UNIVERSIDADE ESTADUAL DO CEARÁ
REDE NORDESTE DE BIOTECNOLOGIA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA
DOUTORADO EM BIOTECNOLOGIA

AÍRIS MARIA ARAÚJO MELO

DESENVOLVIMENTO DE IMUNOSSENSOR ELETROQUÍMICO PARA A DETECÇÃO DE *Salmonella* sp. EM LEITE A PARTIR DE GOMA DE CAJUEIRO CARBOXIMETILADA

FORTALEZA – CEARÁ
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Tese apresentada ao Douturado em Biotecnologia do Programa de Pós-Graduação em Biotecnologia da Rede Nordeste de Biotecnologia da Universidade Estadual do Ceará, como requisitos parcial para a obtenção do título de doutora em Biotecnologia. Área de concentração: Recursos Naturais.

Orientador: Prof. Dr. Carlucio Roberto Alves

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FORTALEZA – CEARÁ

2020

Dados Internacionais de Catalogação na Publicação

Universidade Estadual do Ceará

Sistema de Bibliotecas

Melo, Aíris Maria Araújo .
Desenvolvimento de imunossensor eletroquímico para a detecção de salmonella sp. Em leite a partir de goma de cajueiro carboximetilada [recurso eletrônico] / Aíris Maria Araújo Melo. - 2020.
1 CD-ROM: il.; 4 ¾ pol.

CD-ROM contendo o arquivo no formato PDF do trabalho acadêmico com 105 folhas, acondicionado em caixa de DVD Slim (19 x 14 cm x 7 mm).

Tese (doutorado) - Universidade Estadual do Ceará, Rede Nordeste de Biotecnologia, Doutorado em Biotecnologia, Fortaleza, 2020.
Área de concentração: Recursos Naturais..
Orientação: Prof. Dr. Carlucio Roberto Alves.
Coorientação: Prof.ª Dra. Roselayne Ferro Furtado.

1. Biossensores. 2. Goma de cajueiro carboximetilada. 3. Método rápido. 4. Segurança de alimentos. 5. Salmonella sp. I. Título.

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Aprovada em: 04 de março de 2020

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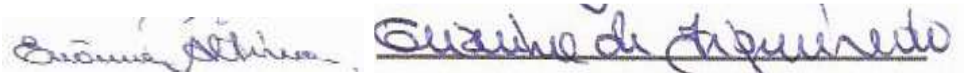

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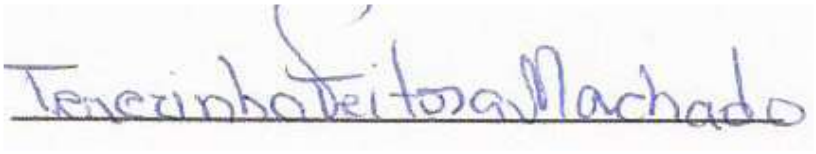

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Aos meus pais, Francisco e Francisca, por toda dedicação e empenho ao ensinar-me o valor do trabalho e do estudo, na busca de uma vida digna e feliz.

AGRADECIMENTOS

A Deus, por colocar no meu caminho pessoas competentes e humanas que tornaram a realização desse trabalho um rico período de crescimento na minha vida.

Ao prof. Dr. Carlúcio Roberto Alves, pela excelente orientação e pelo exemplo de profissional que serve de inspiração para os futuros docentes.

À pesquisadora Dra. Roselayne Ferro Furtado, por ter sido muito além de uma orientadora, contribuindo de maneira fundamental no meu crescimento pessoal e profissional.

À pesquisadora Dra. Maria de Fátima Borges, por todo o suporte na realização das avaliações microbiológicas, além de sua disponibilidade nas orientações e esclarecimentos prestados, que serviram de grande incentivo.

À professora Dra. Evânia Altina Teixeira de Figueiredo, por ter me conduzido nos primeiros passos da pós-graduação, o que me ajudou a definir uma nova jornada profissional.

Às pesquisadoras do laboratório de embalagens, Dra. Selenes Benevides, Dra. Socorro Bastos que juntamente com a Dra Roselayne lideram a equipe de bolsistas e estagiários promovendo um ambiente harmonioso e produtivo.

À bolsista de iniciação científica Lorena Cristina Rodrigues Bezerra, pelo apoio na realização desse trabalho.

À analista Lorena Mara, pelo suporte na realização das análises de espectroscopia.

À equipe da Central Analítica da UFC sob a coordenação do prof. Dr. Antônio Gomes de Souza Filho.

À companheira de pesquisa Maria Roniele Felix de Oliveira, pela cooperação nas etapas iniciais dos experimentos deste estudo.

Aos companheiros de curso, Nadya Peixoto, Rogênio Mendes, Magnólia e Iramaia pela parceria na realização dos trabalhos nas disciplinas realizados com muito empenho e diversão.

Às amigas Elaine Cristina Pereira e Maria de Fátima, pelo apoio e incentivo nos momentos difíceis.

À equipe do laboratório de Tecnologia de Embalagens da Embrapa, Lorena, Airlane, Nádia, Luana Carvalho, Marília, Leônia, Dani, Gabi, Thiago, Williams por toda ajuda, atenção e cooperação durante nossa convivência.

Aos meus irmãos, Arlan, Acrísio e Aila, por acreditarem na minha capacidade, incentivarem o meu crescimento e por comemorarem comigo essa vitória.

Aos laboratórios da Embrapa Agroindústria Tropical com seus respectivos pesquisadores, analistas técnicos, bolsistas e estagiários que sempre estiveram disponíveis e atenciosos no atendimento às demandas experimentais.

À Embrapa, à CAPES e ao CNPq, pelo apoio financeiro.

“—Não é o mais inteligente nem o mais forte que sobrevive e sim aquele que melhor se adapta às mudanças.”

(Charles Darwin)

RESUMO

Imunossensores eletroquímicos são dispositivos bioeletrônicos de resposta rápida com grande potencial para miniaturização e portabilidade, que utilizam moléculas de anticorpo como biorreceptor e transdutor eletroquímico para geração da resposta analítica. O desenvolvimento de imunossensores requer a utilização de plataformas biocompatíveis que permitam a ligação de metais e de biomoléculas simultaneamente. O presente estudo teve como objetivo utilizar a goma de cajueiro carboximetilada (GCCM) como uma nova plataforma para imobilização de anticorpos visando desenvolver, caracterizar e avaliar o desempenho analítico de um imunossensor eletroquímico para a detecção de *Salmonella* sp. em leite. No desenvolvimento do imunossensor, o filme de GCCM foi eletrodepositado na superfície de eletrodos de ouro e serviu de base para a imobilização dos anticorpos policlonais anti-*Salmonella*. Estudos de otimização foram conduzidos e a resposta analítica foi obtida por meio da técnica de cronoamperometria na presença da bactéria *Salmonella enterica* serovar Typhimurium. O imunossensor foi caracterizado através das técnicas eletroquímica, microscopia eletrônica de varredura e espectroscopia de infravermelho com transformada de Fourier. Os parâmetros de desempenho do imunossensor foram determinados, e a aplicabilidade em alimentos foi testada em diferentes amostras de leite comparado com outros métodos já consolidados. A caracterização do imunossensor demonstrou que a GCCM é uma excelente plataforma para a montagem de imunossensores, produzindo uma estrutura estável com coeficiente de variação de 12% após 30 clicos voltamétricos em meio tampão fosfato salino. O imunossensor apresentou uma faixa de linearidade nas concentrações de 10^1 a 10^5 UFC mL^{-1}, a partir daí observou-se uma perda de sensibilidade com provável saturação eletrocatalítica nas concentrações seguintes. O limite de detecção foi de 10 UFC mL^{-1}. Quando testado em amostras de leite, o imunossensor foi capaz de distinguir três concentrações diferentes, comprovando sua funcionalidade na detecção de bactérias numa matriz complexa de alimento. O imunossensor eletroquímico a base de GCCM detectou o patógeno *Salmonella* com um baixo limite de detecção de forma rápida e específica em amostras de leite demonstrando um grande potencial para uso na indústria de alimentos.

Palavras-chave: Biossensores. *Salmonella* sp. Goma de cajueiro carboximetilada. Método rápido. Segurança de alimentos.

ABSTRACT

Electrochemical immunosensors are rapid response bioelectronic devices with great potential for miniaturization and portability that use antibody molecules such as bioreceptor and electrochemical transducer to generate the analytical signal. The development of immunosensors requires the use of biocompatible platforms that allow the binding of metals and biomolecules simultaneously. The present study aimed to use carboxymethylated cashew gum (GCCM) as a new platform for immobilizing antibodies to develop, characterize and evaluate the analytical performance of an electrochemical immunosensor for the detection of *Salmonella* sp. in milk. In the development of the immunosensor, the GCCM film was electrodeposited on the surface of gold electrodes and served as the basis for the immobilization of polyclonal anti-*Salmonella* antibodies. Optimization studies were conducted to improve the performance of the CMCG-based immunosensor. The analytical response was obtained by means of the chronoamperometry technique in the presence of the bacterium *Salmonella enterica* serovar Typhimurium. The immunosensor was characterized by electrochemical, scanning electron microscopy and infrared spectroscopy techniques. Performance parameters of the immunosensor were determined, and food applicability was tested on different milk samples which were also compared to other methods for detection of already established *Salmonella* use. Characterization of the CMCG-based immunosensor demonstrated that CMCG was an excellent platform for mounting the immunosensor producing a stable structure with a 12% coefficient of variation after 30 sweeps in PBS buffer. The chronoamperometric response was proportional to the concentration of *S.* Typhimurium tested and presented a linearity from 10^1 to 10^5 CFU mL^{-1}, from which a loss of sensitivity with probable system saturation was observed at the following concentrations. The limit of detection was 10 CFU mL^{-1}. When tested on immunosensor milk samples it was able to distinguish three different concentrations, proving its functionality in detecting bacteria in a complex food matrix. The GCCM-based immunosensor detected the *Salmonella* pathogen with a low detection limit quickly and specifically in milk samples showing great potential for use in the food industry.

Keywords: Biosensors. *Salmonella* sp. Cashew Gum. Fast Method. Food Safety.

SUMÁRIO

1 INTRODUÇÃO

A segurança de alimentos é uma preocupação crescente dos consumidores e das indústrias de alimentos, devido ao aumento dos surtos envolvendo alimentos contaminados por microrganismos patogênicos. Entre os agentes etiológicos de maior incidência em surtos alimentares encontramos as bactérias *Salmonella* spp., com registros expressivos nos dados epidemiológicos publicados por autoridades mundiais de saúde (BRASIL, 2019; CENTERS FOR DISEASE CONTROL AND PREVENTION, 2019b; EUROPEAN FOOD SAFETY AUTHORITY, 2014). Sendo assim, o monitoramento deste patógeno é uma rotina obrigatoria da indústria de alimentos.

A análise para detecção de *Salmonella* é uma exigência das autoridades sanitárias que regulamentam a segurança de alimentos em seus respectivos países. As técnicas convencionais são consideradas sensíveis e confiáveis, no entanto, dependem de uma sequência complexa de etapas e requerem vários dias para a obtenção do resultado (ANDREWS et al., 2019). Os avanços recentes em tecnologias para detecção, quantificação e identificação de microrganismos, disponibilizaram alternativas mais rápidas, sensíveis e específicas. Porém, quase todas, incluem uma etapa de pré-enriquecimento da amostra aumentando o tempo de análise em aproximadamente até 24 horas (LEE et al., 2015).

Uma alternativa para a detecção rápida de analitos é a utilização de biossensores, dispositivos bioeletrônicos, que nos últimos anos têm ganhado maior visibilidade por apresentarem além da rapidez de resposta, um grande potencial para miniaturização e portabilidade; características desejáveis para uma aplicação industrial. Existem exemplos de aplicação de biossensores em diferentes áreas como no controle de qualidade em indústrias de alimentos e bebidas (MELLO; KUBOTA, 2002), na detecção de patógenos em alimentos e em água (LAZCKA; CAMPO; MUÑOZ, 2007; MORTARI; LORENZELLI, 2014; NAYAK et al., 2009; O'KENNEDY et al., 2005; VELUSAMY et al., 2010), na detecção de fitopatógenos (SKOTTRUP; NICOLAISEN; JUSTESEN, 2008), e inclusive para a detecção de *Salmonella* spp. em alimentos (ALEXANDRE et al., 2018; MELO et al., 2016, 2017; SILVA et al., 2018).

A evolução no desenvolvimento de biossensores é favorecida, pelo aumento da compreensão molecular e bioquímica da resposta analítica e das suas respectivas tecnologias de suporte. Por essas razões é possível encontrar hoje dispositivos miniaturizados, de custo acessível e de fácil uso, a exemplo dos glicosímetros. Existem diferentes tipos de biossensores e os mesmos podem ser classificados pelo tipo de moléculas biológicas imobilizadas (os

biorreceptores), dentre esses destacam-se os genossensores e os imunossensores que utilizam ácidos nucleicos e anticorpos como biorreceptores, respectivamente. Já com relação ao tipo de transdutor utilizado para a obtenção da resposta analítica, os eletroquímicos são os mais amplamente usados.

Uma das etapas críticas na montagem de um biossensor é a seleção de materiais que formam a plataforma de imobilização das biomoléculas. Novos materiais têm sido propostos, com êxito, para essa finalidade, promovendo a melhoria de sensibilidade e/ou seletividade dos dispositivos. A utilização de polímeros naturais vem sendo adotada preferencialmente por razões ambientais e econômicas. Um bom exemplo disso é a goma de cajueiro (GC), que apresenta propriedades interessantes como atoxicidade, biodegradabilidade e biocompatibilidade; esta última é especialmente atrativa, pois favorece a estabilidade de biomoléculas, as quais podem ser imobilizadas sobre estes filmes (PAULA et al., 2012). Estudos têm explorado com sucesso a utilização da goma de cajueiro como plataforma para a montagem de dispositivos bioeletrônicos em nanobiomedicina (ARAÚJO et al., 2012), para imobilização de enzimas (SILVA et al., 2010) e em estudos para o desenvolvimento de compósitos condutores (CASTRO et al., 2017).

O Brasil tem aproximadamente 710.000 ha de área plantada com cajueiros e uma produção média de goma/árvore/ano de 700 g, com um potencial de produção anual de GC superior a 38.000 toneladas (ARAÚJO et al., 2012). O interesse tecnológico na GC está fundamentado em suas similares características reológicas e aplicações industriais a muitos polímeros sintéticos, provenientes principalmente de sua biodegradabilidade e propriedades mecânicas (CUNHA et al., 2009). A ampliação do uso da GC com fins biotecnológicos vai além das fronteiras brasileiras e diversas aplicações já estão em prospecção (PAULA et al., 2012; ARAÚJO et al., 2012; RIBEIRO et al., 2016).

Aliada a importância do gênero *Salmonella* para a saúde pública mundial, a necessidade da detecção rápida no controle de segurança de alimentos das indústrias e a oportunidade de explorar o potencial biotecnológico da goma de cajueiro, o presente estudo teve o objetivo de desenvolver, caracterizar e avaliar o desempenho analítico de um immunosensor eletroquímico para detecção de *Salmonella* sp. utilizando a goma de cajueiro carboximetilada como uma plataforma para a imobilização de anticorpos.

2 OBJETIVOS

2.1 GERAL

O presente estudo teve o objetivo de utilizar a goma de cajueiro carboximetilada (GCCM) como uma nova plataforma para imobilização de anticorpos policlonais anti-*Salmonella* a fim de desenvolver, caracterizar e avaliar o desempenho analítico de um imunossensor eletroquímico para a detecção de *Salmonella* sp. em leite.

2.2 ESPECÍFICOS

Otimizar os parâmetros para a montagem do imunossensor em superfície eletrocatalítica de ouro;
Caracterizar eletroquímica, morfológica e espectroscopicamente o imunossensor;
Avaliar os parâmetros de desempenho do imunossensor;
Testar a aplicabilidade em amostras de leite;
Comparar o desempenho do imunossensor a métodos para detecção de *Salmonella* validados.

3 REVISÃO BIBLIOGRÁFICA

3.1 O GÊNERO *Salmonella*

As bactérias do gênero *Salmonella* apresentam-se em forma de bastonetes, Gram negativos, móveis, não formadoras de esporos, pertencente à família *Enterobacteriaceae* e a tribo *Salmonellae*. Variantes não-móveis incluem *S.* Gallinarum e *S.* Pullorum. O gênero *Salmonella* é composto por duas espécies que podem causar doenças nos seres humanos: *S. enterica* e *S. bongori* (WORLD HEALTH ORGANIZATION, 2007). A espécie *S. enterica* é a que representa maior risco à saúde pública, estando associada à maioria dos casos de doenças transmitidas por alimentos (DTAs). Para propósitos epidemiológicos, os sorotipos de *Salmonella* podem ser divididos em três grupos:

a) sorotipos que infectam somente o homem – *S.* Typhi e *S.* Parathyphi, causadores da febre tifoide e paratifoide, respectivamente;

b) sorotipos adaptados à hospedeiros específicos – alguns são patógenos humanos. Exemplos destes são: *S.* Gallinarum – frango; *S.* Dublin – gado; *S.* Abortus-equi – cavalo; *S.* Abortus-ovis – ovelhas; *S.* Choleraesuis – suínos;

c) sorotipos não-adaptados (sem preferência por hospedeiro) – patogênicos ao homem e outros animais.

As bactérias do gênero *Salmonella* podem causar dois tipos de doenças, de acordo com o sorotipo: a salmonelose e a febre tifoide (além da paratifoide, uma forma mais branda que a anterior). A sintomatologia da salmonelose é caracterizada por náuseas, vômitos, dores abdominais, diarreia, febre e dores de cabeça, podendo durar de dois a sete dias. Apresenta uma baixa taxa de mortalidade, e em organismos jovens e saudáveis é considerada uma doença autolimitada. A salmonelose pode ser causada por todos os demais sorotipos que não sejam S. Typhi e S. Paratiphy. O mecanismo de ação da bactéria permite que ela penetre na mucosa intestinal promovendo sua multiplicação. Em alguns casos, o patógeno consegue atravessar a mucosa intestinal e invade os sistemas linfático e cardiovascular, podendo se disseminar e, eventualmente, afetar outros órgãos. A febre associada às infecções por *Salmonella* ocorre devido à liberação de endotoxinas, quando as células bacterianas são lisadas (HAMMACK, 2012).

As febres tifoide e paratifoide são mais graves e têm uma taxa de mortalidade alta e são causadas pelos sorotipos *S.* Thyphi e *S.* Parathypi, respectivamente. O paciente apresenta febre alta, em torno de 40 °C e cefaleia contínua. A diarreia surge por volta da

segunda semana e a febre tende a diminuir. Normalmente, os sintomas permanecem durante duas semanas. Diferentemente da salmonelose, as bactérias não se multiplicam nas células do epitélio intestinal, ao invés disso, elas podem se multiplicar dentro das células fagocitárias e se disseminar pelo corpo sendo isoladas no sangue, na urina e nas fezes. Nos casos mais graves, podem ocorrer perfurações na parede intestinal, podendo levar o paciente à óbito (HAMMACK, 2012).

O habitat primário da *Salmonella* é o trato gastrointestinal do homem e dos animais, como pássaros, répteis, animais de granja e, eventualmente insetos. A bactéria é normalmente eliminada nas fezes que acabam por contaminar a água tornando-a um dos principais veículos de contaminação. Além disso, os insetos ao entrarem em contato com as fezes contaminadas também se tornam vetores de contaminação. Uma grande variedade de alimentos já foi associada a surtos incluindo carnes, aves, ovos, leite e produtos lácteos, peixes, camarão, as especiarias, o fermento, o coco, molhos, saladas, misturas para bolo, cremes, sobremesas e coberturas que contêm ovos crus, gelatina, manteiga de amendoim, cacau, chocolate, sorvete, frutas e legumes (como tomates, pimentões e melão) (HAMMACK, 2012). Os sorotipos mais comuns em infecções são, *Salmonella* Enteritidis e *Salmonella* Thyphimurium. Porém, nos casos que envolvem leite, como alimento veiculador, o sorotipo Thyphimurium tem se destacado (CENTERS FOR DISEASE CONTROL AND PREVENTION, 2019a).

A salmonelose é a doença veiculada por alimentos de maior incidência no mundo. Na União Europeia são relatados mais de 90.000 casos de salmonelose por ano (EUROPEAN FOOD SAFETY AUTHORITY, 2014). Nos Estados Unidos, estima-se a ocorrência de 1,35 milhão de casos, com 26.500 hospitalizações, e aproximadamente 420 mortes anualmente (CENTERS FOR DISEASE CONTROL AND PREVENTION, 2019b). No Brasil, por exemplo, alimentos contaminados por bactérias do gênero *Salmonella* ocupam o segundo lugar, representando 11,3% dos surtos com agentes etiológicos identificados ficando atrás apenas da espécie *Escherichia coli* nas estatísticas no período de 2009 a 2018 (BRASIL, 2019). No entanto, estima-se que o número de casos seja bem maior do que apresentado nos registros. A subnotificação é muito comum, devido à negligência dos casos domésticos e à falta de identificação do agente etiológico, nos casos onde há hospitalização. Trata-se de um problema global de saúde pública, em razão dos elevados custos envolvendo tratamentos e hospitalizações.

Devido à ampla distribuição e à patogenicidade dessa bactéria as autoridades de saúde têm estabelecido a obrigatoriedade do seu monitoramento em alimentos. No Brasil, a

Resolução RDC nº 12, de 02 de janeiro de 2001 ANVISA (BRASIL, 2001), estabelece os padrões microbiológicos para alimentos e, determina que, o alimento próprio para o consumo humano deve apresentar ausência de *Salmonella* sp., em 25 g ou mL da amostra. As técnicas convencionais, baseadas em métodos clássicos de culturas de bacterias, são consideradas sensíveis e confiáveis, no entanto, dependem de uma sequência complexa de etapas e requerem vários dias para a obtenção do resultado (ANDREWS et al., 2019).

Os avanços recentes em tecnologias para detecção e identificação de microrganismos, disponibilizaram alternativas mais rápidas, sensíveis e específicas em relação aos métodos convencionais. Esses são, geralmente, referidos como métodos “rápidos" ou alternativos, termos geralmente usados para descrever uma variedade de testes que incluem *kits* bioquímicos miniaturizados, ensaios imunológicos, testes baseados em DNA/RNA, e combinações com métodos de cultura bacteriana. Considerando os testes alternativos para detecção de *Salmonella* spp., apesar de “rápidos”, quase todos necessitam de uma etapa prévia de pré-enriquecimento da amostra, aumentando o tempo de análise em aproximadamente 24 horas (LEE et al., 2015; SILVA et al., 2018).

A automatização, a portabilidade e a possibilidade de analisar múltiplas amostras ao mesmo tempo são características, que vêm despertando o interesse, principalmente, das indústrias. Os setores de qualidade e de segurança de alimentos tem demandado às instituições de validação de procedimentos, a aprovação de diversos métodos alternativos para detecção de patógenos em alimentos, tendo em vista a obrigatoriedade de alguns testes determinada pelas regulamentações. Reduzir o tempo de análise dos alimentos em sua rotina aplicando métodos rápidos, confiáveis e específicos é uma questão estratégica que aumenta a competitividade das empresas.

3.1.1 Classificação sorológica do gênero *Salmonella*

Para o gênero *Salmonella*, a divisão em espécies e subespécies apresenta pouca importância na prática, pois sua classificação baseia-se na composição antigênica. O esquema de Kauffmann e White é utilizado como método de identificação, que divide o gênero em tipos sorológicos (WORLD HEALTH ORGANIZATION, 2007). Este sistema baseia-se na presença de antígenos O-somático, Vi-capsular e H-flagelar. Quando o sistema de classificação foi proposto pela primeira vez foram identificados 44 sorotipos. De acordo com Hammack (HAMMACK, 2012) o número de sorotipos descobertos em 2007 já era de 2.579.

