-cTnT/ PPy-CNT/PTE

The analytical performance of the immunosensor was performed through of monitoring of the current values after cTnT sample exposures. The anti-cTnT/PPy-CNT/PTE was incubated with 10 μL of cTnT samples for 30 min at room temperature and maintained in a moist chamber. The label-free electrochemical detection of the antigen-antibody interaction at the sensor interface was obtained through voltammetry differential pulse technique. The analyses of the DPV were recorded from 0 V to 0.4 V with a pulse amplitude of 0.05 V, a width of 0.01 s, and a step potential of 0.01 V in a solution of 0.005 mol L^{-1} $K_3Fe(CN)_6/K_4Fe(CN)_6$ prepared in 0.1 mol L^{-1} KCl. The analytical response to cTnT was obtained taking into account the difference between the peak current (ΔI) of the Anti-cTnT/PPy-CNT/PTE with cTnT and the blank (i.e. without cTnT).

Electrochemical characterization of the Anti-cTnT/PPy-CNT/PTE preparation were performed by using the CV technique in the presence of 0.005 mol L^{-1} $K_3[Fe(CN)_6]/K_4[Fe(CN)_6$ prepared in 0.1 mol L^{-1} of KCl as redox probe. Cyclic voltammograms were obtained by scanning the potential from −0.2 to 0.5 V vs. Ag/AgCl $_{(KCl\ sat)}$ electrode at 0.1 V.

3. Results and discussion

3.1 Construction of PPy-CNT film

The electrochemical polymerization process provides a controllable and reproducible way of obtain polymeric films with different features such as thickness and morphology onto electrode surfaces Thus, the influence of the time of the electropolymerization of the PY and CNT on the Pt electrode was

evaluated in order to standardize the nanostructured platform. Pt electrode was immersed in a PY and CNT solution and submitted to a fixed potential of 0.8 V vs. Ag/AgCl $_{(KCl\ sat)}$ during 20, 40, 60, 80 and 100 s. The voltammograms registered after the electropolymerization step showed a proportional increase of the anodic and cathodic peaks (I_{pa} and I_{pc}, respectively) in relation to time of the electropolymerization **(Figure 2)**. These dates can be ascribed to charge density provided during the time of electrochemical synthesis of the film until obtain a maximal recovered of the sensor surface. The I_{pa} and I_{pc} variations exhibited a optimal response when the electropolymerization time was scheduled for 80 s (**Figure 2**, *inset*).

