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Desenvolvimento de imunossensor para detecção de *Staphylococcus aureus:* estratégias de imobilização de anticorpos


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Caxias do Sul

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# Desenvolvimento de imunossensor para detecção de *Staphylococcus aureus:* estratégias de imobilização de anticorpos

**Dissertação apresentada ao programa de Pós-graduação em Biotecnologia da Universidade de Caxias do Sul, visando à obtenção do grau de Mestre em Biotecnologia.**

**Orientadora: Profª. Drª. Mariana Roesch Ely**

**Co-Orientador: Prof. Dr. Frank Patrick Missell**

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# DESENVOLVIMENTO DE IMOSSENSOR PARA DETECÇÃO DE *S. AUREUS*: ESTRATÉGIAS DE IMOBILIZAÇÃO DE ANTICORPOS

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Orientadora: Profa. Dra. Mariana Roesch Ely

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DISSERTAÇÃO APROVADA EM 18 DE ABRIL DE 2016.

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"... Para cada problema deve haver um método para
resolvê-lo sistematicamente; parte por parte ..."

(René Descartes 1638)

Dedico este trabalho aos meus pais Isabel e Adilone, meu irmão Eduardo, ao meu esposo Alexander, pela confiança depositada e pela paciência e apoio nos momentos difícieis, e à minha filha Verônica pelo seu amor incondicional.

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## LISTA DE ABREVIATURAS

| | |
|---|---|
| Ab | Anticorpos |
| AFM | Microscopia de força atômica |
| AO/EB | Laranja de acridina/ brometo de etídeo |
| CHO | Células do Ovário do Hamster Chinês |
| DMSO | Dimetilsulfóxido |
| DEMEN | Dulbecco's Modified Eagle Medium |
| DTT | Dithiothreitol |
| EDC | 1-etil-3(3-dimetilaminopropil)carbodiimida |
| ELISA | Enzima imunoensaio |
| Fc | Domínio constante |
| Fab | Domínio de ligação ao antígeno |
| FBS | Soro fetal bovino |
| ICP-OES | Espectrometria de emissão óptica com plasma |
| IgG | Imunoglobulina G |
| LB | Langmuir and Blodgett |
| ME | Magneto-elastic |
| MEV | Microscopia eletrônica de varredura |
| MTT | 3-(4,5-dimetiltiazol-2-il)-2,5difeniltetrazólico |
| PBS | Tampão fosfato-salino |
| PEI | Polietilenoimina |
| pI | Ponto isoelétrico |
| PrA | Protein A |
| PrG | Protein G |

| | |
|---|---|
| PRD | Proteínas de reconhecimento de domínos |
| RBS | Rutherford Backscattering Spectroscopy |
| RMS | Raiz do valor quadrático médio |
| SAM | Monocamadas auto organizadas |
| scFv | Anticorpo de cadeia única |
| ΔG | Energia Livre |

## RESUMO

Biossensores são dispositivos que convertem uma resposta biológica em um sinal elétrico e estão acoplados a um elemento de reconhecimento biológico, o qual fornece informações analíticas específicas e quantitativas. Transdutores magneto-elásticos são dispositivos promissores para aplicação como biossensores em diferentes áreas, com destaque na captura de patógenos e monitoramento de contaminantes em amostras. A biomolécula de reconhecimento pode ser disposta sobre o biossensor utilizando diferentes estratégias de imobilização, randômica ou orientada, conforme funcionalização química da superfície. Neste estudo os sensores magneto-elásticos foram inicialmente recobertos com ouro bilateral pela técnica de *sputtering*, essa cobertura foi então avaliada quanto a estabilidade pelas medidas de frequência de ressonância e avaliação da presença de íons no meio por ICP-OES. A melhora da biocompatibilidade pela cobertura de ouro foi determinada pelos ensaios de MTT e coloração de brometo de etídio e laranja de acridina. Os resultados demostraram que a cobertura de ouro foi eficiente para estabilização do sensor quanto a degradação, este também apresentou biocompatibilidade pelo período de 7 dias. Dois métodos de imobilização de anticorpos foram então testados para detecção de *S. aureus*. No método 1 foi utilizado proteína G como captura orientada de anticorpo, enquanto que no método 2 os anticorpos foram imobilizados randomicamente sobre a superfície amino funcionalizada. Os resultados obtidos indicaram melhores rendimentos para imobilização orientada com um limite de detecção de $10^4$ UFC/mL para tiras de 5mm x 1mm x 15 µm, enquanto que a imobilização randômica apresentou um limite de detecção de $10^7$ UFC/mL para sensores de mesmo tamanho. Imagens de microscopia eletrônica de varredura e microscopia óptica com fluorescência mostraram um incremento na captura de *S. aureus* utilizando a imobilização orientada. Um modo de contato intermitente com AFM foi utilizado para análise da interface, o qual evidenciou alterações nas superfícies modificadas em estudo. Os sensores magneto-elásticos são dispositivos promissores para detecção de patógeno e toxinas, gerando resultados rápidos e sensíveis comparados as abordagens convencionais.

## ABSTRACT

Biosensors are devices that convert a biological response into an electrical signal and are coupled to a biological recognition element, which provides specific and quantitative analytical information. Magneto-elastic transducers are promising devices for use as biosensors in different areas, especially in the capture of pathogens and monitoring of contaminants in samples. The recognition biomolecule may be disposed on different biosensor using immobilization strategies, random or oriented, depending on the chemical functionalization of the surface. In this study the magneto-elastic sensors were initially covered with a bilateral gold layer by sputtering technique, this coverage was then evaluated for stability by measures of frequency resonance and evaluation of the presence of ions in the middle by ICP-OES. Biocompatibility by gold coverage was determined by MTT assay and ethidium bromide and acridine orange staining. The results showed that the gold coverage was effective in stabilizing the sensor and degradation, this also presented biocompatibility for a period of 7 days. Two antibody immobilization methods were then tested for the detection of *S. aureus*. In method 1 protein G was used as oriented capture antibody, while in method 2 antibodies were randomly immobilized on the amino functionalized surface. The results indicated best yields for oriented immobilization with a detection limit of $10^4$ UFC/ml for 5 mm x 1mm x 15µm strips, whereas the random immobilization showed a detection limit of $10^7$ UFC/ml for the same size sensors. Images of scanning electron microscopy and optical microscopy with fluorescence showed an increase in the capture of *S. aureus* using the oriented immobilization. An intermittent contact mode AFM was used for analysis of the interface, which showed changes in surface modification under study. The magneto-elastic sensors are promising devices for pathogen detection and toxins, causing rapid and sensitive results compared to conventional approaches

## 1. INTRODUÇÃO

Um biossensor consiste em um dispositivo analítico que contém um componente biológico o qual garante a especificidade gerando uma resposta quantitativa ou semi quantitativa. Também podemos considerar os biossensores como dispositivos de sensoriamento para detecção de contaminantes químicos e biológicos de alta sensibilidade e baixo custo, capazes de fornecer resposta em tempo real. Devido essas características, as pesquisas na área de biossensores têm grandes perspectivas de crescimento a nível global.

O mercado de biossensores é altamente competitivo e impulsionado principalmente pelo setor médico e farmacêutico. Adley *et al* (2014) demostraram em seu trabalho que as receitas globais referentes a investimentos em pesquisas de sensores apresentam crescimento robusto, e devem exceder 14 bilhões de dólares em 2016, estes distribuídos em 47 áreas de aplicação. Os testes específicos para detecção de patógeno também acompanham este crescimento para todos os segmentos a uma taxa composta de crescimento anual de 4,5%, esses dados estão dispostos no trabalho de Sadana Sadana (2015).

Grandes esforços em pesquisa e desenvolvimento já produziram biossensores viáveis para uma vasta gama de aplicações na área médica. No entanto, apenas alguns destes dispositivos são específicos para a detecção de bactérias patogênicas e estão comercialmente disponíveis, ou se aproximam da comercialização. A pesquisa na área de biossensores foi inicialmente explorada pelo trabalho de Clark e Lyons (1962), que utilizou uma combinação de um sensor de oxigênio eletroquímico para medição quantitativa de glicose. Inicialmente, o campo foi voltado para imobilização de enzimas sobre eletrodos, expandindo gradualmente para inclusão de outros elementos tais como

anticorpos, células e os ácidos nucleicos e o termo “eletrodo de enzima” foi transformado em “biossensor”.

Nos últimos anos, várias técnicas empregando biossensores, tais como ressonância de plasma de superfície, microbalança de cristal de quartzo e imunossensor piezoeléctrico, entre outras, têm sido desenvolvidas visando reduzir o tempo de detecção e identificação de patógenos. Biossensores magneto-elásticos (ME) vem sendo estudados para aplicação na detecção de patógenos, e tem demostrado resultados promissores no monitoramento de contaminantes em diferentes amostras (Guntupalli *et al.* 2007, Li *et al.* 2010, Chai *et al.* 2013, Chin *et al.* 2014, Rodriguez-Rodriguez *et al.* 2016). Estes transdutores apresentam promitente aplicação em sensoriamento devido ao fato de não necessitarem utilização de conexões físicas diretas (monitoramento *wireless*), essa e outras propriedades do respectivo dispositivo foram apresentadas em uma revisão publicada por Grimes *et al* (2002).

O desenvolvimento eficiente de biossensores depende das interações das biomoléculas alvo com grupos químicos específicos ou macromoléculas que são imobilizados sobre um material sólido. O uso de macromoléculas é de grande interesse na área de biossensores estas podem ser anticorpos, enzimas, fragmento de DNA, vírus entre outras.

Métodos de imobilização que promovam um desempenho satisfatório na captura da biomolécula alvo são fundamentais para alcançar grandes resultados de diagnóstico. A perda de atividade destas moléculas está associada a algumas limitações na utilização de técnicas de imobilização. Uma das principais razões para tal é atribuída à perda de orientação aleatória das macromoléculas assimétricas sobre as superfícies de apoio. No desenvolvimento de biossensores para detecção de patógenos moléculas de anticorpos

são utilizadas devido sua alta especificidade, porém a orientação desta molécula torna-se imprescindível para uma melhor captura do micro-organismo.

*O Staphylococcus aureus* é considerado um patógeno humano oportunista, colonizando de forma assintomática a nasofaringe, e sendo responsável por uma vasta gama de infecções superficiais e invasivas. As infecções mais comuns envolvem a pele e feridas em sítios diversos. *Staphylococcus aureus* está também associado a mastite bovina em vacas leiteiras, sendo capaz de causar infecções de longa duração, com tendência a se tornarem crônicas, com baixa taxa de cura e grande perda na produção de leite. Algumas estirpes desta bactéria são capazes de produzir toxinas que podem causar intoxicação alimentar, com severos sintomas como náuseas, vômitos, diarreia e desidratação sendo este um fator relevante para produtos derivados do leite.

A detecção *S. aureus* utilizando métodos tradicionais demanda tempo, podendo chegar a 5-6 dias, fator que pode comprometer o avanço e controle da infecção. O desenvolvimento de métodos rápidos para a detecção de *S. aureus* é importante para o diagnóstico precoce e para controle da presença dessa bactéria em alimentos, principalmente no leite.

Este trabalho teve como o objetivo funcionalizar e caracterizar a superfície de sensores magneto-elásticos para a detecção de *S. aureus.* Assim sendo, os objetivos específicos foram: 1) Avaliar os efeitos das modificações nas superfícies de sensores magneto-elásticos; 2) Padronizar e aperfeiçoar a imobilização de anticorpos na superfície dos sensores; 3) Comparar diferentes métodos de imobilização quanto ao desempenho para o desenvolvimento de um sensor ME na detecção de *Staphylococcus aureus,* e 4) Caracterizar topograficamente os diferentes tipos de imobilização testados;

## 2. REVISÃO BIBLIOGRÁFICA

Um biossensor consiste em um dispositivo analítico que contém um componente biológico que garante a especificidade e produz uma resposta que é traduzida pelo componente físico em um sinal detectável. Podemos citar como componentes biológicos: antígenos, anticorpos, enzimas, ácidos nucleicos, receptores, células e suas organelas. Todos estes materiais biológicos são capazes de produzir respostas específicas a vários analitos. Existe uma gama de componentes físicos disponíveis, como fibras ópticas, dispositivos acústicos, cristais piezoelétricos, dispositivos magnéticos, além de diversos tipos de eletrodos modificados quimicamente. Em relação aos dispositivos de sensoriamento para detecção de contaminantes químicos e biológicos, estes apresentam alta sensibilidade e baixo custo, e são capazes de fornecer resposta de análise em tempo real, impulsionado pesquisa na área de biossensores.

