UNIVERSIDADE FEDERAL DE UBERLÂNDIA
INSTITUTO DE BIOTECNOLOGIA
PÓS-GRADUAÇÃO EM GENÉTICA E BIOQUÍMICA

Biossensores para detecção de *Toxoplasma gondii*

Aluna: Lívia Maria Alves

Orientadora: Prof. Dra. Ana Graci Brito Madurro

Co-Orientador: Prof. Dr. João Marcos Madurro

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ALUNA: Lívia Maria Alves

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Ana Graci Brito Madurro

Dedico este trabalho ao meu filho
João Vitor, autor das minhas alegrias.

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LISTA DE ABREVIATURAS

AFM - microscopia de força atômica (*atomic force microscopy)*

AuNPs- nanopartículas de ouro (*gold nanoparticles)*

BSA – albumina de soro bovino (*bovine serum albumin*)

DNA - ácido desoxirribunocleico (*deoxyribonucleic acid)*

ELISA - ensaio de imunoadsorção enzimática (*enzyme-linked immunosorbent assay)*

GRA- antígenos de grânulo denso (*dense granule antigens*)

GRA6 - antígenos de grânulo denso 6 (*dense granule antigens 6*)

GRA7 - antígenos de grânulo denso 7 (*dense granule antigens 7*)

HBA- ácido hidroxibenzóico (*hydroxybenzoic acid*)

HIV - vírus da imunodeficiência adquirida (*human immunodeficiency vírus)*

HPV - papiloma vírus humano (*human papillomavirus*)

Ig (G, A, M ou E) – imunoglobulina (G, A, M ou E) (*immunoglobulin G, A, M ou E*)

PBS- tampão fosfato salino (*phosphate buffered saline*)

PCR- reação em cadeia da polimerase (*polymerase chain reaction)*

PepB3- peptídeo com a sequência “APTGDPSQNSDGNRG”

pI – ponto isoelétrico (*isoelectric point*)

SAG- antígenos de superfície (*surface antigen*)

SSC – tampão citrato de sódio salino (*saline-sodium citrate buffer*)

Stag - antígeno solúvel de *Toxoplasma gondii* (soluble *Toxoplasma gondii* antigen)

ToxG1- oligonucleotídeo sintético (sonda)

ToxG2- oligonucleotídeo sintético (alvo)

VPD – voltametria de pulso diferencial

APRESENTAÇÃO

As doenças parasitárias representam desafios para a saúde humana e animal, principalmente nos países em desenvolvimento. O *Toxoplasma gondii* é um protozoário parasita intracelular responsável pela toxoplasmose, uma zoonose com importância médica e veterinária em todo o mundo.

Um diagnóstico eficiente é muito importante para a prevenção e tratamento dessas doenças parasitárias. O desenvolvimento de biossensores para auxiliar o diagnóstico de doenças é uma ferramenta significativa devido às vantagens que estes dispositivos apresentam, tais como: baixo custo, sensibilidade, seletividade e possibilidade de miniaturização.

Sendo assim, este trabalho visou a construção de dois biossensores para a detecção de *Toxoplasma gondii*, por meio da imobilização de uma sonda específica de DNA (genossensor) e um peptídeo mimético (imunossensor) em eletrodos de grafite modificados com poli (3-hidroxibenzóico).

A apresentação deste trabalho foi realizada segundo as normas da Pós-Graduação em Genética e Bioquímica da Universidade Federal de Uberlândia (Anexo 1). A tese foi dividida em: **Capítulo 1** - Fundamentação Teórica; **Capítulo 2** - Development of direct assays for *Toxoplasma gondii* and its use in genomic DNA sample e **Capítulo 3** - Peptide-based electrochemical immunosensor for anti- *Toxoplasma gondii* immunoglobulins detection

CAPÍTULO 1

FUNDAMENTAÇÃO TEÓRICA

1. Doenças parasitárias

O termo “doenças parasitárias humanas” abrange uma ampla e variada gama de infecções causadas por diversas espécies de protozoários, helmintos e ectoparasitas que formaram uma relação simbiótica com hospedeiros humanos e os efeitos dessas infecções são igualmente diversos (CARRION; HAMER; EVANS, 2017).

Infecções parasitárias devido a protozoários e helmintos são responsáveis por taxas de morbidade e mortalidade consideráveis no mundo e estão fortemente associadas à pobreza causando impacto considerável na saúde e na economia desses países. No passado, muitas dessas infecções foram ligadas predominantemente a áreas tropicais ou subtropicais, mas, atualmente, mudanças climáticas e ecológicas, um aumento significativo nas viagens internacionais, conflitos armados e migração de humanos e animais têm influenciado a transmissão de algumas doenças parasitárias para países desenvolvidos (MOMČILOVIĆ *et al.*, 2018).

Com exceção das mudanças climáticas, os principais fatores determinantes das doenças parasitárias são os determinantes sociais como urbanização, guerras, conflitos, instabilidade política e anticiência. Estima-se que em 2050 poderá haver um aumento na prevalência de doenças parasitárias causadas por helmintos e protozoários, incluindo a toxoplasmose, principalmente em regiões pobres, devido a construções de baixa qualidade, falta de saneamento, degradação ambiental, falta de acesso a serviços de saúde e água potável (HOTEZ, 2018). Sendo assim, o diagnóstico preciso e rápido representa uma arma crucial na luta contra essas infecções parasitárias (MOMČILOVIĆ *et al.*, 2018; SANCHEZ-OVEJERO *et al.*, 2016) e a investigação de novas técnicas de detecção que auxiliem no diagnóstico dessas infecções se torna uma ferramenta importante diante desse cenário.

2. *Toxoplasma gondii*

Toxoplasma gondii (*T. gondii*) é um protozoário pertencente ao Filo *Apicomplexa*, parasita intracelular obrigatório, de ampla distribuição geográfica, que pode ser encontrado em uma grande variedade de hospedeiros vertebrados

(CAKIR-KOC; ÖZDEMIR, 2018; KOCHANOWSKY; KOSHY, 2018), descoberto por Nicolle e Manceaux, na Tunísia, e por Splendore, no Brasil, em 1908 (KOMPALIC-CRISTO, 2005).

O sucesso do *T. gondii* como parasita intracelular em hospedeiros intermediários e em cultura de tecidos depende do reconhecimento de receptores de superfície de células hospedeiras via antígenos de superfície (SAGs) em sua membrana celular, da secreção sequencial de organelas secretoras especializadas e da mobilização de elementos particulares de seu citoesqueleto para invadir células hospedeiras e formar um vacúolo parasitário, dentro do qual se multiplicam. O parasita possui três organelas secretórias especiais que estão relacionadas com a invasão do parasita à célula hospedeira, essas organelas são as micronemas, as róptrias e os grânulos densos (**Figura 1**). Os grânulos densos são organelas esféricas distribuídas por todo o corpo do parasita, com diâmetro médio de 200 nm e secretam proteínas, denominadas GRA (antígenos de grânulo denso), que são moléculas imunogênicas responsáveis pela sobrevivência intracelular do parasita (BLACK; BOOTHROYD, 2000; MERCIER; CESBRON-DELAUW, 2015; NAM, 2009).

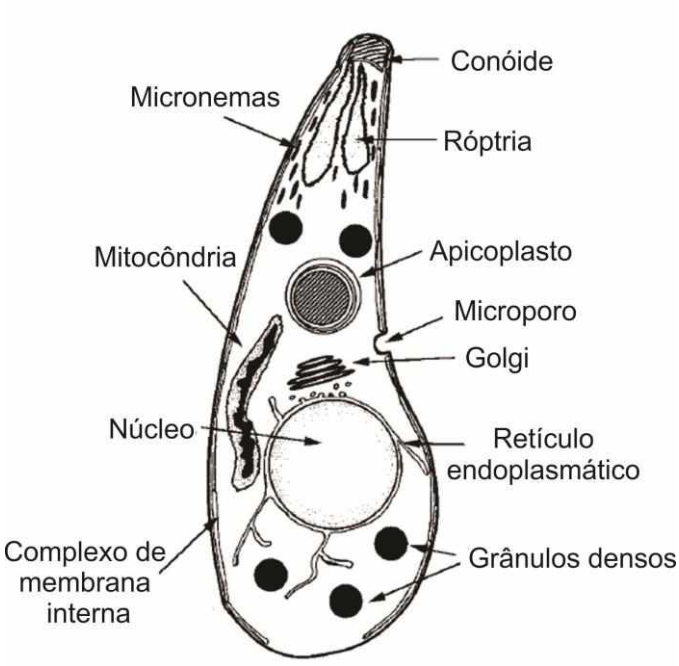

Figura 1. Principais organelas do *T. gondii* em seu estágio taquizoíto. Traduzido de Black; Boothroyd (2000).

2.1. Formas infectantes e ciclo de vida

O parasita possui três formas de vida: taquizoítos, bradizoítos (cistos teciduais) e esporozoítos (dentro dos oocistos). O ciclo do *T. gondii* (**Figura 2**) é heteroxeno, com uma fase sexuada que ocorre nas células intestinais dos hospedeiros definitivos, membros da família *Felidae*, e outra fase assexuada, que pode ocorrer nos hospedeiros definitivos e nos hospedeiros intermediários mamíferos e aves (BOGITSH; CARTER; OELTMANN, 2019; TENTER; HECKEROTH; WEISS, 2000).

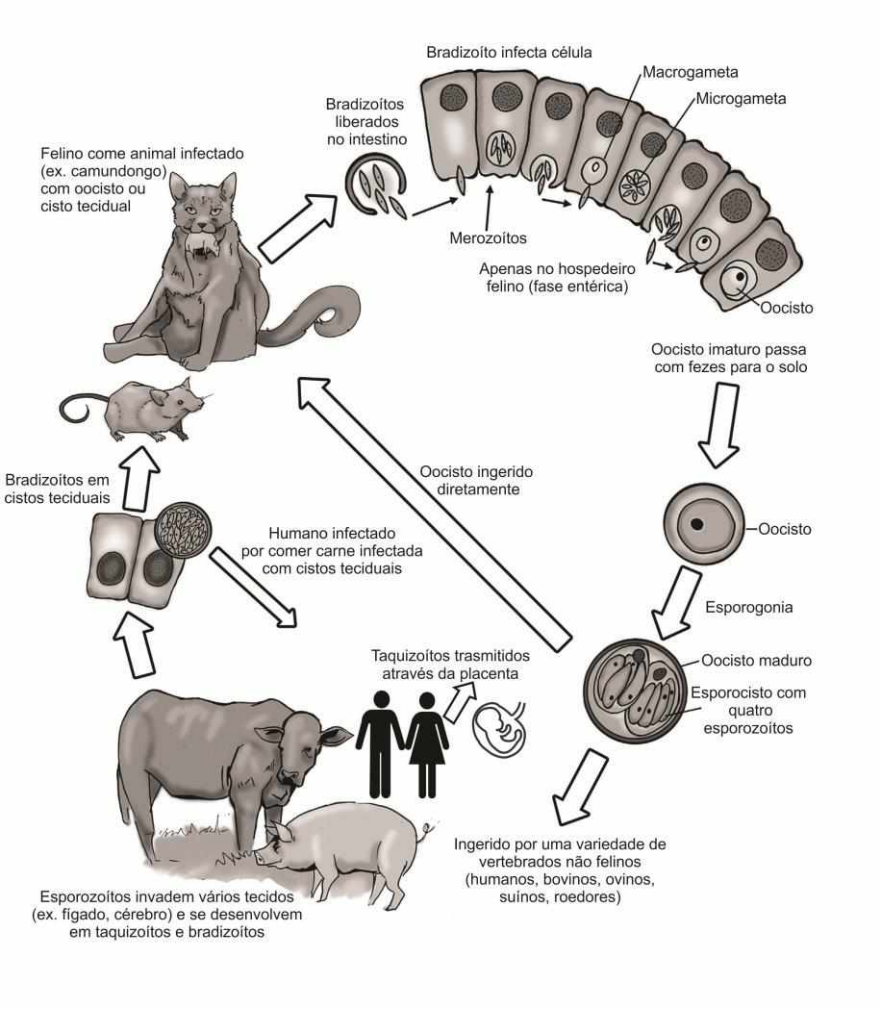

Figura 2. Ciclo de vida do *T. gondii*. Adaptado e traduzido de Bogitsh; Carter; Oeltmann (2019).

Nos enterócitos do gato, o parasita sofre merogonia, formando os merozoítos, que são liberados após o rompimento da célula hospedeira. Esses merozoítos invadem as células epiteliais adjacentes e sofrem gamogonia, formando os

gametócitos. Os gametócitos originam os gametas masculinos (microgametas) e os femininos (macrogametas). Os microgametas, que são formas móveis extracelulares, fecundam os macrogametas localizados dentro dos enterócitos, formando o zigoto, que fica envolto por uma parede resistente, sendo chamado de oocisto. Os felídeos eliminam em suas fezes oocistos imaturos não esporulados, que em condições ambientais propícias como temperatura, umidade e oxigenação ideais, se tornam maduros por esporogonia, podendo ser ingeridos por outros animais, até mesmo pelo ser humano. Cada oocisto esporulado infectante de *T. gondii* possui em seu interior dois esporocistos, cada um contendo quatro células denominadas esporozoítos. O ciclo assexuado ocorre nos hospedeiros intermediários e definitivos, e tem início com a ingestão de oocistos eliminados pelas fezes dos gatos ou cistos teciduais, contendo bradizoítos, presentes na carne crua ou mal cozida de animais infectados. Após ingestão, a parede externa dos oocistos é rompida por degradação enzimática e as formas infectantes, bradizoítos ou esporozoítos, respectivamente, são liberadas no lúmen intestinal onde rapidamente invadem as células do hospedeiro e se diferenciam em taquizoítos, por divisão assexuada. Os taquizoítos penetram em qualquer célula nucleada, formando um vacúolo parasitóforo. No interior do vacúolo sofrem rápidas e sucessivas divisões por endodiogenia, formando novos taquizoítos, que rompem a célula parasitada e invadem novas células. A disseminação do parasita no organismo ocorre através de taquizoítos livres ou intracelulares na linfa, ou no sangue circulante, podendo provocar um quadro polissintomático, cuja gravidade dependerá da quantidade de formas infectantes adquiridas, da cepa do parasita e da susceptibilidade do hospedeiro (NEVES, 2005; TENTER; HECKEROTH; WEISS, 2000).

Em tecidos e tipos de células específicos, os taquizoítos se diferenciam em bradizoítos, encontrados dentro do vacúolo parasitóforo de uma célula, cuja membrana forma a cápsula do cisto tecidual. Cistos contêm muitos bradizoitos e parece que evadem a resposta imune do hospedeiro, permitindo assim que *T. gondii* estabeleça um infecção persistente durante a vida do hospedeiro. Em humanos, o encistamento de *T. gondii* pode ocorrer no cérebro, coração e

músculo esquelético. Em roedores e humanos, o cérebro é o principal órgão para encistamento (KOCHANOWSKY; KOSHY, 2018).

2.2. Diversidade genética

O genoma do *T. gondii* está disponível em http://toxodb.org (GAJRIA *et al.*, 2007; HARB; ROOS, 2014). Apesar da presença de um ciclo sexual e de uma distribuição mundial, a estrutura populacional desse parasita foi inicialmente descrita como sendo altamente clonal e exibindo uma baixa diversidade genética, conclusão resultante de estudos genéticos de cepas isoladas da Europa e dos Estados Unidos, que agruparam essas cepas em três principais linhagens clonais tipos I, II e III. A virulência dessas cepas foi determinada em camundongos, sendo que as cepas de tipo I (exemplo: cepa RH) são consideradas de alta virulência, pois causam infecções letais nesses animais. As cepas de tipo II (exemplo: cepa ME49) são consideradas de moderada virulência e as cepas do tipo III (exemplo: cepa VEG) são ditas de baixa virulência, já que a infecção causada por este tipo clonal é controlada pelo sistema imunológico dos camundongos e a infecção tende a cronificar (ROBERT-GANGNEUX; DARDE, 2012; SIBLEY, L. DAVID; BOOTHROYD, 1992; SIBLEY, L. D. *et al.*, 2009; XIAO; YOLKEN, 2015). Além das cepas clonais existem as cepas recombinantes ou atípicas, que foram relatadas na América Central, América do Sul, Austrália e África (CONTOPOULOS-IOANNIDIS; MONTOYA, 2018).

2.3. Transmissão, epidemiologia e sintomas

O principal meio de adquirir a infecção é por ingestão de carne mal cozida contendo cistos teciduais, alimentos e água contaminados por oocistos (BOGITSH; CARTER; OELTMANN, 2019; DUBEY, 2014). Além disso, em humanos, o *T. gondii* pode ser transmitido da mãe ao feto (infecção congênita) e via transplantes de órgãos contaminados (KOCHANOWSKY; KOSHY, 2018). O termo infecção por *T. gondii* é usado quando se refere à infecção primária ou crônica assintomática, e a toxoplasmose é usada quando a infecção primária ou reativação da infecção crônica causa sinais ou sintomas (CONTOPOULOS-IOANNIDIS; MONTOYA, 2018).

A soroprevalência varia amplamente entre diferentes países e diferentes regiões do mesmo país. Um estudo realizado em 2009 mostrou que os países da América Latina e Sudeste da África com climas quentes e úmidos apresentam altas soroprevalências, nas regiões Sul da Europa soroprevalências moderadas e menores relatadas na América do Norte, Norte da Europa, Sudeste Asiático, China e Coréia (PAPPAS; ROUSSOS; FALAGAS, 2009).

Foroutan-Rad *et al.* (2016) avaliaram a taxa de soroprevalência do *T. gondii* em doadores de sangue (**Figura 3**), e nesse estudo a prevalência total ponderada de exposição ao *T. gondii* em doadores de sangue foi de 33% e o Brasil foi identificado como um país com alta soroprevalência (75%).

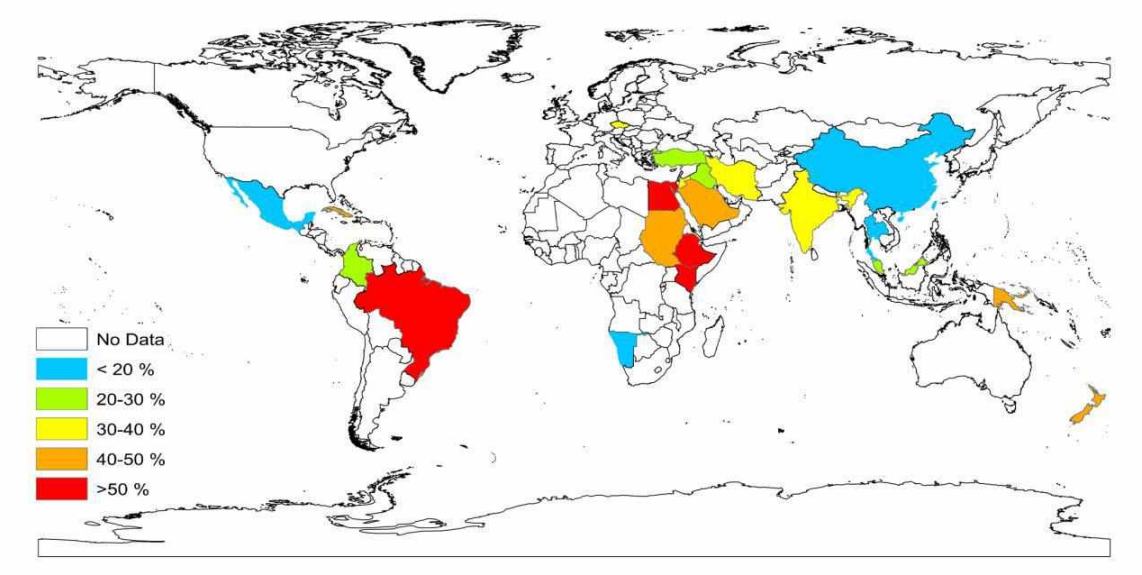

Figura 3. Prevalência global de anticorpos para *T. gondii* em doadores de sangue de diferentes regiões geográficas (FOROUTAN-RAD *et al.*, 2016).

Aproximadamente 30% da população humana no mundo é cronicamente infectada com *T. gondii*. Em geral, a infecção humana é assintomática, mas o parasita pode induzir doença grave em fetos e pacientes imunocomprometidos (GALAT, 2018). As manifestações clínicas associadas à infecção por *T. gondii* são muito diversas e variam de miocardite, toxoplasmose ocular (retinocoroidite, coroidite), encefalite e hidrocefalia a doenças mentais (BLADER *et al.*, 2015). Quadros clínicos de toxoplasmose podem ser agrupados como toxoplasmose

adquirida, toxoplasmose congênita, toxoplasmose em pacientes imunodeficientes e toxoplasmose ocular (CAKIR-KOC; ÖZDEMIR, 2018).

Sinais e sintomas resultam de infecção primária ou reativação, em ambas as formas, a rápida proliferação do taquizoíto e sua resposta imune inflamatória correspondente é responsável por manifestações clínicas. A gravidade pode ser influenciada pela cepa genética, tamanho do inóculo, forma infecciosa, imunocompetência, genética do hospedeiro e a ausência de exame pré-natal ou tratamento no pós-natal. Em aproximadamente 10% dos pacientes, os seguintes sintomas ou síndromes, isoladamente ou em várias combinações já foram relatados na infecção aguda, febre (≤104 °F ou ≤ 40 °C), linfadenopatia, cefaléia, mialgia, artralgia, dor de garganta, rigidez no pescoço, náusea, dor abdominal, anorexia, confusão, sintomas oculares incluindo dor ocular, mal-estar geral e fadiga e ocasionalmente, erupções cutâneas e dor de ouvido. Em surtos comunitários ou em certas áreas tropicais, até 82% das pessoas infectadas são sintomáticas e até 19% podem ter doenças oculares (CONTOPOULOS-IOANNIDIS; MONTOYA, 2018).