Os antígenos somáticos são compostos pelos lipopolissacarídeos (LPS) presentes na face externa da parede celular. Diferenças estruturais na cadeia dos LPS como, por exemplo nos tipos de monossacarídeos ou nas ligações entre eles, levam ao surgimento de diferentes sorotipos. Os antígenos "O" são termoestáveis (100 ou 120 °C / 2 h) e podem ser classificados como maiores e menores, ou ainda, como principais e secundários em função de sua importância no diagnóstico. Os antígenos maiores servem de base para a separação das cepas de *Salmonella* em sorogrupos somáticos e estão presentes apenas em seu grupo característico. Os antígenos menores são aqueles com valor discriminatório, podendo ser encontrados em cepas de um ou mais sorogrupos. A maioria dos antígenos menores surgem de pequenas modificações químicas na sequência da cadeia de polissacarídeo que pode ser frequentemente intermediada por bacteriófagos (HAMMACK, 2012).

Os antígenos capsulares são encontrados em poucos sorotipos de *Salmonella*. Um antígeno capsular bem conhecido é o "Vi", que ocorre em apenas três sorotipos de *Salmonella*, Typhi, Paratyphi e Dublin. As cepas desses sorotipos podem apresentar ou não o antígeno Vi que, se presente, pode mascarar os antígenos somáticos e impedir sua aglutinação com o seu respectivo antissoro. O aquecimento a 100 °C, geralmente, inativa o antígeno Vi, permitindo a reação de aglutinação com o antissoro somático (HAMMACK, 2012).

Os antígenos flagelares "H" são derivados dos flagelos das cepas móveis. São termossensíveis e decorrem de variações na sequência de aminoácidos da proteína flagelar conhecida como flagelina, uma subunidade do filamento helicoidal que forma o flagelo. A maioria das cepas de *Salmonella* pode produzir dois tipos de flagelos, com características antigênicas diferentes, sendo assim, chamados bifásicos. Poucas cepas são monofásicas, produzindo apenas um tipo de flagelo (sorotipos Typhi e Enteritidis) e outros não produzem flagelos, sendo imóveis (sorotipos Pullorum e Gallinarum). Os fatores antigênicos da fase um foram, originalmente, identificados por letras minúsculas, enquanto os antígenos da fase dois são designados por números (HAMMACK, 2012).

Os sorotipos de *Salmonella* são identificados através de uma fórmula composta por números e letras, organizados na seguinte sequência: 1°) antígenos somáticos, maiores seguidos dos menores, 2°) antígenos capsulares, se a presença deste não for constante no sorotipo, será apresentado entre parênteses, 3°) antígenos flagelares da fase um e 4°) os antígenos flagelares da fase dois, se presentes. Exemplo: Sorotipo 9,12, (Vi): d: - significa, fator somático maior O:9, menor O:12, Vi pode estar presente ou não, o antígeno H da fase um é "d" e não possui antígeno H da fase dois. Os sorotipos também podem ser definidos por nomes. O nome do sorotipo pode indicar o tipo de doença do agente causado (Typhi e

Paratyphi), ou o hospedeiro (Gallinarum, Abortus-equis, Abortus-ovis e Choleraesuis), ou ainda o local onde o sorotipo foi identificado pela primeira vez (London, Panama e Montivideo). A forma correta de grafar os nomes dos sorotipos utilizada pela *World Health Organization* (WHO) e C*enters for Disease Control and Prevention* (CDC), é com letra inicial maiúscula sem itálico (WHO, 2007; (HAMMACK, 2012).

De acordo com o Centro de Referência e Pesquisa em *Salmonella* do Instituto Pasteur (França), colaborador da WHO, existem, atualmente 2.579 sorotipos, destes 2.557 pertencem à espécie *S. enterica* e 1.531 à subespécie *enterica*. Os sorogrupos somáticos (O) mais comuns são A, B, C1, C2, D, E1 e E4 e correspondem a aproximadamente 99% das infecções por *Salmonella* causadas em humanos e animais de sangue quente (WORLD HEALTH ORGANIZATION, 2007).

3.2 BIOSSENSORES

De acordo com a definição da *International Union of Pure and Applied Chemistry* (INTERNATIONAL UNION OF PURE AND APPLIED CHEMISTRY, 2014), um biossensor é um dispositivo que utiliza reações bioquímicas específicas mediadas por enzimas, imunossistemas, organelas, tecidos ou células para detectar compostos químicos, geralmente, por meio de sinais elétricos, térmicos ou ópticos. De forma mais prática, pode-se dizer, que um biossensor é uma ferramenta analítica, constituída pelo biorreceptor, camada sensora do transdutor que reconhece o analito, e pelo transdutor que converte o evento de reconhecimento em sinal elétrico mensurável. O biorreceptor pode ser um tecido, microrganismo, organela, célula, enzima, anticorpo ou ácido nucleico. O transdutor pode ser óptico, eletroquímico, termométrico, piezoelétrico, magnético e micromecânico, ou combinações destes (VELUSAMY et al., 2010).

O funcionamento de um imunossensor óptico, por exemplo, está baseado no produto da reação envolvida na interação entre o biorreceptor e o analito, onde haverá uma molécula oxidada ou reduzida provocando mudança na absorção da radiação eletromagnética. As principais vantagens desse transdutor envolvem a rapidez e a reprodutibilidade das medidas, enquanto as principais desvantagens são o alto custo do aparelho utilizado e o fato de não ser recomendado em meios turvos (CHAUBEY; MALHOTRA, 2002; VALADEZ et al., 2009).

O imunossensor piezoelétrico baseia-se no princípio da geração de dipolos elétricos ao submeter um cristal anisotrópico natural a estresse mecânico. A adsorção de

analito aumenta a massa do cristal e altera sua frequência básica de oscilação. Eles são bastante usados para determinação de amônia, óxido nitroso, monóxido de carbono, hidrogênio, metano e uma quantidade indeterminada de compostos organofosforados e mais recentemente, no desenvolvimento de imunossensores (ABAD et al., 1998; SALAM; ULUDAG; TOTHILL, 2013). Como desvantagens podem ser citados o alto custo do equipamento e a necessidade de um profissional altamente qualificado.

A interação do biossensor com o analito resulta na alteração de uma ou mais propriedades físico-químicas (modificação de pH, transferência de elétrons, variação de massa, transferência de calor, liberação de gases ou íons etc.) que são detectadas e mensuradas pelo transdutor. O principal objetivo é produzir um sinal eletrônico proporcional em magnitude e/ou frequência à concentração de um determinado analito ou grupo de analitos que interage com o elemento biorreceptor. Além do transdutor, a parte eletrônica é constituída de um amplificador dos sinais elétricos e de um sistema de processamento dos dados.

O conceito básico do biossensor foi primeiramente proposto por Leyland C. Clark, em 1962, em sua descrição de um "eletrodo enzima". Clark demonstrou que a detecção eletroquímica de peróxido de hidrogênio ou oxigênio poderia ser realizada por instrumentos com enzimas imobilizadas. O exemplo clássico foi o uso da glicose oxidase imobilizada em eletrodo de platina, que transformou este simples eletrodo em um poderoso instrumento analítico para a quantificação de glicose em amostras de sangue humano de pessoas com diabetes (TURNER, 2013).

A demanda do mercado por técnicas rápidas e portáteis está impulsionando o desenvolvimento de biossensores. Repostas rápidas, específicas e de alta sensibilidade são fundamentais para o monitoramento preventivo de patógenos na cadeia produtiva das indústrias de alimentos. Os investimentos realizados para o desenvolvimento de biossensores aplicáveis nesta área convergem para a necessidade urgente de implantação de medidas que garantam a produção de alimentos seguros. A aplicação destes dispositivos reflete em ganhos à saúde pública e à economia dos países, através da redução da ocorrência de DTAs e dos custos associados aos seus tratamentos.

3.2.1 Imunossensores eletroquímicos

Os imunossensores são biossensores que utilizam como biorreceptor moléculas de anticorpo, e são comumente classificados pelo tipo de transdutor utilizado. Uma grande variedade de transdutores foi desenvolvida recentemente para a detecção de patógenos

transmitidos por alimentos. Estes se baseiam em métodos ópticos, onda acústica (piezoelétricos) e eletroquímicos (VELUSAMY et al., 2010).

Os imunossensores eletroquímicos podem ser classificados em amperométricos, potenciométricos, impedimétricos e condutimétricos, com base nos parâmetros observados, como corrente, potencial, impedância e condutância, respectivamente. Embora a detecção eletroquímica tenha várias vantagens como baixo custo, capacidade de trabalhar com amostras com alta turbidez e fácil miniaturização, a presença de interferentes associados deve ser minimizada. Os imunossensores amperométricos são baseados na medida de variação de corrente elétrica, resultante de alterações de oxidação ou redução de espécies eletroativas. Durante as análises, o potencial é mantido constante ao longo do tempo, as alterações de corrente verificadas são correlacionadas diretamente com a concentração das espécies eletroativas presentes, com a sua produção, ou com a taxa de consumo de reagentes envolvidos na reação. Os imunossensores potenciométricos se baseiam na diferença de potencial entre dois eletrodos em condições de corrente elétrica constante ao longo do tempo, enquanto nos imunossensores condutimétricos as mudanças são observadas nas medidas de condutância, resultante de produtos de reação catalítica. Esse tipo de sensor é utilizado, geralmente, empregando enzimas como a urease, cuja atividade origina produtos iônicos (GALLARDO SOTO; JAFFARI; BONE, 2001).

A relação dos princípios voltamétricos com as reações imunológicas possibilita o desenvolvimento de dispositivos analíticos altamente sensíveis e seletivos - os imunossensores amperométricos. A amperometria é um processo dinâmico no qual o fluxo de elétrons para um eletrodo polarizado inerte é medido, mantendo tipicamente um potencial aplicado constante, a fim de direcionar o fluxo de elétrons para ou a partir da molécula redox monitorada. O sistema fundamental de medição usa normalmente três eletrodos: um eletrodo de trabalho onde a reação catalisada desejada ocorre, um eletrodo de referência (por possuir uma área grande não se polariza) que mantém o seu potencial constante para que apenas o eletrodo de trabalho polarize e um contra-eletrodo que transporta o fluxo de corrente para longe do eletrodo de referência (RAMÍREZ; SALGADO; VALDMAN, 2009).

Os imunossensores amperométricos têm sido preferidos pela fácil manipulação e por trabalharem com potencial específico, reduzindo enormemente a interferência de espécies químicas. A concentração do analito é diretamente proporcional à alteração na corrente elétrica. Quando são utilizados como biorreceptores anticorpos ou DNA, a interação com o analito não gera íons redox, sendo necessária a conjugação a uma enzima como, por exemplo, a peroxidase para amplificar a resposta eletroquímica (PIMENTA-MARTINS et al., 2012).

Na medida amperométrica, quando são utilizadas enzimas como a glicose oxidase, que catalisa reações de oxirredução, o material biológico não necessita de um marcador. O transdutor amperométrico é a ferramenta eletroquímica mais comumente usada como método de detecção para agentes patogênicos, tendo uma sensibilidade superior ao método potenciométrico (VELUSAMY et al., 2010).

Uma grande variedade de imunossensores amperométricos para detecção de patógenos pode ser encontrada na literatura (MELO et al., 2016; SILVA et al., 2018). Palenzuela et al. (PALENZUELA et al., 2004) desenvolveram um método para a determinação indireta de contaminação bacteriana com base no metabolismo catabólico de glicose. A determinação da glicose foi realizada por um sistema de injeção em fluxo com detecção amperométrica.

Um imunossensor amperométrico descartável foi estudado por Rao et al. (RAO et al., 2006), para a detecção de *Vibrio cholerae*, empregando o princípio do ensaio indireto de *Enzyme Linked Immunosorbent Assay* (ELISA). Lin et al. (LIN et al., 2008) elaboraram um imunossensor amperométrico descartável para a detecção rápida de *E. coli* O157: H7 baseado em ensaio imunológico ligado à enzima em sanduíche indireto, utilizando eletrodos de carbono impresso com prata comercial e tintas de carbono. O limite de detecção em tampão foi de 6 UFC mL^{-1} e em leite foi de 50 UFC mL^{-1}.

Singh et al. (SINGH et al., 2005) definiram uma metodologia para a montagem de um imunossensor amperométrico renovável, para a detecção de *Salmonella* Typhi utilizando anticorpos específicos do flagelo bacteriano, com um limite de detecção de 10^5 UFC mL^{-1}. Em um outro estudo de Rao et al. (KAMESWARA RAO et al., 2005), desenvolveram um sensor amperométrico para a detecção de anticorpos contra *Salmonella* Typhi no soro dos pacientes através do uso de eletrodos impressos. Lee et al. (LEE et al., 2009) criaram um método combinando um sensor amperométrico com a reação de redução do azul de metileno para a detecção de bactérias do grupo coliformes em leite com um limite de detecção de 10^5 UFC mL^{-1}.

3.2.2 Técnicas utilizadas na montagem do imunossensor eletroquímico

3.2.2.1 Modificação da superfície do eletrodo

Há uma grande diversidade de materiais que pode ser utilizada na montagem de um imunossensor. A composição básica é constituída por um eletrodo base e pelas moléculas

biológicas imobilizadas na superfície do eletrodo. O eletrodo deve apresentar características eletroquímicas apropriadas, além de ser compatível com o método de imobilização adotado. Dentre os materiais mais usados como eletrodo base podemos destacar a platina, o ouro, o carbono vítreo, o grafite epóxi, carbono, a pasta de carbono, óxido de índio-estanho (ITO) (VELUSAMY et al., 2010).

Dentre esses, o ouro tem sido aplicado com maior frequência (RICCI; ADORNETTO; PALLESCHI, 2012), por tratar-se de um metal considerado biocompatível com estruturas celulares e biomoléculas, razoavelmente inerte e não tóxico (YANG et al., 1995). Os protocolos para o desenvolvimento de imunossensores eletroquímicos têm como etapa inicial o pré-tratamento da superfície do eletrodo, considerada fundamental, pois apresenta influência direta nas etapas de montagem do dispositivo. Normalmente, utiliza-se uma limpeza mecânica com uma solução de alumina 0,3 μm, imersão em etanol 96% em banho ultrassônico, solução piranha 1:3 (H_2O_2:H_2SO_4), e uma limpeza eletroquímica com H_2SO_4 e vários ciclos de varredura em uma janela de potencial pré-definida (com evolução de H_2 e O_2). O pré-tratamento deve ser realizado imediatamente antes da modificação da superfície do eletrodo para se obter uma superfície limpa, eletrocatalítica e eliminar imperfeições da superfície metálica (CARVALHAL; FREIRE; KUBOTA, 2005).

3.2.2.2 Imobilização das biomoléculas

Na literatura são descritas várias técnicas para imobilização de biomoléculas, e a escolha deve ser realizada considerando o tipo de transdutor utilizado e a aplicação final do dispositivo. As técnicas mais comuns são a ligação covalente, a ligação covalente cruzada, a oclusão em gel e a adsorção física.

Na imobilização por adsorção, o material biológico é fixado fisicamente na superfície do sensor por meio de forças eletrostáticas, tais como interações do tipo van der Waals, interações iônicas, ligações de hidrogênio e interações hidrofóbicas. Entretanto, as moléculas podem ser facilmente lixiviadas para a solução por fatores como variação da força iônica, solvente e pH do meio. Em geral, esse tipo de imobilização é raramente utilizado no desenvolvimento de imunossensores pois proporcionam baixa estabilidade e sensibilidade (DATTA; CHRISTENA; RAJARAM, 2013).

Na oclusão, o material biológico é confinado na grade de uma matriz polimérica ou em membranas semipermeáveis. Neste processo uma ampla variedade de materiais pode ser usada e altas concentrações de biomoléculas ativas são imobilizadas. As matrizes mais

comumente empregadas são as gelatinas, poliacrilamida, colágeno, triacetato de celulose, alginato e outros (DATTA; CHRISTENA; RAJARAM, 2013).

A imobilização por meio de ligação covalente proporciona uma maior estabilidade aos imunossensores e é mais amplamente utilizada. A ligação é realizada quimicamente por meio de grupos funcionais do material biológico que não sejam essenciais a sua atividade catalítica, sendo necessário certo conhecimento de sua estrutura com os grupamentos ativos do suporte, tais como –OH, -NH_2, -COOH, -SH. A ligação covalente geralmente envolve três etapas: (i) a ativação da superfície do sensor, (ii) o acoplamento do elemento de reconhecimento e (iii) remoção das moléculas fracamente ligadas. As condições experimentais ótimas para cada etapa devem ser determinadas. É importante que os sítios ativos nas biomoléculas não sejam afetados pelo processo de imobilização, para que não ocorra perda da atividade biológica (DATTA; CHRISTENA; RAJARAM, 2013).

Ligações covalentes cruzadas, geralmente, resultam em uma grande quantidade de moléculas biológicas imobilizadas, uma vez que se formam ligações entre estas, além de ligações entre moléculas biológicas-suporte. Porém, apesar de ser um método bastante simples, nem sempre é fácil controlar as condições experimentais e, com isso, a integridade do elemento biológico pode ser alterada (DATTA; CHRISTENA; RAJARAM, 2013).

A proteína A desempenha um papel fundamental no desenvolvimento dos imunossensores, funcionando como um elo de ligação entre a superfície do eletrodo, por meio de uma plataforma polimérica e o anticorpo que detectará o antígeno específico. A proteína A é um receptor de membrana altamente estável, produzido por cepas de *Staphyloccocus aureus* (BABACAN et al., 2000). É bastante utilizada como molécula de orientação de anticorpos ligando-se a fração Fc (região constante do anticorpo com atividade biológica importante no controle da infecção) e deixando a fração Fab (região variável do anticorpo responsável pelo reconhecimento do antígeno) de imunoglobulinas livres sobre a superfície. Essa orientação é importante para o aumento da especificidade, estabilidade e sensibilidade do imunossensor (BERGSTRÖM; MANDENIUS, 2011).

Vários imunossensores têm sido elaborados com a utilização da proteína A como agente de orientação na imobilização de anticorpos, com resultados promissores (DERKUS et al., 2014; LEE et al., 2003; SALMAIN et al., 2012; SU; LI, 2005; SUN; ZHU; WANG, 2011; TANG; YUAN; CHAI, 2008). Pimenta-Martins et al., (PIMENTA-MARTINS et al., 2012) testaram três métodos de imobilização da proteína A, para o desenvolvimento de um imunossensor amperométrico para a detecção de enterotoxinas estafilocócicas. Foram comparadas as respostas eletroquímicas do dispositivos desenvolvidos através da adsorção

direta, utilização de glutaraldeído e estabelecimento de ligações covalente dos grupamentos carboxílicos por ativação com N-hidroxisuccinimida/N-(3-dimetilaminopropil)-N'-etilcarbodiimida (EDC/NHS). O último método apresentou melhor resposta e foi selecionado para a elaboração do imunossensor, o qual apresentou resultados satisfatórios na detecção da enterotoxina A de *S. aureus*.

Uma etapa importante no desenvolvimento de um imunossensor eletroquímico que usa marcação é a escolha do marcador do anticorpo secundário e do procedimento utilizado para a obtenção da resposta analítica. Devido à facilidade de acesso a partir de fontes comerciais e à sua extensa aplicação em ensaios imunológicos, o uso da peroxidase de raiz forte (*Horseradish Peroxidase* - HRP), conjugada ao anticorpo secundário tem sido a enzima preferida desde os exemplos pioneiros de imunossensores eletroquímicos (RICCI; ADORNETTO; PALLESCHI, 2012). A enzima HRP participa das reações de oxi-redução, gerando a resposta que é mensurada através da variação da corrente elétrica. Essa corrente por sua vez, é proporcional a quantidade do analito presente na amostra.

A HRP é, atualmente, usada em uma grande variedade de sistemas analíticos e de diagnósticos importantes em laboratórios clínicos. Ela vem sendo empregada com sucesso no desenvolvimento de diversos imunossensores (DAI; LIU, 2019; NIU et al., 2020). De acordo com Trakhtenberg et al. (2005), trata-se de uma enzima estável por longos períodos de tempo a temperatura ambiente. O pH ótimo para essa enzima varia na faixa de 6,0 a 6,5, sendo estável na faixa de pH entre 5,0 a 9,0 (TRAKHTENBERG et al., 2005). A estabilidade das peroxidases é muito importante para sua utilização em diversas áreas da ciência, pois quanto maior a estabilidade e a atividade enzimática, melhor a capacidade de aplicação da enzima em diversos métodos de análises.

3.2.3 Técnicas eletroquímicas

As técnicas eletroanalíticas envolvem o estabelecimento de relações entre a concentração do analito e algumas propriedades elétricas, tais como, corrente elétrica, potencial elétrico, condutividade elétrica, resistência elétrica e carga elétrica. Será avaliada a variação da corrente elétrica em função do potencial, e todo processo é realizado numa célula eletroquímica (SKOOG; HOOLER; CROUCH, 2009).

As vantagens de utilização desta técnica são citadas a seguir:

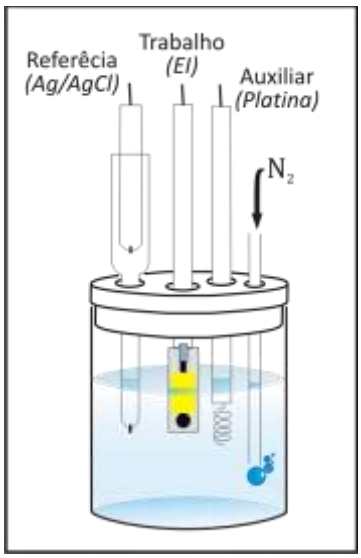
- As medidas eletroquímicas são específicas para cada estado de oxidação de um elemento;

- A instrumentação é relativamente mais barata e manipulação mais simples;

- Fornecimento de informações sobre atividade e concentração de espécies químicas novas aos mais variados meios, sem separações químicas ou tratamentos prévios.

Em geral, estas técnicas utilizam três eletrodos para a realização das análises (FIGURA 1). O eletrodo de trabalho possui potencial variado e podem ser de platina, ouro, mercúrio, grafite, fibra de carbono ou metais com superfícies modificadas. O eletrodo de referência , possui potencial constante e pode ser de prata/cloreto de prata (Ag/AgCl), calomelano saturado (Hg/Hg_2Cl_2) ou hidrogênio (H_2) de acordo com o meio de trabalho.O eletrodo auxiliar ou contra-eletrodo permite a circulação dos elétrons (e-) envolvidos do processo de redução e/ou oxidação e geralmente é um metal inerte (fio ou placa de platina) (SKOOG; HOOLER; CROUCH, 2009).

Figura 1- Ilustração de uma célula eletroquímica.



Fonte:SILVA, 2010

3.2.3.1 Sistema eletroquímico

Entre o eletrodo de trabalho e o eletrodo de referência é observada a diferença de potencial entre o eletrodo auxiliar e o eletrodo de trabalho e são medidas as correntes que originam as curvas de trabalho, variação de potencial.

As correntes elétricas que fluem por um sistema eletroquímico se apresentam como somatório de três componentes.

- Corrente residual (ir);

- corrente faradaica;

- corrente capacitiva.

- Corrente de migração (im);
- Corrente de difusão ou difusional (id).