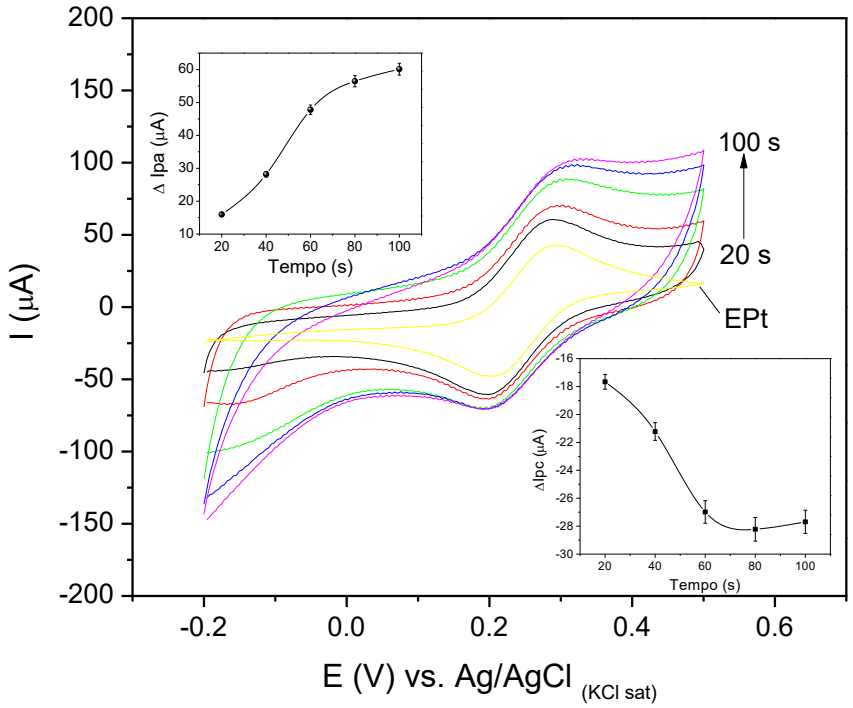


Figure 2. Influence of the time of the electropolymerization on the obtaining of the PPy-CNT film (20, 40, 60, 80 and 100 s). *Inset:* I_{pa} and I_{pc} values *versus* electropolymerization time. Voltammetric measurements performed in 0.005 mol L^{-1} $K_3[Fe(CN)_6]$ /$K_4[Fe(CN)_6]$ solution prepared in 0.1 mol L^{-1} KCl at scan rate of 0.1 V s^{-1}.

The electrical conductivity is one of the most important properties of conducting films. The monomer concentrations and counter-ion used in electropolymerization affect directly the conductivity of the formed film. Thus, optimum PPy and SO^{-4} counter-ion concentrations were evaluated. Different PY concentrations (0.1 to 0.5 mol L^{-1}) were used in the electropolymerization solution. The currents responses exhibited an proportional increase in conductivity according with the PPy concentrations and reached with a plateau a maximum current at 0.3 mol L^{-1} (**Figure S1 (a)**). The conductivity is derived

from the high number of electrons in the formation of longer chain of the PPy in the polymerization process (PATOIS et al., 2010). After optimum PPy concentration, it was not observed a significant increase in conductivity of the film. It can ascribe to a possible saturation of the polymeric chain in relation to the free SO^{-4} in the sensor interface. Since PPy is formed in its oxidized form carrying positive charges, the counter anions incorporated into the film from the electrolyte. Thus, the growth of polymer film is dependent of the counter-ion. The influence of the counter-ion concentration on the building of the PPy-CNT film reveals a plateau in the current values when the counter-ion concentration was 0.35 mol L^{-1}(**Figure S1 (b)).** This was used in all subsequently studies.