A toxoplasmose congênita resulta da infecção transplacentária fetal, que pode resultar em natimortos ou vários defeitos congênitos graves. Aproximadamente, 12% das crianças infectadas nascidas vivas morrem logo após o nascimento, e menos de 20% das sobreviventes são normais aos 4 anos de idade. Anormalidades ocorrem no sistema nervoso central e olhos, com sintomas como icterícia, microcefalia e hidrocefalia, aparecendo no nascimento ou logo em seguida (BOGITSH; CARTER; OELTMANN, 2019). A toxoplasmose ocular causada por infecção congênita e por infecção adquirida, é mais comum na América do Sul e Central, no Caribe e em partes da África tropical, em comparação com a Europa e a América do Norte, e é bastante rara na China. Na América do Sul é mais grave que em outros continentes devido à presença de cepas extremamente virulentas do parasita (PETERSEN, ESKILD *et al.*, 2017; PETERSEN, E.; KIJLSTRA; STANFORD, 2012). Cerebrite, retinocoroidite, pneumonia, miocardite, erupção cutânea, e até morte foram reportados em indivíduos imunocomprometidos (ÜNÜVAR, 2018).

Apesar da toxoplasmose clínica afetar apenas um número limitado de indivíduos, ocasionalmente algumas epidemias ocorrem (BOGITSH; CARTER; OELTMANN, 2019). Surtos de toxoplasmose têm sido registrados nas últimas décadas em todo Brasil (ALMEIDA, 2011; BONAMETTI, 1997; MIORANZA, 2008). No começo de 2018 teve início um surto da doença na cidade de Santa Maria no estado do Rio Grande do Sul, com 771 casos confirmados conforme o último boletim divulgado em setembro pela Prefeitura de Santa Maria e o Governo do Estado, e de acordo com a Sociedade Brasileira de Medicina Tropical os registros contabilizados até o momento devem confirmar a cidade como o local de maior surto da doença no mundo (Relatório de atualização de investigação de surto de toxoplasmose em Santa Maria/RS, 2018; Sociedade Brasileira de Medicina Tropical, 2018).

2.3. Diagnóstico

Os sintomas da infecção por *T. gondii* não são específicos e variam dependendo dos órgãos infectados. Vários métodos devem ser usados para apoiar os sintomas clínicos e estabelecer um diagnóstico final (CAKIR-KOC; ÖZDEMIR, 2018). O diagnóstico da infecção por *T. gondii* e toxoplasmose incluem testes sorológicos, amplificação de sequências específicas de ácidos nucléicos (reação em cadeia da polimerase), exames histopatológicos e isolamento do parasita.

Métodos de diagnóstico histopatológico determinam taquizoítos em amostras de pacientes ou detectam bradizoítos de cistos teciduais em biópsias teciduais (CAKIR-KOC; ÖZDEMIR, 2018). O isolamento de *T. gondii* do sangue ou fluidos corporais estabelece que a infecção é aguda. Tentativas de isolamento do parasita podem ser realizadas por inoculação em camundongos ou inoculação em culturas de tecidos celulares de praticamente qualquer tecido humano ou fluido corporal e constitui um diagnóstico positivo (BOGITSH; CARTER; OELTMANN, 2019; MONTOYA, 2002).

A infecção por *T. gondii* ativa a resposta imune humoral e celular do hospedeiro. A resposta humoral à infecção por *T. gondii* resulta em níveis aumentados de imunoglobulinas circulantes específicas: IgM, IgG, IgA, e IgE **(Figura 4)**. Cada isotipo aparece com um perfil cinético específico após a infecção

primária; IgM e IgA aparecem durante a primeira semana e seus níveis atingem um pico após aproximadamente 1 mês antes de diminuir para níveis indetectáveis após várias semanas até vários anos. Os níveis de IgE atingem o pico após aproximadamente 3 meses e depois diminuem rapidamente, a IgG aparece pela primeira vez aproximadamente 2 semanas após a IgM, atinge um patamar após aproximadamente 2 a 3 meses e, em seguida, diminui de forma constante para um título residual de longa duração (DARD *et al.*, 2016). A detecção sorológica de imunoglobulinas anti-*T. gondii* desempenham um papel crucial no diagnóstico clínico da toxoplasmose.

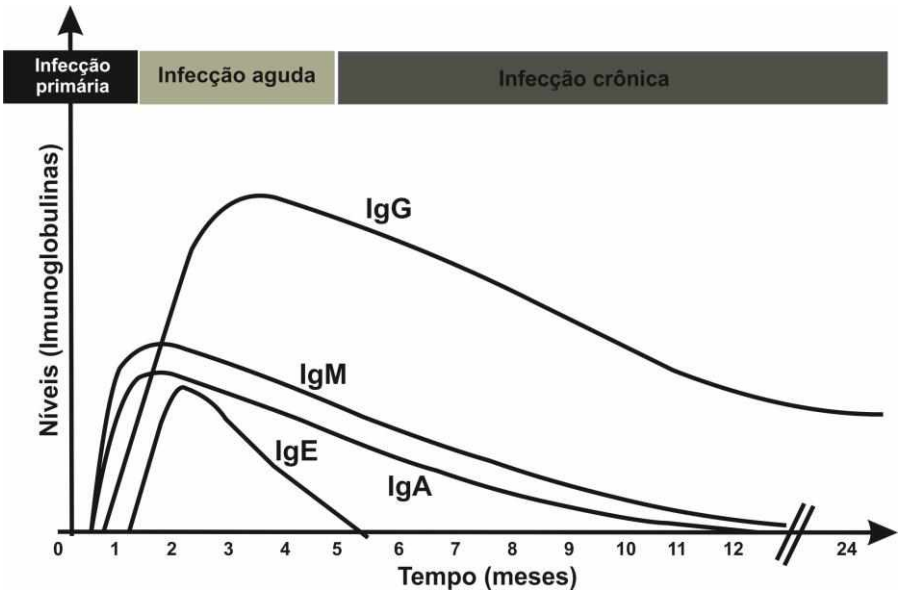

Figura 4. Cinética de imunoglobulinas anti-*Toxoplasma* após infecção primária. Traduzido de Dard *et al.* (2016).

Uma ampla gama de técnicas sorológicas está disponível para a detecção de imunoglobulinas específicas de *T. gondii*, as principais metodologias utilizadas são o ensaio de imunoabsorção enzimática (ELISA), ensaio de aglutinação imunossorvente, ensaios de hemaglutinação indireta, testes de anticorpos fluorescentes indiretos, o teste de aglutinação modificado, Western Blotting e avidez de IgG. O teste de corante Sabin-Feldman, que foi reconhecido como padrão ouro por um longo período, não é mais usado devido às suas dificuldades práticas, particularmente porque são necessários parasitas vivos dentro de seus componentes. Embora o ELISA vem sendo considerado um dos métodos mais

confiáveis para a detecção de anticorpos específicos anti-*Toxoplasma* no soro, é uma técnica demorada e inclui preocupações específicas (DARD *et al.*, 2016; LIU *et al.*, 2015; TEIMOURI *et al.*, 2018; ZHANG, K. *et al.*, 2016).

Avanços no conhecimento do genoma do *T. gondii* tornaram possível a utilização da PCR para a detecção do parasita. O método de PCR é usado para determinar o DNA de *T. gondii* em tecidos e fluidos corporais. A região do gene específico de *T. gondii* deve ser selecionada e amplificada e diferentes pares de iniciadores para replicação *in vitro* dirigidos à diferentes alvos têm sido utilizados. O primeiro a ser testado, amplamente usado para diagnóstico molecular com sensibilidade aceitável é o gene B1 (GenBank:AF179871.1) que se encontra repetido em 35 cópias no genoma do parasita (BELAZ *et al.*, 2015; COSTA, J.-M. *et al.*, 2013; FALLAHI *et al.*, 2014; OKAY *et al.*, 2009). Um outro alvo molecular tem sido amplamente investigado para o diagnóstico de toxoplasmose é a região repetitiva (GenBank: AF146527.1), descrita por Homan *et al.* (2000), constituída de um fragmento de 529 pb, repetido 200 a 300 vezes no genoma de *T. gondii* (FALLAHI *et al.*, 2014; PRATAMA, 2009; WAHAB *et al.*, 2010).

Dentre as proteínas do grânulo denso, a proteína GRA6 (antígeno do grânulo denso 6) de 32kDa, codificada pelo gene GRA6 (um gene de cópia simples altamente polimórfico, GenBank: L33814.1) é utilizada para a genotipagem (BIRADAR *et al.*, 2014; FAZAELI *et al.*, 2000; LECORDIER *et al.*, 1995; MERCIER *et al.*, 2002; NAM, 2009) e vários estudos reportaram o uso de proteínas antigênicas recombinantes como GRA para o diagnóstico sorológico da toxoplasmose (ARAB-MAZAR *et al.*, 2016; HOLEC-GASIOR, 2013; SONG, K. J. *et al.*, 2013). Um ensaio de nested-PCR usando o gene GRA7 como alvo, que também codifica uma proteína granular densa, foi desenvolvido. O teste apresentou sensibilidade e especificidade igual ou maior que os ensaios de PCR padrão para detecção de *T. gondii*, que amplificam a sequência B1 ou a região repetitiva de 529 pb, e dentre os genes testados para o desenvolvimento desse ensaio o gene GRA6 também apresentou um baixo índice de variabilidade genética (COSTA *et al.*, 2016).

3. Biossensores

Os biossensores, dispositivos analíticos que convertem uma resposta biológica em um sinal elétrico mensurável (MEHROTRA, 2016), são constituídos por três componentes principais (**Figura 5**): o elemento de reconhecimento biológico, que identifica o estímulo; o transdutor, que converte este estímulo em um sinal mensurável e o sistema de processamento de sinal, que envolve a amplificação e exibição dos dados em um formato apropriado (PERUMAL; HASHIM, 2014).

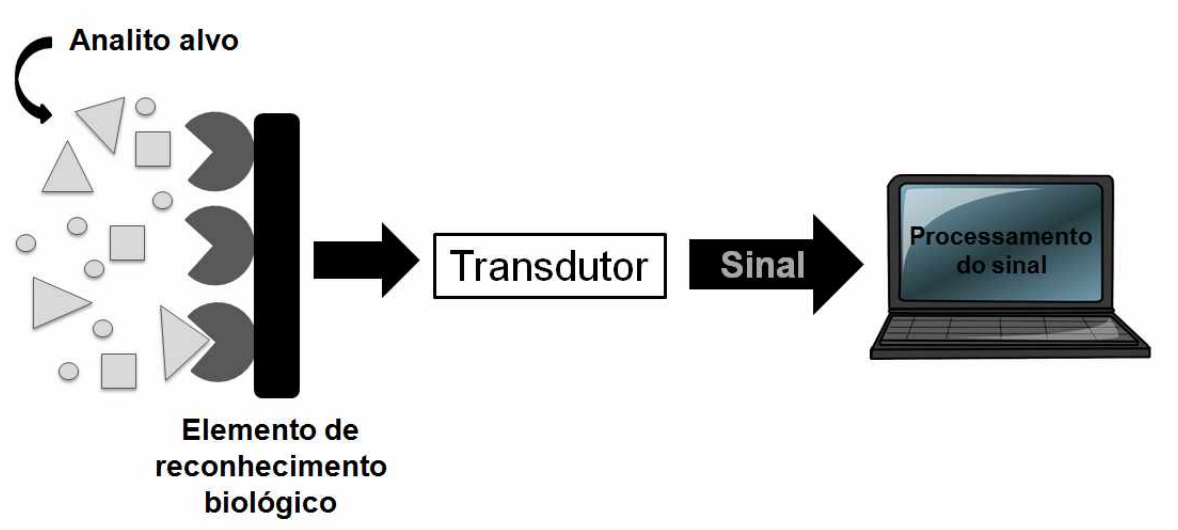

Figura 5. Representação esquemática de um biossensor. Traduzido de Rodovalho *et al.* (2015).

As principais vantagens dos biossensores em relação as técnicas analíticas convencionais são medições rápidas, baixo custo, possibilidade de miniaturização, possibilidade de monitoramento contínuo e, em alguns casos, a capacidade de medir os analito em matrizes complexas com preparação mínima da amostra (BETTAZZI *et al.*, 2017; MADURAIVEERAN; SASIDHARAN; GANESAN, 2018; SANTORO; RICCIARDI, 2016).

Os biossensores têm sido amplamente pesquisados e desenvolvidos como uma ferramenta analítica para diversas áreas, como a médica (JUSTINO; DUARTE; ROCHA-SANTOS, 2016; MURUGAIYAN *et al.*, 2014), alimentar (EVTUGYN, 2016), ambiental (BOSCH-OREA; FARRÉ; BARCELÓ, 2017; WANG, X.; LU; CHEN, 2014) e forense (ARAUJO *et al.*, 2018; YÁÑEZ-SEDEÑO *et al.*, 2014).

Vários parâmetros são utilizados para a classificação dos biossensores (**Figura 6**), tais como o elemento de reconhecimento biológico utilizado e o método de transdução do sinal (BOSCH-OREA; FARRÉ; BARCELÓ, 2017).

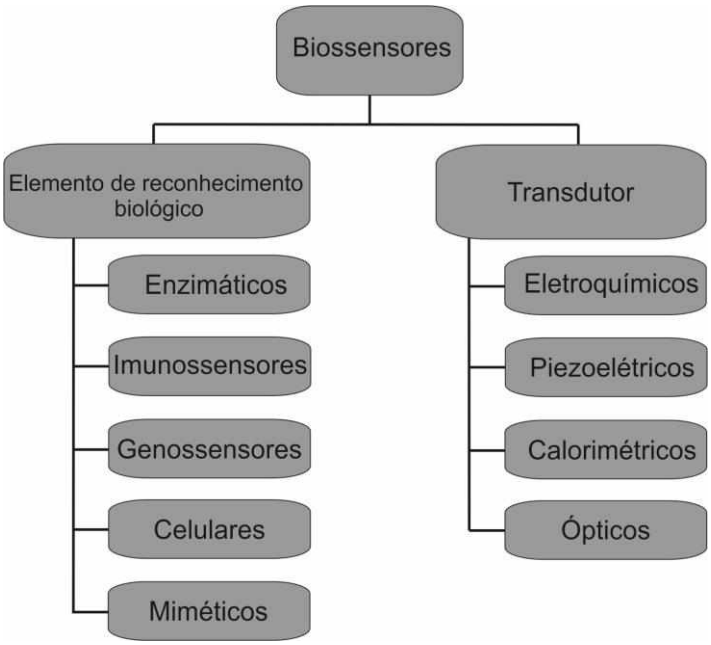

Figura 6. Diferentes categorias de biossensores. Adaptado e traduzido de Perumal; Hashim, (2014).

3.1. Classificação baseada no elemento de reconhecimento biológico

Um elemento de reconhecimento biológico ou biorreceptor geralmente consiste de uma biomolécula imobilizada que é capaz de detectar o analito alvo específico. É crucial um elemento de reconhecimento ser seletivo, sensível e estável para o analito alvo específico a fim de evitar a interferência de outras substâncias presentes nas amostras (PERUMAL; HASHIM, 2014; WANG, X.; LU; CHEN, 2014). Devido aos grandes avanços na biologia molecular, bioquímica, genética, microbiologia e imunologia, uma vasta gama de biomoléculas são comumente utilizadas como elemento de reconhecimento biológico para a construção dos biossensores, cada um com suas próprias vantagens na detecção do analito alvo.

Baseando-se no elemento de reconhecimento biológico, os biossensores podem ser classificados em: enzimáticos, celulares, miméticos, genossensores e imunossensores (PERUMAL; HASHIM, 2014).

3.1.1. Biossensores enzimáticos

As enzimas são biocatalisadores muito eficientes, que possuem a capacidade de reconhecer especificamente os seus substratos e catalisar a sua transformação, estas propriedades únicas tornam as enzimas ferramentas interessantes para o desenvolvimento de dispositivos analíticos, como os biossensores (PERUMAL; HASHIM, 2014). Biossensores enzimáticos podem ser definidos como um dispositivo analítico com uma enzima como biorreceptor integrado ou intimamente associado ao transdutor físico para produzir um sinal digital discreto ou contínuo que seja proporcional à concentração do analito presente na amostra (KARUNAKARAN; MADASAMY; SETHY, 2015). Os biossensores enzimáticos podem ser distinguidos entre aqueles que medem a inibição de uma enzima devido à presença do analito alvo, e aqueles que medem a transformação catalítica do analito alvo por uma enzima específica (FARRÉ; BARCELÓ, 2007).

3.1.2. Biossensores celulares

Biossensores celulares são um tipo de biossensor que utilizam células vivas como o elemento de reconhecimento; baseiam-se na capacidade da célula em detectar as condições do microambiente intracelular ou extracelular e os parâmetros fisiológicos, e produzir uma resposta por meio da interação entre o estímulo e a célula (HANSEN; UNRUH, 2017; PERUMAL; HASHIM, 2014), e sua principal aplicação está no monitoramento ambiental e segurança alimentar (YE; GUO; SUN, 2018). Esses dispositivos também são conhecidos como biossensores microbianos (D'ORAZIO, 2011). Os microrganismos, tais como bactérias e fungos são geralmente utilizados como biorreceptores para a detecção de moléculas específicas ou o estado geral de um ambiente circundante, e proteínas presentes nas células, como as enzimas e podem também ser utilizadas como biorreceptores para a detecção de um analito específico (D'SOUZA, 2001; PERUMAL; HASHIM, 2014).

3.1.3. Biossensores miméticos

Os biossensores miméticos são aqueles que utilizam como elemento de reconhecimento biológico biomoléculas sintéticas ou artificiais que mimetizam uma biomolécula natural. Nesta categoria podemos incluir os biossensores baseados na imobilização de aptâmeros, que são oligonucleotídeos de fita simples que se ligam a seus alvos com alta afinidade e especificidade (GOPINATH *et al.*, 2016; KOZITSINA *et al.*, 2018).

3.1.4. Genossensores

Os genossensores são baseados em processos de reconhecimento de ácidos nucléicos visando a detecção de alvos envolvidos em doenças genéticas, doenças infecciosas. Permitem o monitoramento de danos e interações ao DNA e detecção de interações com proteínas, compostos farmacêuticos, íons metálicos e complexos metálicos, poluentes, radicais livres e radiação ultravioleta (DICULESCU; CHIORCEA-PAQUIM; OLIVEIRA-BRETT, 2016; MURUGAIYAN *et al.*, 2014) e nos últimos anos, têm sido considerados promissores dispositivos de diagnóstico clínico alternativos especialmente para doenças infecciosas (RASHID; YUSOF, 2017).

São desenvolvidos por meio da imobilização de fita simples de DNA, geralmente pequenos oligonucleotídeos sínteticos, sobre a superfície do transdutor, seguida pelo reconhecimento do seu alvo complementar para formação da dupla hélice de DNA. A detecção do alvo complementar e do evento de hibridização pode ser realizada por medida direta ou por medida indireta utilizando um indicador de hibridização (BETTAZZI *et al.*, 2017; PERUMAL; HASHIM, 2014). Os ácidos nucléicos oferecem vantagens para serem utilizados como sonda, pois são estáveis, facilmente sintetizados, altamente específicos e podem ser reutilizáveis (MURUGAIYAN *et al.*, 2014; SANTORO; RICCIARDI, 2016).

3.1.5. Imunossensores

Um imunossensor é um tipo de biossensor no qual o antígeno se liga ao anticorpo e forma um complexo estável, e o antígeno ou anticorpo pode ser imobilizado na superfície de diferentes transdutores (ZHANG; ZHAO, 2019).

A determinação de anticorpos é uma área importante na bioanálise, pois sua presença fornece informações sobre diversas doenças. Infecções induzem uma resposta do sistema imunológico, permitindo o diagnóstico da doença com ensaios de detecção de anticorpos do hospedeiro produzidos em resposta à infecção (HEYDUK *et al.*, 2018).

Como a resposta imune é mediada por anticorpos, ela ocorre por meio do reconhecimento de epítopos específicos; a redução do antígeno para sequências mínimas de peptídeos tem sido frequentemente utilizada (HEYDUK *et al.*, 2018; PAVAN; BERTI, 2011).

Os aminoácidos, unidades fundamentais dos peptídeos e proteínas, são constituídos por um átomo de carbono central, ligado a quatro grupos: um átomo de hidrogênio, um grupo α-carboxílico, um grupo α-amino e um grupo R, que representa as cadeias laterais exclusivas de cada aminoácido e responsáveis pelas suas propriedades químicas individuais. Os peptídeos são formados por polímeros curtos naturais ou sintéticos de aminoácidos que são unidos por ligações peptídicas, também chamadas de ligações amida, formadas pela união do grupo α-carboxílico de um aminoácido ao grupo α-amino de outro aminoácido (DIETZEN, 2018; KATSOYANNIS, 1961). Peptídeos adotam configurações específicas, dependendo de quais grupos R estão próximos uns dos outros em uma cadeia peptídica (PUIU; BALA, 2018).