Os sensores magneto-elásticos vêm sendo estudado para aplicação como dispositivo de sensoriamento em diferentes áreas e proporcionam inúmeras vantagens principalmente devido a utilização de amostras reduzidas e aos resultados serem obtidos em baixo tempo. Na área de alimentos a intensificação da adoção de medidas que diminuam os riscos à saúde e as barreiras sanitárias restritivas ao comércio internacional, têm impulsionado o controle da qualidade de alimentos. A detecção de patógenos em alimentos, bem como em amostras hospitalares por métodos rápidos de baixo custo, são de grande interesse na saúde pública. Na área de alimentos estas técnicas garantem a qualidade do alimento, e na área hospitalar podem reduzir consideravelmente o tempo de internação diminuindo os gastos em saúde pública.

## 2.1 *Staphylococcus aureus*

O *S. aureus* está classificado entre os patógenos mais comuns e importantes causadores de doença em animais e humanos. Foi isolado pela primeira vez em 1880 pelo cirurgião escocês Alexander Ogston de um abscesso cirúrgico. Ogston usou o termo "Staphylococcus" (Staphyle grego, cacho de uvas; Kokkos, baga), que se refere à aparência deste micro-organismo ao microscópico depois da coloração **Figura 1 B**. Em 1884, o médico alemão Friedrich Julius Rosenbach diferenciou as bactérias pela cor de suas colônias: *S. aureus* (do aurum Latina, ouro) e *S. albus* (Latin para o branco). *S albus* foi rebatizado mais tarde com o nome de *S. epidermidis* por causa de sua onipresença na pele humana. O *S. aureus* tem um tamanho aproximado de 1µm **Figura 1 C** e sua colônia mede em média de 1 a 2 mm (Licitra 2013) **Figura 1A**. A importância clínica deste micro-organismo em humanos e animais tem sido conhecida desde o seu isolamento.

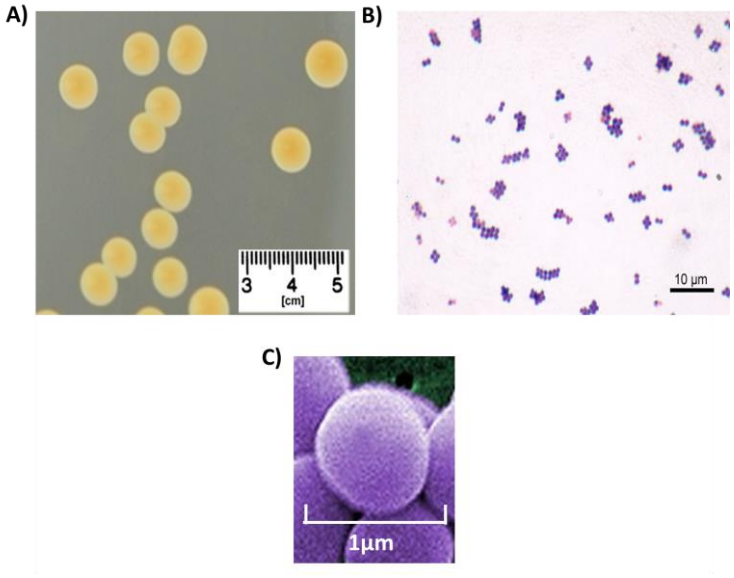


**Figura 1**: Imagens de colônias e disposição de S. aureus A) tamanho aproximado de UFC de colônia e coloração aurea; B) coloração de gram e disposição em forma de Staphyle em cocos gram negativo; C) imagem de microscopia eletrônica evidenciando um coco. (**Fonte:** http://www.microbiologyinpictures.com/staphylococcus%20aureus.html Acesso em 24 março 2016)

A distribuição no ambiente do *S. aureus* é muito ampla, visto que essa bactéria é significativamente capaz de resistir à dessecação e ao frio, podendo permanecer viável por longos períodos em partículas de poeira (Bannerman and Goldblum 2003, Tong *et al.* 2015). Esse micro-organismo pode ser encontrado no ambiente de circulação do ser humano, sendo o próprio homem seu principal hospedeiro, além de estar presente em diversas partes do corpo, como fossas nasais, garganta, intestinos e pele. Desses sítios anatômicos, as narinas possuem o maior índice de colonização, cuja prevalência é de cerca de 40% na população adulta, podendo ser ainda maior dentro de ambientes hospitalares (Cavalcanti *et al.* 2005). De acordo com alguns estudos, o carreamento nasal também contribui para a transmissão da bactéria por disseminação aérea (Zoltner *et al.* 2013).

O *S. aureus* está associado a diferentes patologias de grande importância para a área da saúde. Esse patógeno pode causar infecções graves como bacteremia ou sepse, endocardite e osteomelite (Bergin *et al.* 2015). O patógeno também é um importante causador de mastite em bovinos leiteiros, está entre os agentes etiológicos mais prevalentes nesta doença e o mais importante em termos de frequência e gravidade clínica, outros animais produtores leiteiros podem ser infectados são estes os caprinos e ovinos. Como um agente de infecções intramamária, o *S. aureus* pode contaminar o reservatório de leite a granel, e assim constituir um perigo bacteriológico para o leite e produtos lácteos consumidos (Bergonier *et al.* 2014). Algumas estirpes desta bactéria produzem toxinas que podem causar intoxicação alimentar principalmente por produtos derivados do leite, com severos sintomas como náuseas, vômitos, diarreia e desidratação (Jamison 2001, Tong *et al.* 2015).

Os métodos de identificação desta bactéria passa pelo isolamento de colônias que são posteriormente classificadas através de testes bioquímicos. Os *S. aureus* são

aeróbio e anaeróbio facultativo, oxidase negativo, catalase positiva, não-móveis, fermentativo e não-formador de esporos de bactérias (Santos *et al.* 2007). Porém, muitos destes testes bioquímicos possuem baixa especificidade e sensibilidade. O teste para a produção de hemólise e pigmentação em placas de ágar sangue, por exemplo, representa uma prática simples e rápida, mas, segundo Boerlin et. al., (2003), não muito segura, pois existem isolados de *S. aureus* sem capacidade hemolítica e com pigmentação variável, produzindo resultados falsos negativos.

O método para identificação de espécies do gênero *Staphylococcus* que utilizam sistemas comerciais de teste em miniatura, baseados em imunoensaios e testes bioquímicos, garantem uma maior precisão na identificação de *Staphylococcus* coagulase-positivo (Cercenado *et al.* 2012). Entretanto são testes que necessitam de tempos longos para obtenção dos resultados. Técnicas empregando biossensores, tais como ressonância de plasma de superfície (Nawattanapaiboon *et al.* 2015), microbalança de cristal de quartzo (Olsson *et al.* 2012), imunossensores piezoeléctricos (Boujday *et al.* 2008), veem sendo estudadas para redução do tempo de detecção.

Embora uma variedade de diferentes biossensores tenha sido desenvolvida nas últimas duas décadas, ainda existe a necessidade de dispositivos miniaturizados e descartáveis de baixo custo capazes de detectar rapidamente e identificar de maneira precisa uma ampla gama de contaminantes, toxinas e agentes patogênicos.

**2.2 Biossensores**

Os biossensores estão definidos pela “Union of Pure Apllied Chemistry” (IUPAC - 2001) como “Um biossensor é um instrumento integrado que é capaz de fornecer uma informação analítica específica quantitativa ou semi-quantitativa através do uso de um elemento de reconhecimento biológico (receptor bioquímico) que esta em contato direto com o elemento transdutor”.

Algumas etapas preliminares são cruciais e importantes no desenvolvimento de um biossensor, destacam-se: a) a seleção do componente biológico adequado, de modo que haja um método de imobilização passível a ser aplicado a este, e de modo a manter a seletividade do instrumento, e b) a seleção de sistema de transdução que permita converter adequadamente o produto biológico gerado em um sinal elétrico mensurável e confiável (Lawal and Adeloju 2012). Diferentes transdutores podem ser empregados na construção de um biossensor, tais como óticos (Long *et al.* 2013), magnéticos (Keat Ghee *et al.* 2009), elétricos (Azzouzi *et al.* 2015) térmicos (Kopparthy *et al.* 2015) ou acústicos (Crivianu-Gaita *et al.* 2016).

Os bio-compostos comumente utilizados no desenvolvimento da interface de sensoriamento de sensores biológicos são: enzimas, cofatores, receptores, anticorpos, células de micro-organismos, organelas e tecidos vegetais e animais. Assim, de acordo com o elemento biológico utilizado para a sua construção, os biossensores podem ser divididos em 3 classes; os enzimáticos (Hu *et al.* 2014), os quimiorreceptores (Zhang *et al.* 2015) e os imunossensores (Garcia Marin *et al.* 2015).

Os imunossensores são sensores que utilizam anticorpos como molécula de reconhecimento. Os anticorpos são moléculas com alta especificidade e de grande interesse na aplicação em diferentes técnicas analíticas devido à capacidade dos anticorpos se ligarem ao antígeno de forma peculiar.

No sistema de reconhecimento de um biossensor temos na interface a parte que entra em contato com o sistema e produz um sinal, a molécula de afinidade reconhece e liga-se ao análito de interesse, no caso de imunossensores são os anticorpos que capturam o patógeno por uma região específica. Os anticorpos devem ser escolhidos e validados, estes podem ser monoclonais ou policlonais. Outra etapa é a escolha do tipo de transdutor a ser utilizado baseado nas propriedades físicas destes, técnicas de

imunoensaios podem então ser delineadas. Por fim, o imunossensor deve ser validado quanto ao limite de detecção, linearidade e possíveis interferentes na amostra a ser analisadas. Na **Figura 2** estão representadas as etapas para o desenvolvimento de um imunossensor.

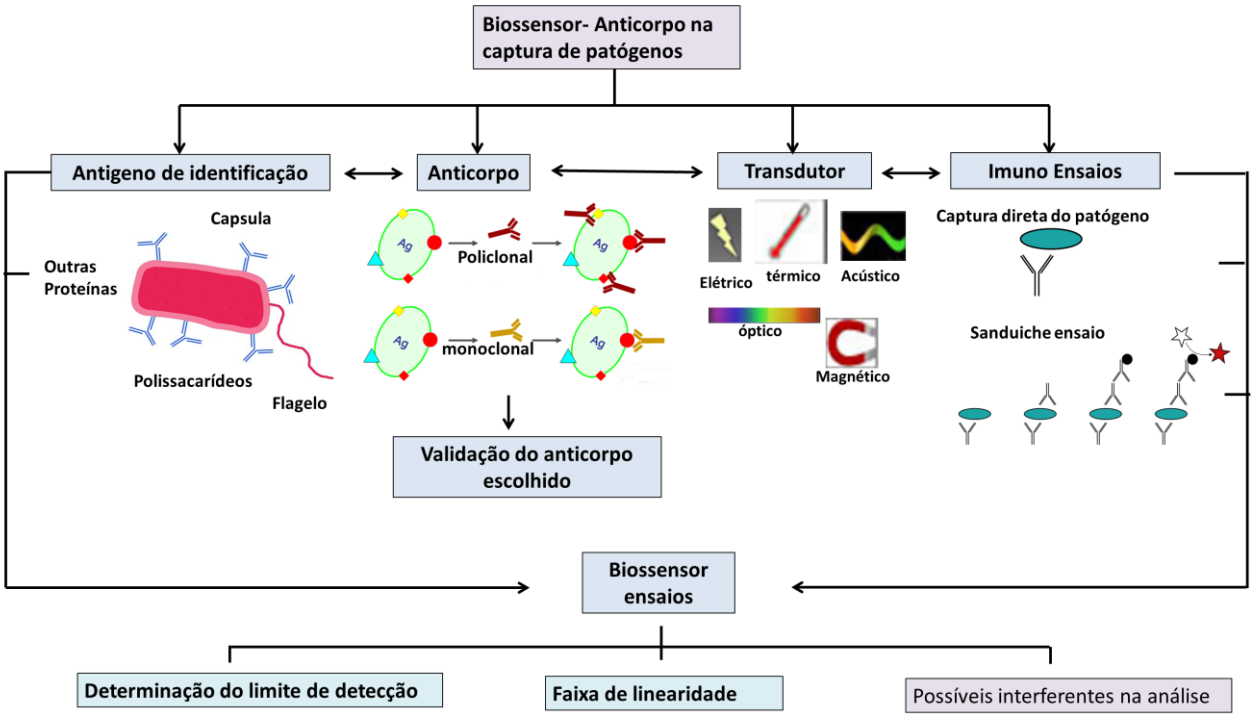


**Figura 2:** Esquema das diferentes estratégias para o desenvolvimento de um imunossensor na detecção de patógenos. Inicialmente é necessário escolher um biomarcador na bactéria para depois escolher o anticorpo que deve ser validado. Após seleciona-se o transdutor a ser utilizado, seguido do ensaio imunológico a ser desenvolvido. E, por fim, a validação da operação do sistema.