Onde:

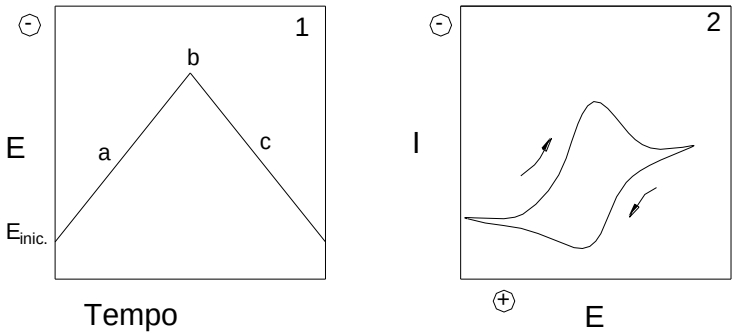
itotal = ir + im + id Equação 1

A corrente residual é composta por duas sub-componentes; a corrente faradaica que é produzida na oxidaçãoe/ou redução de traços de alguma espécie eletroativa indesejável, e a corrente capacitiva que se dá devido à formação da dupla camada elétrica na superfície do eletrodo de trabalho. A corrente de migração é formada a partir da redução ou oxidação da espécie eletroativa que chega a superfície do eletrodo por atração eletrostática, que aumenta com a concentração da espécie eletroativa e depende da carga do íon. Uma das maneiras de "eliminar" é a adição do eletrodo suporte, muito concentrado, tornando a corrente de migração desprezível. A corrente difusional resulta do movimento dos íons em todas as direções devido a um gradiente de concentração existente entre o eletrodo de trabalho e o seio da solução (SKOOG; HOOLER; CROUCH, 2009).

3.2.3.2 Voltametria cíclica – VC

Na voltametria cíclica, uma varredura de potenciais de forma triangular é imposta ao eletrodo de trabalho, de maneira que a varredura parte de um potencial inicial (a) seguindo até o potencial de inversão (b) e retornando, geralmente, ao potencial inicial (c). A Figura 2 apresenta a forma de aplicação do potencial na voltametria cíclica e voltamograma resultante para um sistema totalmente reversível.

Figura 2 - Forma de aplicação do potencial (1) com a) varredura direta, b) potencial de inflexão e c) varredura inversa, e resposta de corrente-voltagem na Voltametria Cíclica (2)



Fonte: SKOOG; HOOLER; CROUCH, 2009

Nas curvas de corrente-voltagem obtidas, chamadas de voltamogramas, as respostas, com relação à corrente são caracterizadas por um pico que é proporcional à concentração da espécie e governada pela equação de Randles-Sevcik:

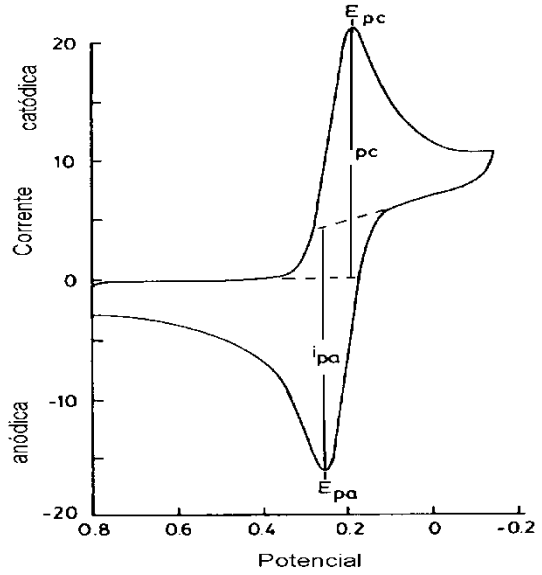
$$i_p = 2{,}69 \times 10^5 \cdot n^{3/2} \cdot A \cdot D^{1/2} \cdot v^{1/2} \cdot C$$

Equação 2

Onde *ip* é a corrente de pico, *n* é o número de e^- envolvidos da reação, *A* é a área do eletrodo de trabalho, *D* coeficiente de difusão da espécie eletroativa, *v* é a velocidade de varredura e *C* é a concentração da espécie no seio da solução (SKOOG et al. 2006).

Se considerarmos a reação onde o potencial é mudado para um valor onde ocorra redução, a forma oxidada (O) é convertida na forma reduzida (R) de acordo com a Equação de Nernst e a corrente resultante é chamada de corrente de pico catódica e por convenção é positiva. Se o processo redox for totalmente reversível, e o potencial for invertido e retornar ao potencial onde ocorre a redução, surge um pico reverso chamado pico anódico. Vejamos a seguir na Figura 03 que apresenta um voltamograma teórico típico de um processo redox totalmente reversível, onde ipa é a corrente de pico anódica proviniente da oxidação da espécie redox e ipc é a corrente de pico catódica, proveniente da redução (SKOOG; HOOLER; CROUCH, 2009.)

Figura 3 - Voltamograma cíclico, teórico, típico de um processo redox totalmente reversível (Epc) Potencial do pico catódico, (Epa) Potencial do pico anódico, (ipc) Corrente do pico catódico, (ipa) corrente do pico anódico



Fonte: SKOOG; HOOLER; CROUCH, 2009.

No desenvolvimento de imunossensores, especialmente nas etapas de montagem e caracterização, a voltametria cíclica é uma técnica bastante aplicada. A imobilização de biomoléculas na superfície do eletrodo promove mudanças na sua área eletroativa. O uso de soluções com espécies redox, como por exemplo o ferricianeto de potássio ($K_3[Fe(CN)_6]$), permite a observação de tais mudanças através da comparação dos voltamogramas obtidos após cada etapa de imobilização. A voltametria cíclica também é bastante útil nas etapas de otimização de concentrações de biomoléculas permitindo selecionar concentrações ótimas com base na observação da resolução dos picos dos voltamogramas (oxidação ou redução) e dos valores de corrente elétrica. Além disso, também fornece informação importantes para a obtenção da resposta analítica do biossensor como, por exemplo, a determinação do potencial que será aplicado na cronoamperometria.

3.2.3.3 Cronoamperometria

A cronoamperometria é uma técnica eletroquímica que determina a corrente que flui pelo eletrodo de trabalho em função do tempo, com potencial aplicado constante. Esse fluxo de corrente é correlacionado com o gradiente de concentração das espécies oxidadas ou reduzidas na superfície do eletrodo de trabalho. Trata-se de um método eletroanalítico no qual

a magnitude do sinal elétrico (corrente, decorrente de reação redox do substrato em estudo) é medida em função do tempo quando um potencial constante é aplicado ao eletrodo de trabalho (DA SILVA et al., 2018; FERREIRA; AVACA, 2008).

Os imunossensores cronoamperométricos são uma classe de biossensores eletroquímicos projetados para medir um fluxo de corrente desencadeado por uma reação eletroquímica em um potencial constante num determinado tempo. A especificidade do reconhecimento molecular do antígeno por anticorpos forma um complexo estável que é o princípio de funcionamento do dispositivo. A resposta amperométrica pode ser direta (não marcada), onde as mudanças físico-químicas causadas durante a formação do complexo antígeno-anticorpo for detectada, ou indireta (marcada) onde marcadores enzimáticos geram o sinal de resposta analítica (MISTRY et al., 2016).

Para o diagnóstico *point of care*, os métodos de detecção amperométrica atraíram atenção devido à configuração simples e de baixo custo. Além disso, os baixos requisitos de energia tornam a detecção eletroquímica altamente atraente. A combinação de sistema amperométrico com um imunoensaio altamente específico mostra grande potencial para criar uma plataforma simples de diagnóstico para detecção de bactérias (HIRAIWA et al., 2016). Tais facilidades tornam essa classe de imunossensores uma das mais aplicadas em estudos para detecção de bactérias (MELO et al., 2016; SILVA et al., 2018).

3.3 GOMA DE CAJUEIRO

Diversas técnicas e materiais são estudadas buscando a produção de novas plataformas para a montagem de dispositivos simples e versáteis, incluindo carboidratos, proteínas, nanoparticulas, corantes, ácidos nucleicos e gomas naturais (EIRAS et al., 2010; FELDHEIM et al., 1996; LADAM et al., 2000; PEI et al., 2001). Existem alguns relatos bem-sucedidos usando quitosana (HERNÁNDEZ-IBÁÑEZ et al., 2016; LAKARD et al., 2011) e goma de cajueiro (ARAÚJO et al., 2012; CASTRO et al., 2017; SILVA et al., 2010). A goma de cajueiro (GC) possui propriedades interessantes como atoxicidade, biodegradabilidade e biocompatibilidade, sendo esta última especialmente atrativa, pois favorece a estabilidade das biomoléculas, que podem ser imobilizadas nesses filmes (PAULA et al., 2012). Neste contexto, os polímeros naturais vêm sendo aplicados com sucesso para o desenvolvimento de imunossensores visando a melhoria de sensibilidade e/ou seletividade, baseados na confecção de sensores modificados química ou biologicamente.

A goma de cajueiro (GC) é um heteropolissacarídeo complexo isolada do exsudado de cajueiros (*Anacardium occidentale* Linn.) como resposta às injúrias e ataques de patógenos. Apresenta alta disponibilidade na região Nordeste do território brasileiro, podendo gerar lucros no período da entressafra do caju. Estudo realizado por De Paula et al., (DE PAULA; HEATLEY; BUDD, 1998) caracterizou a GC extraída do exsudado de árvores da região nordeste como um heteropolissacarídeo ramificado contendo: β-D-galactose (72-73%), α-D-glucose (11-14%), arabinose (4-6,5%), ramnose (3,2-4%) e ácido glucurônico (4,7-6,3%) em porcentagem de massa. A GC possui propriedades interessantes como alta hidrofilicidade e a liberação de íons (polieletrólito) em solução aquosa.

O Brasil tem aproximadamente 710.000 ha de área plantada com cajueiros e uma produção média de goma/árvore/ano de 700 g, com um potencial de produção anual de GC superior a 38.000 toneladas (ARAÚJO et al., 2012). O interesse tecnológico na GC está fundamentado em suas similares características reológicas e aplicações industriais a muitos polímeros sintéticos, provenientes principalmente de sua biodegradabilidade e propriedades mecânicas. A ampliação do uso da GC com fins biotecnológicos vai além das fronteiras brasileiras e diversas aplicações já estão em prospecção (ARAÚJO et al., 2012; PAULA et al., 2012; RIBEIRO et al., 2016).

Silva et al., (SILVA et al., 2010) descrevem o uso do polissacarídeo de goma de caju (CGP) para imobilização da peroxidase (HRP). A imobilização covalente do HRP foi otimizada e permitiu atingir um rendimento de 25,4% quando 98,2 U de HRP foram adicionados a 15 mg de CGP ativado com glutaraldeído. Dessa forma observou-se que o polímero CGP foi utilizado para imobilizar a HRP com uma eficiência muito boa e CGP – HRP constitui um sistema alternativo muito interessante para fins analíticos.

Araújo et al. (2012) relataram uma experiência positiva na utilização de plataformas com GC para biossensores. O grupo de pesquisa estudou a GC para a formação de filmes utilizando a técnica *Layer by Layer* (LbL) para aplicação em dispositivos nanobiomédicos como os sensores eletroquímicos para o neurotransmissor da dopamina. Observou-se que a GC permitiu a obtenção de filmes estáveis com processos redox bem definidos e que podem detectar o analito dopamina com um limite de detecção relevante para a indústria farmacêutica. Morfologicamente, a GC formou filmes finos pontuados por grandes características globulares, exibiu valores de rugosidade bastante baixos, e recobriu completamente o substrato.

Castro et al. (CASTRO et al., 2017) investigaram a eletrossíntese e as características eletroquímicas, morfológicas e topográficas de um novo compósito condutor

de polipirrol / goma de caju (PPy / CG). Os filmes compósitos foram eletrossintetizados na superfície do ouro por voltametria cíclica e cronoamperometria em meio aquoso. Os compósitos evidenciaram a incorporação do polieletrólito GC no filme PPy, que possuía uma morfologia granular e nodular com tamanho de grão variando de 0,7 a 2,0 μm. Além disso, os filmes produzidos por voltametria cíclica e cronoamperometria apresentaram carga anódica de 3,83 e 4,34 mC cm^{-2}, respectivamente. Os dados também mostraram que a rugosidade da superfície do polipirrol foi fortemente afetada pela concentração de GC. Este estudo demonstrou a viabilidade de produzir um filme condutor de base biológica alternativo através da eletrodeposição de PPy / CG e forneceu informações úteis sobre suas propriedades estruturais e eletroquímicas.

Os possíveis métodos de processamento da goma de cajueiro dependem de modificações químicas destinadas a desenvolver características funcionais que tornam este material versátil e útil em diversas aplicações. Modificações químicas, como oxidação, acetilação, hidroxilpropilação, carboximetilação e reticulação, fornecem uma rota eficiente a fim de melhorar as propriedades físico-químicas e introduzir novas propriedades aumentando as possibilidades de aplicação do polímero quimicamente modificado. A goma de cajueiro nativa pode ser modificada em derivados solúveis em água usando grupos reativos para substituir os grupos hidroxila livres ao longo da macromolécula (OLUSOLA; OLUTAYO, 2014).

Modificações químicas da GC são realizadas visando a expansão do seu uso industrial. A carboximetilação de polímeros naturais melhora a eficiência da imobilização de biomoléculas em sua superfície pois, aumenta a quantidade de grupos COOH presentes em pequenas quantidades na superfície de filmes poliméricos não modificados quimicamente, apresentando-se como uma técnica muito promissora para desenvolvimento de dispositivos analíticos com plataformas à base de GC (HEBEISH; KHALIL, 1988a). O uso do complexo N-(3-dimetilaminopropil)- N’-etilcarbodiimida/ N-hidroxisuccinimida (EDC/NHS) para a ativação de grupos COOH e estabelecimento de ligações covalentes entre biomoléculas é bastante consolidado nos estudos de biosensores (RICCI; ADORNETTO; PALLESCHI, 2012). A novidade da presente proposta será a utilização da GC carboximetilada como plataforma de imobilização covalente de biomoléculas a partir da ativação de seus grupos COOH pelo complexo EDC/NHS. Esta aplicação amplia o potencial de aplicações da GC não somente para biossensores, como também para o desenvolvimento de outros produtos biotecnológicos. Além disso, são geradas novas demandas de consumo para GC aumentando seu potencial econômico, nos países produtores.

4 ARTIGOS

CAPÍTULO I Electrochemical immunosensors for *Salmonella* detection in food

Appl Microbiol Biotechnol (2016) 100:5301–5312 DOI 10.1007/s00253-016-7548-y

Electrochemical immunosensors for *Salmonella* detection in food

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Abstract

Pathogen detection is a critical point for the identification and the prevention of problems related to food safety. Failures at detecting contaminations in food may cause outbreaks with drastic consequences to public health. In spite of the real need for obtaining analytical results in the shortest time possible, conventional methods may take several days to produce a diagnosis. *Salmonella* spp. is the major cause of foodborne diseases worldwide and its absence is a requirement of the health authorities. Biosensors are bioelectronic devices, comprising bioreceptor molecules and transducer elements, able to detect analytes (chemical and/or biological species) rapidly and quantitatively. Electrochemical immunosensors use antibody molecules as bioreceptors and an electrochemical transducer. These devices have been widely used for pathogen detection at low cost. There are four main techniques for electrochemical immunosensors: amperometric, impedimetric, conductometric and potentiometric. Almost all types of immunosensors are applicable to *Salmonella* detection. This article reviews the developments and the applications of electrochemical immunosensors for *Salmonella* detection, particularly the advantages of each specific technique. Immunosensors serve as exciting alternatives to conventional methods, allowing "real-time" and multiple analyses that are essential characteristics for pathogen detection and much desired in health and safety control in the food industry.

Keywords: *Salmonella*; immunosensor; food safety; pathogen; rapid detection

Introduction

Salmonella is a major foodborne pathogen in the world and can infect animals and humans resulting in morbidity and mortality (CDC 2012; EFSA 2014). Genus *Salmonella* is composed of two species, *Salmonella enterica* and *Salmonella bongori*, seven subspecies, and more than 2,500 serovars (based on antigenic composition), all of which are believed to be capable of causing human illnesses, such as typhoid fever (serovar Typhi), paratyphoid fever (serovar Paratyphi), and gastroenteritis (all other serovars) (FDA 2012). *Salmonella enterica* serovars Typhimurium and Enteritidis are the most commonly identified in foodborne diseases worldwide (CDC 2012; EFSA 2014). Conventional methods of detecting *Salmonella* in food follow a complex sequence of steps. Typically, it entails a nonselective pre-enrichment step, followed by selective enrichment, isolation on selective agar media, bacterial identification by biochemical testing, and serotyping - the entire process taking at least five days to reach a diagnosis (Andrews; Jacobson; Hammack 2015).

Official food safety agencies, such as U.S. Food and Drug Administration (FDA), U.S. Department of Agriculture (USDA), Association of Official Analytical Chemist International (AOACI), and International Organization of Standardization (ISO) recommend conventional culture methods as the most reliable and accurate techniques for foodborne pathogen detection. Nevertheless, advances in technology and innovations have given microbiological laboratories a variety of kits and instruments based on different mechanisms of detection such as Polymerase Chain Reaction (PCR) and Enzyme Linked Immuno Sorbent Assay (ELISA), which take less time than the conventional methods. Many of these tests have emerged as alternatives to conventional methods to reduce the analysis time (Velusamy et al. 2010). Although easy to perform, most of these alternative tests need 24 h for pre-enrichment in order to increase target bacteria population and reach the detection limits of the tests (Lee et al. 2015). In this context, there is an increased interest in having rapid new methodologies with the advantages of rapid response (without pre-enrichment step), high sensitivity, and ease of multiplexing (readings of many samples simultaneously) in order to address the current challenges in food hygiene inspection.

Biosensors are analytical devices, consisting of three associated elements: a bioreceptor or biological recognition element; a transducer (an electronic part which converts a biochemical signal from the interaction between analyte and bioreceptor into an electronic signal); and a processor, which amplifies and displays the analytical response signal. These innovative bioelectronic devices have a wide range of applications, such as diseases diagnosis, biomedicine, food processing, food safety, environmental monitoring, national defense and security (Velusamy et al. 2010; Su et al. 2011; Holford et al. 2012 Saleem 2013). Currently the most widespread application of this analytical tool is found in health care for the quantification of some substances produced by the human body, such as glucose, lactate, and cholesterol.

Most portable commercial biosensors have electrochemical transducers, which are easier to use in automatic devices (Skládal et al. 2013). Many companies have fabricated this type of biosensors especially for glucose detection. Electrochemical techniques are very sensitive and when associated with biomolecules it is possible to enhance the specificity of analysis. In general, electrochemical signal involved in analytical response depends on electronic movements resulting from oxidation-reduction reactions captured by the transducer. Clark and Lyons (1962) reported the first electrochemical biosensor by immobilizing glucose oxidase on the surface of an oxygen electrode. Since then, numerous types of biosensors have been developed for various substances in different areas. At present, there are biosensors capable of determining molecules involved in food quality control (Arora et al. 2011; Niraj 2012; Mortari and Lorenzelli 2014), biomedical and drug sensing (Vidal et al. 2013; Vilarino et al. 2009) and toxicity analysis in the environment (Gil and Mello 2010; Qureshi et al. 2012; Singh et al. 2014; Burcu and Kemal 2015). Biosensors have improved as a result of improved molecular and biochemical understanding of analytical response and supporting technologies. For these reasons, it is possible to find today biosensors that are very small, cheap and interface-friendly. Currently, different types of biosensors are classified by the type of biological molecules immobilized (genosensors, imunosensors), by interaction with analyte (catalytic or enzymatic), by analytical response (direct or indirect), or by transducer (electrochemical, optical, or acoustic wave).

Antibodies represent one group of biomolecules, which interact readily with different types of analytes, especially biological contaminants such as bacteria and viruses, via specific recognition of their antigens (Holford et al. 2012). An immunosensor is a biosensor having an antibody on the surface as a bioreceptor, and it functions similarly as ELISA, except that it is faster, cheaper and easier to handle as detailed in the next sections. The ELISA has been applied as a ‘gold-standard’ for the validation of all recently developed immunoassays and immunosensors. One of the first papers on the use of immunosensors was written by Vo-Dinh et al. (1987); they demonstrated that antibodies could be engaged *in situ* for chemical carcinogen detection.

Analytical methods must overcome different challenges to detect bacteria efficiently. First, these detection methods have to be rapid to permit adoption as an emergency measure when necessary. Secondly, a high sensitivity is required, since the presence of even a single strain of pathogenic bacterium is able to develop an infection depending on the health status of the infected body and the virulence of the microorganism. Thirdly, detection must be extremely selective, especially in food, because a low number of pathogenic bacteria are often present in a complex matrix with proteins, fat, carbohydrates, hormones and other nutrients. These kinds of molecules can hide the presence of bacteria. All the above challenges can be met by an electrochemical immunosensor, which proves to be a powerful tool in bacteria detection and prevention of bacterial outbreaks. The purpose of this paper is to review the developments and the applications of electrochemical immunosensors for

Salmonella detection in food and to document the procedures for evaluation and characterization of the performance of immunosensors.

Alternative methods for *Salmonella* detection

A wide variety of alternative methods for *Salmonella* detection has been developed, and they can be grouped into several categories. Based on their operational principles, we can distinguish three main groups of techniques, immunology-based assays, nucleic acid-based assays, and biosensors. Among these methods, ELISA and PCR procedures have the specificity and the sensitivity that are almost similar to conventional methods. ELISA assays are able to detect *Salmonella* concentration at the level of 10^5 UFC mL^{-1} while PCR-based assays provide a level of sensitivity at 10^4 UFC mL^{-1} after pre-enrichment step (Lee at al. 2015). The sensitivity and specificity of these methods can be strongly altered by the intrinsic characteristics of the food involved, such as background microbiota, sample matrix, presence of non-culturable cells, and inhibitory substances (e.g., fats, proteins, carbohydrates, heavy metals, antibiotics, and organic compounds) (Mortari and Lorenzelli 2014). Thus, comparative studies are necessary to ensure that a particular assay is effective in analyzing a specific type of food. Some alternative tests have distinctive performance characteristics and are applicable to a restricted array of food.

Several papers reported comparisons between alternative and conventional methods. For example, Margot et al. (2013) compared methods for the detection of *Salmonella* species using pure cultures of *Salmonella* and others bacterial species commonly found in food products and concluded that rapid methods were as sensitive and specific as the conventional methods. Sometimes difficult matrices such as black tea can pose a problem with false negative results due to atypical colony colors. Therefore, rapid methods have some limitations in its application as will be shown in the following discussions on the three major categories.

Immunology-based assays

Immunology-based assays have been often used for the detection of *Salmonella* spp; they generally employ specific antibodies that bind with antigens. This type of assays includes ELISA tests, latex agglutination tests, immunodiffusion, and immunochromatography. As in other rapid tests, these methods have some potential drawbacks for *Salmonella* detection such as the need of a prior pre-enrichment step to recover stressed cells, cross-reactions with closely related antigens, antigen variation, limits in sensitivity for some sample matrices, and high cost for automation and application to industrial scale.

Among immunology-based assays, ELISA has been the most commonly used for *Salmonella* detection with several commercial kits available on the market. ELISA method is a biochemical technique used to detect the presence of an antibody or an antigen in a sample. Briefly, it involves immobilization of a

biomolecule (an antibody or antigen) onto a solid surface – with enzymes being used as markers for the presence of a specific antibody-antigen coupling. As examples, the most used commercial kits for *Salmonella* detection are Assurance GDS™ for *Salmonella* (BioControl Systems, Inc., Bellevue, WA), TECRA *Salmonella* (Tecra International Pty Ltd, French Forest, New South Wales, Australia), *Salmonella* ELISA Test SELECTA/OPTIMA (Bioline APS, Denmark), and Vitek Immuno Diagnostic Assay System (VIDAS) (BioMerieus, Hazelwood, MO) (Lee et al. 2015).