Os peptídeos têm sido utilizados como elemento de bioreconhecimento devido a sua estabilidade contra desnaturação, aquisição simples, especificidade, custo-benefício, protocolo sintético padrão, acessibilidade, fácil modificação, versatilidade química, combinação química e seleção em bibliotecas aleatórias (KARIMZADEH *et al.*, 2018).

Um número crescente de biossensores utilizando peptídeos para detecção de anticorpos têm sido relatados na literatura com uma ampla variedade de campos de aplicação. Peptídeos específicos foram conjugados com o marcador redox azul

de metileno e utilizados no desenvolvimento de um biossensor eletroquímico para a detecção de anticorpos anti- p24, uma proteína presente no nucleocapsídeo do vírus da imunodeficiência humana tipo I (HIV-1) (GERASIMOV; LAI, 2010). Zaitouna; Lai (2014) desenvolveram um sensor para a detecção de anticorpos anti-Ara h 2, uma proteína presente no amendoim, com base também na imobilização de peptídeo conjugado com azul de metileno, Valencia *et al.* (2016) desenvolveram um imunossensor eletroquímico para detecção de anticorpos anti-peptídeos de HPV com base na imobilização de um peptídeo em eletrodos de ouro. Rodovalho *et al.* (2018) e Oliveira *et al.* (2018) utilizaram peptídeos para detecção de imunoglobulinas IgG específicas para artrite idiopática juvenil e artrite reumatoide, respectivamente.

3.2. Classificação quanto à transdução do sinal

Os biossensores podem ser classificados de acordo com o princípio usado na transdução de sinal. O transdutor é um componente do biossensor que desempenha um importante papel no processo de detecção do sinal, por isso o transdutor pode ser definido como um dispositivo que converte uma vasta gama de efeitos biológicos, físicos e químicos, em um sinal elétrico mensurável (PERUMAL; HASHIM, 2014). No que se refere ao tipo de transdutor físico-químico, os biossensores são classificados em piezoelétricos, calorimétricos, ópticos e eletroquímicos.

3.2.1. Biossensores piezoelétricos

Também conhecidos como sensores mecânicos, estes dispositivos detectam a mudança na massa devida à interação do alvo com o elemento de reconhecimento, produzindo um sinal elétrico mensurável. Eles são baseados no efeito piezoelétrico, onde certos materiais sólidos acumulam carga elétrica em resposta a tensões mecânicas. A principal vantagem da abordagem de transdução piezoelétrica inclui a capacidade de realizar medições diretas sem marcações do processo de ligação, incluindo a análise em tempo real da cinética de ligação (SANTORO; RICCIARDI, 2016).

3.2.2. Biossensores calorimétricos

Esses dispositivos exploram a propriedade de absorção ou liberação do calor das reações bioquímicas. O princípio do biossensor calorimétrico ou térmico envolve a medição das variações de temperatura na reação entre o elemento de reconhecimento e um analito alvo; esta alteração de temperatura pode ser correlacionada com a quantidade de reagentes consumidos ou produtos formados, com a medição do calor liberado ou consumido (KARUNAKARAN; RAJKUMAR; BHARGAVA, 2015; RAMANATHAN; DANIELSSON, 2001).

3.2.3. Biossensores ópticos

Nos biossensores ópticos, um sinal óptico que é baseado na mudança na fase, amplitude, polarização ou frequência da luz de entrada, em resposta aos processos de reconhecimento. Podem ser classificados como colorimétricos, fluorescentes, quimioluminescentes, eletroquimioluminescentes e biossensores baseados em ressonância plasmônica de superfície (JU, 2017; LECHUGA, 2005).

3.2.4. Biossensores eletroquímicos

Os biossensores eletroquímicos são baseados no consumo e/ou geração de espécies eletroativas durante uma interação química e biológica; nesse processo o transdutor mensura o sinal eletroquímico produzido por essa interação (MEHRVAR, 2004).

As técnicas eletroquímicas possibilitam o estabelecimento de relações diretas entre a concentração do analito e algumas propriedades elétricas como corrente, potencial, condutividade, resistência ou carga. Como as medidas destas propriedades são facilmente acessíveis experimentalmente, são adequadamente utilizadas na quantificação de espécies de interesse nas diferentes áreas de estudo (SOUZA, 2003). Os biossensores eletroquímicos são os mais comuns e estudados para o uso em análises clínicas (SONG, S.; XU; FAN, 2006)

Estes dispositivos dependem de propriedades elétricas (resistência, corrente, potencial, capacitância, impedância), que são detectadas e medidas usando

diferentes métodos, como potenciometria, condutometria, amperometria ou voltametria (KARUNAKARAN; RAJKUMAR; BHARGAVA, 2015).

Neste trabalho, técnicas voltamétricas foram utilizadas para o desenvolvimento dos biossensores para o diagnóstico de *T. gondii.*

3.2.4.1. Voltametria

Esta técnica foi desenvolvida pela primeira vez por Jaroslav Heyrovsky em 1922, o que resultou no Prêmio Nobel de Química em 1959 (HOUSSIN; SENEZ, 2014). O termo voltametria refere-se às técnicas nas quais a corrente é medida variando-se o potencial em uma faixa definida. A resposta de corrente é geralmente um pico ou um platô proporcional à concentração do analito (BETTAZZI *et al.*, 2017; RONKAINEN; HALSALL; HEINEMAN, 2010). O gráfico de corrente *versus* potencial, chamado voltamograma, fornece informações sobre a reação química e a posição da corrente de pico está relacionada à substância química específica, e os valores de corrente de pico é proporcional à concentração da espécie correspondente (KARUNAKARAN; RAJKUMAR; BHARGAVA, 2015).

Na voltametria, um potencial em função do tempo é aplicado a um eletrodo de trabalho, mudando o seu potencial em relação a um potencial fixo de um eletrodo de referência. A corrente resultante, que flui entre o eletrodo de trabalho e um eletrodo auxiliar, é medido em função do potencial (HOUSSIN; SENEZ, 2014).

O eletrodo de trabalho é o eletrodo onde a reação de interesse ocorre, e o desempenho das medições voltamétricas é fortemente influenciado pelo material do eletrodo de trabalho, que pode ser de vários materiais como carbono vítreo, prata, ouro, mercúrio, tintas impressas, vidro revestido com óxido de índio-estanho, pasta de carbono e grafite. O eletrodo de referência é usado para produzir um potencial constante na célula eletroquímica; o mais comumente usado para soluções aquosas é o eletrodo de prata/cloreto de prata (Ag/AgCl), e o eletrodo auxiliar é o eletrodo utilizado para fechar o circuito na célula eletroquímica e geralmente é feito de um material inerte como platina, ouro, grafite e carbono vítreo (KARUNAKARAN; RAJKUMAR; BHARGAVA, 2015).

Os métodos voltamétricos incluem voltametria de varredura linear, voltametria cíclica, voltametria hidrodinâmica, voltametria de pulso diferencial, voltametria de onda quadrada, polarografia e voltametria de redissolução (RONKAINEN; HALSALL; HEINEMAN, 2010). A escolha da técnica voltamétrica a ser utilizada, está relacionada com o tipo e a qualidade de informação quantitativa e/ou qualitativa que se quer obter a respeito do analito ou do processo que envolve a interação entre o analito e o eletrodo de trabalho (PACHECO, 2013).

Na voltametria cíclica (**Figura 7**) o potencial inicial (E_1) aplicado varia linearmente em um sentido até o potencial final (E_2), então, o sentido da varredura é invertido e o potencial volta ao valor inicial (E_1), seu voltamograma consiste de uma curva fechada. Na presença de compostos eletroativos, podem ser detectados picos de oxidação, em um potencial de pico anódico (E_{pa}) e com corrente de pico anódico (i_{pa}) específicos; e/ou picos de redução, em um potencial de pico catódico (E_{pc}) e com corrente de pico catódico (i_{pc}) específicos. Para as correntes de pico, pode ser realizada a subtração da corrente na base do pico, o que resulta na variação de corrente de picos anódico e catódico (Δi_{pa} e Δi_{pc}) (BARD, 2008).

Geralmente esta técnica não é usada para análise quantitativa devido à sua baixa sensibilidade (KARUNAKARAN; RAJKUMAR; BHARGAVA, 2015).

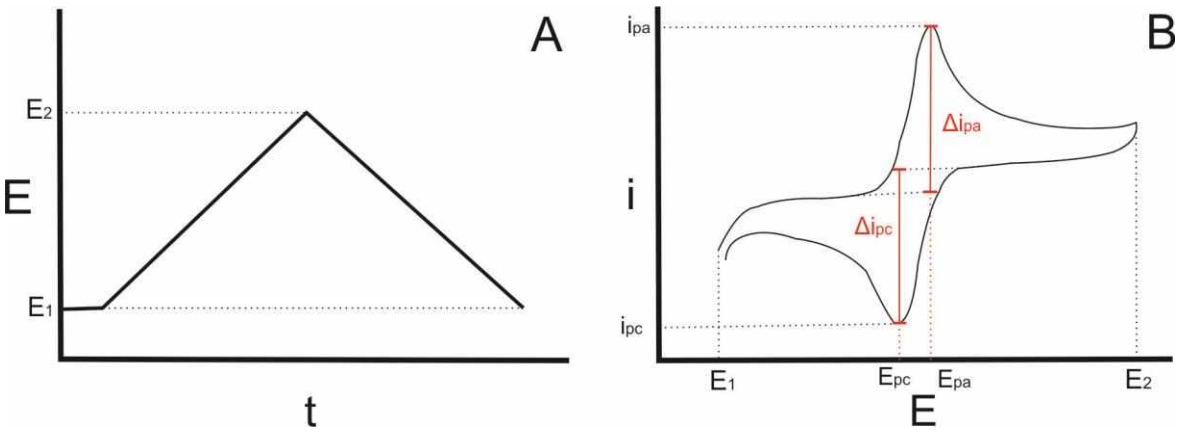

Figura 7. Representações gráficas da voltametria cíclica. A) Gráfico de potencial em função do tempo: em cada ciclo o potencial é variado de E_1 a E_2, em seguida retornando a E1. B) Voltamograma cíclico, com a corrente elétrica em função do potencial. Neste exemplo, é possível observar os picos de oxidação e redução. E: potencial elétrico; i: corrente elétrica; Epc: potencial de pico catódico; Epa: potencial de pico anódico; ipc: corrente de pico catódico; ipa: corrente de pico anódico. O delta (Δ) representa a variação de corrente de pico, com a subtração da corrente de pico pela corrente na base do pico. Adaptado de Karunakaran; Rajkumar; Bhargava (2015).

A voltametria de pulso diferencial (**Figura 8**) é uma técnica mais sensível, onde pulsos de amplitude fixos sobrepostos a uma rampa de potencial crescente são aplicados ao eletrodo de trabalho. A corrente é medida duas vezes, uma antes da aplicação do pulso e outra ao final do pulso. A primeira corrente é instrumentalmente subtraída da segunda, e a diferença das correntes é plotada *versus* o potencial aplicado. O voltamograma resultante consiste de picos de corrente de forma gaussiana, cuja área é diretamente proporcional à concentração do analito (BARD, 2008; KARUNAKARAN; RAJKUMAR; BHARGAVA, 2015; PACHECO, 2013).

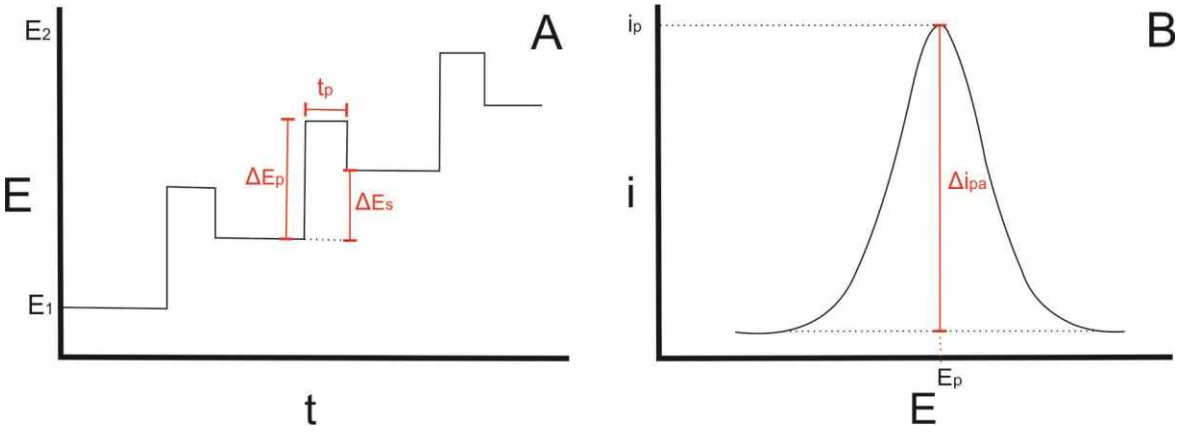

Figura 8. Representações gráficas da voltametria de pulso diferencial. A) Gráfico de potencial em função do tempo: são aplicados pulsos periódicos de potencial. Os pontos destacados representam a amostragem de corrente para um pulso. B) Voltamograma de pulso diferencial, com a corrente elétrica em função do potencial. Neste exemplo, é possível observar um pico de oxidação. E: potencial elétrico; i: corrente elétrica; ΔEp: variação de potencial de pulso; ΔEs: variação de potencial de amostragem; tp: período de pulso; Δip: variação de corrente de pico. Adaptado de Karunakaran; Rajkumar; Bhargava (2015).

3.2.4.2. Mecanismos de detecção eletroquímica

Os ensaios eletroquímicos podem ser diretos ou indiretos. Nos métodos diretos, a ligação do analito ao elemento de reconhecimento é detectado diretamente, sem a necessidade de sondas redox ou indicadores eletroativos. Componentes de ácidos nucléicos, como nucleosídeos e nucleotídeos, são espécies eletroativas que podem ser monitoradas diretamente. O comportamento eletroquímico do DNA e os efeitos de sua adsorção em diversos tipos de eletrodos têm sido amplamente investigados (DRUMMOND; HILL; BARTON,

2003; MUTI; KURALAY; ERDEM, 2012; PALECEK; BARTOSIK, 2012; RASHID; YUSOF, 2017). Oliveira-Brett *et al.* (2004) investigaram a oxidação voltamétrica de todos os nucleotídeos do ácido desoxirribonucleico em eletrodos de carbono vítreo (**Figura 9**), onde observaram que as purinas, guanina (G) e adenina (A) são oxidadas em potenciais positivos muito menores (+ 0,70 e + 0,96 V *vs*. Ag/AgCl) quando comparadas as pirimidinas, timina (T) e citosina (C) cujos potenciais de oxidação (+1,16 e +1,31 V *vs*. Ag/AgCl) estão próximos do potencial de evolução do oxigênio. Além disso, na mesma concentração, as correntes de oxidação das bases pirimídicas são muito inferiores às das bases púricas (ZHANG *et al.*, 2016). Como as bases nitrogenadas redox G e A são mais ativas e mais facilmente detectadas, o monitoramento do sinal de oxidação dessas bases tem sido utilizado para monitorar eventos de hibridização entre sonda e alvo (ERDEM *et al.*, 2004; ERDEM *et al.*, 2006; GOKCE *et al.*, 2016).

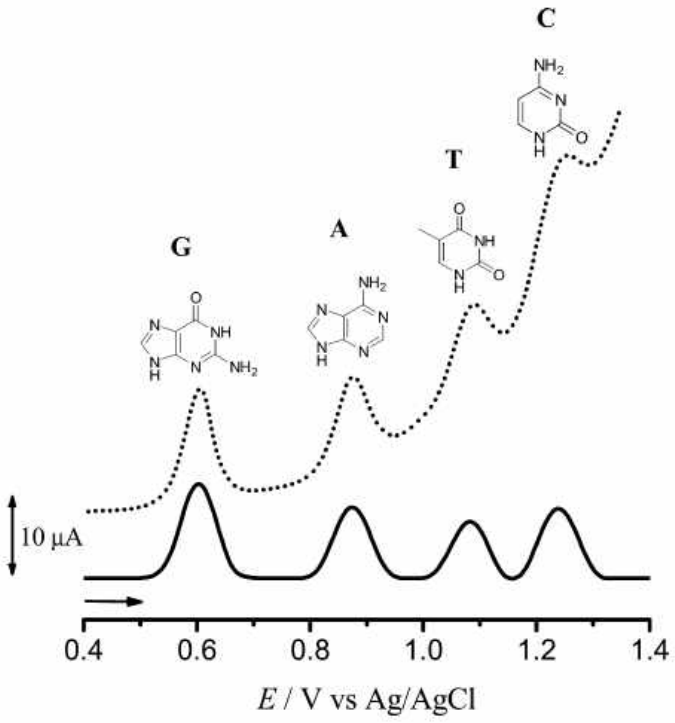

Figura 9. Voltametria de pulso diferencial obtido com um eletrodo de carbono vítreo diâmetro 3 mm para a mistura de $2x10^{-5}$ M guanina (G), adenina (A) e $2x10^{-4}$ M timina (T) e citosina (C) em eletrólito suporte tampão fosfato 0,1M pH 7,4 (...) voltamograma registrado; (-) voltamograma corrigido a linha de base (OLIVEIRA-BRETT *et al.*, 2004).

Nos métodos indiretos, há a utilização de biomoléculas conjugadas com elementos catalíticos (enzimas peroxidase e fosfatase alcalina, por exemplo), indicadores da interação ou moléculas fluorescentes (GRIFFIN, 2009; THÉVENOT *et al.*, 2001). As mudanças que ocorrem na superfície do eletrodo, como a interação sonda e alvo, podem ser observadas por alterações na transferência de elétrons de um sistema redox conhecido. Um indicador de interação muito utilizado é a sonda redox externa ferrocianeto de potássio K4[Fe(CN)$_6$]. O objetivo de usar esta molécula é investigar a área da superfície do eletrodo disponível para sua oxidação: $[Fe(CN)_6]^{4-} \rightleftarrows [Fe(CN)_6]^{3-} + e^-$ (HENNESSEY *et al.*, 2009).

RESENDE *et al.* (2017) descreveram um imunossensor para detecção de proteína C reativa utilizando ferrocianeto como indicador. Rodovalho *et al.* (2018) e Oliveira *et al.* (2018) também utilizaram essa sonda redox para detecção de imunoglobulinas IgG específicas para artrite idiopática juvenil e artrite reumatoide, respectivamente.

3.3. Estratégias de imobilização de biomoléculas

A imobilização do elemento de reconhecimento na superfície do eletrodo de trabalho para reconhecer seu alvo é o passo crucial na construção de sensor eletroquímico. Para a escolha do método de imobilização, alguns fatores devem ser levados em consideração, como o tipo de elemento de transdução, as propriedades físicas do analito e a natureza do biocomponente a ser imobilizado. O elemento de reconhecimento pode ser imobilizado por adsorção física, ligação covalente ou oclusão (SANTORO; RICCIARDI, 2016).

A adsorção física de biomoléculas depende de interações não covalentes, principalmente força eletrostática, ligação iônica, ligação de hidrogênio e interação hidrofóbica, sendo um método simples que gera pouco efeito na bioatividade e com ampla aplicabilidade (JU, 2017; SANTORO; RICCIARDI, 2016).

A imobilização de sondas de DNA na superfície do eletrodo de trabalho para reconhecer seu alvo complementar via hibridização é a etapa crucial na construção dos genossensores. Adsorção é a técnica mais simples para a imobilização de sondas de DNA na superfície do eletrodo de trabalho, pois não requer uso de reagentes químicos e modificação das sondas, e o uso de filmes

poliméricos tem sido utilizado para auxiliar nessa adsorção (RASHID; YUSOF, 2017).

3.4. Polímeros condutores

Os polímeros condutores são polímeros orgânicos que conduzem eletricidade devido à deslocalização de elétrons π. Tais compostos podem ter condutividade metálica ou ser semicondutores e podem ser sintetizados por várias técnicas, incluindo a polimerização eletroquímica (AWUZIE, 2017; RAHMAN *et al.*, 2015), utilizada neste trabalho.

Polímeros condutores têm sido amplamente utilizados no desenvolvimento de biossensores eletroquímicos, devido a várias vantagens, como a presença de grupos funcionais que facilitam a imobilização de biomoléculas (GERARD, 2002; RAHMAN *et al.*, 2015). Além disso, a combinação de materiais poliméricos com biossensores promove um aumento no desempenho analítico do biossensor, aumentando a sensibilidade, seletividade, estabilidade e reprodutibilidade da resposta do eletrodo na detecção de uma variedade de analitos alvo (ATES, 2013).

Polímeros derivados do ácido hidroxibenzóico (HBA) são utilizados no desenvolvimento de filmes poliméricos para a modificação de eletrodos devido a presença de grupos funcionais (ácido carboxílico e ácido hidroxílico), que podem sofrer eletropolimerização e interagir com biomoléculas (FERREIRA, *et al.*, 2014; FERREIRA, *et al.*, 2011) Trabalhos anteriores mostraram que o polímero derivado do (3-HBA) é uma matriz eficiente tanto para a imobilização de oligonucleotídeos (FERREIRA, *et al.*, 2014) quanto peptídeos (OLIVEIRA *et al.*, 2018).