Dispositivos de bio-sensoriamento que proporcionem uma resposta analítica rápida são preferidas em diversas áreas. O desenvolvimento de biossensores para detecção de patógenos vem crescendo nos últimos anos (Lu *et al.* 2013, Abdalhai *et al.* 2014, Bandara *et al.* 2015). Não menos importante, a utilização de anticorpo como molécula de reconhecimento tem se tornado atrativo devido sua alta especificidade, fazendo com que esses dispositivos sejam promissores para uma análise precisa, rápida e sensível. Estudos recentes utilizam técnicas experimentais com diferentes anticorpos

como molécula de reconhecimento na detecção de patógenos (Ahmed *et al.* 2013, Chrouda *et al.* 2013, Barreiros Dos Santos *et al.* 2015) e permitem uma alta performance no desempenho do dispositivo.

### 2.3 Anticorpos

Os anticorpos são glicoproteínas que pertencem à família das imunoglobulinas. Um anticorpo é um homo dímero, ou seja, é composto por duas cadeias pesadas e duas cadeias leves com um peso molecular de aproximadamente 150 kDa. As cadeias pesadas são ligadas em conjunto por pontes dissulfeto, que também os vincula às suas cadeias leves correspondentes. Cada anticorpo contém um domínio constante ou Fc (do inglês "fragment constant") e um domínio de ligação ao antígeno ou Fab (do inglês "fragment antigen-binding"). A região amino terminal do domínio Fab é hiper-variável e responsável pelo reconhecimento do antígeno e contribui para a especificidade da molécula (Abbas *et al.* 2011).

Os anticorpos monoclonais são específicos e liga-se a uma única região definida ou epítopo do antígeno; enquanto que, as preparações de anticorpos policlonais, consistem de uma mistura de anticorpos e podem reconhecer uma grande variedade de antígenos ou epítopos múltiplos em um mesmo antígeno (Abbas *et al.* 2011), e até mesmo de um organismo.

Existem variantes na estrutura de anticorpos, sendo o fragmento variável de cadeia única scFv (do inglês "single-chain variable fragment") o mais empregado em imunoensaios devido a diversidade que apresenta. Estes podem ser gerados por abordagens genéticas ou químicas (Holliger and Hudson 2005). É possível encontrar uma gama de diferentes formas de "anticorpos-like", também conhecidos como anticorpos de domínio único (Sun *et al.* 2014). Estas variantes de menor porte podem proporcionar algumas vantagens para estudos *in vitro* (Sun *et al.* 2014).

O conhecimento das propriedades químicas e funcionais de um anticorpo, juntamente com a natureza química dos suportes sólidos que serão empregados para imobilização do anticorpo, são essenciais para a concepção de uma estratégia de imobilização adequada (Dixit *et al.* 2010). Os sítios de ligação ao antígeno das moléculas de anticorpos devem ser preservados e essa proteção pode ser conseguida pela orientação da ligação do anticorpo as superfícies de imobilização (Holliger and Hudson 2005, Shen *et al*. 2011). Estratégias de imobilização de anticorpos devem ser delineadas levando em conta a orientação destas moléculas sobre a superfície.

## 2.4 Estratégias de Imobilização

A imobilização do material biológico na superfície de biossensores constitui uma das fases cruciais no seu desenvolvimento, pois os sítios ativos da molécula devem ser mantidos, a fim de não prejudicarem a reação com a amostra de interesse (Campanella *et al.* 2008). Diversos métodos de imobilização do material biológico podem ser usados: oclusão (aprisionamento), micro encapsulamento (confinamento em pequenas esferas), adsorção física (interações do tipo iônica, polar, ligação de hidrogênio), e ligação covalente cruzada e covalente simples. Rotineiramente, compostos químicos como glutaraldeído (ligação covalente cruzada), cistamina (ligação covalente), polietilenoimina (PEI) e membrana de acrilamida (aprisionamento), são empregados (Arya *et al.* 2008).

### *2.4.1 Adsorção*

A adsorção é um processo de adesão de biomoléculas sobre as superfícies em consequência da variação de energia livre ($\Delta G$), que deve ser negativo em magnitude. Isto pode ser conduzido pela entropia ou entalpia (Pace et al. 2011). A entropia, que é regida pela segunda e terceira lei da termodinâmica, é uma função de estado de um sistema termodinâmico que descreve a desordem dentro da matéria. Além disso, a

entropia é a medida da quantidade de energia que não pode ser usado para realizar trabalho. De acordo com as leis que regem a termodinâmica, a entropia de um sistema isolado nunca pode diminuir assumindo que o sistema está em seu estado de energia. Portanto, termodinamicamente uma certa quantidade de entropia sempre é tida por um sistema em qualquer momento, e isto regula a reatividade do sistema referido. Considerando-se que a matéria está no estado dinâmico com o seu ambiente, a quantidade de entropia em um dado sistema pode aumentar ou diminuir de acordo com a natureza do sistema com a qual ira interagir (Cornish- Bowden 2002).

Entalpia é o teor total de energia de um sistema que é necessária para manter a sua forma física e química. A alteração na entalpia de um dado sistema regula a estabilidade conformacional e funcional das proteínas no meio. Há sempre uma compensação entre entalpia e entropia. Esta compensação é a relação matemática entre ambos os parâmetros termodinâmicos e é descrito na equação de Gibbs como variação de energia livre (ΔG) (Howard 2002).

$$\Delta G = \Delta H - T\Delta S$$

Onde,

ΔG= Energia livre de Gibbs; ΔH= entalpia; T= temperatura e ΔS=entropia.

Pode ser visto, a partir desta equação, que um aumento linear na entalpia e entropia não altera a energia livre do sistema, portanto, qualquer alteração na química será observada nos parâmetros físicos. E um aumento da entalpia, sem mudar a entropia do sistema, irá introduzir alterações estruturais e químicas no sistema. Esta compensação entalpia-entropia é a expressão termodinâmica mais importante e básica que regem todas as alterações físicas ou químicas de que a matéria sofre (Howard 2002).

Um anticorpo em solução interage de forma contínua com o solvente, superfícies e outras moléculas de anticorpos, através de ligações hidrofóbicas, de Van der Waals, e iônicas. As ligações de ponte de hidrogênio podem também estar presentes, dependendo da natureza química do solvente (Abbas *et al.* 2011). Estas interações anticorpo e solvente estão em estado dinâmicas onde novas ligações são continuamente formadas em substituição das interações anteriores. Por conta da termodinâmica do sistema, esta dinâmica de interação está diretamente regulada pela entalpia e entropia (Roque *et al.* 2009). Portanto, pode-se inferir que as interações dos anticorpos estão em estado dinâmico com seu ambiente e não são eventos isolados. Isto sugere que a entropia das interações anticorpo com superfície poderia aumentar ou diminuir de acordo com a temperatura, o pH, a natureza de solvente e a natureza dos produtos químicos usados (Scholtz *et al.* 2009).

Existem dois tipos diferentes de adsorção física ou química. Adsorção física envolve principalmente interações fracas de Van der Waals e hidrofóbicas. Interações hidrofóbicas ocorrem quando redes hidrofóbicas são geradas em torno de grupos pendentes não polares de um anticorpo solubilizado em água. A exposição de grupos apolares a água resultam em repulsão dos grupos não polares, devido à hidrofobicidade (Irun *et al.* 2001, Yu *et al.* 2015). O mecanismo básico da adsorção química é semelhante à adsorção física onde a interação anticorpo-superfície inicialmente é controlada por forças fracas (Putzbach and Ronkainen 2013). No entanto, no caso da adsorção química, a estabilidade da interação anticorpo e superfície são alcançadas por meio de reações químicas realizadas em um determinado $\Delta G$, que é descrito como o potencial da reação (Irun *et al.* 2001). Imobilização de anticorpos em superfícies carregadas positivamente, tais como filmes de polianilina ou poli-L-lisina, por meio de interação eletrostática, é um exemplo de adsorção química, como mostra na **Figura 3**.

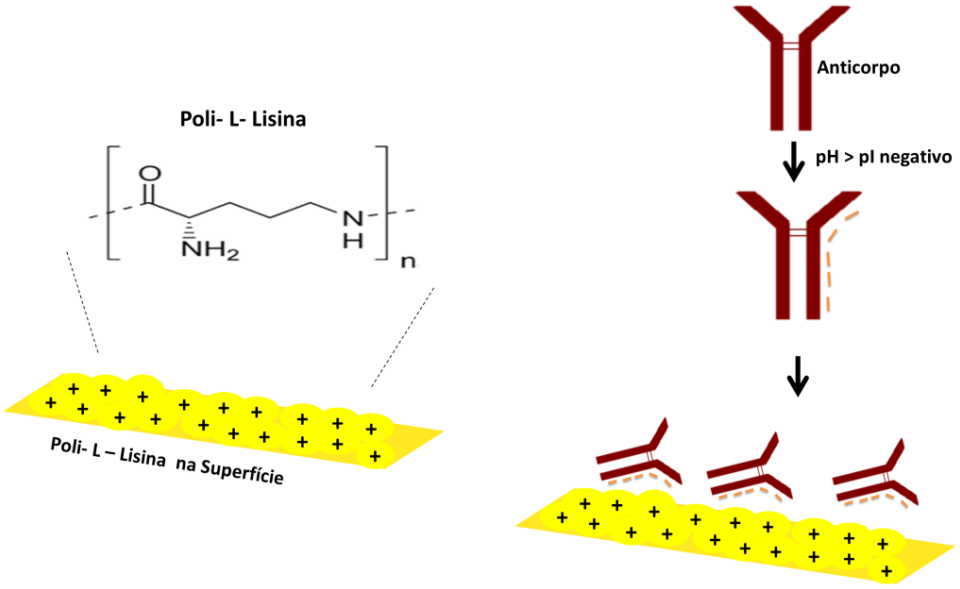


**Figura 3:** Interação eletroquímica do anticorpo com a superfície, em pH alto o anticorpo tem pI negativo e interage com a superfície coberta com poly-L-lisina que torna a superfície positivamente carregada.

O ponto isoelétrico (pI) de uma proteína é o pH no qual há equilíbrio entre as cargas negativas e positivas dos grupamentos iônicos. Assim, a mudança de pH do tampão pode gerar cargas positivas ou negativas na superfície da molécula gerando uma alternativa na ligação destas moléculas em superfícies. O pI abaixo do fisiológico dificulta a imobilização destas proteínas, tendo em vista que a maioria dos tampões utilizados estão na faixa de pH fisiológico. Os valores de pH baixos podem desestabilizar compostos químicos da superfície. Uma alternativa nestes casos é a utilização de ligantes, que serão discutidos posteriormente.