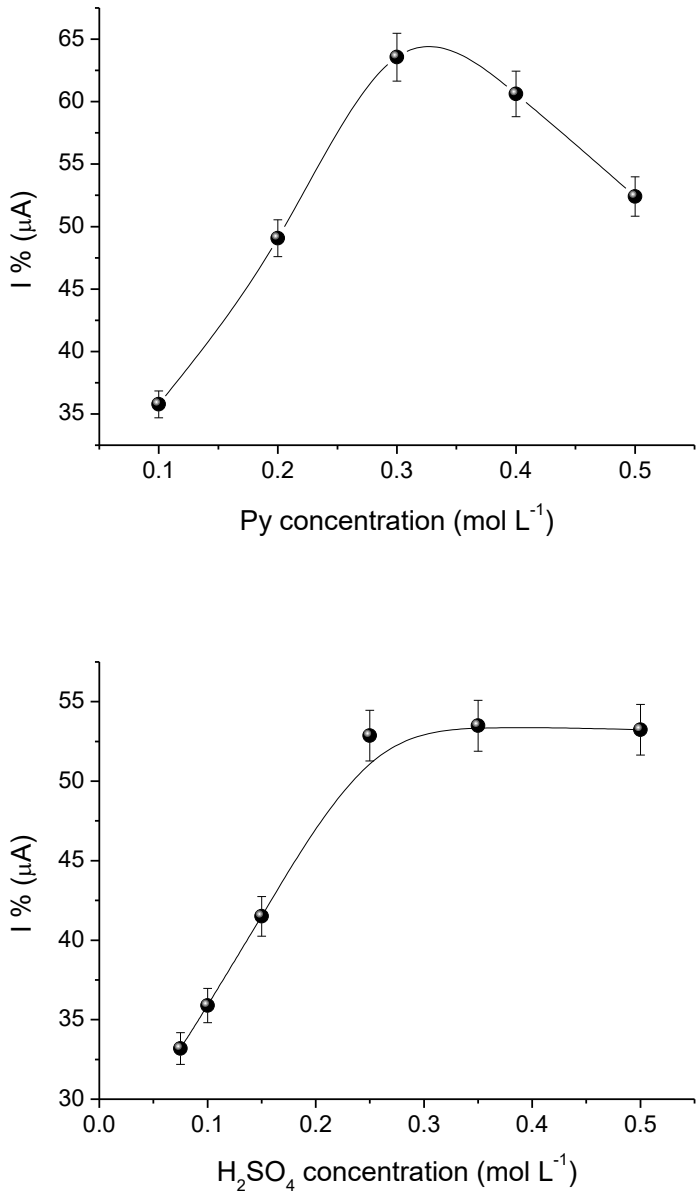


Figure S1. Influence of the PPy and counter-ion concentrations in the electropolymerization process of the PPy-CNT film. Measures performed by CV assay in $K_3[Fe(CN)_6$ /$K_4[Fe(CN)_6]$ (0.005 mol L^{-1}) solution prepared in KCl (0.1 mol L^{-1}) at scan rate of 0.1 V s^{-1}.

Molecular vibration patterns of the PPy-CNT film were investigated through FT-IR analyzes. A control study was analysed when the Pt electrode was modified only carboxylated CNTs, as can see in the **Figure 3 (a)**. This spectrum showed the presence of the molecular stretching of –OH groups at 3350 cm^{-1}, indicating the typical band of the carboxylic groups of the CNTs. Another peak at 1650 cm^{-1} was associated with the C=O stretching derivative functionalized nanotube. The **Figure 3 (b)** showed of the spectrum of the PPy-CNT/PE film, which also was observed the presence of the main peaks describes to the carboxylated CNTs at 3350 and 1650 cm^{-1}. The presence of the polymeric structure of the PPy was assigned to C-N stretching at 1240 cm^{-1} and C=C-H in plane and out of plane bending vibrations at 1050 cm^{-1} and 870 cm^{-1}. This spectrum confirms the interaction of the CNTs and the PPy in the obtaining of the nanocomposites film at the Pt surface. The process of construction of the PPy-CNT/PE film is based on the ionic interaction of the cationic charge of the oxidized Py at specific potential and available carboxylic groups of nanotubes, which allows the formation of a polymeric film around the nanotubes with easily controlled properties at interface sensor in a single step (TAM; HIEU, 2011).

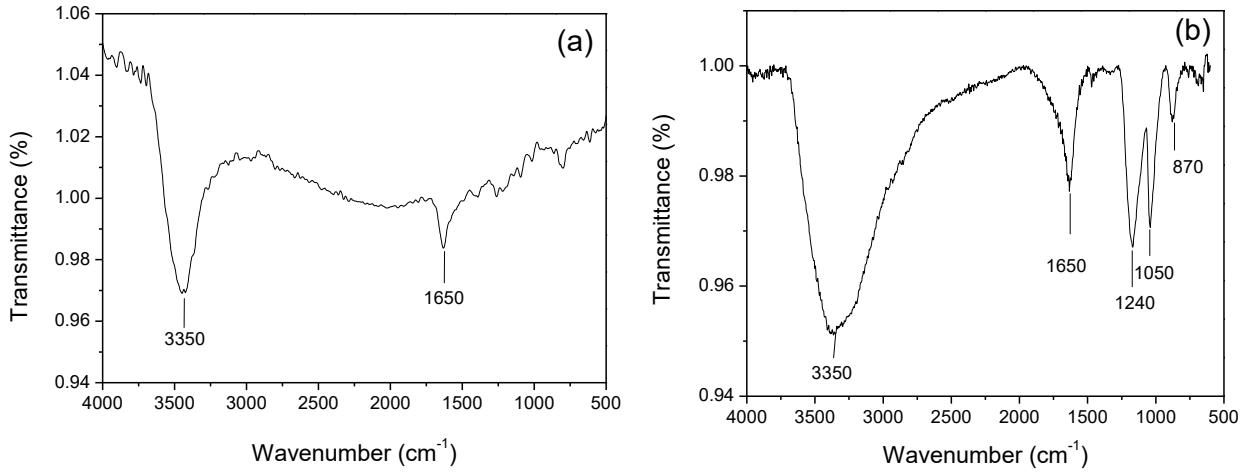


Figure 3. FT-IR spectra of the PTE modified with (a) CNTs and (b) PPy-CNTs.