4. Biossensores para detecção de *Toxoplasma gondii*

Biossensores aplicados a detecção de *Toxoplasma gondii* têm sido descritos na literatura. A maioria dos biossensores para *T. gondii* reportados baseia-se em imunoensaios para a detecção de anticorpos anti-*T. gondii*. Um imunoensaio piezelétrico baseado na aglutinação foi desenvolvido para detectar diretamente imunoglobulinas anti- *T. gondii* em soro e sangue de coelhos infectados. A técnica proposta é baseada na aglutinação específica de nanopartículas de ouro revestidas com antígeno (diâmetro de 10 nm). Na presença do anticorpo

correspondente, que provoca uma mudança de frequência monitorada por um dispositivo piezoelétrico (WANG, H. *et al.*, 2004). Ding *et al.* (2005), desenvolveram um biossensor eletroquímico baseado em uma amplificação catalisada por enzimas. Antígenos de *T. gondii* foram imobilizados na superfície de um eletrodo de ouro, a fim de ligarem-se à IgG anti-toxoplasma, e isso foi seguido pela adição de anti-IgG conjugado com peroxidase, as técnicas utilizadas para a detecção foram microbalança de cristal de quartzo, espectroscopia de impedância eletroquímica e voltametria cíclica.

Um imunossensor eletroquímico foi construído utilizando folhas de grafeno/náfion, nanopartículas de ouro e tionina para produzir uma membrana híbrida depositada em eletrodos de vidro. A detecção de IgM anti-toxoplasma foi realizada através de anti-IgG conjugada com peroxidase e acopladas com nanopartículas magnéticas de óxido de ferro (JIANG *et al.*, 2013). Medawar-Aguilar *et al.* (2018) descreveram um imunossensor fluorescente para determinação de IgG anti-toxoplasma utilizando nanopartículas de óxido de zinco cobertas com quitosana e funcionalizadas com antígenos de *T. gondii,* e a detecção foi realizada utilizando anticorpos anti-IgG marcados com fosfatase alcalina.

Luo *et al.* (2013) usaram dois aptâmeros com alta afinidade para IgG anti-toxoplasma como sondas de detecção para o desenvolvimento de um aptassensor conjugado com quantum dots. Na presença de IgG anti-toxoplasma, um complexo sanduíche de aptâmero-proteína-aptâmero é formado e capturado em microplacas de múltiplos poços, cuja fluorescência pode ser lida usando quantum dots como marcador. Também baseado na detecção fluorimétrica, He *et al.* (2015) descreveu o uso de nanopartículas fluorescentes magnéticas no desenvolvimento de genossensor para a detecção de oligonucleotídeos de DNA de *T. gondii*. Outro genossensor foi desenvolvido a partir da imobilização de oligonucleotídeos modificados com inosina sobre eletrodos de grafite de lápis, e a detecção eletroquímica da formação do duplex em amostras de DNA amplificadas por PCR foi realizada monitorando o sinal da oxidação da guanina utilizando voltametria de pulso diferencial (GOKCE *et al.*, 2016).

Apesar desses vários estudos reportando biossensores para detecção de *T. gondii*, a maioria apresenta metodologias complicadas e plataformas intrincadas e com custo relativamente elevado. O presente trabalho objetivou o desenvolvimento de plataformas mais simples, utilizando eletrodos de grafite como transdutores, sendo os mesmos modificados com um polímero derivado do ácido 3-hidroxibenzóico, facilmente obtido por eletropolimerização.

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CAPÍTULO 2

DEVELOPMENT OF DIRECT ASSAYS FOR *TOXOPLASMA GONDII* AND ITS USE IN GENOMIC DNA SAMPLE

O capítulo 2 foi publicado na revista Journal of Pharmaceutical and Biomedical Analysis **(Anexo 2)**

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Development of direct assays for *Toxoplasma gondii* and its use in genomic DNA sample

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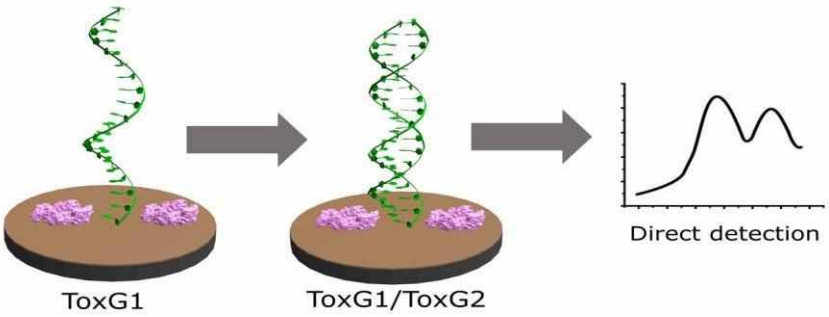
GRAPHICAL ABSTRACT



HIGHLIGHTS

A new biosensor to toxoplasmosis was developed based on modified electrode;

The biosensor was able of detect until 100 ng.mL-1 of the *T. gondii* genomic DNA;

The genosensor showed high selectivity, discriminating non-specific targets;

Optical assays showed significant change in the absorbance peak in presence of *Toxoplasma gondii* genomic DNA.

Abstract

This work describes an approach for the selection and detection of specific DNA probes related to *Toxoplasma gondii*, a protozoan parasite responsible for toxoplasmosis. The detection system was developed on graphite carbon electrode modified with poly (3-hydroxybenzoic acid) sensitized with ToxG1 probe. The hybridization of the specific genomic DNA related to *T. gondii* showed good response by direct detection of guanine residue oxidation using differential pulse voltammetry (DPV). The biosensor was able to distinguish both the complementary and non- complementary targets and detect up to 100 $ng.\mu L^{-1}$ of the *T. gondii* genomic DNA. The hybridization (ToxG1: *T. gondii* genomic DNA) was confirmed by optical measurement. Optical assays using gold nanoparticles:ToxG1 probe showed a significant change in the absorbance peak in the presence of the *T. gondii* genomic DNA according to the electrochemical results. This novel biosensor shows potential as electrochemical transducer and was successfully applied in the biological sample.

Keywords: *Toxoplasma gondii*; DNA; GRA6 gene; biosensor; gold nanoparticles

Resumo

Este trabalho descreve uma abordagem para seleção de sondas de detecção de DNA específicas para o *Toxoplasma gondii*, um protozoário parasita responsável pela toxoplasmose. O sistema de detecção foi desenvolvido em eletrodos de grafite modificados com poli (3-ácido hidroxibenzóico) e sensibilizados com a sonda ToxG1. A hibridização do DNA genômico específico para *T. gondii* mostrou uma boa resposta por detecção direta da oxidação dos resíduos de guanina usando voltametria de pulso diferencial (VPD). O biossensor foi capaz de distinguir entre os alvos complementares e não complementares e detectou até 100 $ng.\mu L^{-1}$ de DNA genômico de *T. gondii*. A hibridização (ToxG1: DNA genômico de *T. gondii*) foi confirmada por medição óptica. Ensaios ópticos usando nanopartículas de ouro:ToxG1 mostrou uma mudança significante no pico de absorção na presença do DNA genômico de *T. gondii,* de acordo com os resultados eletroquímicos. Este novo biossensor apresenta potencial como transdutor eletroquímico e foi aplicado com sucesso em amostras biológicas.

Palavras- chave: *Toxoplasma gondii*; DNA; gene GRA6; biossensor; nanopartículas de ouro

1. Introduction

Toxoplasma gondii is the intracellular protozoan parasite responsible for toxoplasmosis, a zoonosis with medical and veterinary importance worldwide [1]. Most infections are asymptomatic in healthy adults, but severe disease may develop with congenital infections or immunodepression [2]. Estimates show that more than a third of the world's population has been infected with this parasite [3]. Diagnosis and genetic characterization of *T. gondii* infection are crucial for monitoring, prevention and control of toxoplasmosis. Traditional approaches for toxoplasmosis detection rely on immunological diagnosis and imaging techniques [4]. These methods have limitations; as for instance, the identification based on imaging techniques is less sensitive and uncertain. In addition, these methods demand a long time for sample preparation, expensive equipment and the need for skilled personnel.

There is a significant need for improved diagnosis. Biosensors stand out as a promising alternative to the traditional methodologies. Biosensors are analytical devices that combine the specificity of biomolecules with sensible transduction platforms, presenting portability, rapidity and good cost-effectiveness relation. In the literature, sensors developed for the detection of *T. gondii* are generally based on the immobilization of antibodies [5-7], aptamers [8] and DNA probes [9, 10] The detection of specific DNA sequences plays a major role in many fields, especially in clinical diagnostics. Direct monitoring of changes in its electrochemical properties, such as oxidation of guanine and adenine residues, enables the detection of hybridization on the electrode surface [11].

The combination of polymeric materials with biosensors promotes an increase in the analytical performance of the biosensor. Polymers derived from hydroxybenzoic acid are used in the development of polymeric films for the modification of electrodes due to the presence of functional groups (carboxylic acid and hydroxyl acid), which are liable to undergo electropolymerization and to interact with biomolecules [12].

Nucleic acid components, such as nucleosides and nucleotides, are electroactive species that can be monitored. The electrochemical behavior of DNA and the effects of its adsorption onto several types of electrodes have been widely

investigated [13-15]. Oliveira-Brett and collaborators [11] investigated the voltammetric oxidation of all nucleotides of deoxyribonucleic acid on glassy carbon electrodes. Silva et al. [16] successfully described the immobilization of purine and pyrimidine bases on graphite electrodes modified with poly(4-methoxyphenethylamine).

The focus of the current work was the development of a method for the selection of specific DNA probes related to *T. gondii* and a system for the detection of a specific target sequence for the diagnosis of toxoplasmosis based on graphite electrodes modified with poly(3-hydroxybenzoic acid) and sensitized with DNA-oligonucleotide probe (**Fig. 1**). In this work, it was possible to detect double-strand DNA related to *T. gondii* onto the modified surface. To the best of our knowledge, this is the first genosensor that uses specific genomic DNA as a target for the diagnosis of *T. gondii* infection or toxoplasmosis.

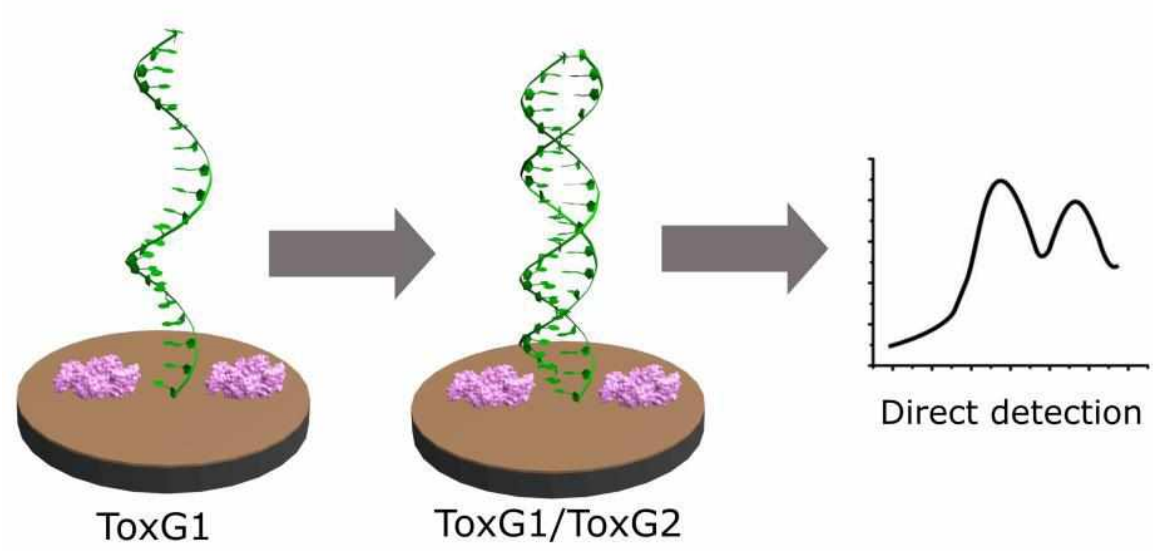


Figure 1. Schematic representation for the immobilization of the ToxG1 probe on modified electrode and its hybridization.

2. Experimental

2.1. Chemicals

All reagents used were of analytical grade without previous purification. Ultra high purity water (Master System, Gehaka, Brazil) was used for the preparation of all solutions. The monomer employed for the polymerization reaction was 3-

hydroxybenzoic acid (3-HBA) 99%, purchased from Sigma–Aldrich (USA). The detections were performed in 0.1 mol.L-1 phosphate buffer (Na_2HPO_4 0.061 mol.L^{-1}, NaH_2PO_4 0.039 mol.L^{-1}, pH = 7.4). All solutions were deoxygenated by ultra-pure nitrogen bubbling for at least 45 minutes before use. Experiments were conducted at room temperature (25 ± 1°C).

Oligonucleotides were synthesized by Invitrogen Life Technologies and their sequences are: ToxG1 (probe): 5'-CTGGGAACGGTGGGAATGAA-3', ToxG1S (probe) 5'-CTGGGAACGGTGGGAATGAA-SH-3', ToxG2 (target): 5'-TTCATTCCCACCGTTCCCAG-3', Non-complementary target: 5'-CCACTCTTCAATTCTCTCCGCC-3'.

The oligonucleotide stock solutions and dilutions were prepared in SSC buffer (pH 7.0, sodium chloride 0.3 mol.L^{-1} and sodium citrate 0.03 mol.L^{-1}, Sigma–Aldrich, USA) and stored at −12ºC.

2.2. Apparatus

The electrochemical studies were performed in a potentiostat from CH Instruments, model 620C, USA. A graphite disk (6.15 mm diameter, 99.9995% purity) from Alfa Aesar was used as working electrode. A platinum plate and silver/silver chloride (3.0 mol L^{-1}) were used as auxiliary and reference electrodes, respectively. Film morphology in the absence or presence of biomolecules was assessed by atomic force microscopy (SPM 9600, Shimadzu). The assays using Gold nanoparticles (AuNPs) were carried out using a spectrophotometer (UV-1650PC, Shimadzu).

2.3. *In silico* probe selection

Nucleotide sequences of GRA6 gene (GenBank: L33814.1), B1 gene (GenBank: AF179871.1) and a repetitive region (GenBank: AF146527.1) from *T. gondii* genome were obtained from the National Center for Biotechnology Information (NCBI) database. DNA probes were selected *in silico* using a pipeline that integrated Primer3 software [17] with algorithms for multi-fast handling and parameter setting, as well as scoring, sorting and formatting of the results.

The developed pipeline took fasta files as input, along with parameters specified in the main script, such as probe size, GC content, scoring scheme, DNA

and salt concentrations, constraints of melting temperature for desirable probes and undesirable secondary structures. Afterwards, Primer3 parameter setting and execution were sequentially conducted. The results obtained were scored with a user-defined scheme that accounted for occurrence of pairs, triplets and quartets of specific base residues, since some of them are preferred for electrochemical direct monitoring in modified carbon electrodes (G and A) [11]. Next, the results were ordered according to the score and formatted for the generation of detailed output files. Finally, the probes obtained were submitted to BLAST alignment with the nucleotide database in order to select the most species-specific probes.

2.4. Parasite preparation and genomic DNA extraction

Strains of *Neospora caninum* (NcLivΔHPT) and *T. gondii* tachyzoites (RH) were maintained by serial passages in HeLa cell line (CCL-2, ATCC, Manassas, VA, USA), cultured in RPMI 1640 medium supplemented with 2 $mmol.L^{-1}$ glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin, at 37°C in 5% CO_2 atmosphere. Parasite suspensions were obtained as previously described [18]. Briefly, tachyzoites were harvested by scraping off the cell monolayer after 2-3 days of infection, passed through a 26-gauge needle and centrifuged at low speed (45 × g) for 1 min, for debris removal. The supernatant containing parasite suspension was collected, washed in phosphate buffered saline (PBS, pH 7.2) and centrifuged at 1,000 × g for 10 min. After the centrifugation, the pellet was kept at -20 °C for DNA extraction.

The genomic DNA was extracted from NcLivΔHPT *N. caninum* and RH *T. gondii* tachyzoites. The parasites were lysed and digested with 200 µL lysis buffer (400 $mmol.L^{-1}$ NaCl, 10 $mmol.L^{-1}$ Tris-HCl, 2 $mmol.L^{-1}$ and EDTA, pH 8.2), 6 µL 10% SDS, 3 µl proteinase K (20 $mg.mL^{-1}$), and incubated at 55°C overnight. After incubation, NaCl (150 µL, 6 $mmol.L^{-1}$) was added into the solution. The mixture was centrifuged at 12,000 × g at 4 °C for 10 min. The supernatant was transferred to the 1.5- mL tube, ethanol (800 µL, 100%) was added and centrifuged (12,000 × g for 10 min). The precipitated DNA was washed with ethanol (1 mL, 75%) and solubilized in 20 µL of Milli-Q water.

The human and mouse genomic DNA was extracted from peripheral blood according to Al-Shuhaib [19].

The concentration and quality of DNA extracted from each sample were analyzed using a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). After that, the DNA samples were stored at −20 °C until use.

2.5. Preparation of graphite electrode modified with poly (3-HBA)

The bare graphite electrodes were polished mechanically with alumina slurry (0.3 μm), sonicated, washed with deionized water and dried in air. The preconditioning was performed in 0.5 mol.L^{-1} $HClO_4$ solution through cyclic voltammetry between + 0.0 and +1.2V, 4 cycles, 50 mV.s^{-1}. The electrodes were modified with polymer derived from 3- hydroxybenzoic acid according to Ferreira and collaborators [12] with modifications. The electropolymerization was conducted on graphite electrodes (10 scans, 0.0 and +1.2 V vs. Ag/AgCl, 50 mV.s-1) from a 3-HBA solution (2.5 mmol.L^{-1}) prepared in $HClO_4$ (0.5 mol.L^{-1}) at room temperature (25 ± 1ºC).

2.6. Effect of probe concentration in the immobilization and hybridization

Optimization studies of the probe concentration were performed by adding 10 μL of different concentrations of ToxG1 (5 μmol.L^{-1}, 15 μmol.L^{-1}, 20 μmol.L^{-1}, 25 μ mol.L^{-1}, 35 μmol.L^{-1} and 50 μmol.L^{-1}) in the modified electrode surface at 37ºC and incubated for 30 minutes. The blocking of non-specific binding was performed with BSA 1% (w/v) at 37ºC for 1 h. After immobilization of the oligonucleotide probes by physical adsorption, 10 μL of different concentrations of ToxG2 target (10 μmol.L^{-1}, 30 μmol.L^{-1}, 40 μmol.L^{-1}, 50 μmol.L^{-1}, 70 μmol.L^{-1} and 100 μmol.L^{-1}) were added onto the modified electrode surfaces.

For the tests using the genomic DNA, the samples were maintained at 98 °C for 5 minutes, in order to de-hybridize the strands; they were then added onto the modified electrodes surfaces and incubated for 15 minutes at 52°C.

After each modification, the electrodes were washed three times with 50 μL of phosphate buffer. Differential pulse voltammetry measurements were performed using phosphate buffer as electrolyte.

2.7. Assay using AuNPs

The AuNPs were produced using the modified Turkevich-Frens method. In this method, gold salt is reduced, occurring the nucleation and growth of gold nanoparticles of 15 to 20 nm, stabilized by citrate ions [20]. After synthesis, the AuNPs were stored at 4°C until the use. The AuNPs sensibilizated with DNA probe were preparated using 200 µL of AuNPs solution added to probe solution, ToxG1S (2 uL, 200 µmol.L^{-1}) and maintained at room temperature for 1 hour. Prior to the hybridization process, the genetic material was digested using the EcoRI restriction enzyme (2 U/L) diluted in the specific buffer (50 mmol.L^{-1} NaCl, 10 mmol.L^{-1} Tris-HCl, 10 mmol.L^{-1} $MgCl_2$, 1 mmol.L^{-1} DTT), pH 7.6. After that, genomic DNA from *N. caninum* (negative sample, 2 uL, 200 ng.µL^{-1}) or *T. gondii* (positive sample, 2 uL, 200 ng.µL^{-1}) were added and maintained at 52ºC for the duplex formation during 30 min.

3. Results and discussion

3.1. Isolation of DNA probe

The repetitive region described by Homan et al. [21] is a fragment of 529 bp, which is repeated 200 to 300 times in the genome of *T. gondii*. This fragment has been widely investigated as a molecular target for the diagnosis of toxoplasmosis [22-24].

The B1 gene is repeated 35 times in the parasite genome and has been commonly used for molecular diagnosis with acceptable sensitivity [24-27].

The dense granule antigens (GRA) are parasitic molecules secreted to the parasitophorous vacuole, being immunogenic and responsible for the parasite intracellular survival. GRA6 (dense granule antigens 6) protein is coded by GRA6 gene (a highly polymorphic single-copy gene) and used for genotyping [28-31]. Several studies have showed the use of recombinant antigenic proteins as GRA for serological diagnosis of toxoplasmosis [32-34].

In the probe selection process, Primer3 considered 5,397 possible probes for GRA6 gene, excluding 3,392 for undesirable secondary structure or long base repeats. The remaining 2,005 probes were sorted and the top scoring was submitted to BLAST alignment. For the repetitive region, from the 1,370 probes

considered, 1,025 were excluded, resulting in 345 probes. For B1 gene, 6,429 probes were considered and 3,025 were excluded, resulting in 3,404 for scoring, sorting and alignment. Finally, the best result for each gene/genomic region was chosen. **Table 1** shows the sequences and highlights some of their features.

Table 1. Selected probes and their features.