Nucleic acid-based assays

Also known as molecular methods, the nucleic acid-based assays are tests that utilize a specific nucleic acid target sequence within the organism's genome (in this specific case, bacterial genome). The most widespread technique in this category of tests is the PCR method, a procedure based on the specific amplification of a short target DNA sequence. In recent years, molecular methods have attracted attention by providing enough specificity and sensitivity for detecting only one molecule of the target DNA in a defined sample. Because of the capability to detect a low concentration of *Salmonella*, enrichment times are considerably shorter to reach the *Salmonella* concentration needed for reliable detection by PCR when compared to other assays. However, tests based on nucleic acids (DNA and RNA) have some limitations, because they are specific in identifying genes and cannot pick out viable bacteria or detect the presence of toxins (Feng et al. 2010). Examples of commercial rapid tests based on PCR for *Salmonella* detection include ABI Prism 7500 (Applied Biosystems, Warrington, UK), Probelia (Sanofi -Diagnostics Pasteur, Marnes-la- Coquette, France), BAX system (DuPont Qualicon, Wilmington, DE), TagMan (PE-Applied Biosystems, Foster City, CA), Gene-Trak (Neogen Corporation, Lansing, MI), iQ-Check™ PCR (BioRad Laboratories, Hercules, CA), LightCycler (Roche Diagnostics, Manheim, Germany), and SmartCycler (Cepheid Inc., Sunnyville, CA) (Lee et al. 2015).

Biosensors

Microbial biosensor represents a rapidly developing research area, and there are numerous publications in this area. Biosensors have the potential to shorten the time between sampling and results, but they need improved selectivity and sensitivities and reduced cost, when compared to other methods. The use of biosensors permits both miniaturization and automation. It is possible to work with sample volumes in the range of nanoliters or less, which implies a lower cost of reagents. Also, multi-analyte analysis can be done in the same device, which shortens the analysis time. Biological recognition elements used in the biosensor application include enzymes, antibodies, nucleic acids, whole cells, tissue/whole organisms, and biomimetic materials. The signal recognition of biosensors is achieved through different types of transducers: electrochemical, optical, thermometric, and piezoelectric. There are many papers related to the development of biosensors for *Salmonella* detection (e.g., Afonso et al. 2013; Dong et al. 2013; Chumyin et al. 2014; Freitas et al. 2014; Hu et al.

2014; Ma et al. 2014). Table 1 lists some immunosensors including their detection limits and detection times.

Table 1 – List of eletrochemical immunosensors for *Salmonella* detection

Method	Limit of detection	Detection time	Reference
Impedimetric	10 CFU mL^{-1}	3 h	Pournaras et al. (2008)
	5 x 10^2 CFU mL^{-1}	6 min	Nandakumar et al. (2011)
	10^5 CFU mL^{-1}	2 h	Mantzila et al. (2008)
	5 x 10^2 CFU mL^{-1}	1 h	Dong et al. (2013)
	10^2 CFU mL^{-1}	40 min	Yang et al. (2009)
	3 cells mL^{-1}	Not reported	Ma et al. (2014)
Amperometric	6 CFU mL^{-1}	Not reported	Zhu et al. (2014)
	10^6 CFU mL^{-1}	3 h	Delibato et al. (2006)
	143 cells mL^{-1}	1.5 h	Afonso et al. (2013)
	20 cells mL^{-1}	Not reported	Salam and Tothil (2009)
	5 x 10^3 CFU mL^{-1}	50 min	Liébana et al. (2009)
	1.95 x 10^2 UFC mL^{-1}	Not reported	Hu et al. (2014)
	13 cells mL^{-1}	1 h	Freitas et al. (2014)
	5 x 10^4 CFU mL^{-1} in BHI; 10^4 CFU mL^{-1} with nanoparticles	1 h	Brandão et al. (2013)
Condutimetric	7.9 x 10 UFC mL^{-1}	10 min	Muhammad-Tahir and Alocilja (2003)
Potentiometric	119 UFC mL^{-1}	Not reported	Dill et al. (1999)

Assembly and evaluation of electrochemical immunosensors

The basic composition of an immunosensor consists of antibodies immobilized on an electrode surface. The surface must have appropriate electrochemical characteristics, in addition to being compatible with the immobilization method. A wide variety of materials can be used for the surface; among them gold has been applied most frequently (Ricci; Adornetto; Palleschi 2012), because it is an inert metal and compatible with cell structures and biomolecules. However, gold and other metals normally do not allow adhesion of biomolecules; therefore, they require some form of surface modification. Self-assembled monolayers (SAMs) are highly ordered molecular assemblies formed spontaneously by chemisorption and self-organization of molecules on the surface (Prashar 2012). This technique has been recently applied to modify the electrode surface and found to be useful in immunosensor assembly because it allows a high degree of control of the composition and thickness of the transducer surface. Surface functional groups (-CN, -NH2 or –SH) on SAMs form covalent bond with biomolecules and metal surface and serve as bridges among them. Short-chain molecules

such as cysteamine can be self-assembled on the electrode and confer a lower degree of blockage for electron transfer than long-chain molecules (Anandam; Gangadharan; Zhang 2009). Thus, the biomolecule which is used in SAM formation must be chosen carefully to match the type of transducers used.

Antibody immobilization on sensor surface is considered a critical point to the sensitivity and specificity determination of immunosensor. There is no perfect immobilization method that provides high sensitivity and superior stability for these devices. Optimization studies for each kind of device must be carried out to produce the best responses. In the literature some methods have been reported that orient the antibodies, leaving the antigen recognizing region (paratope region) free while the Fragment crystallizable (Fc) region of the antibody is surface-bound. Oriented immobilization of antibodies through protein A and protein G has been successfully achieved in the process of immunosensor development (Liu et al. 2012; Ferreira and Sales 2014; Derkus et al. 2014; Cao et al. 2015).

The basic structure of an immunosensor with antibody immobilization oriented by a protein is illustrated in Figure 1.

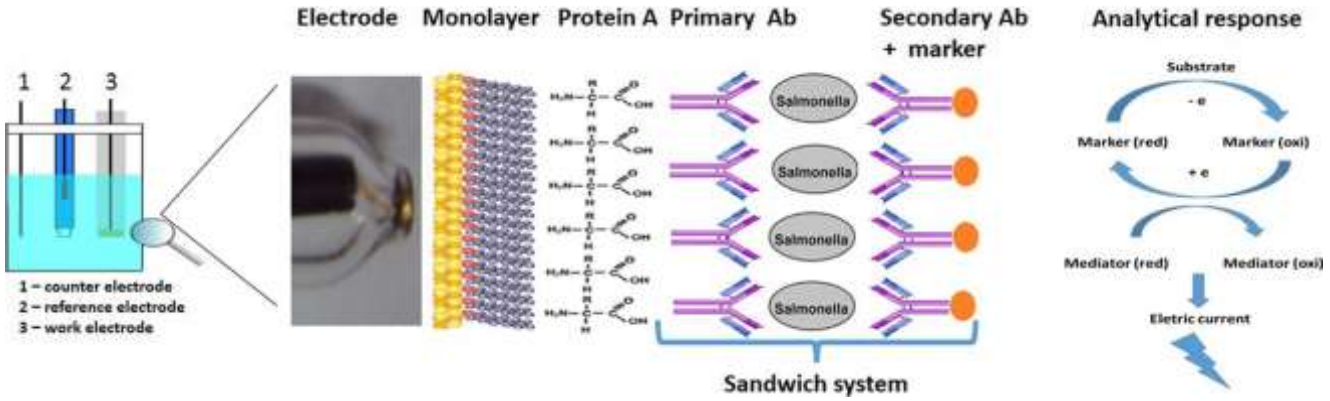

Fig. 1 Schematic diagram of the electrochemical immunosensor and the analytic response

In this figure, we schematically show the immunosensor (work electrode) enclosing an electrochemical cell containing the counter and the reference electrodes, thus forming an electrochemical system. In the next scheme the immunosensor structure is shown sequentially. First, a self-assembled monolayer (SAM) was formed on the electrode surface, and it acted as a bridge between the metal (electrode surface) and protein A linked by a covalent bond. This arrangement provides for protein A to bind to the primary antibody in order to recognize the antigen. A secondary antibody labeled with enzymes is used to generate an analytical response as a sandwich system. The enzymes function as markers because when conjugated to the secondary antibody they catalyze the reaction with their substrates. As products of this reaction, electroactive species emit analytical

response signal for the immunosensor (Figure 1). In indirect immunosensors, different kinds of markers can be utilized beyond the enzymes (Zhao at al. 2016), like biotin (Martín-Yerga et al. 2013), avidin (Kim and Choi 2014), and nanoparticles (Özel et al. 2014).

Mediators are commonly used in this type of biosensors even in commercial ones. They are called biosensors of second generation (Murugaiyan et al. 2014). Mediators are low molecular weight molecules that participate in redox processes with a high rate of electron transfer. During the catalytic reaction, the mediator reacts with the prosthetic group of enzyme and diffuses to the electrode surface in order to receive or transfer electrons (Dominguez-Benetton et al. 2013). Additionally, the work potential is determined by the oxidation/reduction potential. This application is important because in the presence of a mediator, the electrochemical reaction becomes less dependent on the oxygen concentration in the solution. The use of mediators in redox processes is advantageous also because they reduce the operating potential of the device, thus avoiding interference from unwanted redox species.

During immunosensor development, two factors need to be considered. The first factor is characterization, and it is necessary after each stage of assembly to confirm the efficiency of the immobilization set-up. The second factor is the evaluation of the device performance in real food samples, since this is the main goal of device development. These two factors are described below.

Characterization of immunosensors

The immunosensors may be characterized by using the same techniques employed for the analytical response of the device. In this case, each immunosensor is characterized by morphology, topography, electrochemical behavior, or by the presence of functional groups on the surface as determined by attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR). In amperometric immunosensors it is common for the electrochemical characterization to be done by cyclic voltammetry in potassium ferricyanide $K_3[Fe(CN)_6]$ solution. In this case, it is possible to observe the decrease in current electric amplitude of the cathode and anodic peaks due to isolation in the electron flow after each layer is formed on the electrode surface. $K_3[Fe(CN)_6]$ is a redox couple used in the studies of electrochemical characterization (Mantzila et al. 2008). Pimenta-Martins et al. (2012) characterized the changes on the surface during the immunosensor assembly using the redox probe $K_3[Fe(CN)_6/K_4[Fe(CN)_6]$. After pretreatment, the gold electrode was covered with cysteamine and the penetration of the redox probe close to the surface electrode was found to be slightly reduced. Subsequently, the immobilization of protein A on the modified gold electrode left the penetration of the redox probe further reduced. This fact was also validated from the binding of the (antibody-antigen- antibody) sandwich assembly.

Microscopy is often used for morphological and topographic studies (Kaur et al. 2004). This is important because each assembly step for an immunosensor causes changes on the surface that can be

monitored. Canbaz and Sezgintürk (2014) characterized the surface morphologies of the proposed biosensor layer. The modified surface with anti-HER-3 had an almost uniform granular morphology attributed to the dispersion of protein onto the surface. After application of bovine serum albumin (BSA) used for blocking active ends of the surface, the granular morphology of anti-HER-3 changed into an even more granular form due to the three-dimensional structure of BSA. Topographic characterization using atomic force microscopy (AFM) in the contact mode did not rupture the protein surface; thus, images of the protein-coated surfaces could be obtained (Hayes et al. 1998; Coen et al. 2001). Lee et al. (2003) verified that the surface topography was increased by depositing modified protein A, and there were antibodies immobilized onto the self-assembled protein A layers as an aggregate. Moreover, FTIR spectra show spectral features corresponding to the amide II bands of IgGs (β-sheet, main secondary structure element of IgG) indicating the presence of IgGs immobilized onto the film.

FTIR is a non-destructive spectroscopic technique recommended for the characterization of immunosensors in order to obtain information concerning the interfaces and the nature of the bonds in the material and at the interface. Many papers have reported the use of this technique to characterize immunosensor assembly. Sibai et al. (1996) verified that the FTIR spectrum of the antibody obtained was characteristic of a protein, for amide I was present around 1660 cm^{-1}, amide II around 1550 cm^{-1}, as well as the N-H band around 3300 cm^{-1} that was overlapped with O-H band at 3430 cm^{-1}.

Quartz crystal microbalance (QCM) technique can be very useful for characterizing the assembly of immunosensors and providing relevant information about interfaces and surfaces involved in the electrochemical response. This technique consists of mass sensitive detectors which operate on oscillating crystals resonating at the fundamental frequency of the quartz crystal (Babacan et al. 2000). Each change in the surface causes perturbations in the frequency of the crystal, which are associated with binding or desorption of molecules. QCM technique has been used to compare different immobilization methods and determine the best conditions for the biosensor response. Many papers have reported QCM characterization of immunosensors for *Salmonella* detection based on the layer-by-layer or the self-assembly technique for immobilization of the biomolecules (Pathirana et al. 2000; Si et al. 2001; Wong et al. 2002; Olsen et al. 2003). The results provided new perspectives on the development of amperometric sensors using a similar assembly system. Furthermore, the QCM technique has been used to confirm the specificity and applicability of immunosensors during repetitive use after the regeneration step (Prusak-Sochaczewski et al. 1990; Park et al. 2000).

Evaluation of immunosensors in real samples

After it is assembled, the immunosensor should be studied in order to optimize the analytical responses and to improve sensitivity and other operational parameters. Operational parameters include linearity, quantification limit, detection limit, accuracy, precision (reproducibility and repeatability),

and specificity. These parameters can also be influenced by temperature, pH and other environmental conditions. Parallel to these studies, the conditions that can reduce interfering substances present in solution should be verified. Sample preparation should be studied in order to understand the best form to submit to the biosensor. Undoubtedly, the preferred mode is to analyze the sample in nature, i.e., without any type of pretreatment, but for the most part, the sample needs to go through some pretreatments. Like other rapid tests such as immunological tests, the sample can preferably be centrifuged with a solvent in order to remove interfering elements such as fats in foods. For *Salmonella* detection, rapid testing kits usually also require a pre-enrichment step. Biosensors are advantageous in comparison to other methods because in general they do not need any sample treatment. Yet, in the specific case of imunosensors with a marker (i.e., not label-free), the final measurement depends on the addition of a substrate to the marker in a standard solution after the incubation step with the sample. For label-free immunosensors, the response is direct and depends exclusively on the nature of sample (not on fat or pH), if a response is obtained after contact with sample. In both cases, it is necessary to wash the sample after incubation time to eliminate all free substances that are not bound to the antibody.

Electrochemical immunosensors for *Salmonella* detection

Electrochemical immunosensors can be based on potentiometric, amperometric, impedimetric or conductometric transduction principles. A working electrode, a counter electrode, and a reference electrode usually compose the electrochemical sensor (Figure 1). In the specific case of immunosensors, antibodies are immobilized on the working electrode and the signal is generated as a function of electronic transfer which occurs between working electrode and counter electrode. This signal is proportional to analyte concentration present in the sample.

Bacteria present in food can promote reactions, and they can be detected by applying appropriate electrochemical methods. For example, when microorganisms metabolize uncharged substrates to a charged product, such as the conversion of carbohydrates to lactic acid, a change in the conductivity of the medium occurs. Microbial growth can be shown in the same way by an increase in both conductance and capacitance, causing a decrease in impedance. Another evidence of microbial metabolism can be verified by the hydrolysis of specific substances due to enzymatic activity at the microbial layer, and it can be accompanied by the production of protons near the pH electrode. The response comes from the change of electric potential difference between working electrode and reference electrode, which are separated by a selective membrane. Furthermore, the specific interaction between biomolecules, like an enzyme and its substrate or an antibody and its antigen, can produce an electronic transfer capable of generating an electric current in an applied potential, which is related to the concentration of the species in solution. These characteristics can be observed during bacterial presence in food and may be used for the development of different electrochemical

immunosensors for *Salmonella* detection. In the following section, we explore each of these features. Some examples of the techniques for immunosensor are summarized in Table 1.

Amperometric immunosensors

Amperometric measurements are based on electrical current between working and counter electrodes as a function of analyte concentration after applying a constant potential. This technique is preferred in the development of many biosensors. Most of the current commercial biosensors utilize this technique. In the literature, there are many papers with different modes of immobilization and detection using amperometric technique. It is necessary because each biological molecule has a preferred immobilization method and specific steps for analyte detection for any given application. In amperometric immunosensors, the direct sandwich ELISA format is very common in the device assembly, whereas it is possible to find a variety of immobilization methods for the primary antibody and labeling of the secondary antibody (Figure 1).

Amperometric immunosensors represent a modern version of ELISA, which often incurs false negative results due to extremely low amounts of contaminants in sample. Conventional ELISA for *Salmonella* spp. detection provides a limit of detection (LOD) of 10^4-10^5 CFU mL^{-1} (Lee et al. 2015). Some immunosensors reported have a detection limit much lower than the ELISA methods (Salam and Tothil 2009; Freitas et al. 2014; Zhu et al. 2014). As we can see in Table 1, there are amperometric immunosensors with LOD as low as 6 CFU mL^{-1} (Zhu et al. 2014). Another aspect observed is the detection time that varies from 50 min to 3 h in amperometric devices. Moreover, the main advantage of immunosensors using a sandwich system in a solution is the response that is free of interferences, thereby reducing the risks of false positives. This happens because bacteria bind to the primary antibody immobilized on the surface, and the response occurs as a function of the labeled secondary antibody that links to bacteria and remains in immunosensor surface after successive washes of surface. The washes are important in order to remove nonbonding molecules, and an electric current is produced by electron transfer from the substrate reaction (Figure 1). In this kind of immunosensor, it is very common to use mediators, which generally reduce the electrical potential of immunosensors. The decrease in electrical potential is important in reducing the chances of biomolecular denaturation and the interference with other substances in the sample. The main mediator used in biosensors is ferrocene (Morales et al. 2007), but there are other mediators such as ferricyanide and osmium that can be used (Bally and Voros 2009; Alonso et al. 2010; Vashist et al. 2011; Kirsch et al. 2013).

In our laboratory, we have worked with thiol and protein A in order to orientate the primary anti-*Salmonella* antibody and have achieved an excellent detection limit of 10 CFU mL^{-1}. Salam and Tothill (2009) immobilized monoclonal antibody against *Salmonella* Typhimurium using physical and covalent immobilization via amine coupling of carboxymethyldextran on gold surface. A sandwich ELISA format was developed using a polyclonal anti-*Salmonella* antibody conjugated to horseradish

peroxidase (HRP) as an enzyme label. An electron transfer mediator, 3,3,5,5-tetramethylbenzidine dihydrochloride (TMB) with H_2O_2 as substrate system, was utilized. Detection levels of 5 x 10^3 cells mL^{-1} and 20 cells mL^{-1} were achieved respectively for physical and covalent immobilization. Delibato et al. (2006) developed a multichannel electrochemical immunosensor for the detection of *Salmonella*. It consisted of a disposable screen-printed array, coupled with a multichannel pulse monitor, which was assembled in a sandwich system. Croci et al. (2001) preferred to seed the contaminated samples in pre-enrichment broth (buffer peptone water), and samples were taken at different times and analyzed by immunosensor to determine the minimum incubation time needed to detect *Salmonella*. The results showed that this method was efficient and sensitive only after 5 hours of incubation in pre-enrichment broth. It was possible to detect *Salmonella* in meat artificially contaminated with low concentrations of *Salmonella* (1-10 cells 25 g^{-1}).

In addition to the adoption of enzyme labels in this technique, magnetic particles are also widely used. Superparamagnetic particles are highly attractive for use in biosensors for their capability to magnetise under an applied magnetic field. Analytes can be labelled with magnetic beads as an immobilization platform and as a tool to separate measurable molecules found in immunosensors (Wang 2005). The particles can be separated easily from a liquid phase with a small magnet but can be redispersed immediately once this magnet is removed. When coated with recognition molecules, magnetic spheres are ideal for efficient capture and separation of target. Unwanted sample constituents may be washed away, following a simple magnetic separation step. Several procedures other than amperometric technique may be used for subsequent final measurements, such as conventional impendance and wave accoustic assays (Liu et al. 2001; Kim et al. 2003). More recently, magnetic beads have been used not only for labelling and separation of an analyte but also for direct quantification of antibodies with this particular label (Brzeska et al. 2004; Meyer et al. 2007). Gehring et al. (1996) used antibody coated superparamagnet beads in a format termed enzyme-linked immunomagnetic electrochemistry. *Salmonella* Typhimurium was sandwiched between antibody-coated magnetic beads and an enzyme-conjugated antibody. With the aid of a magnet, beads (with or without bound bacteria) were localized onto the surface of disposable graphite ink electrodes in a multi-well plate format. With this technique, a minimum detectable level of 8 x 10^3 cells mL^{-1} of *Salmonella* Typhimurium in a buffer was achieved in about 80 min.

Other nanoparticles such as carbon nanotubes and gold nanotubes have been used on the surface of electrodes in order to increase the active surface area for immobilization of biomolecules and consequently the sensitivity for the biosensor. Chumyim et al. (2014) developed an immunosensor with a detection limit of 10^3 CFU mL^{-1} *Salmonella* based on tyrosinase-amplified labeling platform and the recycling system of catechol/o-quinone redox couple with multiwall carbon nanotubes as an amplified labeling electrochemical sensor.

Impedimetric immunosensors for *Salmonella*

Impedimetric biosensors are less frequently compared to potentiometric and amperometric biosensors; nevertheless, there have been some interesting publications on these immunosensors. Impedimetric immunosensors function by antigen-antibody interaction causing a change in capacitance and electron transfer resistance across a working electrode (Pohanka and Skládal 2008). For these cases, electrochemical impedance spectroscopy (EIS) is often engaged to characterize the surfaces after the immobilization of biomolecules and binding of antigen. The most popular format for evaluating EIS data are the Nyquist and Bode plots (Wang et al. 2012). In the Nyquist plot, the imaginary impedance component (z") is plotted against real impedance component (z'). In the Bode plot, both the logarithm of the absolute impedance (|Z|) and the phase shift (φ) are plotted against the logarithm of the excitation frequency. Impedance immunosensors can be classified into two main categories according to Prodromis (2010): (a) capacitive - where the surface of the electrode is completely covered by a dieletric layer and the whole electrode assembly behaves as an insulator. In this type of sensor, no redox probe is present in the measuring solution and the capacitive current is measured under a small-amplitude sinusoidal voltage signal at low excitation frequencies (typically 10-1000 Hz). The antibody-antigen interactions are expected to cause a decrease in the measured capacitance; and (b) faradic or faradaic impedimetric – where the surface of the electrode, which is covered by an insulating layer, is able to catalyze a redox probe placed in the measuring solution. In this case, the measured parameter is the charge transfer resistance (the real component of impedance at low frequency values, typically 0.1-1.0 Hz) and antibody-antigen interactions are expected to cause an increase in its value as the faradic reaction becomes increasingly hindered. In general, faradic impedimetric immunosensors exhibit a higher sensitivity due to antibody-antigen interaction. However, the redox probes may have an effect on both the stability and the activity of the electrode.

Various types of impedimetric immunosensors for *Salmonella* based on the different types of formation of sensitive layer such as electropolymerization and self-assembly have been proposed. Pournaras et al. (2008) employed functional impedimetric immunosensors based on polytyramine electropolymerized films for the detection of *Salmonella* Typhimurium in real samples. Interestingly, since the detection was performed directly on cultures, it eliminated various centrifugation and washing steps, which were used for the isolation of bacteria cells from culture, thus making the proposed immunosensors promising candidates for on-site applications. Yang et al. (2009) described a capacitive immunosensor for *Salmonella* spp. detection based on grafted ethylene diamine and self-assembled gold nanoparticle on glassy carbon electrode. The antibodies were immobilized on gold nanoparticle and the limit of detection was found to be 1.0×10^2 CFU mL^{-1}. Dev Das et al. (2009) studied self-assembled array for trapping channels on oxidized macroporous silicon substrate for detection of *Salmonella* Typhimurium. It was found that oxidized macroporous silicon substrate with its regular network of pores at 1-2 um diameter is capable of detecting concentrations from 10^3 CFU mL^{-1} to 10^7 CFU mL^{-1} in pure culture of the bacteria.