Certos grupos funcionais de um anticorpo tais como tióis (-SH) e aminas (NH2) tem capacidade de reagir inerentemente com metais ou superfícies revestidas com metais, esta reação é caracterizada como adsorção química (Kausaite-Minkstimiene *et al.* 2010, Putzbach and Ronkainen 2013). Porém, as moléculas de anticorpos ficam muito próximas à superfície podendo gerar impedimentos à ligação ao antígeno. Uma

alternativa é a utilização de agentes químicos que servem como espaçadores, distanciando assim o Ac da superfície. Estas estratégia são obtidas por métodos de imobilização covalente.

*2.4.2 Imobilização covalente*

Na imobilização covalente, moléculas de anticorpo reagem quimicamente através dos grupamentos amino e carboxílico com a superfície modificada, ou não do sensor. Estratégias de imobilização covalentes são geralmente classificadas de acordo com as reações químicas utilizadas (Sassolas *et al.* 2012).

A importância da imobilização covalente de anticorpos na obtenção de ensaios de alta sensibilidade já foi demonstrada em diferentes plataformas de diagnósticos (Lakshmanan *et al.* 2007, Pei *et al.* 2010, Baniukevic *et al.* 2013). A melhora na sensibilidade de detecção do analito pode ser atribuída, pelo menos em parte, a redução das perdas de proteínas, devido à lixiviação e o não bloqueio do sitio de ligação, o que poderia aumentar a cobertura da superfície com a molécula imobilizada (Makaraviciute and Ramanaviciene 2013).

A captura química direta é uma técnica de imobilização covalente que se caracteriza pela reação de pelo menos um dos grupos funcionais da molécula de anticorpo com a superfície. Essa reação ocorre rapidamente sem qualquer ativação ou utilização de mediadores. Estas estratégias são procedimentos de uma única etapa de imobilização (Makaraviciute and Ramanaviciene 2013), e são comumente utilizados agentes como epóxidos e aldeídos.

Diferentes estudos que utilizam esses grupos para imobilização são encontrados na literatura (Abad *et al.* 2002, Liu *et al.* 2010, Freitas *et al.* 2014). Outra possibilidade é a captura direta baseada na geração de grupos aldeídos, que podem ser obtidos pela geração de grupos aldeídos livres no anticorpo ou na superfície de captura. Os aldeídos

ativos podem ser gerados no anticorpo por oxidação do grupo hidroxila ($OH^{-1}$) de hidratos de carbono presentes na região Fc, utilizando periodatos de metais alcalinos, tais como os de sódio ($NaIO_4$) ou de potássio ($KIO_4$). Os grupos resultantes desta ativação podem ser capturados eficientemente em superfícies funcionalizadas com amina (Migneault *et al.* 2004, Migneault *et al.* 2004). No entanto, essa técnica tem como desvantagem a oxidação do anticorpo associada aos produtos químicos utilizados que possuem alta reatividade e podem oxidar os aminoácidos, tais como metionina, triptofano ou histidina em diferentes pontos do anticorpo. Superfícies metálicas e poliméricas são funcionalizadas com aldeídos para capturar anticorpos em aplicações bioanalíticas (Makaraviciute and Ramanaviciene 2013).

A ligação de anticorpos em superfície quimicamente modificada é geralmente mediada por ligantes. Os ligantes são espécies químicas que contêm radicais altamente reativos em uma ou ambas as extremidades e, por consequência, são capazes de criar ligações entre determinados grupos funcionais. Esta reação será gerada pela ligação destes grupos a grupamentos selecionados de um anticorpo, criando intermediários altamente reativos que podem posteriormente ser ligados aos grupos funcionais da superfície. Estes ligantes podem ser categorizados como homo e heterobifuncional (Wong 1991). Ligantes homobifuncional possuem grupos quimicamente reativos em ambas as extremidades, enquanto que os ligantes heterobifuncionais possuem dois diferentes centros reativos nas extremidades (Makaraviciute and Ramanaviciene 2013) (**Figura 4**).

Uma variedade de tais agentes de reticulação encontra-se comercialmente disponível em diferentes combinações (Wong 1991). Por exemplo, amina-amina e sulfidril, sulfidrilas-homo-bifuncionais, e amino sulfidrila e amina-carbóxilo ligantes hetero-bifuncionais. Compostos amplamente utilizados como  glutaraldeído e 1-etil-3

(3-dimetilaminopropil) carbodiimida (EDC), representam categorias homo e hetero-bi-funcional, respectivamente. **(Hermanson 2008, Dixit *et al.* 2010).** A reação amina-carbóxilo obtida pelo agente de reticulação EDC está representadas na **Figura 4**

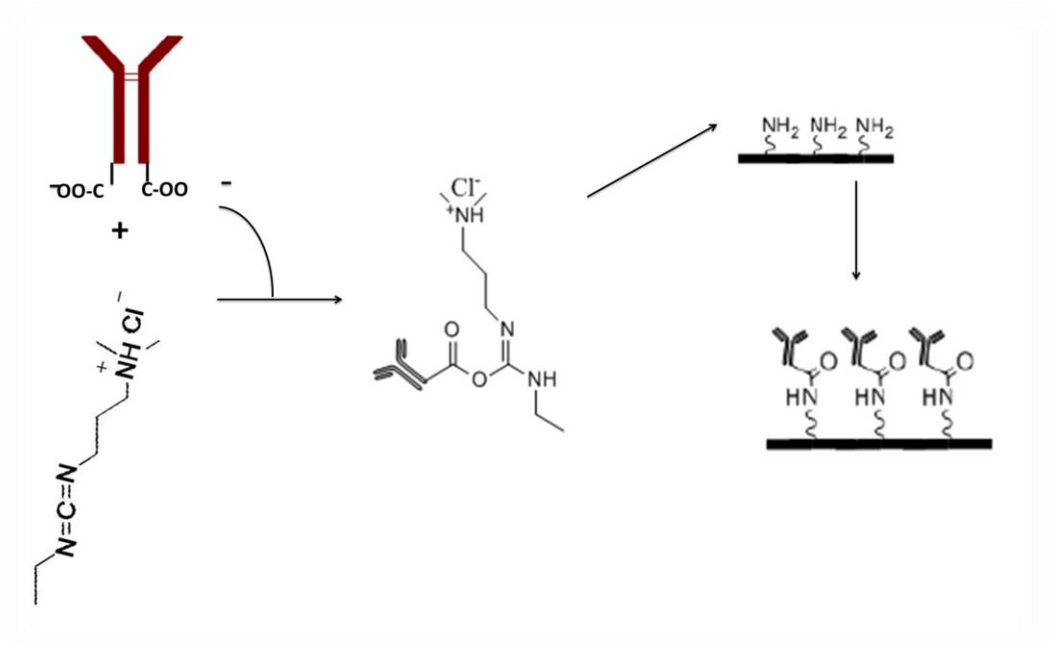


**Figura 4:** Esquema de imobilização utilizando agente reticulante EDC. A formação de uma amida na ativação do grupo carboxila é ligada a superfície amino funcionalizada, por reação de ataque nucleófilo. Adaptado e modificado de (Dixit *et al.* 2010).

Estratégias que orientam as moléculas do anticorpo na superfície são normalmente preteridas pois geram respostas analíticas de alta sensibilidade.

*2.4.3 Imobilização covalente orientada*

A imobilização covalente orientada é baseada em proteínas de reconhecimento de domínio (PRD), tais como proteína A e proteína G, estas moléculas apresentam grande vantagem na orientação de IgG. As PRD tem avidez elevada e específica por domínios do anticorpo e se ligam fortemente, proporcionando orientações dirigidas (**Figura 5**). Além disso, imobilização covalente destas PRD em superfície aumenta a homogeneidade na distribuição do anticorpo sobre a superfície (Kausaite-Minkstimiene

*et al.* 2010) e minimiza a lixiviação da proteína e a variabilidade associada à interface em imunoensaios (Makaraviciute and Ramanaviciene 2013).

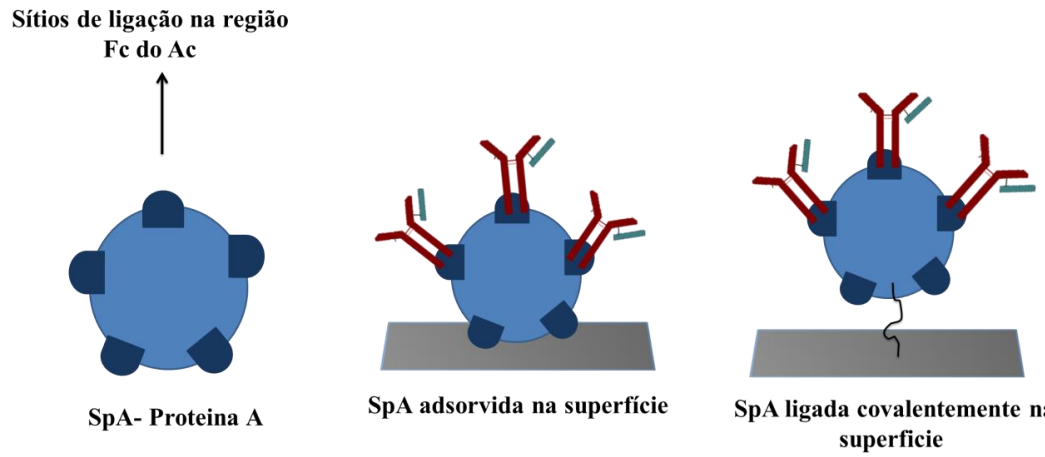


**Figura 5**: Orientação de um anticorpo através da captura por proteína A adsorvida ou covalentemente imobilizada.

No entanto, as PRDs devem ser corretamente orientadas sobre a superfície para proporcionar captura adequada das moléculas de anticorpo. A utilização de PRDs com vários sítios de ligação aos anticorpos, tais como a proteína A e proteína G recombinante, que possuem mais de um sitio de ligação à região Fc do anticorpo, podem minimizar os problemas associados a orientação destas PRDs. A aplicação desta técnica é amplamente utilizada em diferentes bioensaios (Beyer *et al.* 2009, Makaraviciute and Ramanaviciene 2013).

Uma vez conseguida a imobilização da biomolécula a superfície deve ser caracterizada quanto a densidade desta e sua orientação a superfície, bem como a reprodutibilidade da técnica.

## 2.5 Caracterização da interface biológica por métodos de microscopia

A avaliação das superfícies antes e após a formação da interface de reconhecimento permite gerar uma estimativa da densidade de imobilização e

morfologia dos compostos de reconhecimento. A microscopia de força atômica ou AFM (do inglês "atomic force microscopy") é uma técnica amplamente utilizada na avaliação da topografia e a disposição das moléculas sobre a superfície.

A técnica de AFM tem como base a interação da superfície com uma ponta acentuada montada num cantilever, que funciona como uma mola e é suscetível a pequenas variações de forças. Os modos de obtenção das imagens de topografia (modos de varredura) referem-se basicamente a distância mantida entre a ponta de prova e a amostra (Heinrich Hörber 2002)**.** Quando se pretende analisar materiais mais maleáveis e facilmente deformáveis pela ponta, como é o caso de materiais biológicos, polímeros, amostras muito rugosa, utiliza-se o modo intermitente (*tapping*). No modo intermitente, o cantilever oscila à sua frequência de ressonância e durante a sua oscilação, aproxima-se da amostra ocorrendo o contato, que atenua a amplitude de oscilação do cantilever, provocado pelas forças repulsivas que estão presentes no modo de contato (Torre *et al.* 2011). O modo *tapping* é amplamente utilizado na análise de interfaces biológicas de sensores (Farris and Mcdonald 2011, Kim *et al.* 2012, Marciello *et al.* 2014).

O AFM permite a medida da rugosidade de superfícies a raiz do valor quadrático médio ou RMS (do inglês root mean square) é um dos parâmetros utilizado para avaliar a disposição de moléculas de anticorpo acoplados em substratos (Wang *et al.* 2012). Outras técnicas como microscopia eletrônica de varredura (MEV) permitem também a avaliação da interface de reconhecimento.

### 2.6 Transdutores magneto-elásticos

Sensores magnetoelásticos são dispositivos que utilizam como base materiais com propriedadade magnetoelástica que respondem a excitação magnética variável com o tempo o que produz uma onda elástica longitudinal no sensor que pode ser detectada através de técnicas ópticas, acústicas ou magnéticas (**Figura 6**) (Grimes *et al.* 2011).