		Primer3 features						Blast best hit features		
ID	Genomic target	LEN	GC%	TM	SELF-ANY	SELF-END	HAIRPIN	Query cover	Identity	E-value
ToxG1	GRA6 gene (L33814.1)	21	57,14	69,3	0	0	0	100%	100%	0.051
ToxR1	Repetitive region (AF146527.1)	22	54,55	68,83	0	0	0	100%	100%	0.019
ToxB1	B1 gene (AF179871.1)	21	42.62	66.62	0.81	0	0	100%	100%	0.051

* LEN: oligonucleotide length, GC%: GC content, TM: melting temperature, self-any: tendency of any self-complementarity, self-end: tendency of ends self-complementarity, hairpin: tendency of hairpin formation, ToxG1 oligonucleotide sequence: 5'-CTGGGAACGGTGGGAATGAAG-3', ToxR1 oligonucleotide sequence: 5'-GGCGGAGAGAATTGAAGAGTGG-3', ToxB1 oligonucleotide sequence: 5'-AAATGCCAGAAGAAGGGTACG-3'

Among the probes selected, ToxG1 was chosen for immobilization on the modified graphite electrodes due to the higher percentage of guanine (G)-cytosine(C) content in relation to ToxR1 and ToxB1. A probe of higher GC content creates a strong bonder with complementary DNA.

Most tests used for the diagnosis of toxoplasmosis are based on the detection of different antibody classes or antigens. Other DNA-based technologies also use and rely on amplification procedures, based on Polymerase Chain Reaction (PCR) prior to detection. In this method, several multicopy and single-copy genes are used as targets [4]. Costa and collaborators [35] developed a nested-PCR assay using the GRA7 gene as target for prime design, which also encodes a dense granule protein. The test was sensitive and specific compared to other assays, as the methodology presented in this work.

3.2. Immobilization of the DNA probe

Conductive polymers have been widely used in the development of biosensors, due to various advantages, such as the presence of functional groups that facilitate the immobilization of biomolecules [36, 37]. A previous work carried out by our group shows that poly (3-HBA) is an efficient matrix for the oligonucleotide immobilization [12].

Graphite electrodes were modified with polymeric film derived from 3-HBA. A current peak was observed at +1 V, what is attributed to the monomer oxidation, which decreased after the potential scans. After the first cycle, a gradual increase in the current between +0.3 V and +0.9 V can be observed due to the coverage of the electrode with an electroactive material. This modification was confirmed by cyclic voltammetry, in which there is an increase in the current response after the electrode modification with poly (3-HBA).

After the production of the graphite electrode modified with poly(3-HBA), the sensitization with ToxG1 probe was carried out.

Surface density of probe is an important factor that determines the extent to which the immobilized probes are able to capture targets in solution [38-40]. The effect of probe concentration for immobilization (**Fig. 2**) was investigated, by the direct method, using the guanine residue oxidation signal.

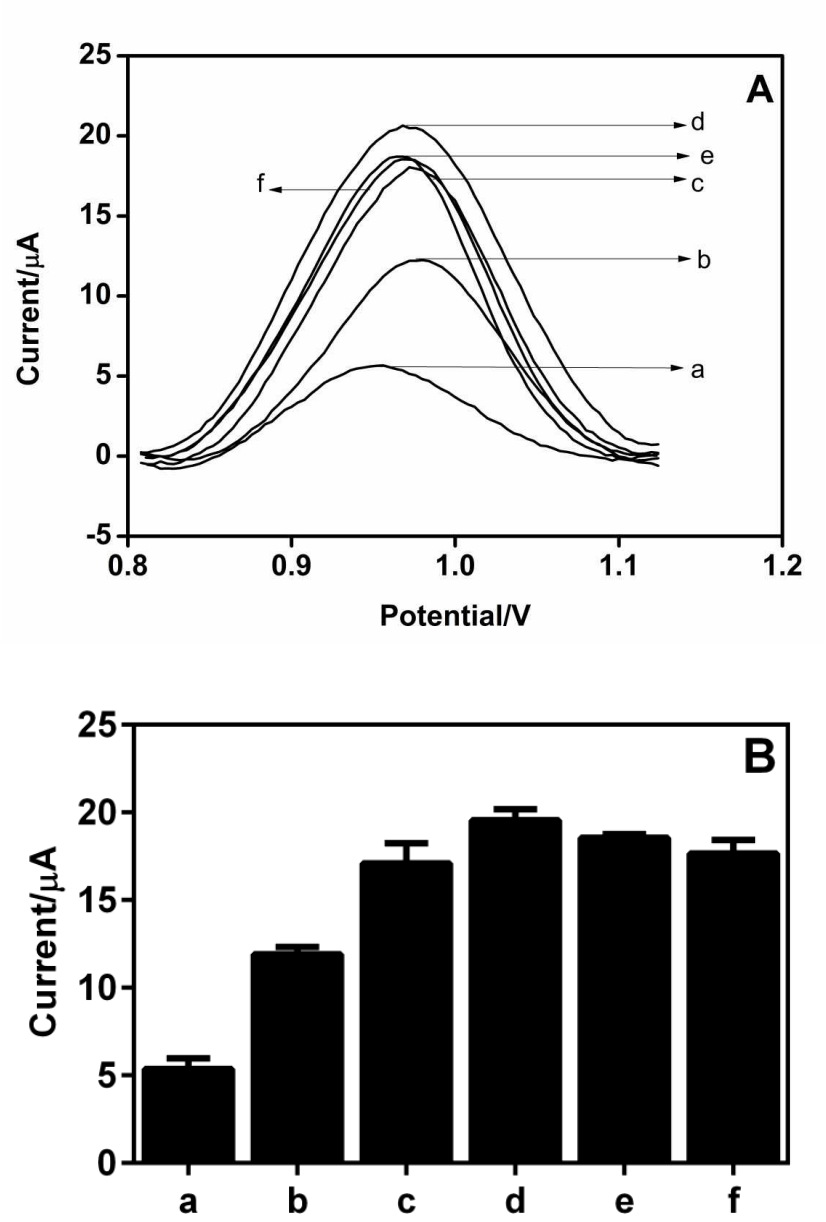


Figure 2. Differential pulse voltammograms (A) and histograms of the peak current response (B) of guanine residue oxidation in graphite electrodes modified with poly (3- HBA) after immobilization of different concentrations of probe (ToxG1): 5 μmol.L^{-1} (a) 15 μmol.L^{-1} (b) 20 μmol.L^{-1} (c) 25 μmol.L^{-1} (d) 35 μmol.L^{-1} (e) and 50 μmol.L^{-1} (f). Modulation amplitude: 25 mV, pulse interval: 0.2 s, scan rate: 20 mV.s^{-1}.

Figure 2A shows that the oxidation peak for guanine residue occurs at +0.96V vs. Ag/AgCl. With the increase in the guanine concentration, the current value of the oxidation peak for guanine increases until 25 μmol.L^{-1}, according to **Figure 2B**. This concentration was used in the hybridization experiments with the

complementary target. These results show that ToxG1 probe was efficiently incorporated to the graphite electrode modified with poly(3-HBA).

3.3. Surface analysis using AFM

The probe immobilization (ToxG1) onto electrode surface was also confirmed using 3D AFM analysis (**Fig. 3**).

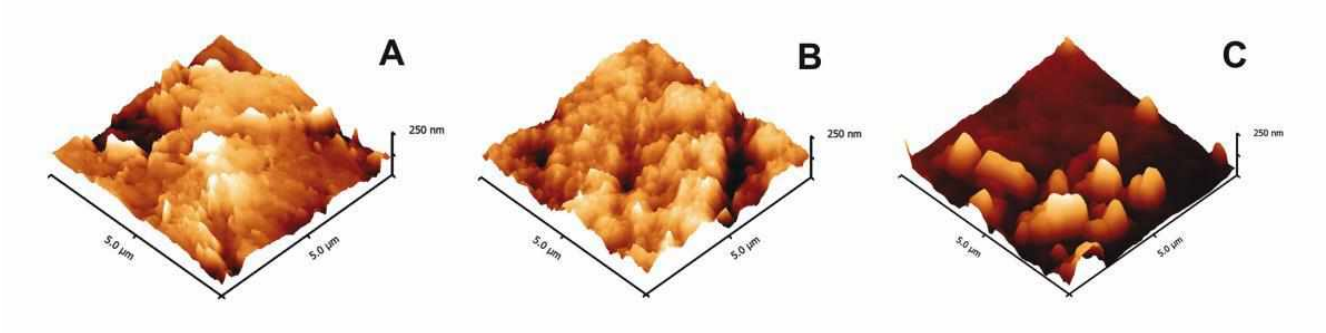


Figure 3. AFM topographical images of graphite electrode (A); graphite/poly (3-HBA) (B); graphite/poly (3-HBA)/probe (C).

Images of graphite electrode, graphite/poly(3-HBA) and graphite/poly(3-HBA)/ToxG1 probe present roughness values of 49.1 ± 2.0 nm, 27.4 ± 1.4 nm and 80.3 ± 3.6 nm, respectively. The surface of the electrode modified with polymer is smoother in comparison to the graphite electrode. Such figures indicate a greater uniformity of the modified electrode surface due to the fact that the polymer fills the valleys contained in the graphite electrode. After probe immobilization, both an increase in roughness and the appearance of clusters between 0.6 and 1.2 nm were observed according to the literature [41-42].

The effect of the probe concentration in the duplex formation is shown in **Figure 4.**

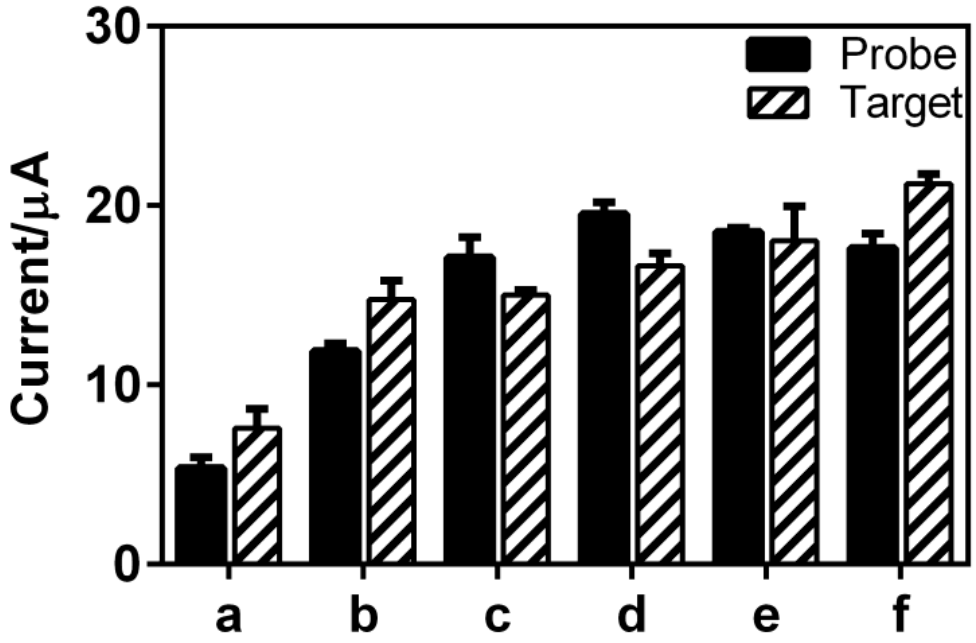


Figure 4. Histogram obtained from current peak response of guanine oxidation in graphite electrodes modified with poly (3-HBA) after immobilization of different concentrations of probe (ToxG1): 5 µmol.L^{-1} (a) 15 µmol.L^{-1} (b) 20 µmol.L^{-1} (c) 25 µmol.L^{-1} (d) 35 µmol.L^{-1} (e), 50 µmol.L^{-1} (f) and after 15 min of incubation with different concentrations of complementary target (ToxG2): 10 µmol.L^{-1} (a) 30 µmol.L^{-1} (b) 40 µmol.L^{-1} (c) 50 µmol.L^{-1} (d) 70 µmol.L^{-1} (e) and 100 µmol.L^{-1} (f).

A decrease in guanine oxidation peak current can be observed after hybridization with probe concentrations of 20 µmol.L^{-1} and 25 µmol.L^{-1}. However, as the difference between the probe (ToxG1) and the probe:target (ToxG1:ToxG2) was higher for 25 µmol.L^{-1}, this probe concentration was used in the experiments. In higher concentrations, it was observed to increase in the current response, probably due to the saturation of the surface by the probe, causing steric hindrance and making it difficult to interact with the complementary target.

The hybridization was monitored by the oxidation of guanine, which is the most redox active nitrogenous base in DNA strands. 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 [43]. Like the bioelectrode produced in this work, several label-free oligonucleotide biosensors have been described in literature [43-46], which are considered as the

simplest method, once it does not require any special reagents or nucleic acid modifications.

3.4. Specificity studies

Specificity studies were conducted using non-complementary oligonucleotide and genomic DNA from *N. caninum*, human and *Mus musculus* as non-specific targets. ToxG2 or *T. gondii* were used as positive control (**Fig. 5**).

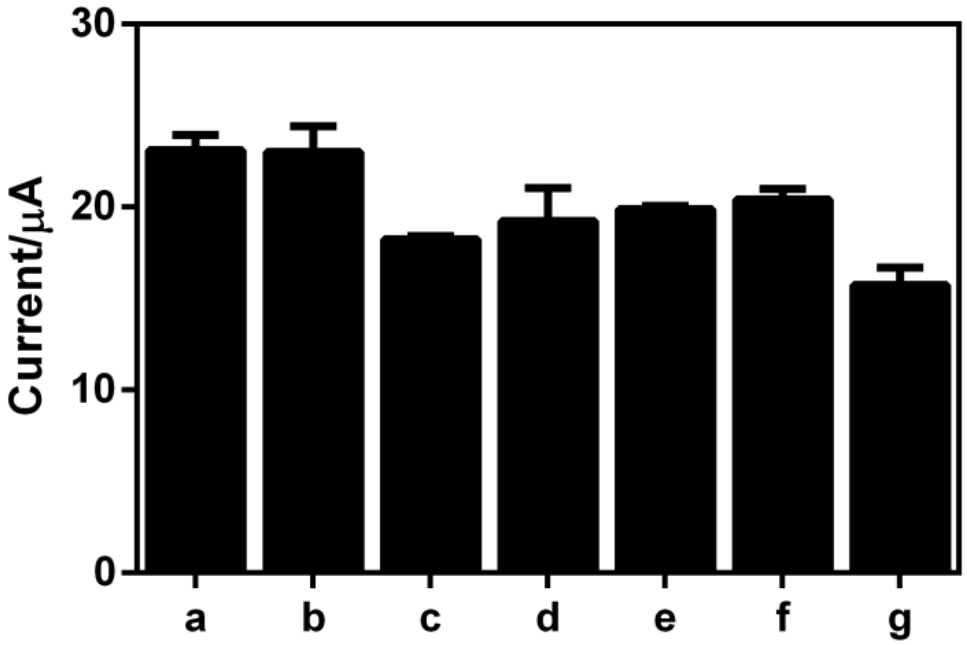


Figure 5. Bar chart obtained from differential pulse voltammograms of guanine residue onto graphite electrode modified with poly (3-HBA) containing ToxG1 probe, before hybridization (a) and after hybridization with: non-complementary oligonucleotide (b), complementary target oligonucleotide (ToxG2) (c), *Neospora caninum* genomic DNA (d), human genomic DNA (e), *Mus musculus* genomic DNA (f) and *Toxoplasma gondii* genomic DNA (g). Electrolyte: phosphate buffer (0.10 mol.L^{-1}), pH 7.4, modulation amplitude: 25 mV. Pulse interval: 0.2 s; Scan rate: 20 mV.s^{-1}.

When the non-specific targets (oligonucleotide or genomic DNA from *N. caninum*, human and *Mus musculus*) were added, the current values observed were similar to the probe. A decrease of about 20% in the guanine signal was observed due to the extent of hybridization between the ToxG1 probe in the presence of ToxG2 or *T. gondii* RH strain genomic DNA. This decrease is due to the formation of hydrogen bonds between the probe and the complementary target

during the hybridization, which hampers the oxidation of nitrogenous bases [11, 16, 41- 43].

Figure 6 shows a decrease in current peak response with the increase in genomic DNA concentration and a linear relationship of the current peak with the genomic DNA concentration given by I/μA = -0.01817X + 19.81 (r = 0.9826), in the range of 100 ng.μL^{-1} to 400 ng.μL^{-1}. It was possible to detect up to 100 ng.μL^{-1} of the genomic DNA of *Toxoplasma gondii*.

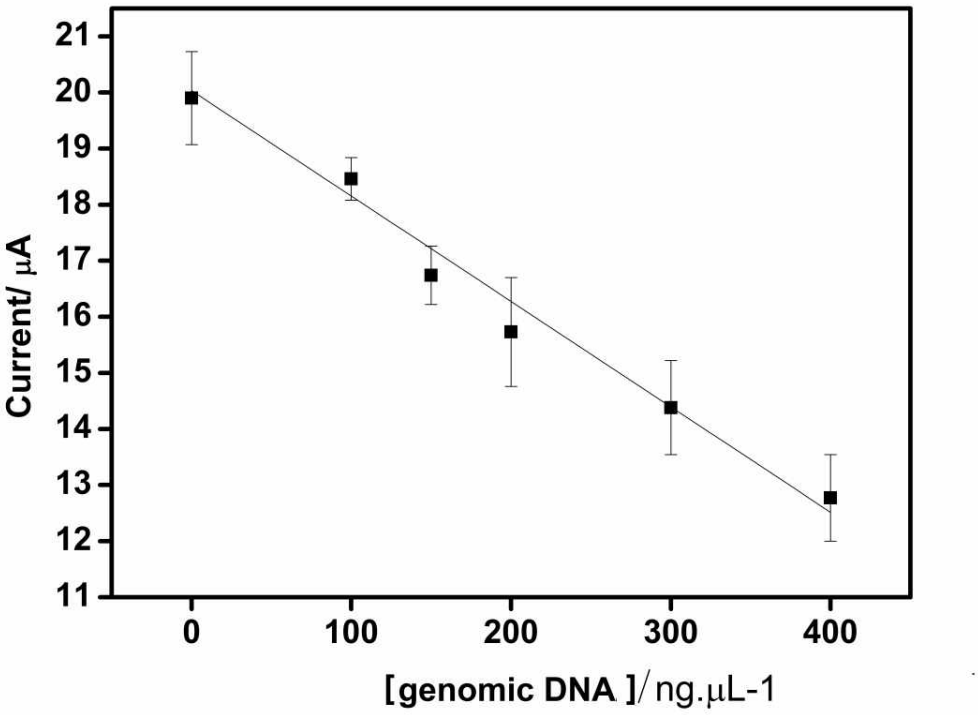


Figure 6. Calibration curve for the oxidation signal of guanine residue obtained from the differential pulse voltammograms onto graphite electrode modified with poly(3- HBA), containing ssDNA (TOXG1) before and after hybridization with different concentrations of *Toxoplasma gondii* RH strain genomic DNA (100 ng. μL^{-1} to 400 ng. μL^{-1}). Electrolyte: phosphate buffer (0.10 mol.L^{-1}), pH 7.4. Modulation amplitude: 25 mV. Pulse interval: 0.2s; 20mVs-1.

3.5. Analysis using AuNPs

Gold nanoparticles are a scaffold for DNA binding. The functionalization of AuNPs with oligonucleotides was used for the electrochemical analysis of the hybridization between ToxG1 probe: *T. gondii* DNA (positive sample) and ToxG1 probe: *N*. caninum DNA (negative sample) (**Fig. 7**).

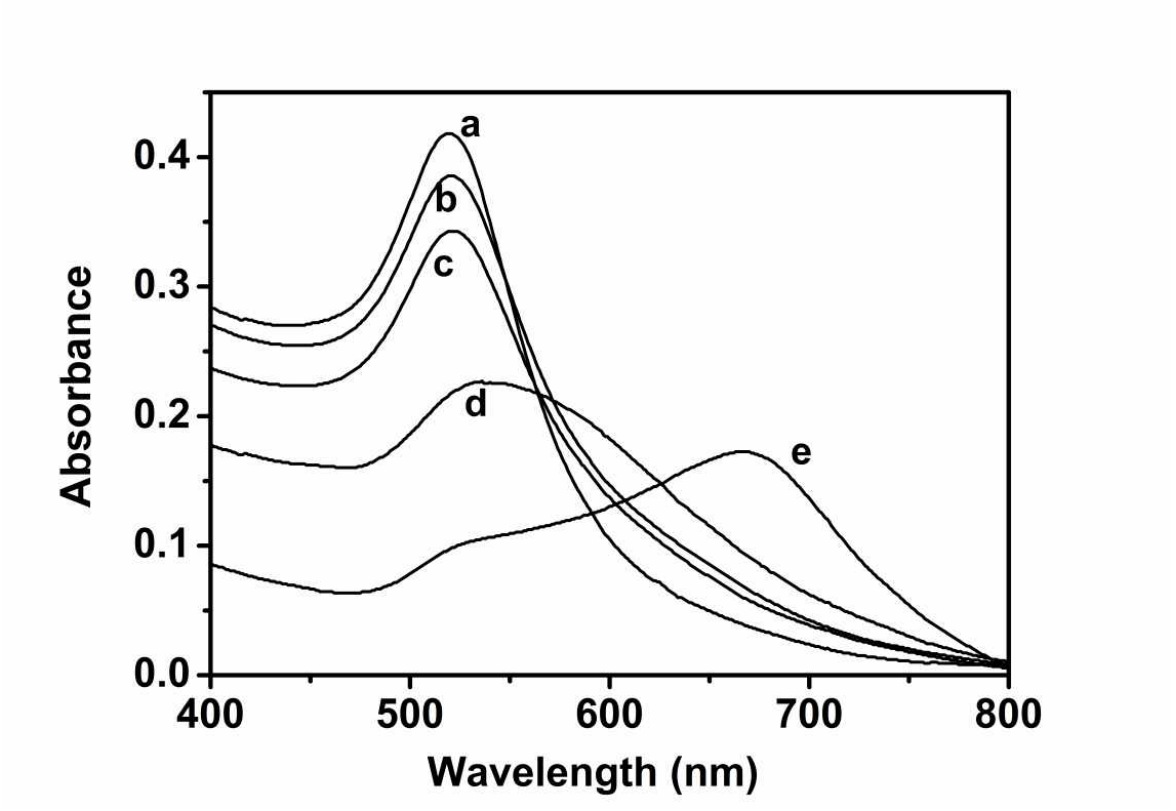


Figure 7. Absorption spectrum in the UV-vis of: AuNPs (a), AuNPs/TOXG1/NaCl (saturated) (b), AuNPs/ToxG1/ *Neospora caninum*/NaCl (saturated) (c) AuNPs/ToxG1/ *Toxoplasma gondii*/ NaCl (satured) (d) and AuNPs/NaCl (saturated) (e).