The development of faradic impedimetric immunosensors for the detection of *Salmonella* Typhimurium in milk also were reported (Rickert et al. 1996; Mantzila et al. 2008). The alteration of the interfacial features of the electrodes due to different modification or recognition steps was measured in the presence of hexacyanoferrate (II)/ (III) redox couple. A substantial amplification of the measured signal was achieved by performing the immunoreaction directly in culture samples. The efficiency of the immunosensors was evaluated in a series of standard culture samples over the final concentration range of 10^2-10^6CFU mL^{-1} for *Salmonella* Typhimurium (Mantzila et al. 2008). However, the hexacyanoferrate (II)/(III) system was found to damage SAMs or to reduce the activity of the immobilized protein according to Rickert and collaborators (1996) who developed a mixed self-assembled monolayer of a synthetic peptide and 11-hydroxyundecanethiol. Faradic impedance spectroscopy is usually considered more sensitive as compared to capacitance measurements at electrically blocked electrodes.

Although promising results have been achieved with impedimetric immunosensors for *Salmonella* detection, some papers reported in the literature have highlighted the complexity of the analytical procedures (Nandakumar et al. 2008; Nguyen et al. 2014).

Conductometric immunosensors

This kind of immunosensor is based on the consumption or production of charged species, thus leading to conductance changes resulting from the antibody-antigen interaction. This immunosensor can be labelled free or not free. In the former case, the use of enzymes is often adopted because a large number of enzymes are known to produce changes in the conductivity of the sensor surface (Table 2). Some enzymes produce ionic products that increase the conductivity, but there are also those, for example, glucose oxidase, whose products induce a decrease in conductivity (Soto et al. 2001). Immunosensors based on conductometric principle present some advantages: they do not require any reference electrode; driving voltage can be sufficiently low to decrease significantly the power consumption; and transducers are not light sensitive (Jaffrezic-Renault and Dzyadevych 2008). Nevertheless, in the literature, there are relatively few papers on this subject, especially involving *Salmonella* detection. Muhammad-Tahir and Alocilja (2003) proposed an immunosensor for *Escherichia coli* O157:H7 and *Salmonella* spp. based on sandwich assay and using polyaniline as the antibody label. The signal (change in conductance) was due to the presence of polyaniline, a conductive polymer, which increased its signal intensity when *Salmonella* was present. This immunosensor provided a specific, sensitive, low-volume and near real-time detection mechanism for the lower limit of detection of 7.9×10^1 UFC mL^{-1} within a 10-min process. However, it was limited for use at high concentrations of *Salmonella* because binding sites may be over-occupied with the antigen, thus obstructing the charge transfer within the conductive polymer structure. In general, the wide application of conductometric immunosensors has been hindered by specific difficulties, such as low specificity of the technique and the need to employ certain experimental conditions (e.g., buffer

concentrations and dissolution of ingredients in solution). The latter conditions are prompted by the need to avoid reduction in signal/noise ratio (which should be greater than 2%).

Table 2 - Relation of enzymes-label for immunosensors that cause conductivity changes.

Enzymes	Source of changes in conductivity
Amidases	Generation of ion group
Dehydrogenases and decarboxylases	Separation of different charge
Esterases	Ion migration
Kinases	Change in level of ion particles association
Phosphatases and sulfatases	Change in size of charged groups

Fonte: (Lawrence and Moores, 1972; Jaffrezic-Renault and Dzyadevych, 2008).

Potentiometric immunosensors

Potentiometric transducers measure the potential difference between working and reference electrodes. Potential changes may be caused by electrochemical, chemical or biological interactions. Thus, changes in pH, ionic, or redox at the surface influence the response of potentiometric sensors. Examples of potentiometric sensors are the solid state Ion Selective Field Effect Transistors (ISFETs) and pH electrode- based Ion Selective Electrodes (ISE's). These are used for pH, ion, chemical or gas sensing and are marketed by iStat Corp, and others. There are few examples of potentiometric biosensors that are generally applicable to enzymes or immunosensing system. An example is Light-Addressable Potentiometric Sensor (LAPS) used in the Molecular Diagnostics cytosensor and Threshold System (Dill, 1999; Purvis et al., 2003). On the other hand, potentiometric transducers for *Salmonella* detection in food are rarely reported in the literature. A notable report is a LAPS used in the Threshold System for *Salmonella* detection in chicken carcass capable of detecting levels as low as 119 CFU mL-1 (Dill, 1999).

Conclusion

Immunosensors offer an exciting alternative to the more traditional assay methods, allowing rapid "real-time" and multiple analyses that are essential for the detection of *Salmonella* and other microorganisms in food, especially in perishable or semi-perishable foods. Although conventional methods in the detection of *Salmonella* and other microbial contaminants can be very sensitive and inexpensive, they require several days to yield results. Thus, immunosensors represent a promising and faster alternative tool to ensure food safety. It is worth noting that the device performance and the commercial prospect for future sensor systems may vary depending on transducer's properties,

improvements in optimization responses, and operational parameters. Future immunosensors with increased sensitivity, lower costs and easier handling are highly desirable. Such immunosensors will be very useful for the rapid and routine detection of *Salmonella* in foods both in the field and in laboratories.

Acknowledgments. The authors acknowledge the financial support of the CNPq (Award number: 475174/2012-7) and CAPES Brazilian agencies. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

Ethical statement .This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest. The authors (Airis Maria Araújo Melo; Dalila L Alexandre; Roselayne F Furtado; Maria F Borges; Carlúcio R Alves; Atanu Biswas; Huai N Cheng; Evania Altina T Figueiredo) declare that they have no conflict of interest.

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CAPÍTULO II Preparation and characterization of carboxymethyl cashew gum grafted with immobilized antibody for potential biosensor application

Carbohydrate Polymers 228 (2020) 115408 DOI:
https://doi.org/10.1016/j.carbpol.2019.115408

Preparation and characterization of carboxymethyl cashew gum grafted with immobilized antibody for potential biosensor application

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ABSTRACT

This report details the design of carboxymethylated cashew gum (CG) as a platform for antibody (Ab) immobilization, which can then be used as a biosensor for bacteria detection. The CG was isolated and characterized, followed by conversion to carboxymethyl cashew gum (CMCG). The CMCG film was a viable support for antibody immobilization; it was electrodeposited on gold surface using the cyclic voltammetry technique, applying a potential sweep from -1.0 V to 1.3 V with a scan rate of 50 mV s^{-1} and 10 scans. The COOH groups on the surface of the film were critical in promoting Ab bonding. The immobilization of the Ab was mediated by protein A (PrA) for recognition of the antigen. Voltammetry studies were used to monitor the antibody immobilization. Finally, the analytical response of the CMCG-PrA-Ab system was evaluated with the chronoamperometry technique and was found to detect *Salmonella* Typhimurium bacteria rapidly and efficiently.

KEYWORDS: Amperometric immunosensor; Bacterial detection; *Anacardium occidentale* L.; Chemical modification; Electrodeposition.

1. Introduction

The cashew gum (CG) is an exudate extracted from *Anacardium occidentale* L. tree, a member of the family Anacardiaceae. The CG has received a great deal of attention from researchers, mainly due to its similarity to gum Arabic (viz., molar masses, uronic acid content, and same type of monosaccharides units) as well as its availability and potentially low cost as a by-product of the cashew industry (Paula, Sombra, Cavalcante, Abreu, & De Paula, 2011). The CG from Brazil is a branched acidic heteropolysaccharide of low viscosity, comprising β-D-galactose (72 %), α-D-glucose (14 %), α-L-arabinose (4.6 %), α-L-rhamnose (3.2 %) and β-D-glucuronic acid (4.5 %) (De Paula, Heatley, & Budd, 1998). However, the proportions of the monosaccharides vary, depending on the seasonality of *A. occidentale*, e.g., source, age of the tree, time of exudation, and climatic conditions (De Paula & Rodrigues, 1995).

The technological and commercial interest in cashew gum comes mainly from its biodegradability and particularly its biocompatibility. There have been numerous studies of CG polysaccharides that explored potentially new applications. Some notable examples include its use in the preparation of nanoparticles for the purpose of transport and delivery of substances (Da Silva, Feitosa, Paula, & de Paula, 2009), microcapsule synthesis for use as lipid delivery and storage vehicles (Da Silva et al., 2018; Gomez-Estaca, Comunian, Montero, Ferro-Furtado, & Favaro-Trindade, 2016), synthesis of microcapsules for larvicides (Paula et al., 2011), potential use as a chromatographic matrix and bioaffinity ligand for proteins (LIMA et al., 2002), new polypyrrole/CG composite grown on gold surface (CASTRO et al., 2017), CG films with potential application in nanobiomedical devices development (ARAÚJO et al., 2012), and a possible novel platform for enzymes immobilization (Silva, Santiago, Purcena, & Fernandes, 2010).

In some cases, CG in its isolated natural form does not have adequate properties for the desired function, so it is common to employ chemical modification strategies in order to improve its properties. Carboxymethylation is one of most used reactions for polysaccharide derivatization (Heinze & Koschella, 2005; Silva et al., 2004). The product obtained is usually a polyelectrolyte that can be applied in a wide variety of fields, e.g., in the chemical, food, pharmaceutical, and cosmetic industries. Other advantages of carboxymethylation reactions include the ease of processing, the low cost of the chemicals used for modification, and the non-toxicity of the products (VERRAEST et al., 1995). Carboxymethylation of cashew gum has been previously reported (Olusola, Toluwalope, & Olutayo, 2014; Silva et al., 2004). The CG in its natural form has carboxylic groups in its structure; however, to improve the binding sites for biomolecules, it is necessary to increase the amount of COOH via carboxymethylation.

The detection of bacteria during the production process is a mandatory routine in the food industry, as determined by national and international regulatory entities (MELO et al., 2016). It is a laborious, time-consuming and complex process that often entails a large laboratory facility and

expensive materials. In the case of the *Salmonella* genus, there is a need for a pre-enrichment step of the sample that takes around 24 hours. The bacterial level determined by international health surveillance authorities for this pathogen is below detection in 25 g or mL of food sample (Melo et al. 2016). Thus, the pre-enrichment step is necessary to raise the population to a level of 10^4 CFU mL^{-1}, considered the detectable lower limit for *Salmonella* detection methods in both rapid tests and conventional culture methods (Lee, Runyon, Herrman, Phillips, & Hsieh, 2015). In contrast, biosensors are recognized as quick and practical analytical tools, especially in the bio-medical area. The efficiency of its use for the detection of bacteria in food has already been proven by several studies (ALEXANDRE et al., 2018; MELO et al., 2016, 2017; POLTRONIERI et al., 2014; RUBAB et al., 2018). In the development of such devices, a convenient platform is desired that allows the integration of the biomolecules to a conductive surface, which captures the analyte detection signal and results in an analytical response.

This study proposes the use of carboxymethyl cashew gum (CMCG) as a platform for antibody (Ab) immobilization. We carried out a systematic study, where the polysaccharide from CG was isolated and partially characterized, followed by carboxymethylation to form CMCG. Electrodeposition was then conducted on the gold surface, and antibody immobilization carried out. Finally, the analytical response of the CMCG-Ab assembly was evaluated in the presence of the bacterium *Salmonella* Typhimurium, with the purpose of using the bio-sensing assembly as a food safety tool.

2. Experimental section

2.1. Apparatus and electrodes

Electrochemical measurements were carried out with an AUTOLAB potentiostat (Ecochemie, The Netherlands) using the software Nova 2.0 (Metrohm Autolab B.V., The Netherlands). A 10-mL glass electrochemical cell was utilized in the experiments, consisting of a gold electrode (Φ = 0.02 cm^2) as working electrode, Ag|AgCl|KCl 3 M as reference electrode and a platinum (Pt) wire as counter electrode (Φ = 0.04 cm^2).

2.2. Reagents and biological materials

Horseradish peroxidase (HRP) (250 U mg^{-1}), glutaraldehyde (25 %), N-hydroxysuccinimide (NHS), N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), protein A, bovine serum albumin (BSA), hydroquinone, and hydrogen peroxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). The culture medium, brain heart infusion agar (BHI agar), brain heart infusion broth (BHI broth), nutrient agar, and nutrient broth were acquired from Difco (Becton, Dickinson and Company, Sparks, MD, USA). All solutions and culture medium were prepared with ultrapure water from Direct-Q® 3 UV purification unit (Millipore Corporation, Billerica, MA, USA).

Salmonella Typhimurium (ATCC 51812) was purchased from Microbiologics (Saint Cloud, MN, USA). The lyophilized strains were activated in BHI broth at 35 °C for 24 h and sub-cultured in BHI agar at 35 °C for 24 h. Cultures were maintained in BHI agar inclined and stored at 4 °C until used.

Stock cultures were maintained in BHI broth supplemented with 25 % glycerol at - 80 °C. Polyclonal rabbit antisera, Difco™ *Salmonella* Antiserum Poly A-I & Vi, were properly rehydrated and used as recommended by the manufacturer. The antibodies were purified by precipitation with $(NH_4)_2SO_4$ at 45 % saturation (Green & Hughes, 1955), and the concentration was determined by Bradford method (BRADFORD, 1976). The antibody solutions were prepared by dilution in phosphate buffer saline (PBS) (10 mM). The secondary antibody was conjugated to HRP according to Avrameas (2003).

2.3. Origin and isolation of CG

CG was obtained from exudate of *Anacardium occidentale* L., collected from native trees of the Experimental Field of Embrapa Agroindústria Tropical. The polysaccharide isolation from CG was performed using the methodology previously described by Torquato et al. (2004) with modifications. The exudate was initially triturated in a knives mill. Then, the triturated sample was solubilized in water in the proportion of 300:1 (g/L), filtered and centrifuged (10,000 rpm for 10 min at 25 °C) for residue removal. After removal of the residues, the supernatant was precipitated in 96 % ethanol in a ratio of 1:3 (v/v) for 24 h under refrigeration. Ethanol was removed and the precipitate was dried in an air-circulating oven at 60 °C for complete drying. Thereafter, the sample was milled, and the isolated CG was obtained. Finally, the isolated CG was submitted to the freeze-drying process and stored in vacuum sealed packages until use.

2.4. CG Characterization

2.4.1. Centesimal composition and phenolic compounds

The humidity determination was performed according to the AOAC Method 934.01 (AOAC, 2005), total nitrogen/protein according to Method 984.13 (AOAC, 2005), ash content according to Method 923.03 (AOAC, 2005), and ethereal extract according to Method 945.38 (AOAC, 2005). The determination of the total phenol content present in the ethanol was made by means of UV/visible spectroscopy (λ = 740 nm) using the Folin-Ciocalteu method with modifications (SANCTIS, 2004).

2.4.2. Metal Characterization

The sample was subjected to nitric-perchloric acid digestion to obtain the extract according to the procedure described by Miyazawa et al. (2009), followed by quantification of phosphorus,

potassium, calcium, magnesium, sulfur, sodium, copper, iron, manganese and zinc by inductively coupled plasma optical emission spectrometry (ICP-OES).

2.4.3. Molar mass by GPC

The molar mass distribution was determined by gel permeation chromatography on a Shimadzu LC-20AD equipment coupled to a refractive index detector (RID-10A). For analysis, a linear polysep column, 300 x 7.8 mm, using $NaNO_3$ (aq) 0.1 mol L^{-1} as the eluent was used. The measurement was made at 30 °C with a flow rate of 1 mL min^{-1} and an injected volume of 50 μL. The molecular mass (M) was converted from elution volume (Ev) with the following relationship: Log M = 14.40967 – 1.1392 Ev.

2.5. Carboxymethylation of cashew gum

Carboxymethyl cashew gum (CMCG) was prepared following a protocol reported previously by Silva et al (Silva et al., 2004). The purified gum (5 g) was mixed with 5 mL of deionized water until a homogeneous paste was formed. A 10 M NaOH solution (8.3 mL) was added and the mixture was kneaded for 10 min. After that, monochloroacetic acid (MCA) (2.6 g) was mixed in thoroughly with the paste. The mixture was heated at 55 °C for 3 h. The product was neutralized with 1 M HCl and dialyzed against deionized water until the reagents or salts were eliminated (monitored by water conductivity). Finally, the CMCG was submitted to the freeze-drying process and stored in vacuum-sealed packages.

2.5.1. Attenuated total reflection/Fourier transform infrared (ATR–FTIR)

ATR–FTIR analysis was performed on isolated CG and CMCG to confirm the carboxymethylation reaction. The measurement was made by pressing the electrode against the ATR crystal on a FTIR spectrometer (model FTLA 2000–102, ABB-BOMEN, USA). All spectra were recorded in the range from 600 to 4000 cm^{-1} at 4 cm^{-1} resolution, averaging over 128 scans.

2.5.2. Nuclear magnetic resonance (NMR)

NMR spectra were obtained using a 600 MHz Agilent DD2 instrument (Santa Clara, CA, USA) equipped with a reverse-detection 5 mm internal diameter (HF/15N-31P) “One-Probe” probe and field gradient in the "z" direction. Samples were prepared by dissolving approximately 10 mg of the CG samples in 0.55 mL D_2O containing 1 % sodium trimethylsilyl propionate, (TSP-d_4, v/w). The one-dimensional ^1H spectrum was acquired at 80 °C with a 10 s time between each acquisition, accumulation of 64 transients, with a spectral window of 16 ppm and 32k data points. The one-dimensional ^{13}C spectrum was obtained with a 1 s time between each acquisition, accumulation of 30k transients, with a spectral window of 251.3 ppm and 32k data points. The ^{13}C peaks of the anomeric carbon of cashew gum in the region between 102 and 106 ppm and the carboxyl resulting from

carboxymethylation between 165 and 180 ppm were integrated to obtain the relative percentage of both in the sample.

2.6. CG electrodeposition

All the samples were evaluated by cyclic voltammetry technique in 4 mM $K_3[Fe(CN)_6]$ and 1 M KCl solution using a potential that ranged from - 0.30 V to 0.75 V and a scan rate of 100 mV s^{-1}. First, the effect of the use of isolated CG (ICG) versus CMCG was observed for the immobilization of the antibody. Thereafter, the optimal concentration for the electrodeposition of the CMCG film on the gold electrode surface was found. Finally, the impact of the use of protein A in the antibody immobilization was checked.

2.7. Antibody immobilization on CMCG

Initially, the gold electrode surface was cleaned by polishing with alumina (3 μm) for 5 min, followed by immersion in 96 % ethanol and sonicated for 5 min in an ultrasonic bath. The cleaned gold surface was modified by CMCG electrodeposition using the cyclic voltammetry technique, applying a potential sweep of -1.0 V to 1.3 V with a scan rate of 50 mV s^{-1} for 10 scans. The modified electrode was immersed in N-hydroxysuccinimide/N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC/NHS) solution (2 mM / 5 mM) for 1 h. After washing with PBS buffer (pH 7.4), the electrode was immersed in the protein A solution for 1 h. Finally, the electrode was incubated overnight in an anti-*Salmonella* solution. Protein A and anti-*Salmonella* concentrations were determined during previous optimization studies with the cyclic voltammetry method. The non-specified sites of the modified electrode were blocked with 1 % BSA for 1 h.

2.8. Bacteria detection

The analytical response for bacteria detection was obtained by the chronoamperometric technique. Initially, the modified electrode (CMCG-prA-Ab-BSA) was immersed in 100 μL *S.* Typhimurium (10^6 CFU mL^{-1} in 0.1 mol L^{-1} PBS, pH 7.4) for 1 h, and PBS buffer was used as the control. The electrode was incubated with HRP-labeled secondary antibodies for 1 h, forming a sandwich system. After each incubation step, the electrode was rinsed with PBS. The amperometric response was carried out in the electrochemical cell (10 mL) containing 0.1 mol L^{-1} PBS (pH 7.4), 300 mM H_2O_2 and 3 mM hydroquinone. The response was determined by polarizing the gold electrode at -75 mV until a stable baseline (steady state) was reached in 120 s. All measurements were carried out at room temperature.

A scanning electron microscope (SEM; Quanta 450 FEG System: FEI Company, USA) was used to observe the surface morphology of the CMCG-PrA-Ab system before and after the *S.* Typhimurium detection. The images were obtained using a scanning voltage of 20 kV.

2.9 Milk Analysis

Skimmed Ultra High Temperature (UHT) milk was purchased from a local market. Milk samples were first spiked with *S.* Typhimurium at a concentration of 10^1 CFU mL^{-1}. The amperometric response was obtained according to Section 2.8 above, after immersion of the immunosensor in the spiked milk samples for one hour at room temperature.

3. Results and Discussion

3.1. CG characterization

Materials from plant origin are known to show variation in their compositions because they are susceptible to the influence of geography, biotic and abiotic factors (De Paula & Rodrigues, 1995). Therefore, it is important to characterize the crude cashew gum used in this work.

The following determinations were conducted on the crude cashew gum: centesimal composition, amounts of phenolic compounds, metal profile, and molar mass. The centesimal composition and the phenolic content are summarized in Table 1.

Table 1 - Centesimal composition and phenolic compounds content of the isolated cashew gum.

Sample	Centesimal composition (%)					Phenolic compounds
ICG*	Protein	Water	Ether extract	Ash	Carbohydrate	(mg 100 g^{-1})
	0.76	4.43	0.09	0.63	94.09	143.85

* ICG = Isolated cashew gum

In general, plant gums are amorphous substances containing mostly carbohydrates. The centesimal composition of cashew gum used in this study displayed values close to those reported in the literature, viz., 7.4-11.1 % water, 0.15-0.75 % protein, 0.06 % lipid, 0.9-1.7 % ash and ca. 95 % carbohydrates (ANDERSON; BELL; MILLAR, 1974; DE PINTO et al., 1995; LIMA et al., 2002). In many cases small amounts of nitrogen were detectable that may be traced to the proteinaceous debris arising from enzymes, such as oxidases (peroxidases and polyphenol oxidases), found in cashew gum and generally associated with the response of the plant to infection by pathogens (Marques & Xavier-Filho, 1991). The presence of phenolic compounds was also detected, which are known to be related to the defense mechanisms of plants, and their concentrations in ICG are similar to those reported in the literature (Marques & Xavier-Filho, 1991).

The metals (TABLE 2) and molar mass (TABLE 3) associated with the ICG sample were also determined. Metals can interact with enzymes by modifying their activity and thereby interfering with

the response of a biosensor. The molar mass data of ICG indicate a distribution of molecular weight with a polydispersity index (PDI) of 2.61.

Table 2 – Profile of metals determined in the isolated cashew gum sample.

Sample	Metals									
	g Kg^{-1}						mg Kg^{-1}			
ICG*	P	K	Ca	Mg	S	Na	Cu	Fe	Zn	Mn
	0.01	1.03	1.46	1.44	0.02	0.21	1	3	1	38

* ICG = Isolated cashew gum

Table 3 – Molar mass (g mol^{-1}) of the isolated cashew gum sample.

Sample	Mp[a]	Mn[b]	Mw[c]	PDI[d]
ICG[e]	2.13×10^4	8.16×10^3	2.13×10^4	2.61

[a] Mp = most probable molecular weight; [b] Mn = number-average molecular weight; [c] Mw = weight-average molecular weight; [d] PDI = polydispersity index; [e] isolated cashew gum

3.2. Carboxymethyl CG Characterization

The ICG was subjected to carboxymethylation reaction (Fig. 1a) in order to increase the amount of carboxylic groups on its structure. This reaction is based on the Williamson synthesis, whereby the polysaccharide alkoxide is reacted with monochloroacetic acid (EGE, 1989) and substituted with carboxymethyl groups. Fig. 1 shows the carboxymethylation reaction and the FTIR and ^{13}C NMR spectra of starting and modified cashew gum.