Grimes *et.al.* (2002) apresenta um trabalho de revisão abrangente da física operacional destes sensores, abordando o processo de desenvolvimento e aplicação. Sensores magnetoelásticos são feitos de fitas metálicas amorfas ou fios, com uma frequência de ressonância característica inversamente proporcional ao comprimento. A respota remota de frequência de ressonância está relacionada a diferentes parâmetros físicos, incluindo a tensão, pressão, temperatura, velocidade de escoamento, viscosidade do líquido, campo magnético, e carga de massa (Grimes *et al*. 2011). As tiras (lâminas com pouca espessura) magnetoelasticas são produzidas a partir de ligas de material ferromagnético amorfo. Sendo estas tiras fabricadas pelo processo de *melt spinning*, onde o material metálico fundido é derramado em um disco de resfriamento com rotação controlada (Diény 2005).

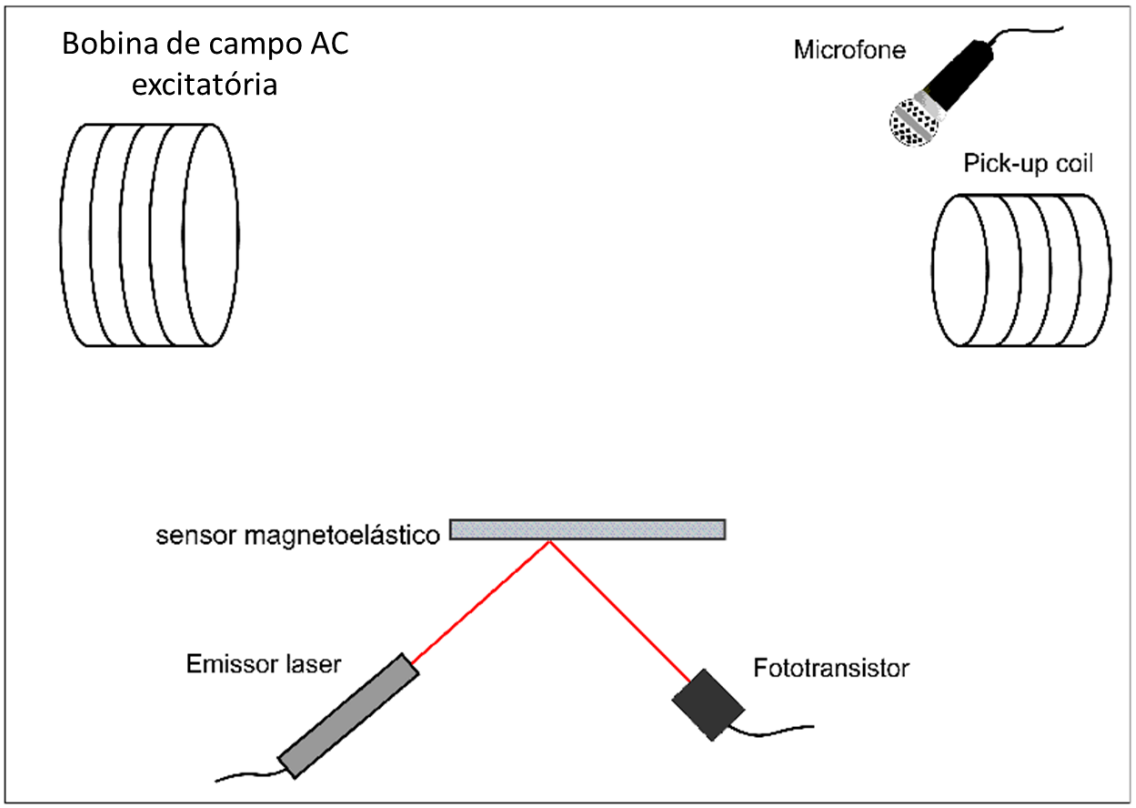


**Figura 6**: Representação de operação do sensor magnetoelástico. Adaptado e modificado (Grimes *et al.* 2011).

*2.6.1 Princípios físicos de funcionamento*

A frequência de ressonância do sensor está relacionada com a vibração elástica do material e está intimamente ligada as suas dimensões, especificamente ao seu comprimento. De acordo com Liang *et. al.* (2007), para uma fita de comprimento *L*, largura *w* e espessura *t*, vibrando longitudinalmente, sua frequência de ressonância fundamental $f_0$ é descrita pela Equação 1, onde *E* é o módulo de Young, ρ é a densidade de massa da fita e ν é o coeficiente de Poisson.

$$f_0 = \frac{1}{2L}\sqrt{\frac{E}{\rho(1-\nu)}} \quad (1)$$

O sistema tem seu funcionamento baseado no efeito magnetostrictivo e magneto-elástico do material amorfo. A excitação das ondas longitudinais é efetuada através de um campo magnético aplicado por uma bobina de Helmholtz e a resposta é captada pela mesma bobina. Podemos usar bobinas diferentes para excitar e captar sinais, dependendo da montagem e do que está sendo medido.

Outro fator que deve ser considerado é aplicação de um campo DC uniforme na direção do sensor. Obtem-se um viés no alinhamento dos domínios que se relaciona com o módulo de elasticidade, permitindo uma melhora no sinal de resposta. Existe um ponto ótimo de operação, que depende do material do qual o sensor é feito e das dimensões envolvidas. Consequentemente, pode-se utilizar o campo DC para sintonizar o sensor em um ponto otimizado de operação, sendo que este campo DC uniforme também influencia o efeito ΔE (Cullity 2011, Grimes *et al.* 2011).

*2.6.2 Sensibilidade de massa*

De acordo com *Grimes et.al.* (2002) e Shen et al. (2009), pequenos carregamentos de massa $\Delta m$, uniformemente distribuídos sobre o sensor, por exemplo, células de patógenos alvos depositadas na superfície, resultará em uma diminuição da frequência de ressonância inicial $f_0$ para uma frequência de ressonância final $f_c$ em um montante $\Delta f$. Para um sensor de massa $m_0$ e frequência de ressonância $f_0$, a resposta esperada da frequência de ressonância causada pelo aumento de massa na superfície do sensor é dado por (Equação 2):

$$\Delta f = -\frac{f_0}{2}\frac{\Delta m}{m_0} \tag{2}$$

Onde $m_0$ é expresso na Equação 3:

$$m_0 = L * w * t * \rho \tag{3}$$

Sendo que a relação entre a massa depositada e a frequência de ressonância é chamada de sensitividade de massa $S_m$, que é expressa pela equação 4.

$$S_m = -\frac{\Delta f}{\Delta m} = -\frac{1}{2\rho L^2 wt}\sqrt{\frac{E}{\rho(1-v)}} \tag{4}$$

Para sensores em que $w = L/5$ (relação 5:1) (Grimes *et al.* 2011), a equação pode ser ligeiramente simplificada (Equação 5):

$$S_m = -\frac{5}{2\rho L^3 t}\sqrt{\frac{E}{\rho(1-v)}} \tag{5}$$

Pode-se também determinar o limite de massa mínimo detectável ($\Delta m_{mín}$), através da frequência de ressonância mínima detectável ($\Delta f_{mín}$), que está relacionada ao limite do sistema de detecção (sensor e equipamento eletrônico), expressa pela Equação 6.

$$\Delta m_{mín} = \frac{\Delta f_{mín}}{S_m} \tag{6}$$

Através da massa mínima detectável e da massa da célula do patógeno alvo, seria possível determinar a quantidade mínima de células para obter a detecção. Porém, geralmente relacionamos uma variação na frequência de ressonância $\Delta f$ com a massa

(das bactérias) depositada no sensor através da sensitividade $S_m$. É difícil conhecer o valor correto de ($\Delta f_{mín}$).

*2.6.3 Desenvolvimento e aplicação como biossensores*

Biossensores magnetoelasticos utilizados na detecção biológica são baseados na afinidade de compostos de reconhecimento, tal como um anticorpo, proteína receptora, material biomimetrico, ou DNA na interface do transdutor de sinal, que juntos referem à concentração de um analito a um sinal eletrônico mensurável (Katz and Willner 2004). Diferentes modelos de estudos baseados na imobilização de elementos de bio-reconhecimento vêm sendo realizados (ZENG e GRIMES, 2007; XIE *et al.*, 2009 e CHAI *et al.*, 2013b).

Neste tipo de biossensor, a presença dos micro-organismos é detectada por uma variação gradual na frequência de ressonância conforme o aumento de massa sobre o sensor. Li *et. al.* (2010) desenvolveu um sensor ME capaz de detectar *Salmonella* sobre a superfície de tomates; que é baseado no princípio de patógeno alvo se ligando ao elemento de bio-reconhecimento molecular neste caso um fagos. Um resumo do funcionamento do sensor pode ser observado na **Figura 7.**

Porém, essa é uma plataforma de sensoriamento que necessita de maior pesquisa dos efeitos da modificação biológica na superfície, pois estes ainda são pouco estudados. Recentemente Possan *et.al.* estudou o efeito da rugosidade das superfícies de sensores magneto-elásticos Metglas®2826MB3 demostrando que superfícies lisas têm um melhor desempenho (Possan *et al.* 2016). Diversas pesquisas com transdutores de material magneto-elástico foram realizadas utilizando anticorpos como agentes de captura. Estas partem do principio que a variação de massa sobre o sensor altera a frequência de ressonância (Berkenpas *et al.* 2006, Guntupalli *et al.* 2007, Zourob *et al.* 2007, Chen *et al.* 2014).

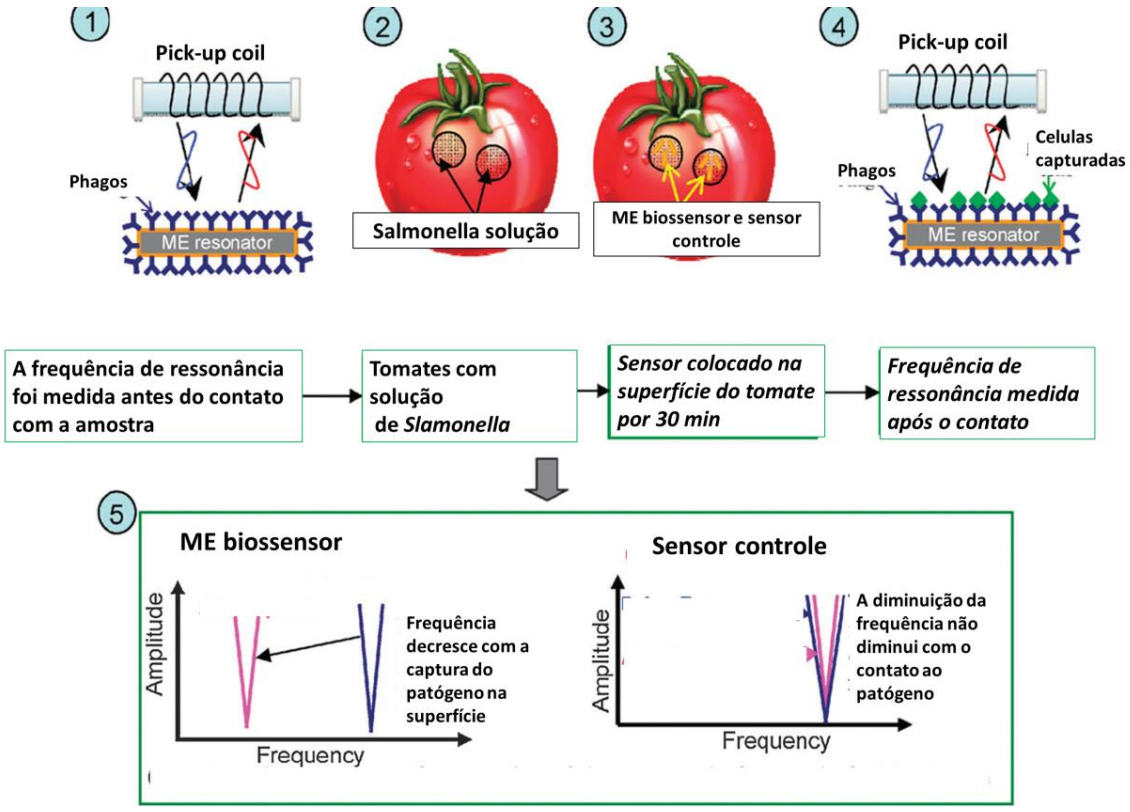


**Figura 7**: Funcionamento de biossensor magneto-elástico para detecção de patógeno trabalho realizado por Li *et.al.* (Li *et al.* 2010) no qual o sensor permite a detecção direta sobre os alimentos de *Salmonella*. 1) fagos (molécula de bio- reconhecimento) imobilizada na superfície do sensor com método de detecção de sinal através de uma bobina; 2) uma solução foi adicionada sobre o alimento; 3) os Biossensores e um sensor controle foram expostos à solução por 30 min; 4) a frequência foi medida novamente; 5) a captura do patógeno resultou na diminuição da frequência de ressonância enquanto no sensor controle a frequência se manteve estável. Modificado e adaptado de (Li *et al.* 2010).