In order to confirm the synergic integration of the CNTs in the PPy matrix, SEM analyses were performed. The image in **Figure 4 (a)** showed the morphology of the PE modified only PPy. It was observed an irregular film with some globular forms which is profile of the polymeric film on the sensors surfaces (MAHORE; BURGHATE; KONDAWAR, 2014). The SEM images of the PE modified with PPy-CNT, in **Figure 4 (b)**, showed a significant morphological difference when compared to the PPy/PE. This micrograph exhibited a several globular structures uniformly distributed on the PE surface. The presence of the globular forms is attributed to the PPy surrounded to the CNT. According to Valentini et al (2013), the CNTs act as nucleation centres for electrodeposition of the pyrrole monomers(VALENTINI et al., 2013). At more close magnitude the SEM images reveals the CNTs like

the spaghetti structures homogeneously distributed on the electrode surface, showing that the PPy was integrated with successful in the nanocomposite (**Figure 4 (c)**).

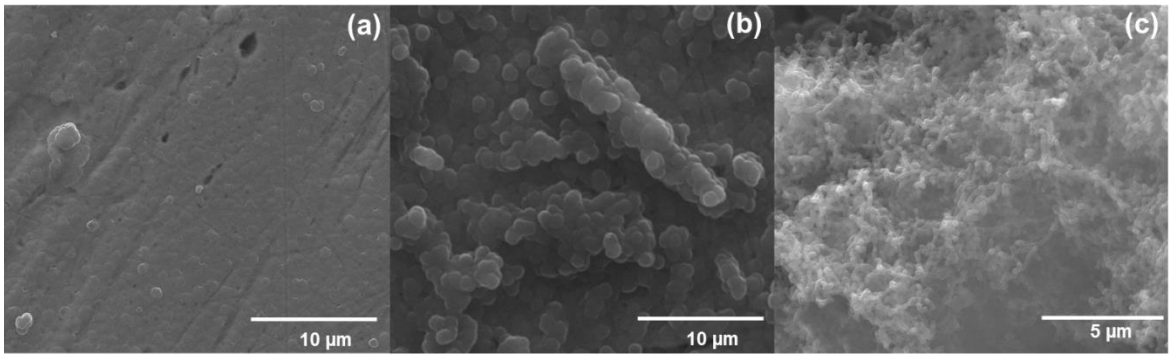


Figure 4. SEM images of the sensor surface modified with (a) PPy and (b and c) PPy-CNT.

3.2 Electrochemical characterization of the immunosensor

The voltammograms of the stepwise of the immunosensor can be seen in **Figure 5**. The clean EPt in **Figure 5 (a)** exhibited Ip_a and Ip_c value approximately equal ($Ip_a/Ip_c = 1$) and a separation of the anodic and cathodic potential (Ep_a e Ep_c, respectively) of the 70 mV. These information shows that the electron transfer on Pt interface is not compromised for any contaminant, which can be influencing the reversibility of redox peaks(ROGERS et al., 2000). An increase of Ip_a and Ip_c values were observed after the electropolymerization of the PPy-CNT film on the clean Pt, **Figure 5 (b)**. For anti-cTnT immobilization, the nanostructured film was activated with EDC/NHS chemistry and a reduction in the currents peaks was showed in **Figure 5 (c)**. This is due to the formation of an layer with negative charges resulted of the conversion of the -COOH free of the PPy-CNT film in amino-reactive NHS esters. These groups are susceptible to nucleophilic attack from groups antibody structure amine (PEI et al., 2010), enabling the covalent immobilization of biomolecules in nanostructured film. In **Figure 5 (d)**, a slight reduction in the amplitude of the redox current in was observed after the electrode incubation with anti-cTnT antibody. This phenomenon can be attributed to obstruction of the electron transfer kinetics on the interface electrode resulting from the insulating nature of the antibody that prevents the diffusion loads to the electrode surface (YUN et al., 2007). The same profile was observed in **Figure 5 (e)** after the addition of the blocking agent.