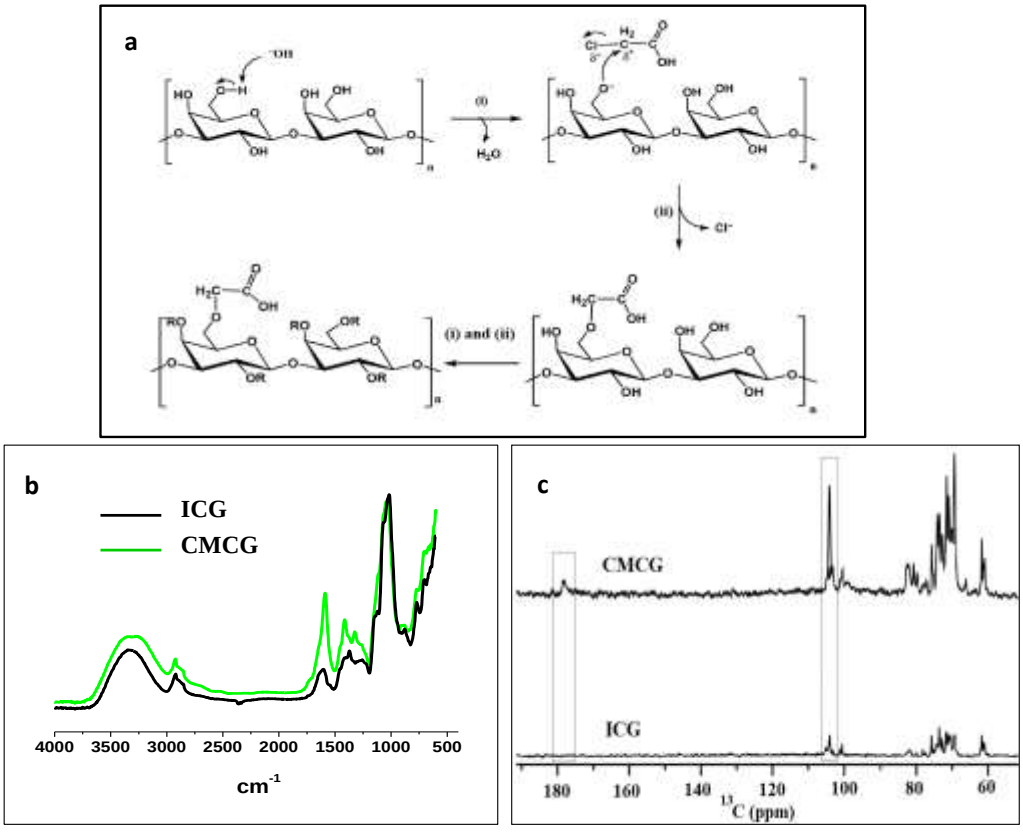

Fig. 1. a) Carboxymethylation reaction for a polysaccharide. R may be H or CH_2COOH group, depending on the progress of carboxymethylation (Silva et al., 2004). b) ATR-FTIR spectra of isolated cashew gum (ICG) and carboxymethyl cashew gum (CMCG). c) ^{13}C NMR spectra in D_2O of ICG and CMCG.

ATR-FTIR spectra (Fig. 1b) showed the presence of a band around 1730 cm^{-1} in the ICG spectrum for C=O stretching vibration of the carboxyl moiety of glucuronic acid (De Paula et al., 1998). A substantial increase in the absorbance of C=O vibration was observed in the CMCG spectrum caused by the introduction of more carboxylic groups after the reaction with monochloroacetic acid.

^{13}C NMR spectroscopy is a sensitive tool to evaluate chemical modification of polysaccharides. Figure 1c gives the ICG and CMCG spectra. ^{13}C NMR spectrum of CMCG showed a carboxyl peak at 178 ppm, as evidence of the carboxymethylation reaction (De Paula et al., 1998). An increase in peak intensities between 85 and 70 ppm attributed to carbons from C-2 to C-5 of monosaccharide residue might be due to CH_2 groups of – CH_2COOH of MCA and also to the shift of primary carbons (C-6) from the region around 60 to 66–69 ppm, after the substitution of –CH_2COOH group on the primary carbon.

3.3. Voltammetry studies for CG electrodeposition

The cyclic voltammogram performed according to the strategies of using CG as a support for immobilization of the antibody are summarized in Fig. 2. The ICG film was electrodeposited onto the surface of the gold electrode and subsequently exposed to antibody immobilization (ICG-Ab). The same procedure was also applied for CMCG electrodeposition and for antibody immobilization (CMCG-Ab). In the voltammogram for the antibody immobilization step, a greater reduction in the cathodic and anodic peak currents for CMCG-Ab was verified, while in the ICG-Ab voltammogram the peaks had a lower current reduction because there was a smaller hindrance to the flow of electrons. Thus, the cathodic and anodic peak reduction would be related to the amount of antibody molecules immobilized on the surface of CMCG, which was proportionally higher than in ICG.

This result corroborates the data presented in Fig. 1, where it was observed in the CMCG a greater amount of COOH clusters, a result promoted by the reaction of carboxymethylation. The carboxymethylation of natural polymers has been shown to increase the efficiency of immobilization of biomolecules on their surfaces and seems to be a promising technique for the development of analytical devices with CG-based platforms (Hebeish & Khalil, 1988; El-Sheikh, 2010); in addition, it increases the adsorption of the CG film on the gold surface

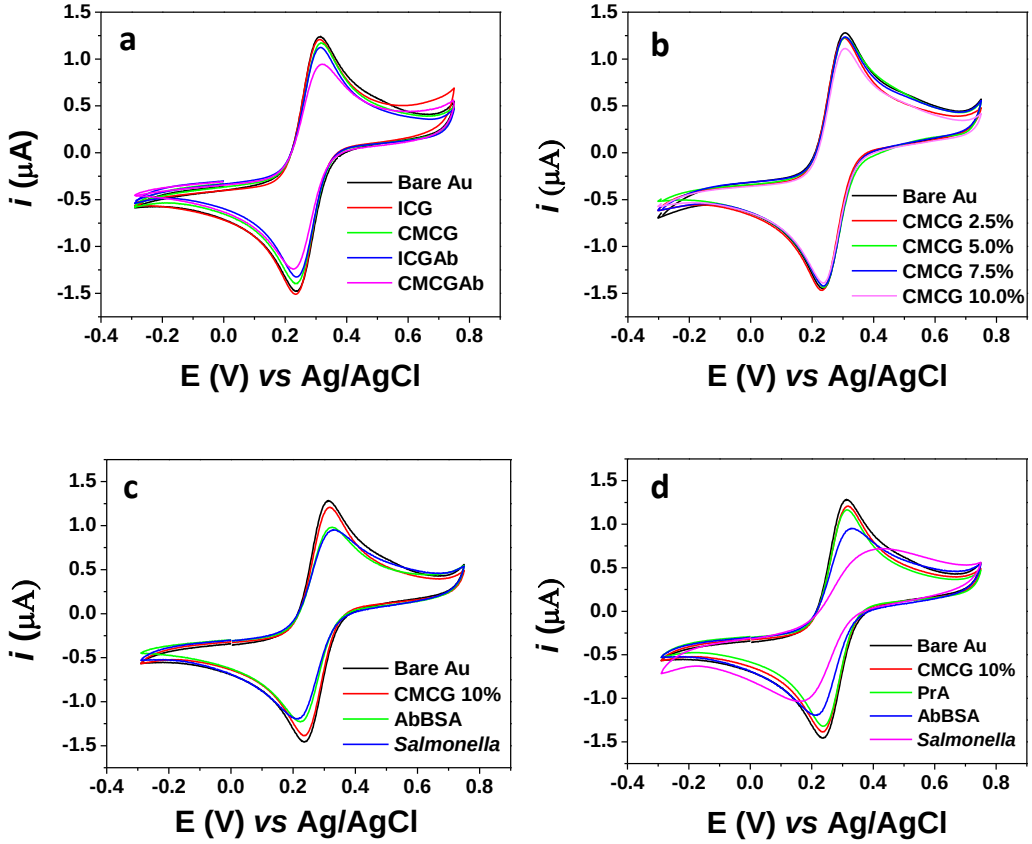

Fig. 2 a) Cyclic voltammograms in 4 mM $K_3[Fe(CN)_6]$ and 1 M KCl solution at each step of ICG and CMCG electrodeposition and antibody immobilization (ICG-Ab and CMCG-Ab). b) Cyclic voltammograms in 4 mM $K_3[Fe(CN)_6]$ and 1 M KCl solution of CMCG electrodeposition in different concentrations (2.5 to 10 %). c) Cyclic voltammograms in 4 mM $K_3[Fe(CN)_6]$ and 1 M KCl solution at each step of biosensor assembly without protein A, d) assembly with protein A.

(PAIK et al., 2003), thus improving its performance as a support for biomolecules immobilization on metallic surfaces. The activation of COOH groups (to become COO^-) on the surface and the Coulombic interaction between this functional group and the amino group of the biomolecules (NH_2) was facilitated via the EDC/NHS treatment (RICCI; ADORNETTO; PALLESCHI, 2012). The voltammograms of Fig. 2a show clearly that the use of CMCG for the immobilization of the antibody was more efficient than the use of ICG; thus, our remaining studies were focused on CMCG only.

Different concentrations of CMCG were electrodeposited on gold surface and their voltammograms are shown in Fig. 2b. When electrodeposited on the surface, the CMCG film promotes the reduction of the electric current of the anodic and cathodic peaks of the voltammograms as evidence of the modification of the gold surface. An estimate of the percentage coverage of the surface (θ) can be made with the $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ probe using Eq. (1):

$$\theta = 1 - (Ip_{modified\ electrode}/Ip_{cleaned\ electrode}) \times 100 \quad (1)$$

where Ip is the electric current peak of the modified and cleaned electrode obtained from voltammograms shown in Fig. 1b. By varying the peak current, we can estimate the surface coverage (ALEXANDRE et al., 2018). In view of the different concentrations tested, the result that best illustrated the modification of the gold surface was the 10 % CMCG because it was the concentration with the best coverage, estimated at 5.7 % on the surface of the gold electrode. Good surface coverage is generally related to efficient immobilization of biomolecules as observed in previous studies (ALEXANDRE et al., 2018; MELO et al., 2017).

The optimum experimental conditions for the immobilization of the antibody (Ab) molecules on the 10 % CMCG film were investigated next. Fig. 2c gives the voltammograms of the biosensor assembly without protein A, and Fig. 2d gives the voltammograms of the assembly using protein A molecule in order to facilitate the orientation of the Ab immobilization. In the absence of protein A, there was a minimum reorganization of the *Salmonella* antigen; this probably occurred because the COOH groups of the CMCG were indistinctly bound to the Ab molecule and possibly blocked the Fab (the fragment antigen-binding) region of the Ab. This is evidenced by the voltammogram in the presence of *Salmonella* being practically in the same position of the Ab voltammogram (Fig. 2c). This behavior demonstrates the importance of protein A for both antibody immobilization and antigen interaction. In Fig. 2d, the voltammogram of *Salmonella* shows smaller peak currents than the antibody voltammogram. Such a reduction indicates that the bacterium *Salmonella* is bound to the antibody. This happens because the protein A binds specifically to the Fc region (the crystallizable fragment) of Ab, leaving the Fab region free for antigen recognition.

Earlier studies have shown that oriented antibodies on the electrode surface produced improved specificity and sensitivity for the *Salmonella* Typhimurium detection (GOPINATH et al., 2014). Danczyk et al., (2003) compared the antibody functional behavior using different immobilization methods and concluded in their studies that protein A is able to orient the antibodies, allowing for greater antigen capture per antibody present on the surface. By improving the immobilization of primary antibodies on the surface, the bio-device becomes more cost-effective by reducing the amount of antibodies that are blocking the surface rather than capturing antigen. In our research group, successful experiments have been previously conducted through the use of protein A in the development of *Staphylococcus aureus* toxin immunosensor (PIMENTA-MARTINS et al., 2012) and detection of *Salmonella* bacteria (ALEXANDRE et al., 2018; MELO et al., 2017).

3.4. Bacteria detection

Our biosensor's ability to detect the presence of *S.* Typhimurium was verified using the chronoamperometry technique. The results are summarized in Fig. 3.

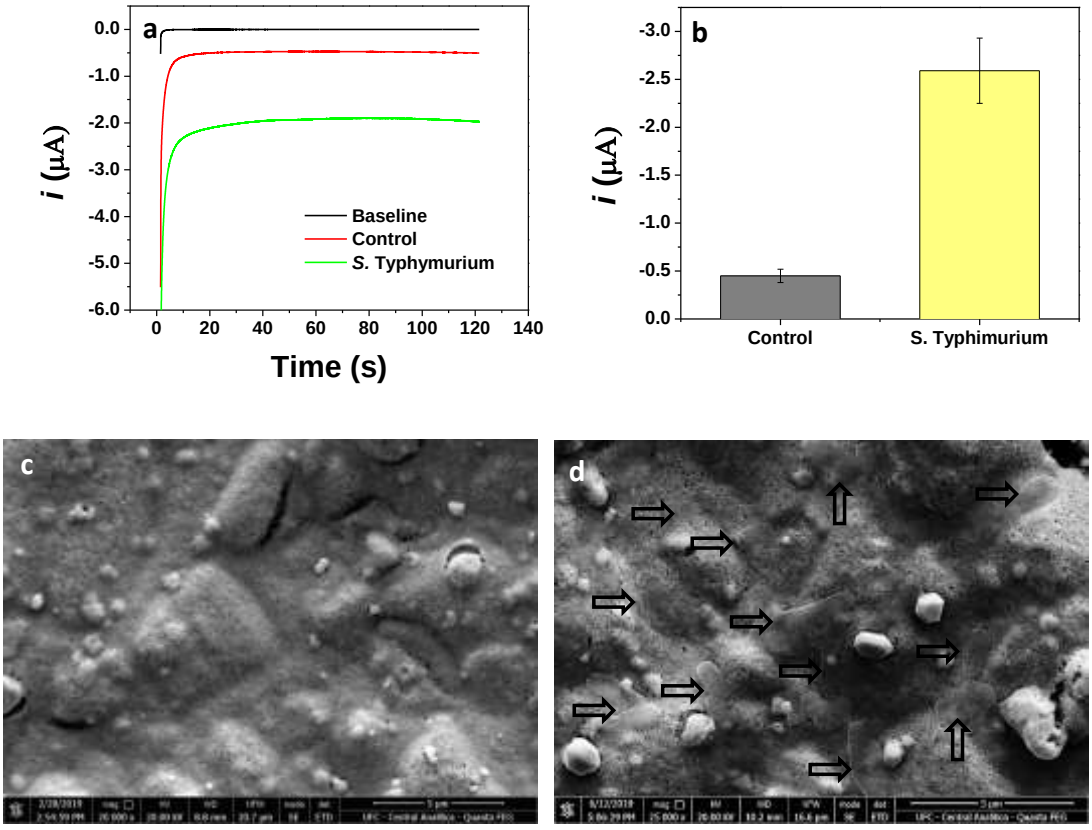

Fig. 3 - Current response of the biosensor. a) Chronoamperometric data obtained for baseline, control (PBS), and three measurements of *S.* Typhimurium 10^6 CFU mL^{-1} in PBS solution (pH 7.4) containing 3 mM hydroquinone and 300 mM H_2O_2. b) Graphical representation of the analytical response. The amperometric measurements were determined by polarizing the electrode at −75 mV until a stable baseline (steady state) was reached in 120 s. c) Scanning electron photomicrograph of the CMCG-PrA-Ab system. The arrows indicate the *S.* Typhimurium captured by the antibody immobilized on the CMCG film surface.

The chronoamperometry technique was used to verify the analytical response of the CMCG-PrA-Ab system in the presence of *S.* Typhimurium (10^6 CFU mL^{-1}). The measurements were conducted in triplicates. Fig. 3a shows the typical chronoamperograms representing the baseline signal, the control (PBS), and measurements of *S.* Typhimurium in the range of concentrations tested. The baseline represents the noise of the electrochemical system, being obtained from the CMCG-PrA-Ab system in the electrochemical cell with PBS without hydrogen peroxide and hydroquinone. In the *S.* Typhimurium chronoamperograms, a fast degradation of H_2O_2 was observed in the first 20 s. After that, the current stabilized, indicating that most of the hydrogen peroxide present in the system had been consumed by the HRP enzyme. It is clear in Figures 3a and 3b that the CMCG-PrA-Ab system developed was effective in detecting *S.* Typhimurium bacteria in the PBS buffer.

Additionally, the immunosensor presented a limit of detection (LOD) of 10 CFU mL^{-1} for S. Typhimurium; this was lower than the previous limit reported by Melo et al. (2016). The LOD is one of the most important performance parameters of a biosensor, and it demonstrates the device's ability

to detect the lowest analyte concentration in a sample. The LOD was obtained from Eq. (2) where $\bar{x}$ is the average of multiple determinations, t is the student distribution factor, and SD is the standard deviation.

$$LOD = \bar{x} + t(n-1, 1-\alpha) * SD \quad (2)$$

Moreover, the detection time was 125 min, which included the incubation time for recognition of antigen in the PBS, the time for the binding of HRP-labeled antibody, and the time for amperometric measurement. This result makes the current system a promising bio-device to be used as a viable method for *Salmonella* detection in the food industry, especially for the rapid emergency screening of contaminated samples.

Furthermore, the CMCG-PrA-Ab system was studied by scanning electron microscopy (SEM) before and after the recognition of the antigen and binding of the HRP-labeled antibody. *S.* Typhimurium cells were clearly observed on the surface as further evidence of the antigen-antibody interaction (Fig. 3c), again demonstrating the effectiveness of the detection system. These results confirm the utility of cashew gum in the development of bioelectronic devices as demonstrated earlier by Araújo et al. (2012) who developed a device for the detection of dopamine and by Silva et al. (2010) who immobilized the peroxidase enzyme on the cashew gum.

3.5 Application in Milk Samples

The feasibility of the CMCG-based immunosensor to detect bacterial contamination in a complex food matrix was evaluated in the absence and the presence (10^1 UFC mL^{-1}) of *S.* Typhimurium in skimmed milk samples (Fig 4).

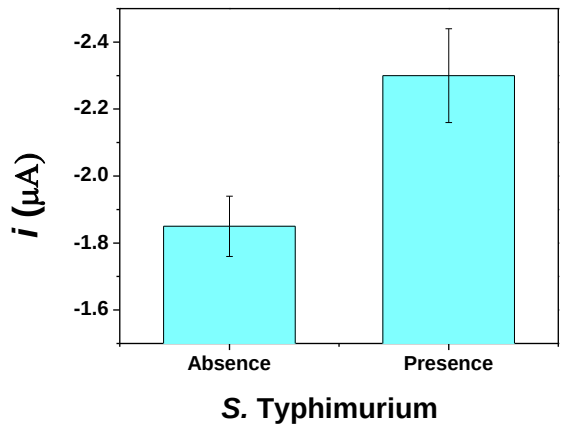

Fig. 4. Amperometric response of the CMCG-based immunosensor the absence and in the presence (10^1 UFC mL^{-1}) of *S.* Typhimurium in skimmed milk samples. Results were obtained in 10mM PBS buffer (pH 7.4) in the presence of H_2O_2 (300 mM) and hydroquinone (3 mM), with 75 mV potential for 120 s.

The analysis was performed in a detection time of 125 min. It is important to note that most commercial alternative methods for *Salmonella* detection in food (such as immunology assays, nucleic acid tests, and miniaturized biochemical assay) require a 24-hour pre-enrichment step in order to elevate the *Salmonella* concentration to 10^4 - 10^5 CFU mL^{-1}, which is the limit of detection for those procedures (Lee, Runyon, Herrman, Phillips, & Hsieh, 2015). The device developed in this study is very rapid because it does not require sample pre-enrichment and thus can provide the desired result in about 2 h. Furthermore, in view of automated integration and portability of the device, this methodology represents a simple, fast, easily handled, and accurate tool for bacterial detection in order to ensure food safety.

4. Conclusions

One of the thrusts in our laboratories is to develop biosensors for food applications, e.g., the design of self-assembled thiol monolayers for *Salmonella* detection (ALEXANDRE et al., 2018; MELO et al., 2017). The present work represents an example of the use of a polysaccharide extracted from renewable sources for the development of bio-devices, substituting synthetic polymeric platforms with the cashew gum derivative. Thus, a new biosensor has been designed based on the CMCG platform, where a polyclonal antibody (Ab) was immobilized on CMCG with the help of protein A (PrA). This biosensor is capable of detecting the presence of the *S.* Typhimurium bacteria dispersed in PBS buffer. This result opens up a range of possibilities for the use of CG and its derivatives to immobilize biomolecules that will function as bioreceptors in the future development of biosensors and bio-devices for rapid tests for various analytes.

Acknowledgements The authors would like to thank the Brazilian agencies, CNPq, and CAPES, for their financial support, Embrapa Tropical Agroindustry and National Center of Energy and Materials Research (CNPEM). Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

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CAPÍTULO III Rapid detection of *Salmonella* sp. in food by a novel assembly platform based on modified cashew gum

Submetido ao periódico Food Control

Rapid detection of *Salmonella* in food by a novel assembly platform based on cashew gum

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ABSTRACT
This study was conducted to develop and evaluate the analytical performance of an electrochemical immunosensor for the detection of *Salmonella* sp. in milk using chemically modified cashew gum (CG) as a new platform for immobilization of biomolecules. The carboxymethilated (CM) CG (CMCG) film was electrodeposited on the surface of gold electrodes and served as the basis for the immobilization of polyclonal anti-*Salmonella* antibodies. Optimization studies were conducted to improve the performance of the CMCG-based immunosensor. The analytical response was obtained by means of the chronoamperometry technique in the presence of the bacterium *Salmonella* enterica serovar Typhimurium. The CMCG was an excellent platform for mounting the immunosensor that displayed a stable structure with a 12% coefficient of variation after 30 sweeps in PBS solution. The immunosensor response was proportional to the concentration of S. Typhimurium tested and presented a linear calibration curve up to 10^5 CFU mL^{-1}. The calculated limit of detection was 10 CFU mL^{-1}. When tested on whole and skimmed milk samples, it could distinguish three different concentrations, proving its efficacy in detecting bacteria in a complex food matrix. The CMCG -based immunosensor detected the *Salmonella* pathogen quickly and specifically in milk samples with a low detection limit, thereby showing great potential for use in the food industry and especially for emergency response.

KEYWORDS: Biosensors, pathogen, immunosensor, fast method, food safety.

1. Introduction

According to World Health Organization (WHO) the consumption of food and water contaminated by pathogenic microorganisms causes 1.8 millions of deaths per year worldwide (SHEN et al., 2014), and the various *Salmonella* serotypes are the more predominant cause of foodborne diseases (DONG et al., 2013; LEE et al., 2015).

Due to its great representativeness in public health, analysis for *Salmonella* in food is a requirement of the authorities that regulate food safety in their respective countries (MELO et al., 2016). Conventional techniques are considered the most sensitive and reliable, however, depend on a complex sequence of steps that require several days to obtain the result (ANDREWS et al., 2019). Recent advances in microorganism detection and identification technologies have provided faster, more sensitive and more specific alternatives. However, almost all include a sample pre-enrichment step increasing the analysis time by up to 24 hours (LEE et al., 2015).