Diante do exposto, cabe salientar que sensores magneto-elásticos são ferramentas promissoras para desenvolvimento de dispositivos de detecção rápida de patógenos. Na área de alimentos, métodos de detecção de contaminantes químicos e biológicos de alta sensibilidade e baixo custo, capazes de fornecer resposta da análise em tempo real, têm impulsionado pesquisas nesta área. Na área médica, esses dispositivos podem proporcionar uma redução nos gastos em saúde diminuindo o tempo de internação hospitalar e ou prevenindo agravos em quadros de infecções. A necessidade de mais estudos sobre o efeito da modificação nas superfícies de ME sensores é necessária para gerar sistemas estáveis e de ótimo desempenho. Esse transdutor é propício para desenvolvimento de testes de baixos custos, pois é um

dispositivo barato, além de possibilitar o monitoramento sem fio o que é uma característica importante para diferentes testes analíticos em diversas áreas.

## 3. RESULTADOS E DISCUSSÃO

Os resultados deste trabalho estão apresentados na forma de capítulos os quais correspondem a três artigos científicos. O primeiro artigo, uma revisão intitulada **Antibody-based magneto-elastic sensor: potential device for detection of pathogens and associated toxins,** foi publicado na revista *Applied Microbiology and Biotechnology 14: 6149-6163 (2016).* O segundo, um artigo intitulado **Biocompatibility and degradation of gold-covered magneto-elastic biosensors exposed to cell culture** foi publicado na revista *Colloids and Surfaces B: biointerfaces 143: 111-117 (2016).* E o terceiro, **Effect of distinct antibody immobilization strategies on the analytical performance of a magneto-elastic immunosensor for *S. aureus* detection** será submetido a revista *Biosensor and Bioeletronics*.

# 3.1 Capítulo I

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MINI-REVIEW

# Antibody-based magneto-elastic biosensors: potential devices for detection of pathogens and associated toxins

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**Abstract** This work describes the design and development process of an immunosensor. The creation of such devices goes through various steps, which complement each other, and choosing an efficient immobilization method that binds to a specific target is essential to achieve satisfactory diagnostic results. In this perspective, the emphasis here is on developing biosensors based on binding antigens/antibodies on particular surfaces of magneto-elastic sensors. Different aspects leading to the improvement of these sensors, such as the antibody structure, the chemical functionalization of the surface, and cross-linking antibody reticulation were summarized and discussed. This paper deals with the progress of magneto-elastic immunosensors to detect bacterial pathogens and associated toxins. Biologically modified surface characterization methods are further considered. Thus, research opportunities and trends of future development in these areas are finally discussed.

**Keywords** Biosensors · Imunossensor · Magneto-elastic sensor · Phatogens · Antibody · Immobilization

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## Introduction

The interest in the development of functional sensors to detect different analytes has emerged in the last decade. Magneto-elastic (ME) sensors have been widely studied as biosensors (Gao et al. 2009; Huang et al. 2008a; Zourob et al. 2007) due to their ability to be queried wirelessly, which brings a great advantage in the microbiology area (Grimes et al. 2011). This resonator is constructed from an iron-based, amorphous alloy with magnetostrictive properties. Magnetostrictive materials undergo a change in shape when subjected to an applied magnetic field. If the magnetic field is aligned along the length direction of the resonator and varied at the proper frequency, the structure can achieve resonance (Garcia-Arribas et al. 2014; Grimes et al. 2002). Upon contact with the specific target bacteria, the biorecognition element on the sensor surface captures the target bacterial cells, causing the overall sensor mass to increase, which results in a decrease in the resonant frequency. The resonant frequency is remotely and wirelessly measured using a pick-up coil (Chin et al. 2014; Guntupalli et al. 2007a).

Biosensor-based assay techniques depend on the interactions of biological target with specific chemical groups or macromolecules that are immobilized on a solid material. The latter classes of molecules are affinity ligands, which may be bonded (immobilized) to a solid material (stationary phase) in a variety of ways (Arkan et al. 2015; Basu et al. 2015; Duangkaew et al. 2015; Guo et al. 2015). The fabrication of an immunosensor goes through various steps that complement each other. Choosing an efficient immobilization method, which can produce binding to a specific target, is essential to achieve satisfactory diagnostic results.

Antibodies (Ab) are molecules of interest due to their high specificity and affinity. An example is immunoglobulin G

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(IgG) that is widely used in immunosensors. The application of antibodies is studied on different surfaces and covers distinct fields such as purification, immunoassays, and other diagnostic techniques (Mahara et al. 2014; Rocha et al. 2015; Vashist et al. 2014b). These methods should respect the limitations of the sensor as well as the chemical composition of the surface and possible interference in the transducer signal. In physical adsorption, for example, the antibody might be encapsulated in a porous polymer material (Butler et al. 1992). However, this immobilization is poor and pH dependent, and the buffer used to perform the test may remove the proteins. Another widely used option would be entrapment in a molecular electrodeposited polyphenol, polythiophene, or polyaniline layer (Barton et al. 2009; Tully et al. 2008). In this case, the molecules have to diffuse from inside to outside. For this diffusion to occur rapidly, which accelerates the response, thinner layers are preferred, leading to an improvement in the detection efficiency.

The coupling of Ab to surfaces is possible using different strategies. Apparently, the covalent immobilizations are preferred (Kausaite-Minkstimiene et al. 2010; Mustafaoglu et al. 2015) since they allow the orientation of the molecules. Different chemical compounds can be used in covalent immobilization, such as cross-linking molecules, which should not increase the chemical steric hindrance around the fragment antigen-binding (Fab) antigen recognition site nor reduce the recognition activity of the IgGs. Furthermore, strategies should preferably align the Fab regions for effective capture of the antigen (Cohn et al. 2015; Kumar et al. 2015).

There are some limitations on the use of antibodies, which are associated with loss of biological activity. One of the main reasons for this loss is attributed to random orientation of the macromolecules on the asymmetric support surfaces (Fuentes et al. 2005). Currently, there are different techniques that enable the characterization of these molecules for guidance and validation of immobilization on the surface: atomic force microscopy (AFM) (Jahanshahi et al. 2014; Niu et al. 2015) and Fourier transform infrared spectroscopy (FTIR-Raman) (Kengne-Momo et al. 2012), for example. It is also important to evaluate the kinetics of the immobilized protein in capturing antigens. Techniques, such as surface plasmon resonance, (SPR) have been widely used in the evaluation of kinetics (Lee et al. 2013; Sohn and Lee 2014). But techniques such as enzyme-linked immunosorbent assay (ELISA), radioimmunoassay, immunofluorescence, and chemiluminescence can also be employed to evaluate the performance of the immobilized antibodies.

In this review, the stages of a biosensor development will be addressed. Initially, an overview of the construction of an immunosensor will be given, followed by a consideration of antibodies as detection elements and strategies of IgG protein immobilization. Different techniques of characterization for antibody conjugation will be explored and, finally, a magneto-elastic immunosensor detection technique for capturing pathogens and associated toxins will be presented.

## Construction, structure, and operation of an immunosensor

Biosensors can usually be placed into different categories, based on the chemical interactions between the sensor and the substance to be analyzed. Immunosensors are affinity-based assays where the analyte identification is highly selective, and the capture is accomplished by high specificity between the analyte and the surface of the functionalized device. These types of tests are based upon specific antigen and antibody interactions (Arlett et al. 2011).

When designing an immunosensor, the main challenge is to achieve optimum performance for both metrics—low analysis time and sensitivity at the picomolar or lower level. A review of different transducers and their performance has recently been given by Arlett et al. (2011). Figure 1 offers a representation of the main steps in developing an immunosensor. This device requires specific antibodies as biological recognition elements to bind with antigens from the sample.

Other recognition elements have been reported with used in pathogen detections biosensors. Poltronieri et al. (2014) showed different biosensors for food pathogen detection, and biomarkers application in infections has been recently revised by Tegl et al. (2015).

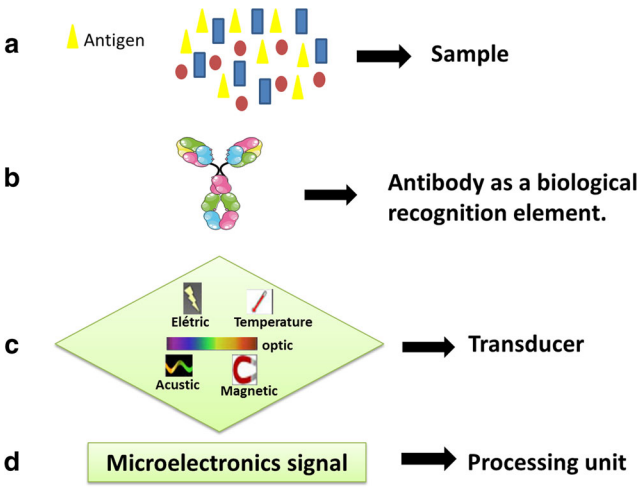


**Fig. 1** Biosensor construction steps: **a** define the conditions to be analyzed and possible interfering parameters in the sample; **b** should read antibody as a biological recognition element; **c** transducer type, can be set by the sensitivity taking into account the type of sample to be analyzed and; **d** signal processing unit, capable of amplifying the signal emitted by the transducer, generating results

## Antibody as the detection element

The antibodies or immunoglobulins are divided into five classes or isotypes: IgG, IgA, IgM, IgD, and IgE (Schroeder and Cavacini 2010). Immunoglobulin G (IgG) is the most abundant in normal serum and most extensively used for biofunctionalization of surfaces (Ahmed et al. 2013; Escamilla-Gómez et al. 2008). The basic structure of an IgG molecule consists of two identical light and heavy chains that are linked together by disulfide bridges (see Fig. 2) having a molecular weight of about 150 kDa and an average size of $14.5 \times 8.5 \times 4\ nm^3$ (Amit et al. 1986).

The light chains contain a variable region (VL) and a constant domain (CL), whereas heavy chains have one variable region (VH) and three constant domains (VL-VH). Altogether, the four protein chains are assembled in such a way as to present a "Y-shaped" geometry. IgG are bifunctional molecules, and the two antigen binding sites are located at the end of the arms of the "Y." These two antigen-binding ends, also known as amino-terminal regions of the Ab molecule, are called fragment antigen-binding (Fab) elements. The stem of the Y or carboxyl terminal end is the fragment crystallizable (Fc) region (see Fig. 2a). This region of the shank (Y) of the molecule ensures that each antibody generates an adequate immune response to a given antigen to trigger effector functions. Ab binds to other components of the immune system leading to different responses, such as complement-mediated lysis, enhancing phagocytosis or allergy response mechanisms (Amit et al. 1986; Braden et al. 1998).

There are fragment variants that can be produced in the antibody structure, such as fragment antigen binding (Fab) and single chain variable fragment (scFv), which may be generated by genetic or chemical approaches (Holliger and Hudson 2005). It is possible to find a range of different forms of "antibody-like" structures, also known as nanobodies and single-domain antibodies (Sun et al. 2014). These unique domains are usually obtained from camels and fish, especially shark. Such structures present a smaller size compared to whole antibodies, are naturally occurring, and have similar affinities for the antigen molecule (Holliger and Hudson 2005). These smaller variants may provide some advantages for immunosensors and in vitro studies (Sun et al. 2014). The application and development of these antibody fragments were recently reviewed by Conroy et al. (2009).