Figure 7a shows the absorption spectrum of the AuNPs. The peak of maximum absorbance at 520 nm is in accordance with Verissimo and collaborators [47].

When the ToxG1-S probe was incubated with the negative control (*N. caninum* DNA), both the absorbance peak and the displacement are similar to the probe (**Fig. 7b**), but with a lower intensity of ToxG1-S (**Fig. 7c**). However, when the ToxG1-S probe was incubated with the positive control (*T. gondii* DNA) (**Fig. 7d**), a significant change in the absorbance peak was observed, as well as widening and bathochromic shift, assuming a peak absorbance near 540 nm. The spectra in **Figure 7d** (shoulder in 580 nm) and **Figure 7e** (peak in 680 nm) suggest that the particles are close to each other, indicating an agglomeration process. These results indicate that the process of target recognition (genomic DNA) occurs by means of the biosensor, corroborating with the electrochemical results.

4. Conclusions

An approach for the selection of DNA probes related to *T. gondii* was shown in this study. Poly(3-hydroxybenzoic acid) was effective for the immobilization and detection of DNA fragments of *T. gondii*.

The immobilization of the ToxG1 and the hybridization of the oligonucleotide (ToxG2) and genomic DNA showed good responses by direct detection of guanine residue oxidation. A decrease of about 20% in the guanine signal was observed due to the hybridization between the ToxG1 probe in the presence of ToxG2 or *T. gondii* genomic DNA. In the presence of non-specific targets (*N. caninum*, human and *Mus musculus*), the current values observed were similar to the probe.

Hybridization was also confirmed by optical assays using AuNPs. When the ToxG1S probe is incubated with the *T. gondii* genomic DNA, there is a significant change in the absorbance peak, widening and shifting to a longer wavelength in accordance with the electrochemical results.

The results obtained contribute to the further development of the rapid diagnosis of *T. gondii* infection or toxoplasmosis.

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CAPÍTULO 3

PEPTIDE-BASED ELECTROCHEMICAL IMMUNOSENSOR FOR ANTI-*TOXOPLASMA GONDII* IMMUNOGLOBULINS DETECTION

O capítulo 3 está de acordo com as normas da revista International Journal of Biological Macromolecules

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Peptide-based electrochemical immunosensor for anti-*Toxoplasma gondii* immunoglobulins detection

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Abstract

Toxoplasma gondii is the intracellular protozoan parasite responsible for toxoplasmosis. The serologic detection of anti-*T. gondii* immunoglobulins plays a crucial role in the clinical diagnosis of toxoplasmosis. In this work, a novel electrochemical immunosensor for detecting anti-*T. gondii* immunoglobulins in infected mouse serum was developed based on the immobilization of an *in silico* predicted peptide (PepB3), obtained from a membrane protein of *T. gondii*, as recognition biological element, on the surface of graphite electrode modified with poly(3-HBA) and verified by atomic force microscopy (AFM) and electrochemical techniques. The infection and the binding specificity of peptide PepB3 were confirmed by indirect ELISA. The produced biosensor was able to discriminate different periods of infection using infected mouse serum samples by differential pulse voltammetry, presenting limit of detection in diluted samples of 1:100 (v/v). The biosensor showed selectivity, discriminating infected and uninfected mouse and high stability provided about 75% of its initial response after 30 days of storage.

Keywords: *Toxoplasma gondii*; mimetic peptide; poly (3-HBA); biosensor

Resumo

Toxoplasma gondii é o protozoário parasita intracelular responsável pela toxoplasmose. A detecção sorológica de imunoglobulinas anti- *T. gondii* desempenham um papel crucial no diagnóstico clínico da toxoplasmose. Neste trabalho, um novo imunossensor eletroquímico para detecção de imunoglobulina anti- *T. gondii* em soro de camundongo infectado foi desenvolvido com base na imobilização de um peptídeo predito *in silico* (PepB3), obtido de uma proteína de membrana de *T. gondii*, como elemento de reconhecimento biológico, na superfície do eletrodos de grafite modificado com poli (3-HBA) e verificado por microscopia de força atômica (AFM) e técnicas eletroquímicas. A infecção e a especificidade de ligação do peptídeo PepB3 foram confirmadas por ELISA indireto. O biossensor produzido foi capaz de discriminar diferentes períodos de infecção utilizando amostras de soro de camundongo infectado por voltametria de pulso diferencial, apresentando limite de detecção em amostras diluídas de 1: 100 (v/v). O biossensor mostrou seletividade, discriminando camundongos infectados e não infectados e alta estabilidade mantendo cerca de 75% de sua resposta inicial após 30 dias de armazenamento.

Palavras- chave: *Toxoplasma gondii*; peptídeo mimético; poli (3-HBA); biossensor

1. Introduction

Toxoplasmosis is a common parasitic disease which affects animals and humans caused by the intracellular protozoan parasite *Toxoplasma gondii* (*T. gondii*) [1, 2]. The principal means of acquiring the infection is either by ingestion of inadequately cooked meat containing tissue cysts or food or water contaminated by oocysts, and congenital or transplacental [3, 4]. Approximately 30% of the human population worldwide is chronically infected with *T. gondii*. In general, human infection is asymptomatic, but the parasite may induce severe disease in fetuses and immunocompromised patients [5].

The humoral response to *T. gondii* infection results in increased levels of specific circulating immunoglobulins, as IgM, IgG, IgA, and IgE, but each isotype is induced with a specific kinetic profile after primary infection [1] and the serologic detection of anti-*T. gondii* immunoglobulins plays a crucial role in the clinical diagnosis of toxoplasmosis.

The detection of antibodies is an important area in bioanalysis because their presence provides information about several pathologies. A wide range of serological techniques is available for the detection of specific *T. gondii* immunoglobulins, being the main methodologies used the enzyme-linked immunosorbent assays (ELISA), immunosorbent agglutination assay, indirect hemagglutination assays, indirect fluorescent antibody tests, the modified agglutination test, Western blotting and IgG avidity. The Sabin-Feldman dye test, which was recognized as gold standard assay for a long time, it is now no longer used due to its practical difficulties, particularly because it is required live parasites within its components. Even though ELISA has been considered one of the most reliable methods for the detection of specific anti-*Toxoplasma* antibodies in sera, it is time consuming and includes specificity concerns [1, 6-8].

It has been described recently that antibodies can be also detected and quantified using immunosensors [9]. An immunosensor is a type of biosensor in which the antigen binds to the antibody, forming a stable complex, and the antigen or antibody can be immobilized on the surface of different transducers. Electrochemical immunosensors have been explored in several analyses since

they are specific, simple, rapid, portable, and generally disposable and can carry out *in situ* or automated detection [10-12].

As antibody-mediated immune response occurs via the recognition of specific epitopes, reduction of the antigen to minimal sequences of peptides has often been addressed [13, 14]. Peptides have been utilized as biorecognition elements due to their stability against denaturation, simple acquisition, specificity, cost effectiveness, standard synthetic protocol, accessibility, easy modification, chemical versatility, chemical combination and selection in random libraries [15]. Several studies reported the application of peptides in detection of immunoglobulins [16-18]. Biosensors applied to toxoplasmosis diagnosis have been described in the literature [19-26], however, they do not use peptides as biorecognition probe.

In this work, a novel electrochemical immunosensor was constructed using an *in silico* predicted peptide for detecting anti-*T. gondii* immunoglobulins in infected mouse serum.

2. Experimental

2.1. Chemicals

All reagents used were of analytical grade, without previous purification. Solutions were prepared with deionized water (Gehaka, 18 MΩ cm) and bubbled with ultrapure N_2 prior to electrochemical measurements. The monomer employed for the polymerization reaction was 3-hydroxybenzoicacid (3-HBA) 99%, purchased from Sigma–Aldrich (USA). Buffer solutions were saline phosphate buffer (PBS, 0.01 mol L^{-1}, pH 7.4, containing 10 mmol L^{-1} NaCl) and phosphate buffer (0.1 mol L^{-1}, pH 7.4). The detections were performed in 5.00 mmol.L^{-1} $K_4Fe(CN)_6$ containing 0.10 mol.L^{-1} KCl solution. Experiments were conducted at room temperature (25 ± 1°C).

The PepB3 (APTGDPSQNSDGNRG) prediction and selection (data not shown) was performed using resources available online. Briefly described ahead, the sequence of SAG related sequence 52 A (SRS52A), a membrane protein of *T. gondii*, was obtained from ToxoDB (www.toxodb.org/toxo) [27, 28]. The whole protein was analyzed using B cell epitope prediction score resources, available at

Immune Epitope Database and Analysis Resource (IEDB – www.iedb.org) [29], searching for sequences of 15 aminoacids with high B cell prediction score. Prediction of linear epitopes was performed by Bepipred linear prediction method using the minimum score of 1.5 [30]. Several peptides were selected and chemically synthetized as described elsewhere [31] and tested against infected mice serum and the PepB3 had the best results. PepB3 isoelectric point (pI) was predicted by PepCalc.com tool [32], its three-dimensional structure was predicted by I-Tasser suite [33]. For electrochemical assays PepB3 was diluted in PBS buffer (10 μg mL^{-1}). Blocking solution was prepared with glycine (10 mM, Merck, 99%) in phosphate buffer.

2.2. Apparatus

The electrochemical measurements were performed with a CHI 760C potentiostat (CH Instruments, USA). A graphite disk (6.15 mm diameter, purity 99.9995 %) from Alfa Aesar was used as working electrode. A platinum plate and silver/silver chloride (3.0 mol L^{-1}) were used as auxiliary and reference electrodes, respectively. Surface morphology in the absence or presence of biomolecules was assessed by atomic force microscopy (AFM 5100N, Hitachi), using silicon cantilevers with constant force 3.1–37.6 N m^{-1} and resonant frequency of 140–390 kHz, measurements were performed in intermittent mode.

2.3. STAg production

Soluble *Toxoplasma gondii* antigen (STAg) was produced using tachyzoites obtained *in vitro* [34]. Tachyzoites of RH strain were maintained by serial passages in HeLa cell line (CCL-2, ATCC, Manassas, VA, USA), which were cultured using RPMI 1640 medium supplemented with glutamine (2 mmol L^{-1}), penicillin (100U/mL) and streptomycin (100 μg/mL), at 37ºC in 5% CO_2 atmosphere. Briefly, the parasite suspensions were submitted to freeze-thawing and sonication cycles in the presence of protease inhibitors, centrifuged, the supernatant collected and the protein content was determined by Bradford method [35].

2.4. Infected mouse serum

Female *Swiss* mice were infected with 10 cysts of ME49 strain, intraperitoneally, and blood samples were collected on the day of infection and after 15, 30, 45, 60, 75 and 100 days, and centrifuged at 67 × g for 10 min at room temperature. Animals were maintained under standard conditions in the Bioterism Center and Animal Experimentation, Federal University of Uberlândia, Brazil. All procedures were conducted according to institutional guidelines for animal ethics and the study received approval of the Ethics Committee for Animal Experimentation of the Institution (CEUA-UFU) under the protocol 109/2016.

The infection and the binding specificity of peptide PepB3 were confirmed by indirect ELISA protocols described elsewhere [34]. Briefly, high-binding microtiter plates (Corning Laboratories 3590, New York, USA) were coated with a solution of STAg or PepB3 in carbonate buffer (10 µg/mL, pH 9.6), 50 µL/well, followed by overnight incubation at 4ºC. Next, the plates were washed with 200 µL/well of PBS-Tween 0.05% (PBST) three times. The remaining binding sites in wells were blocked with 100 µL of PBST supplemented with 5% skim fat milk (PBSTD) for or with a solution of PBST plus 0,5% BSA for 1 hour at room temperature. The plate was washed and mice serum samples were diluted in PBSTD or PBS+BSA in the dilution of 1:50, and each well received 50 µL of sera. After the plates had been incubated for 1 hour at 37ºC, they were washed and dilutions of antibody against mouse Fc-specific IgG labeled with peroxidase (Sigma–Aldrich, USA)) were applied to the wells (50 µL/well of 1:2000 conjugated antibody), followed by 1 hour of incubation at 37ºC. The plate was washed and the reaction revealed using 50 µL/well of commercial developing solution ABTS (0.03% H_2O_2 and 0.01 M of chromogen 2,2'–azin-biz-3-ethil-benzothiazoline sulfonic acid; Sigma–Aldrich, USA). The optical density (OD) was determined at 405 nm in a plate reader (Tecan Spectra Classic, Grödig, Austria).

2.5. Preparation of graphite electrode modified with poly (3-HBA)

The bare graphite electrodes were polished mechanically with alumina slurry (0.3 µm), sonicated, washed with deionized water and dried in air. The preconditioning was performed in 0.5 mol L^{-1} $HClO_4$ solution through cyclic

voltammetry between +0.0 and+1.2 V, 4 cycles, 50 mV s^{-1}. The electrodes were modified with polymer derived from 3-hydroxybenzoic acid according to Ferreira and collaborators [36], with modifications. The electropolymerization was conducted on graphite electrodes (10 scans, 0.0 and +1.2 V vs. Ag/AgCl, 50 mV s^{-1}) from a 3-HBA solution (2.5 mmol L^{-1}) prepared in $HClO_4$ (0.5 mol L^{-1}).

2.6. Preparation of the peptide-modified electrode

After the production of the graphite electrode modified with poly (3-HBA), the sensitization with PepB3 probe was carried out. The solution containing 10 µg mL^{-1} of peptide probe was dropped onto the electrode surface at 37 °C and incubated for 30 min. The blocking of non-specific binding was performed with glycine (10 mM) at 37ºC for 1 h. After immobilization of the peptide probes by physical adsorption and surface blocking, 10 µL of infected mouse serum (0, 15, 30 and 60 days after *T. gondii* infection) diluted (1:25) in phosphate buffer (0.1 mol L^{-1}, pH 7.4) were added and incubated for 30 min. After each modification, the electrodes were washed three times with 50 µL of phosphate buffer. Differential pulse voltammetry measurements were performed using potassium ferrocyanide solution as electrolyte. The biosensor performance was evaluated with varying dilutions of infected mouse serum (30 days of infection) in phosphate buffer (1:100, 1:75, 1:50, 1:25, 1:10). Finally, the biosensor performance was also evaluated after storage at 10 °C for 0, 5, 10, 20 and 30 days.

2.7. Statistics and data presentation

All experiments were performed in triplicate and numeric results are presented as arithmetic means ± standard deviation. For each measurement, a new electrode was used. Voltammograms were plotted with Origin 8 and peaks were corrected and analyzed with Peak Analyzer tool in program. Graphs and simple linear regression analysis were performed with GraphPad Prism 6. The numerical data (days of infection and stability) were analysed statically and compared using the one-way-ANOVA followed by Dunnett's test, using freeware R [37]. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. PepB3 probe immobilization

Conducting polymers have been widely used in the areas of bioanalytical science, due to various advantages, as increase of stability, speed, sensitivity and facilitate the immobilization of biomolecules due the presence of functional groups [38-41]. Previous works shows that poly (3-HBA) is an efficient matrix for the oligonucleotide [25, 36] and peptide [18] immobilization.

Graphite electrodes were modified with polymeric film derived from 3-HBA and the PepB3 peptide immobilization was evaluated through differential pulse voltammetry and atomic force microscopy (**Fig. 1**).

The interactions that occur on the electrode surface can be observed by alterations in the electron transfer of a known redox system. The peptide immobilization was monitored using the external redox molecule $[Fe(CN)_6]^{4-}$. **Fig. 1A** shows the differential pulse voltammograms of $[Fe(CN)_6]^{4-}$oxidation in graphite electrodes modified with poly (3-HBA) in the absence and presence of the pepB3. We observed oxidation peaks at E_p = +0.27 V, with Δi_p = 224.77±5.80 µA for graphite electrodes modified with poly (3-HBA) and E_p = +0.21 V, with Δi_p = 148.13±0.81 µA after the immobilization of pepB3. The oxidation peak was detected at the same potential reported in other works for graphite electrodes modified with poly (3-HBA) [18, 36].

A decrease of about 1.5 times **(Fig. 1B)** was observed in the current values in presence of the peptide, indicating that the biomolecule was immobilized, possibly due to interactions between the immobilized negatively-charged peptide (predicted isoelectric point of 4.12) and the anionic redox molecule in solution making it difficult and reducing the electron transfer of the redox couple to the electrode.

AFM images for the graphite electrodes modified with poly (3-HBA) **(Fig. 1C)** and modified with PepB3 **(Fig. 1D)** present roughness values of 35.56 ± 0.88 and 154.8 ± 3.67 nm, respectively. After immobilization of the peptide there was an increase in the roughness values, indicate that the surface was modified with the probe and corroborating with the analysis by differential pulse voltammetry.

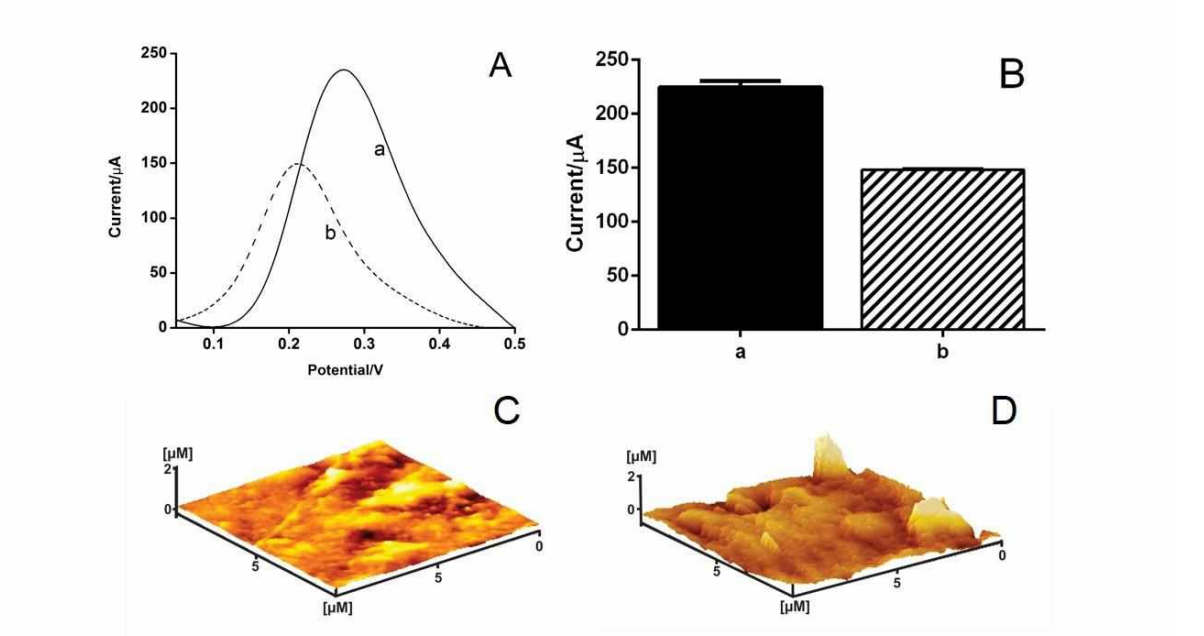

Figure 1. Differential pulse voltammograms (A) and histogram (B) obtained from current peak response of $[Fe(CN)_6]^{4-}$oxidation in graphite electrodes modified with poly (3-HBA) before (a) and after (b) immobilization of pepB3. Electrolyte: potassium ferrocyanide solution. Modulation amplitude: 25 mV. Pulse interval: 0.2 s; Scan rate: 20 mV s^{-1}. AFM topographical images of graphite/poly (3-HBA) (C); graphite/poly (3-HBA)/PepB3 (D).

3.2. Infected mouse serum tested by ELISA

The samples of infected animals were tested by ELISA against STAg and PepB3. The samples tested against pepB3 showed crescent absorbance values until the 30th day of the infection, and against STAg until the 45th day of infection and after remaining constant **(Fig. 2).** Both groups showed low rates of false positive and false negative (<5%; data not shown).