An alternative for the rapid detection of analytes is the use of biosensors, bioelectronic devices that in recent years have gained greater visibility because of their rapid response, great potential for miniaturization and portability; desirable characteristics for an industrial scale application. There are examples of biosensors developed for use in different areas such as quality control in food and beverage industries (MELLO; KUBOTA, 2002); detecting pathogens in food and water (LAZCKA; CAMPO; MUÑOZ, 2007; MORTARI; LORENZELLI, 2014; NAYAK et al., 2009; O'KENNEDY et al., 2005; VELUSAMY et al., 2010); in the detection of phytopathogens (SKOTTRUP; NICOLAISEN; JUSTESEN, 2008).

Although, there are already several studies that prove the effectiveness of different types of biosensors in the detection of *Salmonella* spp. (CINTI et al., 2017; MELO et al., 2016; SILVA et al., 2018). New materials have been successfully proposed for the development of biosensors to improve sensitivity and / or selectivity based on the manufacture of chemically or biologically modified sensors. The cashew gum (GC) has interesting properties such as low cost, non-toxicity, biodegradability and biocompatibility, the last one is especially attractive because it favors the stability of biomolecules, which can be immobilized on these films (PAULA et al., 2012).

Studies have successfully explored the use of cashew gum as platforms for the assembly of bioelectronic devices in nanobiomedicine (ARAÚJO et al., 2012), in studies for the development of conductive composites (CASTRO et al., 2017) a possible novel platform for enzymes immobilization (SILVA et al., 2010) and most recently, in our research group, a promissor result for antibody immobilization based on carboxymethylated cashew gum (MELO et al., 2020) The carboxymethylation reaction of cashew gum is ease, low cost and the final cashew gum is non-toxicity (VERRAEST et al., 1995). In addition, it increases the adsorption of the CG film on the gold surface (PAIK et al., 2003), thus improving its performance as a support for biomolecules immobilization on metallic surfaces.

Gathering the importance of the genus *Salmonella* for global public health, the need for rapid detection in food safety control in industrial scale and the opportunity to explore and expand the biotechnological potential of the cashew gum this study aims to develop and evaluate the analytical performance of the electrochemical immunosensor for *Salmonella* sp. detection in milk samples. The immunosensor was assembled from modified cashew gum as a new platform for the immobilization of the polyclonal antibodies against *Salmonella*. Finally, this immunosensor performance in milk was confronted with recognized and validated fast and conventional methods.

2. Methods

2.1 Cashew gum (CG) preparation

CG was obtained from exudate of *Anacardium occidentale* L., collected from native trees of the Experimental Field of Embrapa Agroindustria Tropical. The polysaccharide isolation was performed using the methodology previously described by Silva et al. (CARVALHO DA SILVA et al., 2018). Finally, the isolated CG was submitted to the freeze-drying process and stored in vacuum sealed packages until use.

Carboxymethyl cashew gum (CMCG) was prepared following a protocol reported previously by Silva et al (SILVA et al., 2004). The purified gum (5 g) was mixed with 5 mL of deionized water until a homogeneous paste was formed. A 10 M NaOH solution (8.3 mL) was added and the mixture was manually stirred for 10 min. After that, monochloroacetic acid (MCA) (2.6 g) was mixed in thoroughly with the paste. The mixture was heated at 55 °C for 3 h. The product was neutralized with 1 M HCl and dialyzed against deionized water until the reagents or salts were eliminated (monitored by water conductivity). Finally, the CMCG was submitted to the freeze-drying process and stored in vacuum-sealed packages. The characterization studies of the isolated CG and CMCG were reported by Melo et al. (MELO et al., 2020).

2.2. Optimizations for immunosensor assembly

All the tests to verify the best conditions for the use of CMCG as an antibody immobilization platform were evaluated by cyclic voltammetry technique in 4 mM $K_3[Fe(CN)_6]$ and 1 M KCl solution using a potential that ranged from - 0.30 to 0.75 V and a scan rate of 100 m V s^{-1}.

Firstly, it was verified the best concentration for the electrodeposition of the CMCG film on the gold electrode surface. In the sequence, the steps of immunosensor assembly were optimized. In this stage, cyclic voltammetry studies were performed at a scan rate of 100 mV s^{-1}, equilibration time of 5 s, and a range of potential from - 0.4 to 0.4 V in 0.1 mol L^{-1} PBS (pH 7.4). Concentrations of protein A, 2.5, 5.0, 7.5 and 10 mg mL^{-1} and anti-*Salmonella* antibodies, 25, 50, 75 and 100 mg mL^{-1} were tested.

2.3. Immunosensor assembly

For the pre-treatment the gold electrode surface was cleaned by polishing with alumina (3 μm) for 5 min, followed by immersion in 96% ethanol and sonicated for 5 min in ultrasonic bath (MELO et al., 2017). The cleaned gold electrode surface was modified by CMCG electrodeposition using the cyclic voltammetry technique, applying a potential sweep of -1.0 V to 1.3 V with a scan rate of 50 mV s^{-1}, 10 scans. The modified electrode was immersed in N-hydroxysuccinimide / N- (3-dimethylaminopropyl) -N'-ethylcarbodiimide (EDC / NHS) solution (2 mM / 5 mM) for 1 h. After washing with PBS buffer (pH 7.4), the electrode was immersed in protein A solution for 1 h. Finally, the electrode was incubated overnight in an anti-*Salmonella* antibodies solution. Protein A and anti-*Salmonella* antibodies concentrations were determined during previous optimization studies with the cyclic voltammetry method. The non-specified sites of the modified electrode were blocked with 1% bovine serum albumin (BSA) for 1 h.

2.3.1. Analytical response

The analytical response was obtained by chronoamperometric technique. Initially, the immunosensor was immersed in 100 μL *S.* Typhimurium (10^6 CFU mL^{-1} in 0.1 mol L^{-1} PBS, pH 7.4) for 1 h. The electrode was incubated with HRP-labeled secondary antibodies for 1 h. After each incubation step, the electrode was rinsed with PBS (0.1 mol L^{-1} PBS, pH 7.4). The amperometric responses were carried out in the electrochemical cell (10 mL) containing 0.1 mol L^{-1} PBS (pH 7.4), 300 mM H_2O_2 and 3 mM hydroquinone. The response was determined by polarizing the gold electrode at - 75 mV until a stable baseline (steady state) was reached in 120 s. All measurements were carried out at room temperature. Electrochemical measurements were performed using potentiostat/galvanostat Autolab/PGSTAT12 and the GPES software (Eco Chemie, The Netherlands).

2.4. Immunosensor characterization

Each step of the immunosensor assembly was electrochemically characterized by cyclic voltammetry, using a solution of 4 mM $K_3[Fe(CN)_6]$ and 1 M KCl and applying a potential range from - 0.30 to 0.75 V and a scan rate of 100 mV s^{-1}. Studies were also carried out to evaluate the stability of the biosensor assembly by 30 consecutive scanning cycles in PBS buffer (pH 7.4) containing 300 mM H_2O_2 and 3 mM hydroquinone.

Attenuated total reflection/Fourier transform infrared (ATR–FTIR) analysis was performed in order to confirm the surface modification. ATR-FTIR analysis of the modified surface was measured directly by pressing the electrode against the ATR crystal in a spectrometer (model FTLA 2000-102, ABB-BOMEN, USA). All spectra were recorded in the range of 600 to 4000 cm^{-1} at 4 cm^{-1} resolution over 128 scans.

A scanning electron microscope (SEM; Quanta 450 FEG System: FEI Company, USA) was used to characterize the surface morphology of the imunossensor after the *Salmonella* recognition by secondary antibody. The images were obtained using a scanning voltage of 20 kV.

2.5. Immunosensor performance parameters

2.5.1. Calibration curve

For the calibration curve, the analytical response was obtained by immersing the immunosensor (average of three immunosensors) in 100 μL S. Typhimurium (ATCC 51812) dilutions (10^1 to 10^7 CFU mL^{-1} in 0.1 mol L^{-1} PBS, pH 7.4) for 1 h, and PBS buffer was used as the control. The electrode was incubated with labeled secondary antibodies for 1 h. After each incubation step, the electrode was rinsed with PBS.

Tests of the amperometric response were carried out in the electrochemical cell (10 mL) according to conditions detailed in section 2.3.1. From the calibration curve the following performance parameters will be obtained: Limit of detection, time of detection, coefficient of linearity and range of linearity.

2.5.2. Accuracy

In this study the accuracy was calculated based on the repeatability expressed quantitatively in terms of the dispersion characteristic of the results and was obtained through the analysis of the relative standard deviation (DPR) based on Eq. (1).

DPR = (standard deviation / mean) x 100 (1)

2.5.3. Immunosensor Cross-reactivity

Escherichia coli (ATCC® 10536), *Staphylococcus aureus* (ATCC® 12600) and *Citrobacter freundii* (ATCC® 43864) strains were used to examine the specificity of the amperometric imunosensor assay. Dilutions were prepared with 10^6 CFU mL^{-1} in 0.1 mol L^{-1} phosphate buffered saline (PBS), pH 7.4 for each bacterium. In mixed bacteria solutions, equal proportions of each bacterial dilution were homogenized, and the final concentration was maintained at 10^6 CFU mL^{-1} in all tested samples. The analytical response was obtained according to the Section 2.3.1.

2.6. Tests in milk samples

The feasibility of applying the immunosensor from CMCG platform for the *Salmonella* detection in a complex matrix of food was studied in different types of milk. Raw milk, whole and skimmed Ultra High Temperature (UHT) milk samples were purchased in local producers and supermarkets. The samples were previously inoculated with *S.* Typhimurium at two different concentrations of the calibration curve range: a low (10^1 CFU mL^{-1}) and a high (10^3 CFU mL^{-1}) concentration. The amperometric response of the biosensor in skimmed milk was tested directly in the contamined sample while in raw and whole milk samples, centrifugations were performed to remove the fat. After centrifugation of the inoculated raw and whole milk (4500 rpm at 25 °C for 30 min), the

pellets were suspended in PBS (pH 7.4), and the amperometric response was obtained. The control samples were raw, skimmed and whole milk samples with no bacteria.

2.7. Immunosensor validation

The immunosensor performance in milk samples was confronted to two other methods: Conventional method of detecting *Salmonella* in liquid milk described in Bacteriogical Online Analytical Manual (BAM) determined by the Food and Drug Administration (FDA) (ANDREWS et al., 2019) and the 1-2 Test® Kit manufactured by Biocontrol, AOAC method 989.13, (AOAC, 2005).

3. Results and discussion

3.1. Immunosensor assembly

Fig. 1 presents a summary of the immunosensor structure assembled from the CMCG and the analytical response of *Salmonella* Typhimurium detection. The CMCG was electrodeposited on the gold (Au) electrode forming a thin film that provides a platform with free COOH groups on the electrode surface. Then, the COOH groups were activated by EDC/NHS reaction for provide the protein A binding. The primary polyclonal antibodies against *Salmonella* were oriented by covalent bonds with protein A. In the next step, the immunosensor recognized the *Salmonella* cells, and HRP-labeled antibodies bound to them forming a sandwich assay. The analytical response signal of the immunosensor was obtained by chronoamperometric technique and, the measured electrical current (i) was generated from the electronic transfer of oxidation-reduction reactions involved in the hydrogen peroxide/hydroquinone system (PIMENTA-MARTINS et al., 2012).

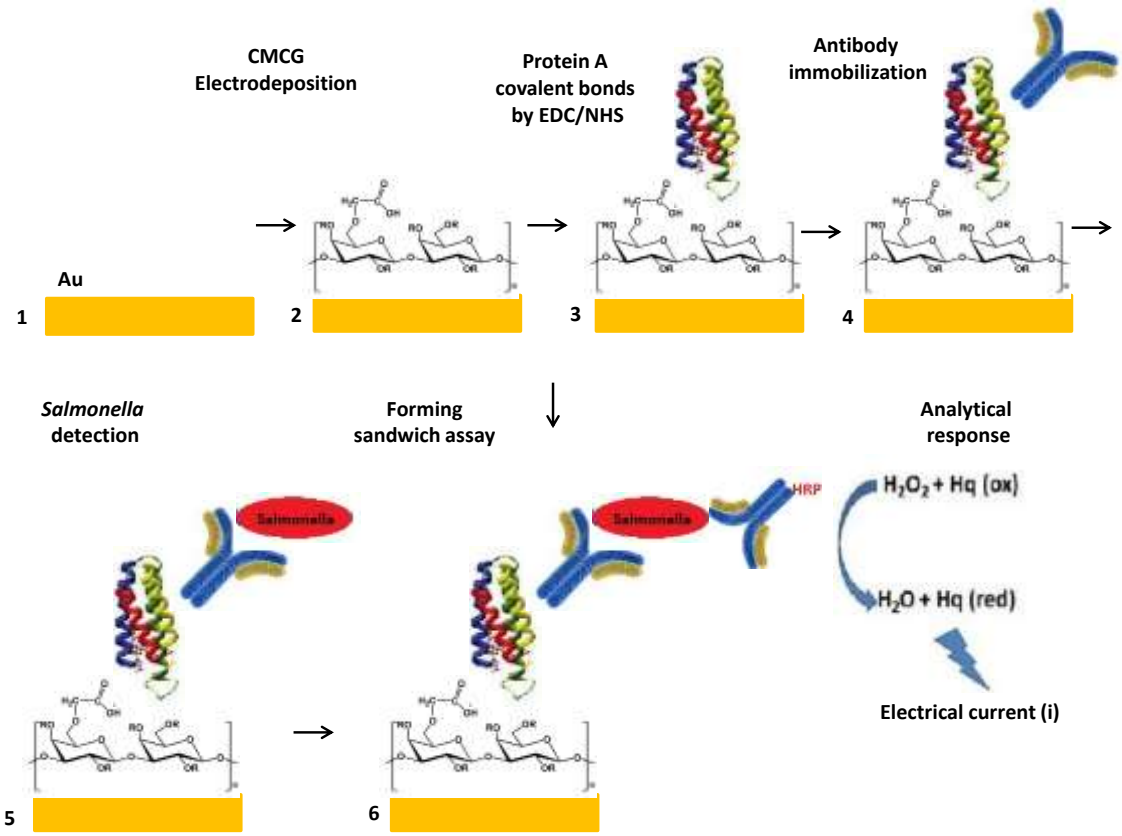

Fig. 1. Immunosensor assembly based on CMCG and the analytical response. (ox) oxidation (red) reduction (Au) gold.

3.1.2. Optimizations studies

Three parameters were optimized by cyclic voltammetry for immunosensor assembly. CMCG concentration for film formation by electrodeposition, the protein A (prA) and the primary antibody (Ab) concentrations. The voltammograms are presented in Fig. 2.

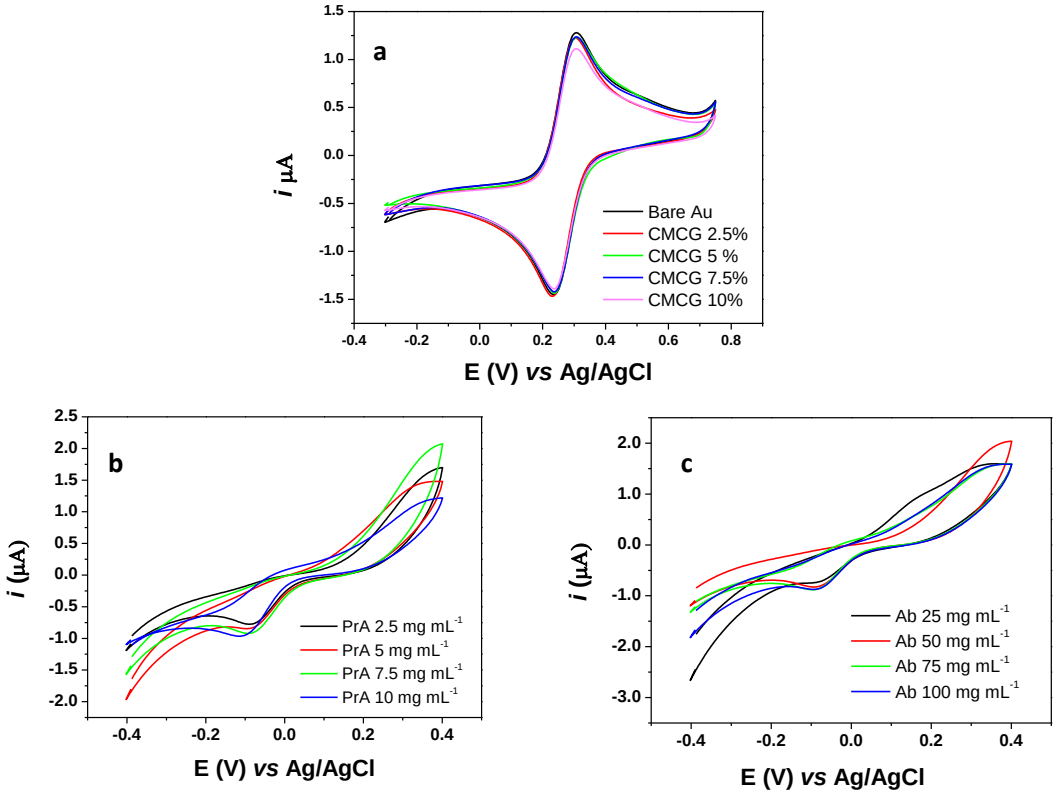

Fig. 2. Cyclic voltammograms (CV) generated during the study of optimization parameters a) CV in 4 mM $K_3[Fe(CN)_6]$ and 1 M KCl solution of CMCG electrodeposition in different concentrations (2.5 to 10% w/ v). b) CV in PBS solution (pH 7.4) containing 4 mM hydroquinone and 600 mM H_2O_2, PrA concentrations of 2.5, 5.0, 7.5, and 10 mg mL^{-1} . c) Anti-*Salmonella* Ab concentrations of 25, 50, 75, and 100 mg mL^{-1}.

Various concentrations of CMCG were electrodeposited on gold surface and their voltammograms are shown in Fig. 2a. When electrodeposited on the surface, the CMCG film promotes the reduction of the electric current of the anodic and cathodic peaks of the voltammograms as evidence of the modification of the gold surface. An estimate of the percentage coverage of the surface (θ) can be made with the $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ probe using Eq. (2):

$$\theta = 1 - (\text{Ipmodified electrode}/\text{Ipcleaned electrode}) \times 100 \quad (2)$$

where Ip is the electric current peak of the modified and cleaned electrode obtained from voltammograms shown in Fig. 2a. By varying the peak current, we can estimate the surface coverage (ALEXANDRE et al., 2018). In view of the different concentrations tested, the result that best illustrated the modification of the gold surface was the 10% CMCG because it was the concentration with the best coverage, estimated at 5.7 % on the surface of the gold electrode. Good surface coverage is generally related to efficient immobilization of biomolecules as observed in previous studies (ALEXANDRE et al., 2018; MELO et al., 2017).

In order to further enhance the immunosensor performance, optimization studies involving concentrations of biomolecules and methods of immobilization are often reported in the literature (BABACAN et al., 2000; MANTZILA; MAIPA; PRODROMIDIS, 2008; PIMENTA-MARTINS et al., 2012). In the present paper, experiments were carried out to establish the optimal concentrations for protein A and antibody in the immunosensor assembly. Thus, cyclic voltammetry studies were performed varying the protein A and antibody concentrations in the presence of 300 mM H_2O_2 and 3 mM hydroquinone in PBS solution (pH 7.4). It was observed in Fig. 2b that the peaks in the voltammograms for PrA concentrations of 7.5 and 10 mg mL^{-1} exhibited the highest values with a similar behavior, with very close peak current values about 0,93 and 0,95 μA, respectively, although the 7.5 mg mL^{-1} peak was more defined and resolute . In contrast, for 2.5 and 5.0 mg mL^{-1} concentrations of protein A, the peak currents were smaller, 0.73 and 0.86 μA. Althougth a variation of the redox reactions from any protein A concentrations have been verified in this work, 7.5 mg mL^{-1} of protein A was considered the minimum for an efficient immunosensor performance, and therefore, it was selected to evaluate the next optimization procedure.

Figure 2c gives the voltammograms as a function of antibody concentration. In this case, the peak current values increased up to 75 mg mL^{-1} antibody concentration, showing the highest current value of 0.86 μA. The antibody concentration of 100-mg mL^{-1} presented the same peak current value, in this point probably reached system saturation. The Ab concentrations of 50 mg mL^{-1} presented a lower peak current 0.81 μA ,while, at Ab concentration of 25 mg mL^{-1} there was no peak formation. So the 75 mg mL^{-1} concentration was considered the most appropriate parameter for immunosensor assembly.

3.2. Immunosensor characterization

The CMCG-based immunosensor was characterized by electrochemical, infrared spectrometry and scanning electron microcopy techniques. The results are summarized in Fig. 3 and Fig. 4.

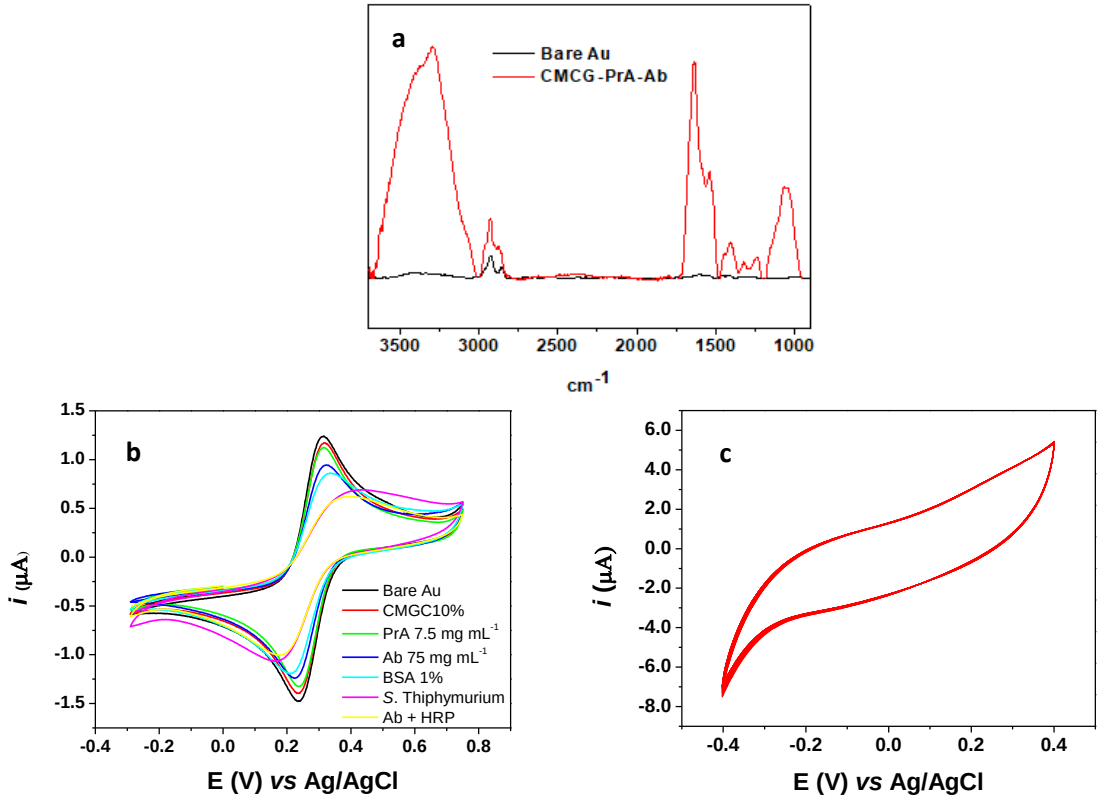

Fig. 3. a) ATR-FTIR spectra of the surfaces of cleaned electrode and electrode modified with immunosensor assembly. b) Cyclic voltammograms in 4 mM $K_3[Fe(CN)_6]$ and 1 M KCl solution at each step of immunosensor assembly. c) Stability study of the imunosensor assembly by 30 consecutive scanning cycles in PBS buffer (pH 7.4) containing 600 mM H_2O_2 and 4 mM hydroquinone.