Knowing the structure and available chemical groups of Abs is essential in setting up immobilization strategies. Fig. 2 shows a schematic representation of the chemical groups arranged in the functional areas of an Ab. Fig. 2a presents the general structure of an immunoglobulin and its constant and variable domains. The light and heavy chain of an immunoglobulin has an amino group in the Fab region and a carboxyl group at the opposite ends, which are linked by disulfide bridges in the hinge region (Fig. 2b). Sulfhydryl groups can be generated after reduction of disulfide bonds with dithiothreitol (DTT) (Konigsberg 1972) or tris (2-carboxyethyl) phosphine (TCEP) (Liu et al. 2010b).

The first step in proceeding an experimental design is selecting an appropriate antibody, polyclonal or monoclonal. Polyclonal antibodies are generally raised in rabbits, sheep, or goats (Hanly et al. 1995), they are often selected according to the immunosensor application for pathogen detection. Polyclonal antibodies can recognize different epitopes in a single cell. Monoclonal antibodies represent a large group of recombinant proteins and present different advantagens using CHO cells as reported by Kunert and Reinhart (2016). However, sensitivity of an antibody must be evaluated to detect and quantify a small amount of cells present in the pathogen. Also, the antibody should be able to distinguish specific strains of interest, presenting a good specificity. Thus, the selection of a highly specific epitope of the pathogen is an important consideration, since many of the bacterial strains share homologous proteins, which can lead to the detection of multiple types of cells of a single antibody. Therefore, it is recommended that a constitutively expressed antigen, which is specific for the species be indicated (Byrne et al. 2015). Wherever possible, the expression of the target antigen should

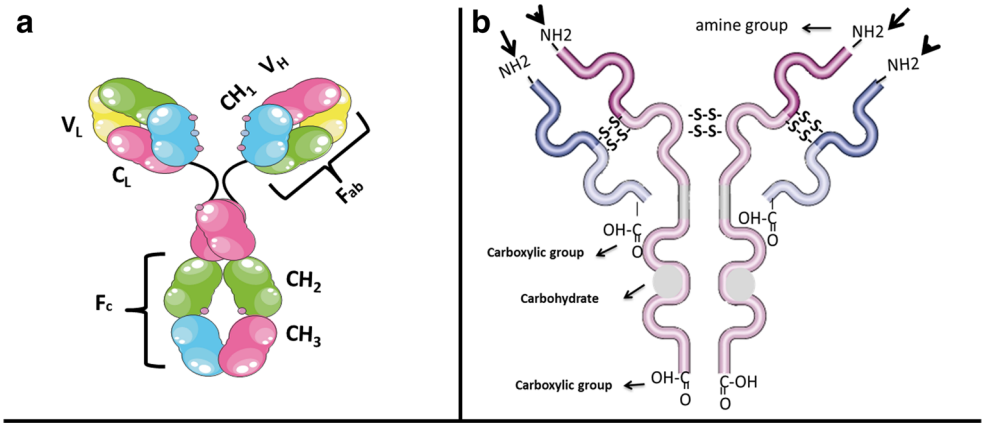


**Fig. 2** Structure of an antibody and its available groups. **a** Structure of an immunoglobulin and its Fc and Fab. **b** Model of a typical IgG with their functional groups. There are two heavy chains (*arrows*) and two light chains (*arrows head*). Each chain has carboxyl (-COOH) and amine ($NH_2$) groups. The light chain is linked to the heavy chain by one disulfide bridge

not be highly dependent on pathogen growth phases. Finally, the antibody must present high affinity properties and connect with the target appropriately.

Western Blot and/or ELISA are the most commonly used methods for determining the specificity and cross reactivity of antibodies (Bordeaux et al. 2010; Mendonca et al. 2012; Pennacchio et al. 2015). Both these methods identify antibodies that detect partially or completely denatured proteins that may or may not be good for capturing or detecting proteins on biosensing platforms. The identification of a candidate antibody that satisfies these requirements will lead to a reduced number of potential antibodies as candidate, with the best affinity for the target epitope. This antibody can then be further selected for incorporation into an immunosensor platform.

## Immobilization of immunoglobulins

The reaction between the biological molecule immobilized on the sensor surface and the analyte in the solution phase is an interfacial reaction. As a result, the way in which the recognition element and the interface are configured will play a large role in the performance of the final device. The recognition element on the interface must be maintained active on the transducer surface to increase the system stability. Reproducibility is also required once experiments are processed.

The best strategies are those based on the process, the transducer surface, and performance in combination with the interface (Escamilla-Gómez et al. 2008; Tran et al. 2012; Wang et al. 2008). Different studies for immobilizing Ab have utilized physical adsorption (Gao et al. 2011; Lu et al. 2012). However, the covalent immobilization has the advantage of orientation of the molecule. Strategies that choose specific groups as targets allow supervision of biomolecule orientation. Different methods of immobilization can result in random or specific orientations of Abs (Fig. 3) and are dependent on the capacity for self-organization of immunoglobulins, which can be controlled for specific reactive groups on the surface. The specific Ab orientation is not easily achieved, since antibodies usually carry several copies of reactive groups.

Langmuir and Blodgett (Blodgett 1935; Langmuir 1917) developed an advanced technique for forming thin layers of a polymer, which accelerates the diffusion of molecules and increases response. They employed layers or multi-layers of molecular thickness to attach amphipathic molecules onto glass or silicon surfaces. The Langmuir and Blodgett (LB) film techniques for immobilizing proteins on a surface can be suitable to associate with IgG surface-binding methods (Cerrutti et al. 2015; Miyajima et al. 2011). However, some proteins may be denatured when the film is dispersed in a water-air interface and tend to provide poorly defined and fragile arrays of biomolecules (Davis and Higson 2005).

The terminal residues are limited in number compared to the large total number of amino acids in the protein. The more common functional targets for the immobilization of protein molecules are the amine and carboxyl groups. The amine group exists in the N-terminus of each polypeptide chain (called alpha-amine) and the side chain lysine residues (Lys, K) (called the epsilon amine). Due to their positive charge under physiological conditions, primary amines are generally outwardly facing (Pauling et al. 1951); so these are generally accessible for conjugation without denaturing the protein structure. The amino groups are deprecated for immobilization of proteins, but in Abs, this grouping is placed in an antigen recognition region. An alternative is to use carboxyl or carbohydrate groups in the Fc region.

Some microorganisms have as a virulence factor a protein capable of binding to the Fc region of antibodies, resulting in the inhibition of immune response. Such proteins may be used in surface immobilization of these molecules; one example is the protein A (PrA) present in *Staphylococcus aureus* (Graille et al. 2000) that is widely used in immunological assays.

## Oriented immobilization for direct binding

Strategies using molecules that enable the capture through specific locations on the Ab such as Protein A (PrA) or Protein G (PrG) are interesting alternatives to bind to the Fc immunoglobulin region (Bjorck and Kronvall 1984; Gronenborn and Clore 1993). Guiding aldehyde groups introduced to the carbohydrate moiety of the $CH_2$ domain via a crosslinking agent (crosslinker) is widely used to search targeted assets. Another possibility is to use these agents for the carboxylic acid immobilization via the Fc portion of the antibody (O'Shannessy et al. 1984).

The native Protein A is a cell wall component produced by several strains of *S. aureus*, but the recombinant PrA is produced in *Escherichia coli*. With a molecular weight MW of ~46 kDa and isoelectric point (pI) of 5.16, it contains five high affinity binding sites (Ka = 108 $mol^{-1}$), which are capable of binding to the Fc region of immunoglobulin G (IgG) from several species such as rabbit and human (Yang et al. 2003). Native PrA molecule can bind to a maximum of two Abs due to spatial constraints (Graille et al. 2000). However, Protein A and IgG binding is not equivalent for all species and even for the subclasses of a particular IgG. This orientation strategy results in a superior analytical performance compared to protocols using exclusively IgG.

Similarly, Protein G is another molecule that binds to the Fc region. This is a bacterial cell wall protein from group

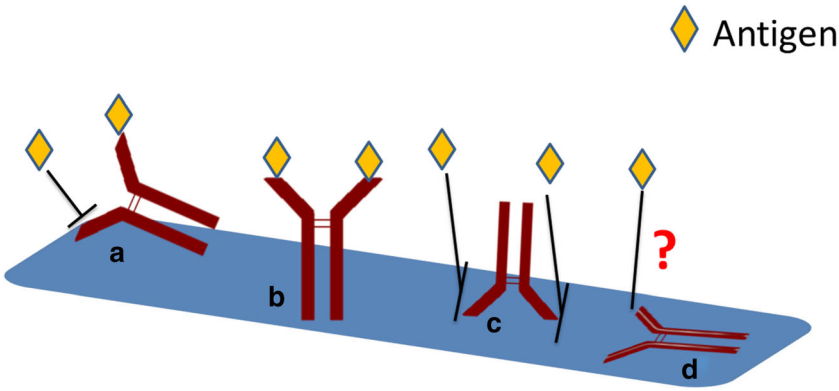


**Fig. 3** Possible coupling orientations of antibodies on surfaces for random immobilizations. Antibodies can acquire different orientations on the surface of the biosensor, binding laterally to the surface (**a**), coupling exclusively to a heavy chain carboxyl terminal (**b**), or Fab antigen recognition region (**c**) and, finally, lying over the surface in any possible position (**d**). The orientation presented in "B" is considered the best strategy for immobilization over IgGs surface

*Streptococcus G*. The native PrG has two IgG-binding domains apart from sites for albumin and cell surface binding, but these nonspecific binding sites have been eliminated from recombinant Protein G (MW = ~22 kDa and pI = 4.5), produced commercially from *E. coli*. Protein G has higher affinity than Protein A for most mammalian IgGs, but it does not show any binding to human IgM, IgA, and IgD. The optimal binding of Proteins A and G occurs at pH of 8.2 and 5, respectively, but is still sufficient at pH 7.0–7.6 (Kato et al. 1995). Because of the inherent differences in the binding properties of these Fc binding proteins, assessment of their binding to specific capture Abs is a prerequisite for a particular bioanalytical application. Indeed, this problem has been solved by the production of Protein A/G, which results from the gene fusion of the Fc-binding domains of Proteins A and G. It binds to all human IgG subclasses with the exception of IgA, IgE, IgM, and to a lesser extent IgD. Besides its stronger affinity, the binding is less pH-dependent with optimum Ab binding at a range of pH 5–8. Apart from the Fc binding proteins A and G, Protein L (MW = ~36 kDa and pI = 4.5) isolated from *Peptostreptococcus magnus* has been employed as an intermediate protein (Kastern et al. 1992). Protein L binds to the kappa light chains of a wider range of IgG classes and subclasses without interfering with the antigen-binding sites of the Ab (Nilson et al. 1992). Furthermore, it binds to the Fab fragments and single-chain variable fragments.

Although these proteins have been widely used for direct binding assays, they are not widely employed for conventional sandwich immunologic assay. Then, the secondary Abs can bind to the vacant binding sites on these intermediate proteins, which leads to a higher background signal and nonspecific binding. The chemical modification of a surface may be obtained through an organized nanomolecular layer with a defined orientation for better reproducibility, durability, and accuracy. These are aliphatic long-chain molecules that functionalize metal or glass surfaces, forming stable covalent bonds between their terminal grouping with metals, metal oxides, or silicates (Culp and Sukenik 1994).

## Self-assembled monolayer (SAM) chemical functionalization

Self-assembled monolayers are important for the development of biosensing interface because they provide a control at molecular level over how the interface is formed (Eggers et al. 2010), how the recognition molecule is immobilized, and how other sample molecules can interact with sensing interface (Mandler and Turyan 1996). The layers formed by SAMs extend the chains of orthogonal hydrocarbons to the surface (Fig. 4), this spontaneous molecular organization is referred to as a SAM whose thickness depends on the length and orientation of the hydrocarbon chain. Examples include monolayers of long-chain alcohols on glass, long-chain amines on platinum (Nuzzo and Allara 1983), alkyl trichlorosilane on silicon (Vuillaume et al. 1993), and long-chain thiols, thiones, thioethers, and alkyl disulfides on gold surfaces (Bigelow et al. 1947) (Fig. 4).