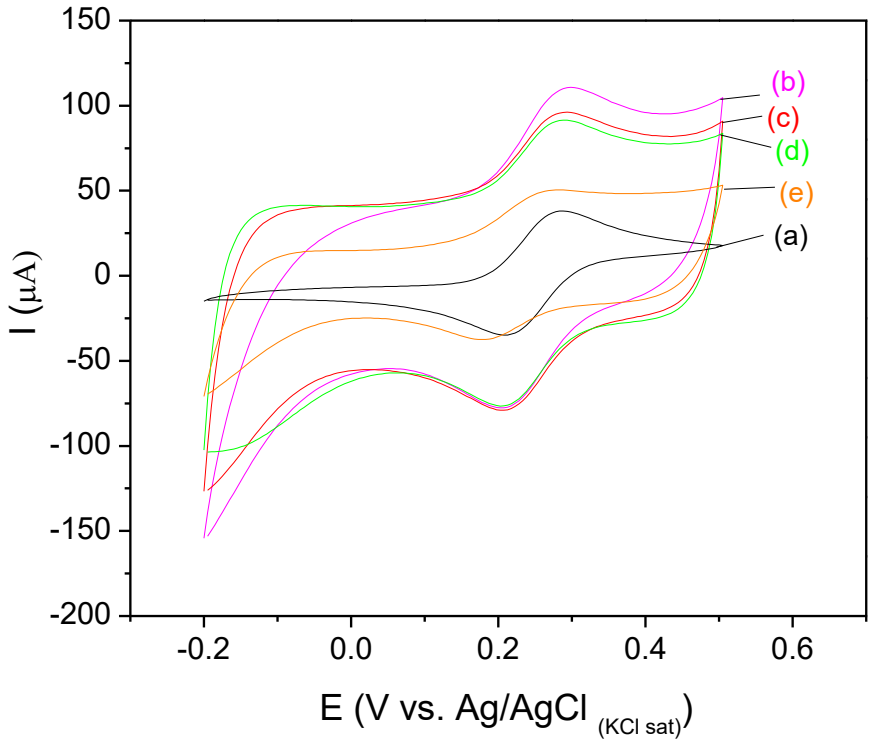


Figure 5. Cyclic voltammogram of the stepwise of the immunosensor: (a) clean EPt, (b) PPy-CNT-COOH/EPt; (c) PPy-CNT-COO^-/EPt; (d) Anti-cTnT/PPy-CNT-COO^-/EPt and (e) Glycine/ Anti-cTnT/PPy-CNT-COO^-/EPt. Measurements performed by CV assay in $K_3[Fe(CN)_6]$ /$K_4[Fe(CN)_6]$ (0.005 mol L^{-1}) solution prepared in KCl (0.1 mol L^{-1}) at scan rate of 0.1 V s^{-1}.

3.3 Optimization of the immunosensor

The influence of the anti-cTnT immobilized on the PPy-CNT film was evaluated in order to obtain the optimal analytical sensitivity. The PPy-CNT-COOH/EPt pre-activated with EDC/NHS chemistry was incubated during 60 min with different anti-cTnT concentrations (2.5; 5.0 and 100.0 µg mL^{-1}) diluted in PBS solution (0.01 mol L^{-1}, pH 7.4). The electrodes were submitted against the cTnT antigens by successive incubations (0.01 ng.mL^{-1} cTnT) to form an analytical curve. The responses obtained using DPV technique by relative variations in the I_{pa} value (ΔI_{pa}) of the PPy-CNT-COOH/EPt before and after immobilization step were calculated . According to table 1, the immunosensor presented a low sensitivity at 2,5 and 5.0 µg mL^{-1} anti-cTnT. The responses to the cTnT at 2,5 µg mL^{-1} anti-cTnT were irregular and inconsistent, since was not possible to calculate the slope and R^2. At 5,0 µg mL^{-1} anti-cTnT, the analytical curve presented a negative value of slope and low R^2 , indicating a poor analytical sensitivity. It can be attributed to the little quantity of the anti-cTnT offered to capture the antigens and cause the significant amperometric changes. Contrary, increasing the load antibody offered

on the electrode surface, for instance at 100 μg mL^{-1} anti-cTnT, the immunosensor shows a better sensitivity, indicating that the zone of equivalence was achieved. Then, the remained experiment was performed at 100 μg mL^{-1} anti-cTnT.