The electrode surface was evaluated by infrared technique before and after the modifications and the obtained spectra are shown in Fig. 3a. On the modified surface, observed after the formation of the CMCG-PrA-Ab-BSA immunosensor a broad band was observed at 3300 cm^{-1} that was attributed to NH stretching vibration of amide A, indicating the presence of amino acids (BARTH, 2007). The bands between 2900 and 2800 cm^{-1} are assigned to C–H stretching; on the modified surface. An increase in the intensity in this region can be observed due to the accumulation of alkyl chains from immobilized proteins. The band at 1600 cm^{-1} is related to amide I and attributed to C=O stretching. The bands at 1500, 1400, and 1350 cm^{-1} probably indicate CN stretching and NH bending of amides II and III, respectively (KONG; YU, 2007). Thus, ATR-FTIR results substantiate the presence of proteins on the modified electrode surface, indicating antibody immobilization on the CMCG film surface.

The electrochemical characterization of the immunosensor was performed by obtaining voltammograms of the cleaned surface and their respective modifications using the redox species $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$ using the cyclic voltammetry technique. Potassium ferrocyanide ($K_3[Fe(CN)_6]$) is a chemical compound with defined redox properties and was found to be very useful

in evaluating surface modifications (DIJKSMA et al., 2002; JUNG et al., 1997). Cyclic voltammetry can provide useful information on the changes of the electrode behavior after each assembly step. As shown in Fig. 3b, the redox probe $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ reveals a reversible cyclic voltammogram at a bare gold electrode. After the CMCG was electrodeposited on pretreated gold electrode forming a thin film layer on the electrode surface, the penetration of the redox probe close to the surface electrode was slightly reduced. Then, a decrease in the current response was observed (Fig. 3b). After the immobilization of protein A on the modified gold electrode the penetration of the redox probe was further reduced. This fact was also verified after the binding of the first Ab–*Salmonella*– HRP-labeled Ab. The immunosensor stability was evaluated after 30 consecutive cycles of cyclic voltammetry in PBS solution at pH 7.4. The current biosensor showed a coefficient of variation of 12%, indicating good stability (Fig 2c).

Furthermore, the biosensor surface was studied by scanning electron microscopy (SEM), before (Fig. 4a) and after (Fig. 4b) recognition of the antigen and binding of the HRP-labeled antibody. The *S.* Typhimurium cells were clearly observed on the surface as further evidence of the antigen-antibody interaction (Fig. 4b).

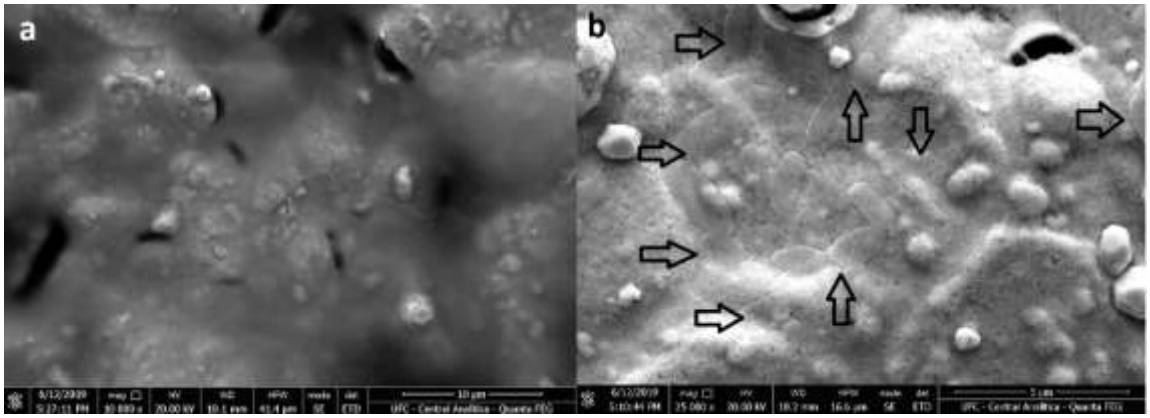

Fig. 4. Scanning electron photomicrograph of the CMCG-PrA-Ab system before (a) and after (b) antigen recognition. The arrows indicate the *S.* Typhimurium captured by the antibody immobilized on the CMCG film surface.

3.3. Immunosensor performance parameters

3.3.1. Calibration curve

The chronoamperometric response of the immunosensor in the presence of low and high *S.* Typhimurium concentrations in PBS pH 7.4 at 3 mM hydroquinone and 300 mM hydrogen peroxide is shown in Fig. 5.

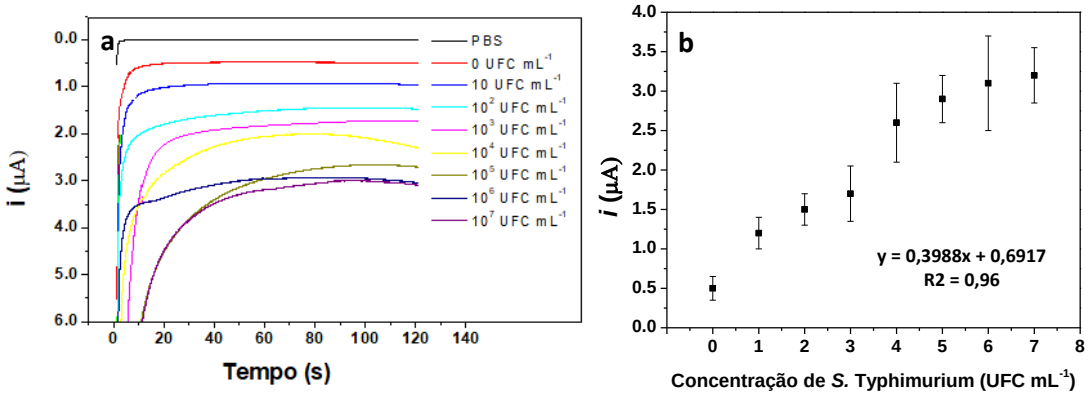

Fig. 5. Current response of the immunosensor. a) Chronoamperogram obtained for *Salmonella* concentrations 10^1 to 10^7 CFU mL^{-1} in PBS solution (pH 7.4) containing 3 mM hydroquinone and 300 mM H_2O_2. b) Response curve of the biosensor (0 to 7 log CFU mL^{-1}). Amperometric measurements were determined by polarizing the electrode at - 75 mV until a stable baseline (steady state) was reached in 120 s.

It was possible to verify in the calibration curve a linear tendency from 10^1 to 10^5 CFU mL^{-1}. From 10^6, a loss of sensitivity with system saturation was observed. The CMCG-based immunosensor was able to distinguish clearly the control (PBS) from *Salmonella* dilutions with statistic difference ($p < 0.05$). Additionally, the immunosensor presented a LOD of 10 CFU mL^{-1} for S. Typhimurium detection; this was much lower than previously reported in other amperometric biosensor studies (MELO et al., 2016). The LOD was obtained from Eq. (2) where $\bar{X}$ is the average of multiple determinations, *t* is the student distribution factor, and SD is the standard deviation.

$$LOD = \bar{X} + t(n-1, 1-\alpha) * SD \quad (2)$$

Moreover, the detection time was 125 min, which included the incubation time for recognition of antigen in the PBS, the time for the binding of HRP-labeled antibody, and the time for amperometric measurement. For whole milks it is necessary one additional step, to remove the fat by centrifugation, this take around 30 min. This result makes the current system a promising biodevice to be used as a viable method for *Salmonella* detection in the food industry, especially for the rapid emergency screening of contaminated samples. In addition, the CMCG platform can be tested for immobilization of other biomolecules and thus expanding the detection possibilities of other analytes of interest in food and other areas.

The relative standard deviation (RSD) of electrical current values for six electrodes was 6.2%, suggesting good precision and reproducibility. Thus, considering that the immunosensor was assembled using various steps of biomolecules immobilization, the current device showed an acceptable and reproducible behavior.

3.3.2. Specificity and selectivity assay

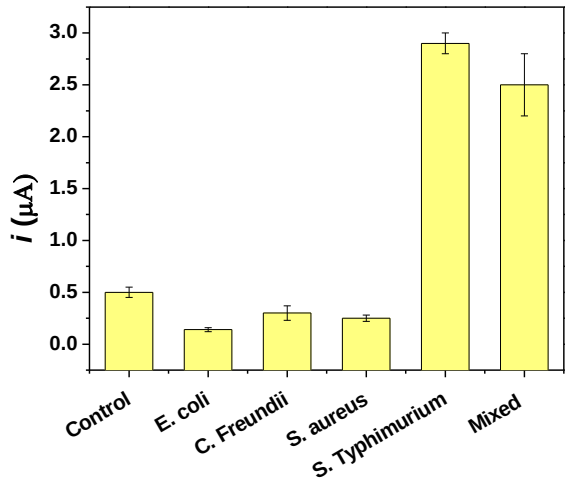

Fig. 6. Specificity of the immunosensor for control sample (PBS pH 7.4), pure, and mixed cultures of *S.* Typhimurium, *S. aureus*, *E.coli* and *C. freundii* (10^6 CFU mL^{-1}).

The specificity of the amperometric immunosensor was also investigated. *Citrobacter freundii*, *Escherichia coli* and *Staphyloccocus aureus* were selected to verify cross-reactivity. *C. freundii* and *E. coli* were chosen because these are groups phylogenetically related to *Salmonella* genus (KNIREL et al., 2002; PÉTERFI et al., 2007), while *S. aureus* is a contagious pathogen that causes mastitis in dairy cattle (BOSS et al., 2016; CREMONESI et al., 2015), and is an opportunistic pathogen in humans and many other animal species (NÜBEL et al., 2011; SANGVIK et al., 2011; ZADOKS et al., 2011). As shown in Fig. 6, the current (*i*) values measured in the *S. aureus*, *E. coli* and *C. freundii* dilutions were lower than the control (PBS pH 7.4), indicating that the response induced by the non-specific bonding did not occur. Even more important was the result of mixed culture, where an inoculum with the four bacteria was tested and the signal was very close to the result of S. Typhimurium individually. This result indicates an adequate performance in the evaluation of unprocessed foods, ie, in the reception of raw materials, where the target bacteria can be masked by the presence of other one. This selectivity and specificity test demonstrated that the immunosensor was clearly able to distinguish *S.* Typhimurium from *E. coli* and *C. freundii*. This specificity indicates a great potential for the application of this immunosensor in food safety control of *Salmonella* contamination.

3.4. Assessment of the immunosensor response in the milk samples

To evaluate the feasibility of the immunosensor proposed for the detection of S. Typhimurium in a complex matrix, we inoculated *S.* Typhimurium into skimmed and whole UHT milk samples at two different concentrations (Fig. 7).

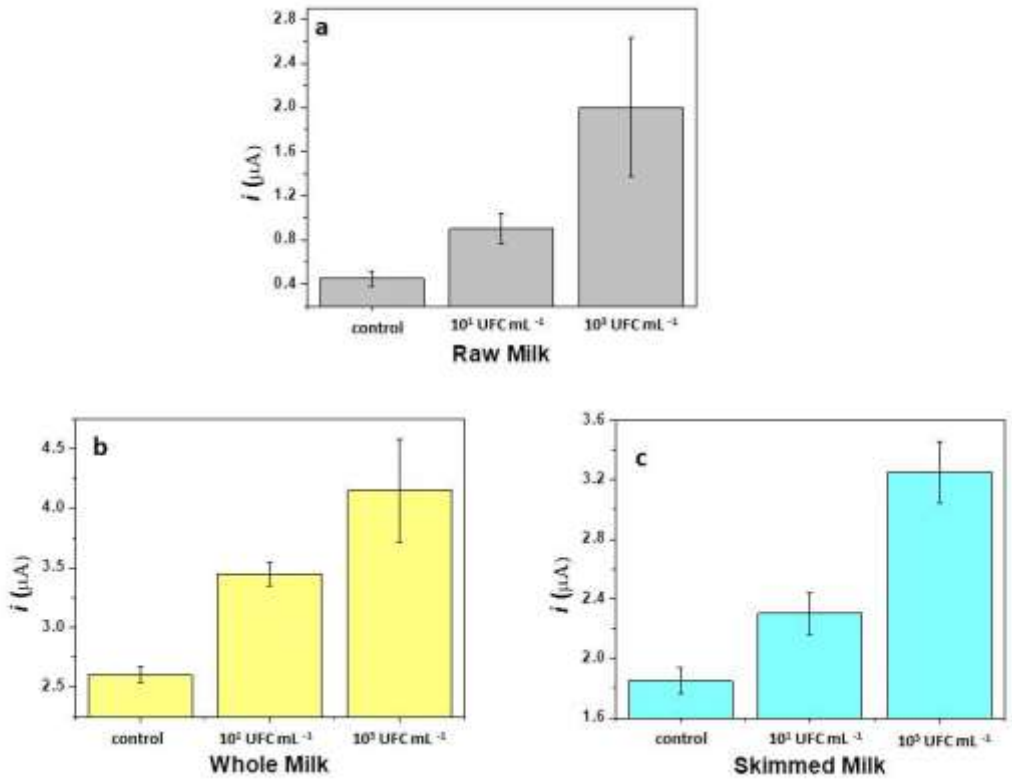

Fig. 7. Amperometric response of the immunosensor at two concentrations of *S.* Typhimurium in raw, skimmed and whole milk samples. Results obtained in 10 mM PBS buffer (pH 7.4) H_2O_2 (300 mM) and hydroquinone (3 mM), potential 75 mV for 120 s.

Satisfactory results were obtained and the immunosensor detection limit remained at 10 CFU mL^{-1} for all kind of milk evaluated in this work. For skimmed milk, the analysis was performed directly on the inoculated sample, and there was no change in the detection time of 125 min. However, for the whole milk, it was necessary to include a sample centrifugation step before submitting it for detection. In the latter case, the detection time was increased to 155 min including the time to remove the fat in milk. Without this last procedure of sample preparation the performance of the immunosensor would be severely affected.

It is important to note that most commercial alternative methods for *Salmonella* detection in food (such as immunology assays, nucleic acid tests, and miniaturized biochemical assay) require a pre-enrichment step in order to elevate the *Salmonella* concentration to 10^4–10^5 CFU mL^{-1}, which is the limit of detection by those procedures (LEE et al., 2015). The pre-enrichment step requires about 24 h to increase the bacterial population to the detectable level. In this connection, the device developed in this study is very rapid because it does not require sample pre-enrichment and thus can provide the result in just over 2 h. Furthermore, in view of automated integration and portability of the device, this methodology represents a simple, fast, easily handled, and accurate tool to carry out the analysis of dairy products to ensure food safety.

3.5 Immunosensor validation

The milk samples analyzed by the immunosensor were also submitted to detection of *Salmonella* by two other methods, a conventional method (BAM online) and a rapid method of *Salmonella* analysis (1-2 Test® Biocontrol) (TABLE 1).

Table 1 – Detection of *Salmonella* sp. in milk (skimmed UHT, whole and raw UHT) by applying 3 different methods, the immunosensor, the conventional method defined by BAM and the rapid test 1-2 Test® Biocontrol.

Milk samples	Method	*S.* Typhimurium concentration (UFC mL^{-1})		
		0	10^1	10^3
Raw, whole and skimmed	Imunosensor	Absence	Presence	Presence
	BAM	Absence	Presence	Presence
	1-2 Test®	Absence	Presence	Presence

The CMGC-based immunosensor reproduced the same results obtained by other two methods tested for the samples of skimmed milk, whole and raw, as well as for all *S.* Typhimurium concentrations inoculated in each type of milk. This evaluation demonstrated detection efficiency for the developed immunosensor comparable to both methods tested. Both conventional and fast are recommended by AOAC is widely used in microbiological food analysis. Through 1-2 Test® Biocontrol, the detection of *Salmonella* in all three types of milk was completed in 54 h, because as with the conventional method, it is also necessary 24 h for pre-enrichment sample. This step is extremely important as it allows the elevation of the bacterial population to a level of 10^4 CFU mL^{-1}, amount required for the detection by conventional methods and most rapid methods (LEE et al., 2015).

Analysis of milk samples by immunosensor was performed at a time of 56 times smaller than the conventional method (BAM) and 21 times smaller than the fast method (1-2 Test® Biocontrol). For skim milk this time was even shorter because it did not require centrifugation step prior to immunosensor analysis, which reduced the total time to 125 min, while for whole and raw milks it took a total of 155 min. They were centrifuged for 30 min to remove fat before immunosensor analysis. The comparison of these methods demonstrated that the immunosensor developed was the most efficient, drastically reduced the time required for detection and requires no pre-enrichment step. And yet, it used a structure simple laboratory system, with a reduced amount of reagents and materials besides ease of handling.

4. Conclusions

Carboxymethylated cashew gum has been shown to be an efficient platform for immobilization of the anti-*Salmonella* antibody and could be tested for immobilization of other biomolecules. The CMCG -based immunosensor detected *Salmonella* bacteria quickly and specifically in PBS buffer and milk samples, demonstrating great potential for routine use in the food industry.

Optimized parameters allowed the development of the CMCG -based immunosensor with rapid, sensitive and quantitative detection of the *Salmonella* pathogen. The characterization performed by the techniques of cyclic voltammetry, scanning electron microscopy and infrared spectroscopy demonstrated that the assembly procedure produced a stable and efficient immunosensor. The immunosensor had a rapid response with one of the smallest detection limits among *Salmonella* immunosensors developed so far. The accuracy of the analytical response yielded a result compatible with similar studies.

A high specificity and selectivity of the biosensor in the detection of *Salmonella* sp. it has been proven in isolated and mixed cultures of *E. coli, C. freundii* and *S. aureus*. The applicability of the immunosensor in different types of milk has been confirmed and is faster, easier to handle, and has a lower detection limit than the conventional method and the fast method tested. The use of immunosensor eliminates the pre-enrichment step of the analytical sample and its response tends to linearity against concentrations of S. Thyphimurium, suggesting its use for quantification assays.

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Acknowledgements

The authors would like to thank the Brazilian agencies, CNPq, and CAPES, for their financial support, Embrapa Tropical Agroindustry and National Center of Energy and Materials Research (CNPEM). Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

Conflict of interests

The authors declare no competing financial interest.

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5 CONCLUSÕES

Os parâmetros otimizados permitiram o desenvolvimento do imunossensor a base de GCCM com detecção rápida, sensível e quantitativa do patógeno *Salmonella* sp.

A caracterização realizada pelas técnicas de voltametria cíclica, microscopia eletrônica de varredura e espectroscopia de infravermelho, demostraram que o procedimento de montagem utilizado produziu um imunossensor estável e eficiente.

O imunossensor apresenta uma resposta rápida com um dos menores limites de detecção dentre os imunossensores para detecção de *Salmonella* desenvolvidos até o momento. A precisão da resposta analítica apresenta um resultado compatível com o método de montagem equiparando-se a estudos semelhantes.

Uma alta especificidade do biossensor na detecção de *Salmonella* sp. foi comprovada em culturas isoladas e culturas mistas de *E. coli*, *C. freundii* e *S. aureus*.

A aplicabilidade do imunossensor para análise em diferentes tipos de leite foi confirmada e demonstra-se mais rápida, de manipulação mais fácil, além de apresentar menor limite de detecção em relação ao método convencional e ao método rápido testados.

O uso do imunossensor dispensa a etapa de pré-enriquecimento da amostra analítica e sua resposta apresenta uma tendência à linearidade para diferentes concentrações de *S.* Thyphimurium, sugerindo seu uso para ensaios de quantificação.

A goma de cajueiro carboximetilada demonstrou-se uma eficiente plataforma para imobilização do anticorpo anti-*Salmonella*. O imunossensor a base de GCCM detectou a bactéria *Salmonella* de forma rápida e específica em tampão PBS e em amostras de leite, demonstrando um grande potencial para uso na rotina da indústria de alimentos.

6 PERSPECTIVAS

O resultado promissor da CMCG como plataforma de montagem de imunossensores encoraja a realização de estudos para testes com outras biomoléculas, como ácidos nucleicos, enzimas, na perspectiva de desenvolvimento de outros tipos de biossensores, ampliando também os testes de aplicabilidade a outras matrizes de alimentos.

Estudos mais específicos com foco em modificações químicas na GC podem eliminar o uso da proteína A como molécula de orientação, tornando a montagem do dispositivo mais rápida, prática e de custo mais acessível.

Esta aplicação amplia o potencial de aplicações da GC não somente para biossensores, como também para outros tipos de sensores. Além disso, são criadas oportunidades para novas demandas de consumo para GC aumentando seu potencial econômico, nos países produtores.

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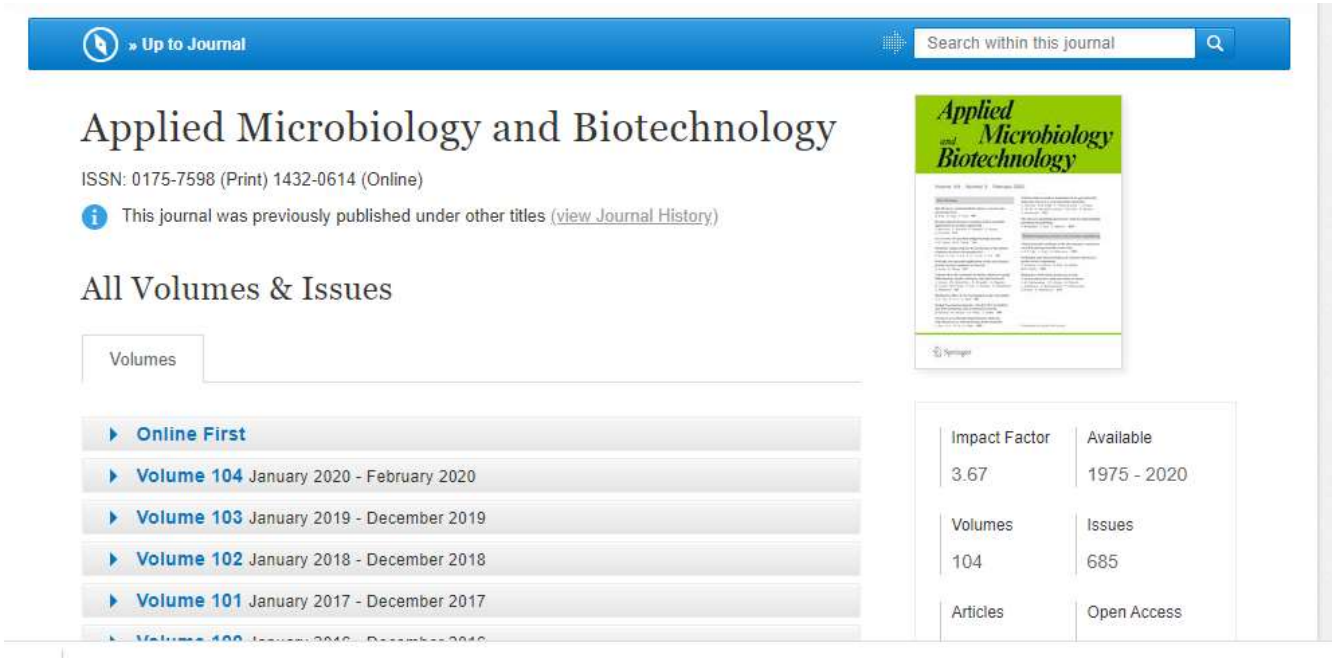
ANEXOS

ANEXO A - Applied Microbiology And Biotechnology – ISSN 0175-7598

QUALIS – A2

FATOR DE IMPACTO – 3,67



ANEXO B - Carbohydrate Polymers – ISSN: 0144-8617

QUALIS – A1

FATOR DE IMPACTO – 6,044