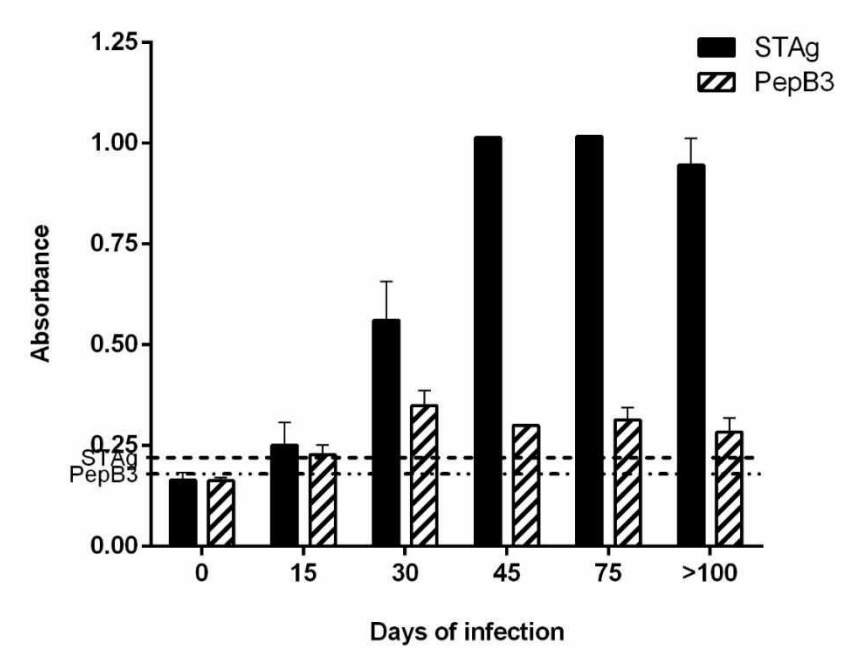

Figure 2. Indirect ELISA results of experimentally infected samples. The dotted lines represent the cut-off value of STAg and PepB3 assays.

3.3. Detection of specific immunoglobulins in infected mouse serum

Mouse serum samples after *T. gondii* infection were applied to the biosensor to evaluate its selectivity. Detection was carried in potassium ferrocyanide solution by differential pulse voltammetry as shown in **Fig. 3.**

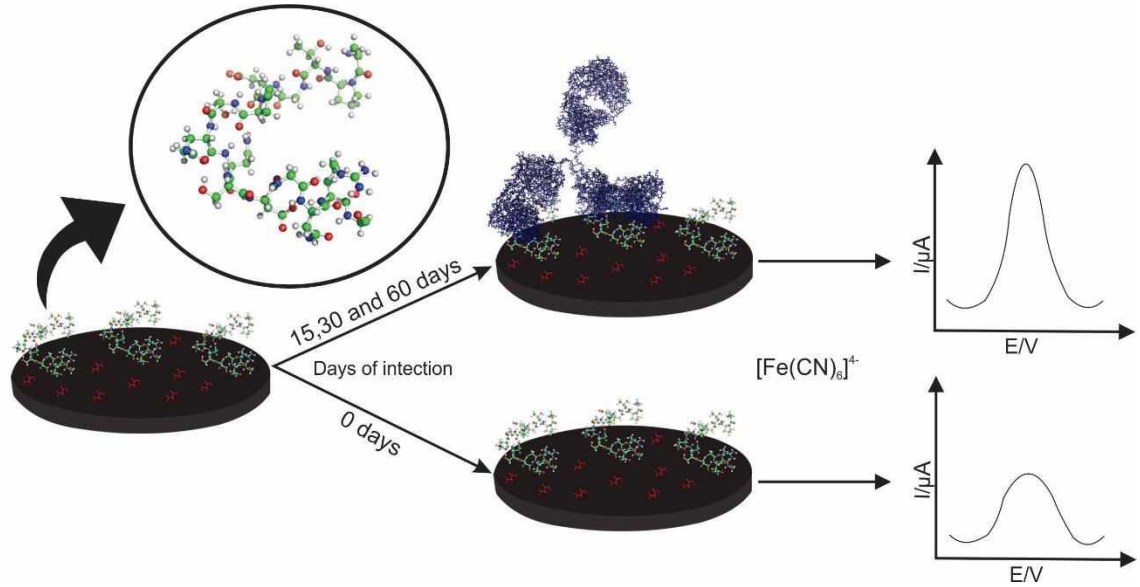

Figure 3. Schematic representation of the immunosensor. Highlighted: predicted structure of PepB3 peptide (APTGDPSQNSDGNRG); Glycine (red) and immunoglobulin (blue).

As showed in **Fig. 4** an increase in current intensity for 15, 30 and 60 days was observed when compared to 0 day (negative control), that increase suggests an interaction between the PepB3 peptide and anti- *T. gondii* immunoglobulins present in the sample.

For the positive sample, the increase of the current values observed after the interaction of the peptide with antibodies occurred, possibly, due to alteration in the three-dimensional structure of the immunoglobulin, causing an exposure of some basic amino acid residues (lysine, arginine and histidine), favoring the electronic transfer for the redox pair. Similar effect it was observed in the literature [42, 43].

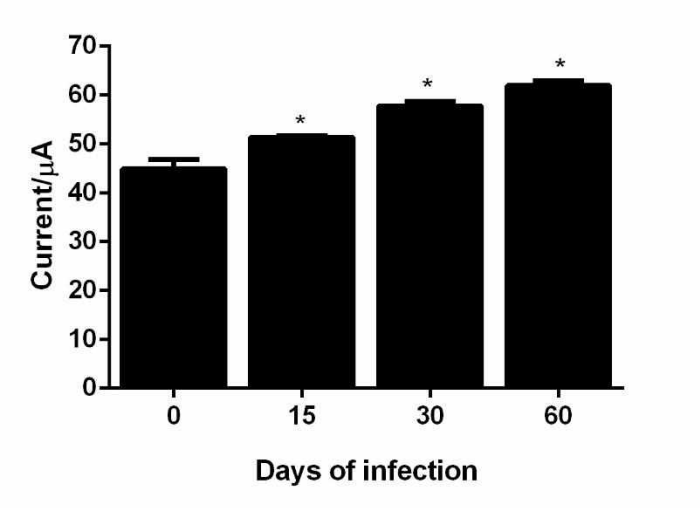

Figure 4. Histogram obtained from current peak response of $[Fe(CN)_6]^{4-}$ oxidation in graphite electrodes modified with pepB3 and after 30 min of incubation with mouse serum samples from 0 to 60 days after *T. gondii* infection. Electrolyte: potassium ferrocyanide solution. Modulation amplitude: 25 mV. Pulse interval: 0.2 s; Scan rate: 20 mV s^{-1}. (*) Significant differences between the negative control (0 day) and 15, 30 and 60 days ($P < 0.05$; ANOVA).

3.4. Calibration curve and stability of the biossensor

The biosensor response to different dilutions of mouse serum from 30 days after *T. gondii* infection (1:10, 1:25, 1:50 and 1:100) was evaluated and an increase in current peak response with the decrease in dilution was observed. From these data a linear relation of the current values in function of the inverse of the dilution factor given by I/μA = 335.3 (1/Dilution factor) + 43.23, r = 0.9967) was found **(Fig. 5).** It was possible to detect up to 1: 100 dilution of mouse serum sample.

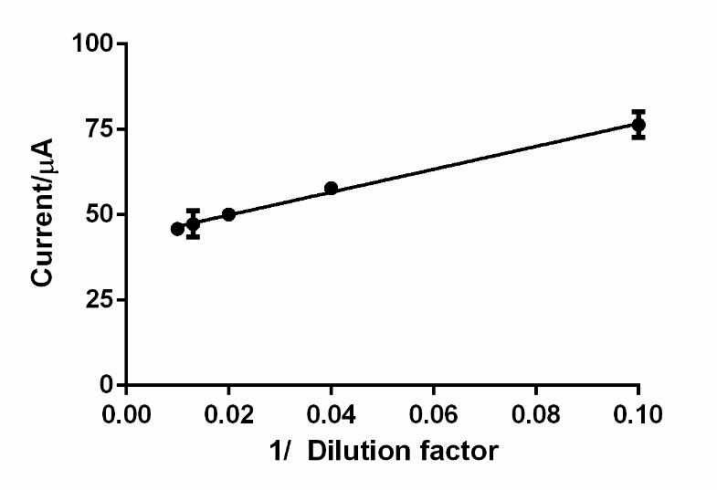

Figure 5. Relation between current and the inverse of dilution factor (1:10, 1:25, 1:50 and 1:100) of infected mouse serum (30 days of infection). Electrolyte: potassium ferrocyanide solution. Modulation amplitude: 25 mV. Pulse interval: 0.2 s; Scan rate: 20 mV s^{-1}.

The response after 30 days of storage indicates that the biosensor maintained about 75% of its initial response under the conditions tested (dry, at 10 °C) **(Fig. 6).** The use of peptides as a recognition probe contributes to the stability of the immunosensor, since peptides with short chains of amino acids generally have better chemical and conformational stability than proteins [44] peptide-based biosensors exhibit excellent properties, and offer opportunity for the production in scale large [15]. In addition, the use of platforms based on polymer films contribute to increase the stability of biosensors [39].

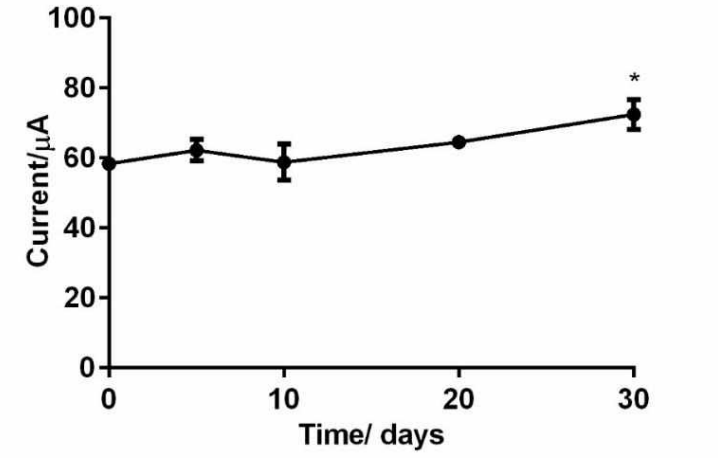

Figure 6. Storage stability profile of immunosensor at 10°C during 30 days. Electrolyte: potassium ferrocyanide solution. Modulation amplitude: 25 mV. Pulse interval: 0.2 s; Scan rate: 20 mV s^{-1}. (*) Significant differences compared to the 0 day of storage ($P < 0.05$; ANOVA).

4. Conclusion

A novel electrochemical immunosensor using an *in silico* predicted peptide, as recognition biological element was proposed, where poly (3-hydroxybenzoic acid) showed effective for the immobilization of PepB3 probe. The immunosensor developed enabled the discrimination of infected and uninfected mouse using the ferrocyanide as indicator. The biossensor for anti-*T. gondii* immunoglobulins was reproducible, specific and detected until 1:100 (v/v). The obtained results contribute to the further development of the diagnosis for point of care of *T. gondii* infection and toxoplasmosis.

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ANEXOS

ANEXO 1

Ministério da Educação e do Desporto
Universidade Federal de Uberlândia
Instituto de Biotecnologia
Programa de Pós-Graduação em Genética e Bioquímica

NORMAS PARA CONFECÇÃO DA VERSÃO FINAL DO MESTRADO/DOUTORADO

(Disponível em: http://www.ppggb.ingeb.ufu.br/)

I.Capa
deve constar:

- Universidade Federal de Uberlândia
- Instituto de Biotecnologia
- Pós-Graduação em Genética e Bioquímica
- Título da Tese
- Nome do aluno
- Uberlândia-MG
- Ano da Defesa

II. Papel: Tamanho A4

Margens

Superior 3,0cm
Inferior 2,5cm

Esquerda 3,0cm

Direita 2,5cm ou 24 pt

Fonte: Arial 12, espaço 1,5

II.1. Numeração

Os números das páginas devem aparecer no canto inferior direito da página e seguem a seguinte organização:

1. Na capa não aparece número, porém ela é contada na numeração que irá aparecer a partir da contra capa;
2. Da contra capa até o índice/sumário, a numeração é romana minúscula (ii, iii, iv ...) (não esqueça de numerar a partir do número ii pois o número i refere-se a capa, conforme descrito no item 1 acima);
3. A partir da APRESENTAÇÃO, a numeração é arábica (1, 2, 3, 4 ...)

III. Ordenação do Conteúdo

O Mestrado e o Doutorado serão escritos sob a forma de Capítulo(s). Tanto o boneco quanto a versão final deverão ter a seguinte forma:

- **1 Capa** (Anexo I)
- **1 Contra capa identificando:**
  Curso, Título, Aluno, Orientador,Informes sobre a titulação, cidade e ano. (Anexo II)

- **Ficha Catalográfica:**
  Deve ser colocada nas costas da CONTRA CAPA (Anexo III). A ficha catalográfica será elaborada pela Biblioteca do Stª. Mônica.

- **Palavras-chave:**
  Devem ser colocadas abaixo da Ficha Catalográfica na CONTRA CAPA (Anexo III)

- **Folha de Rosto com:**
  Curso, Título, Aluno, Comissão Examinadora, Cidade e Data (Anexo IV)

- **Dedicatória**
- **Agradecimentos**
- **Índice/Sumário;**
- **Apresentação**
  1. deverá ser uma síntese do tema pesquisado, situando-o em um contexto geral sobre o conhecimento atual do assunto.
  2. Não deverá trazer citações bibliográficas.
  3. Será finalizada com a apresentação do(s) capítulo(s) com enfoque no(s) objetivo(s) do(s) capítulo(s).
- **Capítulo I Fundamentação Teórica**
  Este capítulo é composto pela fundamentação teórica e suas referências bibliográficas

- **Capítulo(s)**:
  - ✓ Título
  - ✓ resumo (com palavras chaves) e abstract (com key words) referentes ao capítulo
  - ✓ Se o Capítulo foi publicado, pode ser apresentado tanto na forma de separata quanto na forma do texto original do editor de texto, se foi ou está para ser submetido, constar a Revista.
  - ✓ Cada Capítulo (01 ou mais) deve ser escrito em conformidade com uma Revista de Divulgação Científica escolhida pelo Aluno e seu Orientador.
  - ✓ O Capítulo deverá ser escrito em Português **ou** Inglês.
- **Opcional**

  1. Anexar, ao final da Dissertação/Tese, os Protocolos utilizados em Metodologia para desenvolvimento da Pesquisa

  2. Fazer um Capítulo com RESULTADOS COMPLEMENTARES - para resultados que ainda serão completados para fins de publicação, mas que são relevantes e merecem uma Short Communication (nota prévia)

ANEXO 2

Journal of Pharmaceutical and Biomedical Analysis 145 (2017) 838–844

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Development of direct assays for *Toxoplasma gondii* and its use in genomic DNA sample

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ABSTRACT

This work describes an approach for the selection and detection of specific DNA probes related to *Toxoplasma gondii*, a protozoan parasite responsible for toxoplasmosis. The detection system was developed on graphite carbon electrode modified with poly(3-hydroxybenzoic acid) sensitized with ToxG1 probe. The hybridization of the specific genomic DNA related to *T. gondii* showed good response by direct detection of guanine residue oxidation using differential pulse voltammetry (DPV). The biosensor was able to distinguish both the complementary and non-complementary targets and detect up to 100 ng μL^{-1} of the *T. gondii* genomic DNA. The hybridization (ToxG1: *T. gondii* genomic DNA) was confirmed by optical measurement. Optical assays using gold nanoparticles:ToxG1 probe showed a significant change in the absorbance peak in the presence of the *T. gondii* genomic DNA according to the electrochemical results. This novel biosensor shows potential as electrochemical transducer and was successfully applied in the biological sample.

1. Introduction

Toxoplasma gondii is the intracellular protozoan parasite responsible for toxoplasmosis, a zoonosis with medical and veterinary importance worldwide [1]. Most infections are asymptomatic in healthy adults, but severe disease may develop with congenital infections or immunodepression [2]. Estimates show that more than a third of the world's population has been infected with this parasite [3]. Diagnosis and genetic characterization of *T. gondii* infection are crucial for monitoring, prevention and control of toxoplasmosis. Traditional approaches for toxoplasmosis detection rely on immunological diagnosis and imaging techniques [4]. These methods have limitations; as for instance, the identification based on imaging techniques is less sensitive and uncertain. In addition, these methods demand a long time for sample preparation, expensive equipment and the need for skilled personnel.

There is a significant need for improved diagnosis. Biosensors stand out as a promising alternative to the traditional methodologies. Biosensors are analytical devices that combine the specificity of biomolecules with sensible transduction platforms, presenting portability, rapidity and good cost-effectiveness relation. In the literature, sensors developed for the detection of *T. gondii* are generally based on the immobilization of antibodies [5–7], aptamers [8] and DNA probes [9,10]. The detection of specific DNA sequences plays a major role in many fields, especially in clinical diagnostics. Direct monitoring of changes in its electrochemical properties, such as oxidation of guanine and adenine residues, enables the detection of hybridization on the electrode surface [11].

The combination of polymeric materials with biosensors promotes an increase in the analytical performance of the biosensor. Polymers derived from hydroxybenzoic acid are used in the development of polymeric films for the modification of electrodes due to the presence of functional groups (carboxylic acid and hydroxyl acid), which are liable to undergo electropolymerization and to interact with biomolecules [12].

Nucleic acid components, such as nucleosides and nucleotides, are electroactive species that can be monitored. The electrochemical behavior of DNA and the effects of its adsorption onto several types of electrodes have been widely investigated [13–15]. Oliveira-Brett and collaborators [11] investigated the voltammet-

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0731-7085/

ric oxidation of all nucleotides of deoxyribonucleic acid on glassy carbon electrodes. Silva et al. [16] successfully described the immobilization of purine and pyrimidine bases on graphite electrodes modified with poly(4-methoxyphenethylamine).

The focus of the current work was the development of a method for the selection of specific DNA probes related to *T. gondii* and a system for the detection of a specific target sequence for the diagnosis of toxoplasmosis based on graphite electrodes modified with poly(3-hydroxybenzoic acid) and sensitized with DNA-oligonucleotide probe (Fig. 1). In this work, it was possible to detect double-strand DNA related to *T. gondii* onto the modified surface. To the best of our knowledge, this is the first genosensor that uses specific genomic DNA as a target for the diagnosis of *T. gondii* infection or toxoplasmosis.

2. Experimental

2.1. Chemicals

All reagents used were of analytical grade without previous purification. Ultra high purity water (Master System, Gehaka, Brazil) was used for the preparation of all solutions. The monomer employed for the polymerization reaction was 3-hydroxybenzoic acid (3-HBA) 99%, purchased from Sigma–Aldrich (USA). The detections were performed in 0.1 mol L^{-1} phosphate buffer (Na_2HPO_4 0.061 mol L^{-1}, NaH_2PO_4 0.039 mol L^{-1}, pH = 7.4). All solutions were deoxygenated by ultra-pure nitrogen bubbling for at least 45 min before use. Experiments were conducted at room temperature (25 ± 1 °C).

Oligonucleotides were synthesized by Invitrogen Life Technologies and their sequences are: ToxG1 (probe): 5′-CTGGCAACGCTGGGAATGAA-3′, ToxG15 (probe) 5′-CTGGCAACGGTGGGAATGAA-SH-3′, ToxG2 (target): 5′-TTCATTCCCACCGTTCCCAG-3′, Non-complementary target: 5′-CCACTCTTCAATTCTCTCCGCC-3′.

The oligonucleotide stock solutions and dilutions were prepared in SSC buffer (pH 7.0, sodium chloride 0.3 mol L^{-1} and sodium citrate 0.03 mol L^{-1}, Sigma–Aldrich, USA) and stored at −12 °C.

2.2. Apparatus

The electrochemical studies were performed in a potentiostat from CH Instruments, model 620C, USA. A graphite disk (6.15 mm diameter, 99.9995% purity) from Alfa Aesar was used as working electrode. A platinum plate and silver/silver chloride (3.0 mol L^{-1}) were used as auxiliary and reference electrodes, respectively. Film morphology in the absence or presence of biomolecules was assessed by atomic force microscopy (SPM 9600, Shimadzu). The assays using Gold nanoparticles (Au NPs) were carried out using a spectrophotometer (UV-1650PC, Shimadzu).

2.3. In silico probe selection

Nucleotide sequences of GRA6 gene (GenBank: L33814.1), B1 gene (GenBank: AF179871.1) and a repetitive region (GenBank: AF146527.1) from *T. gondii* genome were obtained from the National Center for Biotechnology Information (NCBI) database. DNA probes were selected *in silico* using a pipeline that integrated Primer3 software [17] with algorithms for multi-fast handling and parameter setting, as well as scoring, sorting and formatting of the results.

The developed pipeline took fasta files as input, along with parameters specified in the main script, such as probe size, GC content, scoring scheme, DNA and salt concentrations, constraints of melting temperature for desirable probes and undesirable secondary structures. Afterwards, Primer3 parameter setting and execution were sequentially conducted. The results obtained were scored with a user-defined scheme that accounted for occurrence of pairs, triplets and quarters of specific base residues, since some of them are preferred for electrochemical direct monitoring in modified carbon electrodes (G and A) [11]. Next, the results were ordered according to the score and formatted for the generation of detailed output files. Finally, the probes obtained were submitted to BLAST alignment with the nucleotide database in order to select the most species-specific probes.