Table 1. Effect of anti-cTnT concentrations on the analytical response. Slope and R square calculated by DPV in assay in K3[Fe(CN)6 /K4[Fe(CN)6] (0.005 mol L-1) solution prepared in KCl (0.1 mol L-1).

Concentration Anti-cTnT (μg mL^{-1})	**Slope ± Standard Error**	**Coefficient of variation of slope**	**Adjust. R square**
2,5	ND	ND	ND
5	-22,48 ±12,06	53%	0,382
100	9,25 ± 0,99	10%	0,944

ND = non determined.

The optimal incubation time of anti-cTnT – cTnT was determined by submitting the PPy-CNT-COOH/EPt against a fixed cTnT antigen concentration (0.05 ng mL^{-1}). Different electrodes were incubated with cTnT antigen during 10, 20, 30, 40, 50 and 60 min. A proportional increase in the ΔI_{pa} value in relation to the time of reaction was observed in this study (**Figure S1**). The immunosensor response reached a plateau in 30 min and this was applied in the analytical performance of the immunosensor.

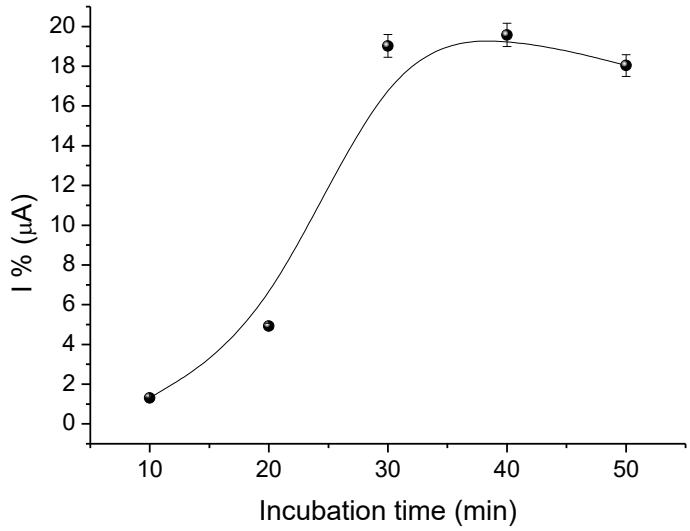


Figure S1. Time of reaction of the anti-cTnT-cTnT on the I_{pa} of the immunosensor. Measures obtained of VCs assay in $K_3[Fe(CN)_6]$ /$K_4[Fe(CN)_6]$ (0.005 mol L^{-1}) solution prepared in KCl (0.1 mol L^{-1}) at scan rate of 0.1 V s^{-1}.

3-Analytical performance of the PPy-CNT/PE

The **Figure 6** showed the analytical curve of the PPy-CNT/PTE obtained by successive cTnT incubations (0.01 ng mL^{-1} cTnT). The increase of cTnT concentrations were proportional to the changes of amperometric responses measured by DPV in $K_3[Fe(CN)_6]$/ $K_4[Fe(CN)_6]$ (0.005 mol L^{-1}). The relative difference of current response exhibited a linear relationship with a regression equation: $\Delta I\% = 54.51 + 9.25\, C_{cTnT}$, with a correlation coefficient of 0.944 ($p << 0.01$, $n = 8$) and a low relative error (<<1%). The limit of detection was approximately of 0.006 ng mL^{-1} cTnT, which allows a detection of acute myocardial infarction in the clinical levels. The linear range was according to ELISA test and similar to other reported immunosensors (Silva at al., 2013)(SILVA et al., 2013).

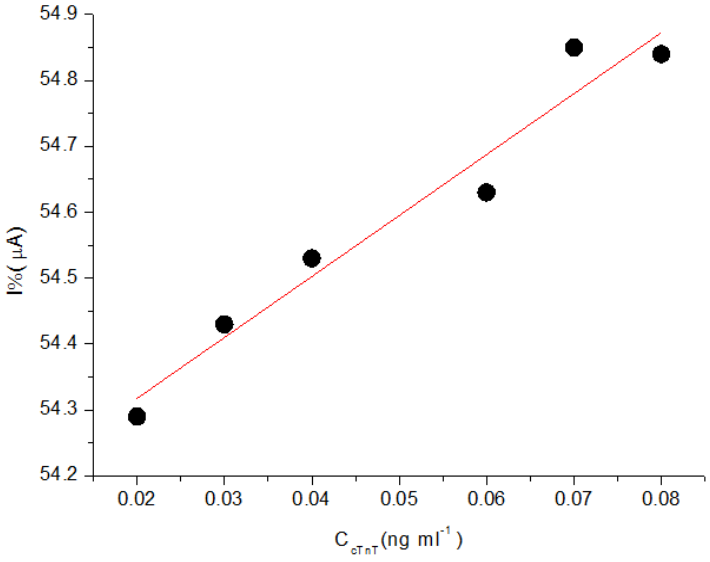


Figure 6. Analytical curve of the PPy-CNT/PE immunosensor for successive cTnT incubations (0.01 ng mL^{-1} cTnT) diluted in PBS (0.01 mol L^{-1}; pH 7.4). Measurements obtained through voltammetry differential pulse in $K_3[Fe(CN)_6]$/ $K_4[Fe(CN)_6]$ (0.005 mol L^{-1}) in KCl (0.1 mol L^{-1}) solution.

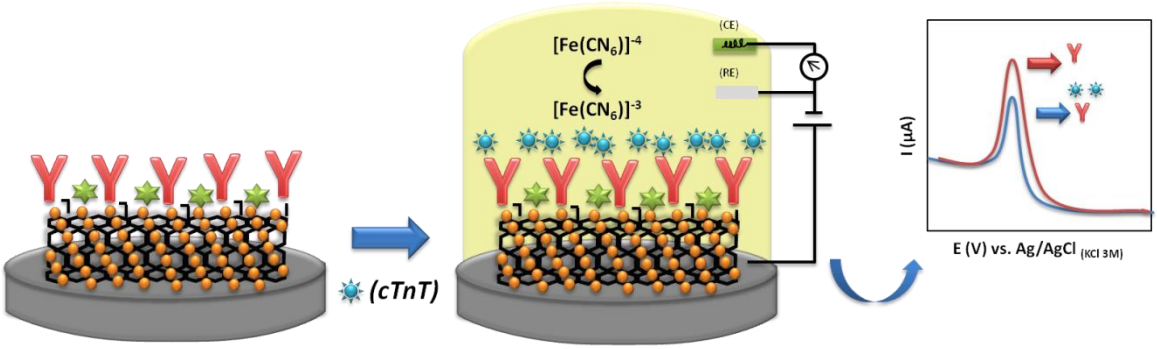
4. Conclusions

The immunosensor developed presented a easy method to obtain an one-step conductive and nanostructured film of the PPy-CNT. Additionally, a synergic effect was observed resulting in high sensitivity providing a tool for cTnT with relevant clinical range for acute myocardial infarction diagnostic.

Acknowledgments

This work was supported by the National Council of Technological and Scientific Development (CNPq) agency from Brazil. Cybelle E. Silva thanks to FACEPE Brazil foundation for the scholarship during this work. The assistance of the Center of Strategic Technologies Northeast (Recife, Brazil) is also acknowledged.

Graphical abstract



Highlights

- ➔ An ultrasensitive label-free immunosensor was developed to cardiac troponin T.
- ➔ A synergism of the pyrrole and carbon nanotube as nanocomposite film is described.
- ➔ A sensible nanocomposite film was obtained in a one-step by chronoamperometry.
- ➔ A low limit of detection 0.006 ng mL^{-1} human cardiac troponin T is achieved.

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