2.4. Parasite preparation and genomic DNA extraction

Strains of *Neospora caninum* (NcLivΔHPT) and *T. gondii* tachyzoites (RH) were maintained by serial passages in HeLa cell line (CCL-2, ATCC, Manassas, VA, USA), cultured in RPMI 1640 medium supplemented with 2 mmol L^{-1} glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin, at 37 °C in 5% CO_2 atmosphere. Parasite suspensions were obtained as previously described [18]. Briefly, tachyzoites were harvested by scraping off the cell monolayer after 2–3 days of infection, passed through a 26-gauge needle and centrifuged at low speed (45 × *g*) for 1 min, for debris removal. The supernatant containing parasite suspension was collected, washed in phosphate buffered saline (PBS, pH 7.2) and centrifuged at 1000 × *g* for 10 min. After the centrifugation, the pellet was kept at − 20 °C for DNA extraction.

The genomic DNA was extracted from NcLivΔHPT *N. caninum* and RH *T. gondii* tachyzoites. The parasites were lysed and digested with 200 μL lysis buffer (400 mmol L^{-1} NaCl, 10 mmol L^{-1} Tris-HCl, 2 mmol L^{-1} and EDTA, pH 8.2), 6 μL 10% SDS, 3 μL proteinase K (20 mg mL^{-1}), and incubated at 55 °C overnight. After incubation, NaCl (150 μL, 6 mmol L^{-1}) was added into the solution. The mixture was centrifuged at 12,000 × *g* at 4 °C for 10 min. The supernatant was transferred to the 1.5-mL tube, ethanol (800 μL, 100%) was added and centrifuged (12,000 × *g* for 10 min). The precipitated DNA was washed with ethanol (1 mL, 75%) and solubilized in 20 μL of Milli-Q water.

The human and mouse genomic DNA was extracted from peripheral blood according to Al-Shuhaib [19].

The concentration and quality of DNA extracted from each sample were analyzed using a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA). After that, the DNA samples were stored at −20 °C until use.

2.5. Preparation of graphite electrode modified with poly (3-HBA)

The bare graphite electrodes were polished mechanically with alumina slurry (0.3 μm), sonicated, washed with deionized water and dried in air. The preconditioning was performed in 0.5 mol L^{-1} $HClO_4$ solution through cyclic voltammetry between +0.0 and +1.2 V, 4 cycles, 50 mV s^{-1}. The electrodes were modified with polymer derived from 3-hydroxybenzoic acid according to Ferreira and collaborators [12] with modifications. The electropolymerization was conducted on graphite electrodes (10 scans, 0.0 and +1.2 V vs. Ag/AgCl, 50 mV s^{-1}) from a 3-HBA solution (2.5 mmol L^{-1}) prepared in $HClO_4$ (0.5 mol L^{-1}) at room temperature (25 ± 1 °C).

2.6. Effect of probe concentration in the immobilization and hybridization

Optimization studies of the probe concentration were performed by adding 10 μL of different concentrations of ToxG1 (5 μmol L^{-1}, 15 μmol L^{-1}, 20 μmol L^{-1}, 25 μ mol L^{-1}, 35 μmol L^{-1} and 50 μmol L^{-1}) in the modified electrode surface at 37 °C and incubated for 30 min. The blocking of non-specific binding was performed with BSA 1% (w/v) at 37 °C for 1 h. After immobilization of the oligonucleotide probes by physical adsorption, 10 μL of dif-

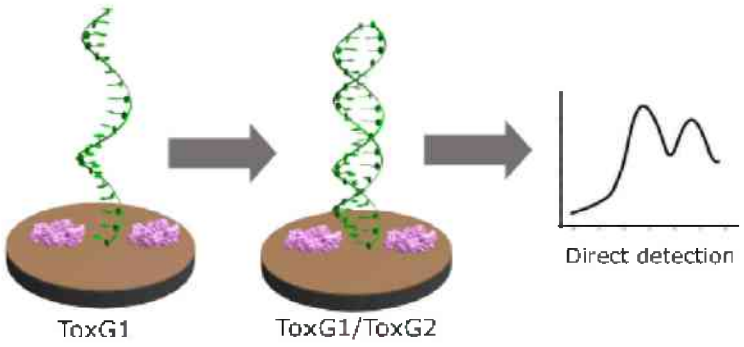

Fig. 1. Schematic representation for the immobilization of the ToxG1 probe on modified electrode and its hybridization.

ferent concentrations of ToxG2 target (10 μmol L^{-1}, 30 μmol L^{-1}, 40 μmol L^{-1}, 50 μmol L^{-1}, 70 μmol L^{-1} and 100 μmol L^{-1}) were added onto the modified electrode surfaces.

For the tests using the genomic DNA, the samples were maintained at 98 °C for 5 min, in order to de-hybridize the strands; they were then added onto the modified electrodes surfaces and incubated for 15 min at 52 °C.

After each modification, the electrodes were washed three times with 50 μL of phosphate buffer. Differential pulse voltammetry measurements were performed using phosphate buffer as electrolyte.

2.7. Assay using AuNPs

The AuNPs were produced using the modified Turkevich-Frens method. In this method, gold salt is reduced, occurring the nucleation and growth of gold nanoparticles of 15–20 nm, stabilized by citrate ions [20]. After synthesis, the AuNPs were stored at 4 °C until the use. The AuNPs sensibilizated with DNA probe were preparated using 200 μL of AuNPs solution added to probe solution, ToxG15 (2 uL, 200 μmol L^{-1}) and maintained at room temperature for 1 h. Prior to the hybridization process, the genetic material was digested using the EcoRI restriction enzyme (2 U/L) diluted in the specific buffer (50 mmol L^{-1} NaCl, 10 mmol L^{-1} Tris-HCl, 10 mmol L^{-1} $MgCl_2$, 1 mmol L^{-1} DTT), pH 7.6. After that, genomic DNA from *N. caninum* (negative sample, 2 uL, 200 ng μL^{-1}) or *T. gondii* (positive sample, 2 uL, 200 ng μL^{-1}) were added and maintained at 52 °C for the duplex formation during 30 min.

3. Results and discussion

3.1. Isolation of DNA probe

The repetitive region described by Homan et al. [21] is a fragment of 529 bp, which is repeated 200–300 times in the genome of *T. gondii*. This fragment has been widely investigated as a molecular target for the diagnosis of toxoplasmosis [22–24].

The B1 gene is repeated 35 times in the parasite genome and has been commonly used for molecular diagnosis with acceptable sensitivity [24–27].

The dense granule antigens (GRA) are parasitic molecules secreted to the parasitophorous vacuole, being immunogenic and responsible for the parasite intracellular survival. GRA6 (dense granule antigens 6) protein is coded by GRA6 gene (a highly polymorphic single-copy gene) and used for genotyping [28–31]. Several studies have showed the use of recombinant antigenic proteins as GRA for serological diagnosis of toxoplasmosis [32–34].

In the probe selection process, Primer3 considered 5397 possible probes for GRA6 gene, excluding 3392 for undesirable secondary structure or long base repeats. The remaining 2005 probes were sorted and the top scoring was submitted to BLAST alignment. For the repetitive region, from the 1370 probes considered, 1025 were excluded, resulting in 345 probes. For B1 gene, 6429 probes were considered and 3025 were excluded, resulting in 3404 for scoring, sorting and alignment. Finally, the best result for each gene/genomic region was chosen. Table 1 shows the sequences and highlights some of their features.

Among the probes selected, ToxG1 was chosen for immobilization on the modified graphite electrodes due to the higher percentage of guanine (G)-cytosine(C) content in relation to ToxR1 and ToxB1. A probe of higher GC content creates a strong bonder with complementary DNA.

Most tests used for the diagnosis of toxoplasmosis are based on the detection of different antibody classes or antigens. Other DNA-based technologies also use and rely on amplification procedures, based on Polymerase Chain Reaction (PCR) prior to detection. In this method, several multicopy and single-copy genes are used as targets [4]. Costa and collaborators [35] developed a nested-PCR assay using the GRA7 gene as target for prime design, which also encodes a dense granule protein. The test was sensitive and specific compared to other assays, as the methodology presented in this work.

3.2. Immobilization of the DNA probe

Conductive polymers have been widely used in the development of biosensors, due to various advantages, such as the presence of functional groups that facilitate the immobilization of biomolecules [36,37]. A previous work carried out by our group shows that poly (3-HBA) is an efficient matrix for the oligonucleotide immobilization [12].

Graphite electrodes were modified with polymeric film derived from 3-HBA. A current peak was observed at +1 V, what is attributed to the monomer oxidation, which decreased after the potential scans. After the first cycle, a gradual increase in the current between +0.3 V and +0.9 V can be observed due to the coverage of the electrode with an electroactive material. This modification was confirmed by cyclic voltammetry, in which there is an increase in the current response after the electrode modification with poly (3-HBA).

After the production of the graphite electrode modified with poly(3-HBA), the sensitization with ToxG1 probe was carried out.

Surface density of probe is an important factor that determines the extent to which the immobilized probes are able to capture

Table 1
Selected probes and their features.

	Primer3 features						Blast best hit features		
ID	Genomic target	LEN	GC%	TM	SELF-ANY	SELF-END	HAIRPIN	Query cover	Identity	E-value
ToxG1	GRA6 gene (L33814.1)	21	57.14	59.3	0	0	0	100%	100%	0.051
ToxR1	Repetitive region I (AF146527.1)	22	54.55	58.83	0	0	0	100%	100%	0.019
ToxB1	B1 gene (AF179871.1)	21	42.62	56.62	0.81	0	0	100%	100%	0.051

* LEN: oligonucleotide length, GC%: GC content, TM: melting temperature, self-any: tendency of any self-complementarity, self-end: tendency of ends self-complementarity, hairpin: tendency of hairpin formation. ToxG1 oligonucleotide sequence: 5'-CTGGGAACGGTGGGAATGAAG-3', ToxR1 oligonucleotide sequence: 5'-GGCGGAGAGAATTGAAGAGTGG-3', ToxB1 oligonucleotide sequence: 5'-AAATGCCAGAAGAAGGGTACG-3'.

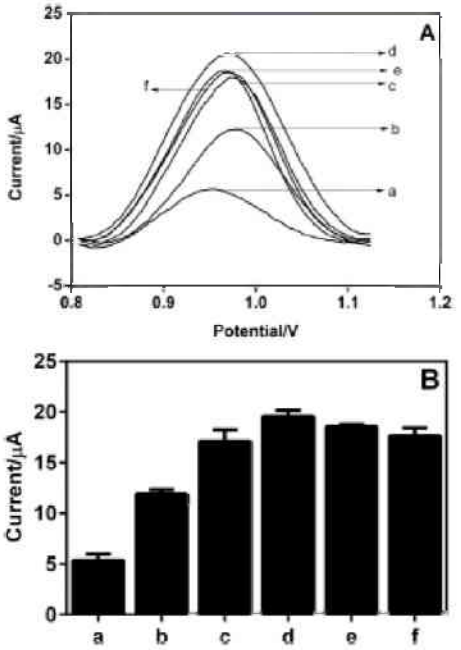

Fig. 2. Differential pulse voltammograms (A) and histograms of the peak current response (B) of guanine residue oxidation in graphite electrodes modified with poly (3-HBA) after immobilization of different concentrations of probe (ToxG1): 5 μmol L^{-1} (a) 15 μmol L^{-1} (b) 20 μmol L^{-1} (c) 25 μmol L^{-1} (d) 35 μmol L^{-1} (e) and 50 μmol L^{-1} (f). Modulation amplitude: 25 mV, pulse interval: 0.2 s, scan rate: 20 mV s^{-1}.

targets in solution [38–40]. The effect of probe concentration for immobilization (Fig. 2) was investigated, by the direct method, using the guanine residue oxidation signal.

Fig. 2A shows that the oxidation peak for guanine residue occurs at +0.96 V vs. Ag/AgCl. With the increase in the guanine concentration, the current value of the oxidation peak for guanine increases until 25 μmol L^{-1}, according to Fig. 2B. This concentration was used in the hybridization experiments with the complementary target. These results show that ToxG1 probe was efficiently incorporated to the graphite electrode modified with poly(3-HBA).

3.3. Surface analysis using AFM

The probe immobilization (ToxG1) onto electrode surface was also confirmed using 3D AFM analysis (Fig. 3).

Images of graphite electrode, graphite/poly(3-HBA) and graphite/poly(3-HBA)/ToxG1 probe present roughness values of 49.1 ± 2.0 nm, 27.4 ± 1.4 nm and 80.3 ± 3.6 nm, respectively. The surface of the electrode modified with polymer is smoother in comparison to the graphite electrode. Such figures indicate a greater uniformity of the modified electrode surface due to the fact that the polymer fills the valleys contained in the graphite electrode. After probe immobilization, both an increase in roughness and the appearance of clusters between 0.6 and 1.2 nm were observed according to the literature [41,42].

The effect of the probe concentration in the duplex formation is shown in Fig. 4.

A decrease in guanine oxidation peak current can be observed after hybridization with probe concentrations of 20 μmol L^{-1} and 25 μmol L^{-1}. However, as the difference between the probe (ToxG1) and the probe:target (ToxG1:ToxG2) was higher for 25 μmol L^{-1}, this probe concentration was used in the experiments. In higher concentrations, it was observed to increase in the current response, probably due to the saturation of the surface by the probe, causing steric hindrance and making it difficult to interact with the complementary target.

The hybridization was monitored by the oxidation of guanine, which is the most redox active nitrogenous base in DNA strands. 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 [43]. Like the bioelectrode produced in this work, several label-free oligonucleotide biosensors have been described in literature [43–46], which are considered as the simplest method, once it does not require any special reagents or nucleic acid modifications.

3.4. Specificity studies

Specificity studies were conducted using non-complementary oligonucleotide and genomic DNA from *N. caninum*, human and *Mus musculus* as non-specific targets. ToxG2 or *T. gondii* were used as positive control (Fig. 5).

When the non-specific targets (oligonucleotide or genomic DNA from *N. caninum*, human and *Mus musculus*) were added, the current values observed were similar to the probe. A decrease of about 20% in the guanine signal was observed due to the extent of hybridization between the ToxG1 probe in the presence of ToxG2 or *T. gondii* RH strain genomic DNA. This decrease is due to the formation of hydrogen bonds between the probe and the complementary target during the hybridization, which hampers the oxidation of nitrogenous bases [11,16,41–43].

Fig. 6 shows a decrease in current peak response with the increase in genomic DNA concentration and a linear relationship of the current peak with the genomic DNA concentration given by $I/\mu A = -0.01817X + 19.81$ ($r = 0.9826$), in the range of 100 ng μL^{-1} to 400 ng μL^{-1}. It was possible to detect up to 100 ng μL^{-1} of the genomic DNA of *Toxoplasma gondii*.

3.5. Analysis using AuNPs

Gold nanoparticles are a scaffold for DNA binding. The functionalization of AuNPs with oligonucleotides was used for the

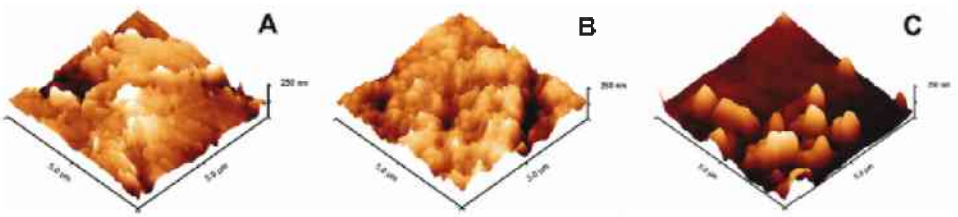

Fig. 3. AFM topographical images of graphite electrode (A); graphite/poly (3-HBA) (B); graphite/poly (3-HBA)/probe (C).

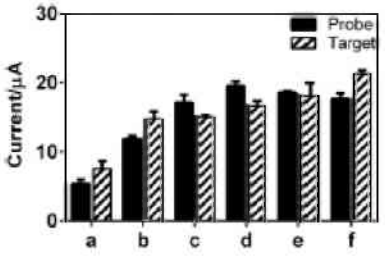

Fig. 4. Histogram obtained from current peak response of guanine oxidation in graphite electrodes modified with poly (3-HBA) after immobilization of different concentrations of probe (ToxG1): 5 μmol L^{-1} (a) 15 μmol L^{-1} (b) 20 μmol L^{-1} (c) 25 μmol L^{-1} (d) 35 μmol L^{-1} (e), 50 μmol L^{-1} (f) and after 15 min of incubation with different concentrations of complementary target (ToxG2): 10 μmol L^{-1} (a) 30 μmol L^{-1} (b) 40 μmol L^{-1} (c) 50 μmol L^{-1} (d) 70 μmol L^{-1} (e) and 100 μmol L^{-1} (f).

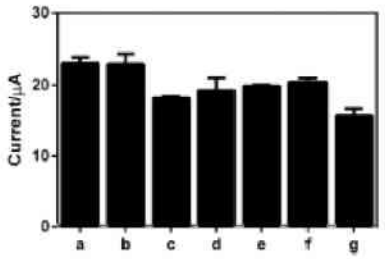

Fig. 5. Bar chart obtained from differential pulse voltammograms of guanine residue onto graphite electrode modified with poly (3-HBA) containing ToxG1 probe, before hybridization (a) and after hybridization with: non-complementary oligonucleotide (b), complementary target oligonucleotide (ToxG2) (c), *Neospora caninum* genomic DNA (d), human genomic DNA (e), *Mus musculus* genomic DNA (f) and *Toxoplasma gondii* genomic DNA (g). Electrolyte: phosphate buffer (0.10 mol L^{-1}); pH 7.4; modulation amplitude: 25 mV. Pulse interval: 0.2 s; Scan rate: 20 mV s^{-1}.

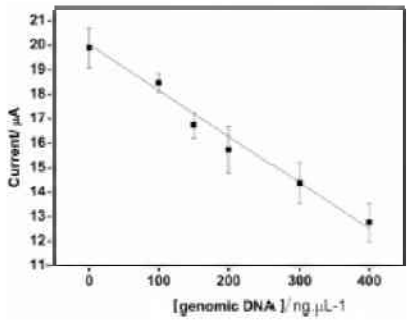

Fig. 6. Calibration curve for the oxidation signal of guanine residue obtained from the differential pulse voltammograms onto graphite electrode modified with poly(3-HBA), containing ssDNA (TOXG1) before and after hybridization with different concentrations of *Toxoplasma gondii* RH strain genomic DNA (100 ng μL^{-1} to 400 ng μL^{-1}). Electrolyte: phosphate buffer (0.10 mol L^{-1}), pH 7.4. Modulation amplitude: 25 mV. Pulse interval: 0.2 s; 20 mV s^{-1}.

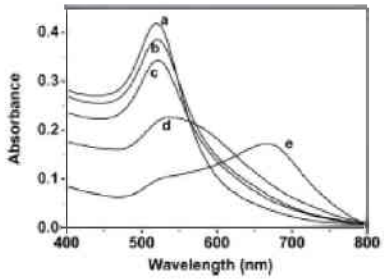

Fig. 7. Absorption spectrum in the UV-vis of: AuNPs (a), AuNPs/TOXG1/NaCl (saturated) (b), AuNPs/ToxG1/*Neospora caninum*/NaCl (saturated) (c) AuNPs/ToxG1/*Toxoplasma gondii*/NaCl (satured) (d) and AuNPs/NaCl (saturated) (e).

electrochemical analysis of the hybridization between ToxG1 probe: *T. gondii* DNA (positive sample) and ToxG1 probe: *N. caninum* DNA (negative sample) (Fig. 7).

Fig. 7a shows the absorption spectrum of the AuNPs. The peak of maximum absorbance at 520 nm is in accordance with Verissimo and collaborators [47].

When the ToxG1-S probe was incubated with the negative control (*N. caninum* DNA), both the absorbance peak and the displacement are similar to the probe (Fig. 7b), but with a lower intensity of ToxG1-S (Fig. 7c). However, when the ToxG1-S probe was incubated with the positive control (*T. gondii* DNA) (Fig. 7d), a significant change in the absorbance peak was observed, as well as widening and bathochromic shift, assuming a peak absorbance near 540 nm. The spectra in Fig. 7d (shoulder in 580 nm) and Fig. 7e (peak in 680 nm) suggest that the particles are close to each other, indicating an agglomeration process. These results indicate that the process of target recognition (genomic DNA) occurs by means of the biosensor, corroborating with the electrochemical results.

4. Conclusions

An approach for the selection of DNA probes related to *T. gondii* was shown in this study. Poly(3-hydroxybenzoic acid) was effective for the immobilization and detection of DNA fragments of *T. gondii*.

The immobilization of the ToxG1 and the hybridization of the oligonucleotide (ToxG2) and genomic DNA showed good responses by direct detection of guanine residue oxidation. A decrease of about 20% in the guanine signal was observed due to the hybridization between the ToxG1 probe in the presence of ToxG2 or *T. gondii* genomic DNA. In the presence of non-specific targets (*N. caninum*, human and *Mus musculus*), the current values observed were similar to the probe.

Hybridization was also confirmed by optical assays using AuNPs. When the ToxG1S probe is incubated with the *T. gondii* genomic DNA, there is a significant change in the absorbance peak, widening and shifting to a longer wavelength in accordance with the electrochemical results.

The results obtained contribute to the further development of the rapid diagnosis of *T. gondii* infection or toxoplasmosis.

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