SAM application has been reported in recent publications with biosensors (Liu et al. 2014; See et al. 2015; Tack et al. 2015). The method offers advantages in the assembly of a self-organized layer of planar surfaces, which can be readily prepared in the laboratory. The protocol consists in immersing the substrate in a dilute organic molecule solution for a specific period of time, followed by exhaustive washing with the same solvent and final drying process. The formation of this layer requires a very small amount of chemical, about $2 \times 10^{-7}$ g cm$^{-2}$ (Davis and Higson 2005). Detailed reviews of protocols for practical SAM preparation, as well as the thermodynamics and kinetics governing the assembly process are available (Love et al. 2005; Samanta and Sarkar 2011; Schwartz 2001).

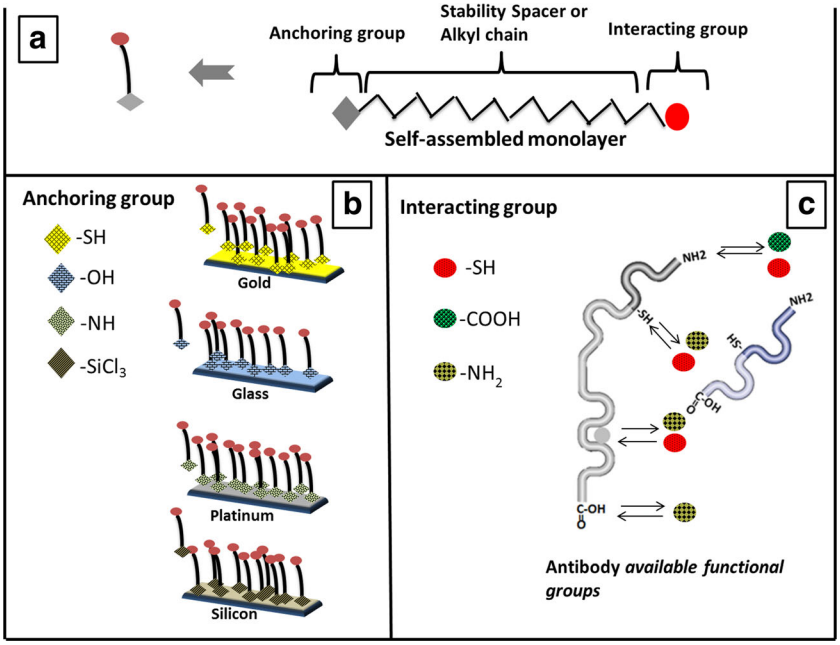


**Fig. 4** The structure of a SAM surface interaction and binding groups for immobilization of Abs. **a** SAM structure, comprised of an anchoring group, alkyl chain and interacting group. **b** Anchoring group, different chemical compositions (-SH, -OH, -NH, -$SiCl_3$) will present affinity for a specific surface (gold, glass, platinum, silicon). **c** Interacting group, at the other end of the chain, which interacts with the functional groups on the antibody and is conjugated to different chemical groups (-SH, -COOH, -$NH_2$). Sufidril and carboxyl groups interact with the amine of the Fab region. Sufidril and amine group interact of the Fc portion carbohydrate. Amine groups interact with the carboxyl terminal of the Fc region in the heavy chain and light chain

Silane SAMs on glass or silicon surfaces oxidized with an active silanol group (Si-OH) was first reported by Sagiv (1980). The covalent binding Ab is widely applied on silicon surfaces, for example, silicates (Dugas and Chevalier 2003; Hu et al. 2006) and antibodies covalently linked to monocrystalline silicon are highly stable and retain functional activity for more than 3 months (Zaitsev et al. 1991). Similarly, highly uniform Ab coupling was achieved on layers mounted with APTES-functionalized glass (Gikunoo et al. 2014). To assemble these layers, trichlorosilane is commonly used. This group is highly reactive, which is incompatible with aqueous solutions and their hydrocarbon straight chain is functionalized with -OH or -$NH_2$. In immobilization processes of antibodies, the substrate can be modified using terminal amino silane (Ruan et al. 2008) such as 3-aminopropyltriethoxysilane (APTES) (Li et al. 2015; Vashist et al. 2014a, 2015), (3-aminopropyl) dimethylethoxysilane (APDMES) (Yang et al. 2015) or terminal thiol mercaptomethyldimethylethoxysilane silane (MDS) (Carrigan et al. 2005), and 3-mercaptopropyltrimethoxysilane (MPTMS) (Chiang et al. 2012). Recently, Vashist et al. (2014a) reviewed the use of silanes for functionalization of analytical platforms with antibodies and enzymes.

The SAMs on gold have a significant application in different sensing devices. Gold is a material of great interest because of its chemical stability, biocompatibility, and unique optical properties, together with a range of suitable processing technology possibilities. Oxide-free surfaces can be easily modified using thiols, not only in gas phase but also in liquid media at ambient conditions. A SAM molecule consists of three parts (Fig 4a): an anchoring group that forms a strong covalent bond with the substrate, the hydrocarbon chain (variable length), which stabilizes the SAM through Van der Waals interactions, and the interacting group, which may have different features (Allara and Nuzzo 1985; Gates et al. 2005). A small change in the terminal group can be enough to alter the physicochemical properties of the compounds (Allara and Nuzzo 1985; Gates et al. 2005). Thus, $CH_3$ and $CF_3$ make the surface of the hydrophobic SAM metallophobic and highly nonstick, while COOH, $NH_2$, or OH produce hydrophilic surfaces with efficient protein binding (Burshtain and Mandler 2006; Toworfe et al. 2009).

The most important and studied SAMs placed on metal surfaces are those of alkanethiols, and to a lesser extent, of arenethiols, alkanedithiols, and arenedithiols (Love et al. 2003, 2005). The Van der Waals interactions existing between adjacent groups guarantee the stability of the packaging and allow wide monolayer applicability (Meyerson et al. 2014; Newton et al. 2013; Vericat et al. 2005). Various techniques, such as X-ray diffraction or Helium atom and X-ray photoelectron spectroscopy, have resulted in the conclusion that sulfur (S) binds to the gold surface forming possibly an RS-Au thiolate (Ayyad et al. 2005), and with a hexagonal structure

(L H Dubois and Nuzzo 1992). Adsorption occurs in places where the S atom in coordination with three gold atoms from the surface (Sek 2007).

Although SAMs based on thiol are stable when stored in an ultra-high vacuum and in the absence of light (Noh et al. 2006), degradation has been observed after 1–2 weeks at room temperature in air (Lee et al. 1998; Srisombat et al. 2011), and more than 70 % of the surface thiol is lost by simple immersion in tetrahydrofuran (THF) at room temperature for 24 h (Schlenoff et al. 1995). The chemical stability of thiol SAMs is one of the most serious problems for their application in aqueous environments and environmental studies (Vericat et al. 2010). Stability improvements were made by changes in the nature of the gold surface (Vericat et al. 2008), the use of longer chain thiols (Tam-Chang et al. 1995), and applying additives (Yang et al. 2004). Also, films prepared from the diazo compounds provide greater stability but, without good control over the monolayer, deposition of a multilayer should be required (Chinwangso et al. 2011). Recently, Crudden et al. (2014) described the generation of carbene SAMs based on an N-heterocyclic gold substrate, which showed considerably higher heat resistance using chemical reagents based on thiol structures. The increased stability observed was associated with increased strength in gold-carbon bond in relation to a gold sulfur bond.

Chemical modification of surfaces by SAMs is of great value to the development of an immunosensor and has good applicability in the transformation of different transducers for biological sensing. Study of the behavior of its kinetic adsorption stability is fundamental for the standardization of surface protocols for chemical functionalization. Recent reviews on these molecules and their properties are available in the literature (Hasan and Pandey 2015; Vericat et al. 2014; Yan and Evans 2014).

## Cross linking antibody reticulation

Crosslinking agents are applied for binding Ab molecules on chemically modified sensor surfaces (Crudden et al. 2014). These linkers are chemical species containing highly reactive radicals at one or both ends and, therefore, are able to create linkages between certain functional groups. These ligands have the characteristic of facilitating the reaction of groups of an antibody by creating highly reactive intermediates, which can then be linked to groups available on the surface. The choice of the linker depends on the chemical reactivity of the chemical grouping from the chosen antibody and the surface. These ligands may be categorized as homo- and hetero-bifunctional (Wong 1991). Homo-bifunctional linkers possess chemically reactive groups at both ends and react with the same target molecules, while the hetero-bifunctional linkers have two different reactive centers at the end (Makaraviciute and Ramanaviciene 2013).

For protein immobilization, it is necessary to maintain the native structure of the complex as previously discussed, and the crosslinking is often the connection used. The choice of agent should be based on connection type, pH, and buffer conditions that do not interfere with the catalytic site, accordingly, the crosslinking procedure should be validated (Vashist et al. 2011). Furthermore, the optimal molar ratios of crosslinker-protein should be established. The degree of conjugation is an important factor, for example, when preparing immunogenic conjugates of a high degree of conjugation. Immunogenicity of the antigen should be increased. However, a low to moderate degree of conjugation must be optimized for an antibody or an enzyme, to maintain biological activity of the protein. The number of functional groups on the protein surface is also an important factor to be considered. If there are numerous target groups, a lower proportion of crosslinking agent in relation to protein can be used. For a limited number of potential targets, a larger proportion of protein-crosslinker may be required. Furthermore, the number of components should be kept to a minimum because conjugates that consist of more than two components are difficult to analyze and provide less information on the spatial arrangement of protein subunits.

An example of a crosslinking agent for Ab widely used is N-ethyl-N- (3-dimethylaminopropyl) carbodiimide (EDC) in combination with N-hydroxysuccinimide (NHS), which generates the NHS ester in the protein (Hermanson 2008; Subramanian et al. 2006). These active esters can be reacted with the antibody modified amino support or amino-modified surface. These agents can also be used for reaction surfaces containing carboxylic groups with amine groups of proteins.

EDC is a crosslinking agent providing the connection of carboxylic acid and amino groups, which first react with the carboxylic group, and an o-shaped acylisourea intermediate (Hermanson 2008). This intermediate rapidly reacts with an amino group to form an amide bond and release an isourea by-product. O-acylisoureia intermediate is unstable in aqueous solutions and hydrolysis generating and releasing N-substituted urea. Therefore, NHS or sulfo-NHS is necessary to stabilize as they react with the unstable reactive intermediate acylisourea O-ester to form a semi-stable amino-reactive NHS ester, which remains stable for several hours at pH 7.4. The best results are obtained when the EDC-activated NHS crosslinking antibodies are used immediately for the reaction with the amine surface of the substrate (Wang et al. 2011). Vashist (2012) tested EDC, EDC-NHS, and EDC-sulfo-NHS for crosslinking anti-antibodies in HFA APTES-functionalized platforms. Crosslinking of antibodies using

EDC alone was more efficient in comparison to the EDC-NHS and EDC-sulfo-NHS in pH 7.4.

## Interface characterization techniques

Different techniques can be used to evaluate the surface roughness before and after signaling and bioconjugation. These techniques also allow an estimate of the immobilization density and morphology. Atomic force microscopy (AFM) provides useful assessments of medium height and roughness of an organic film (Nilson et al. 1992). For biological testing, contactless mode is frequently employed in the 3D structure analysis of bound biomolecules (Ramanaviciene et al. 2012; Tan et al. 2011). Although the biomolecular bonds can be quantified, the AFM does not provide relevant information such as an evaluation of the density of the biomolecules. This information can be obtained by other techniques such as surface plasmon resonance (SPR) (Tan et al. 2011) and ellipsometry (Sun et al. 2015). The structure of the locking interface can be confirmed by photoelectron spectroscopy (XPS) (Liu et al. 2010a), since the homogeneous binding proteins are usually probed by optical microscopy in the near-field in reflection or fluorescence mode.

XPS can be used to identify and quantify the presence of biomolecules immobilized on the film from the increased 1 s signals in the regions C, C 1 s, and O 1 s. Measurements of angle resolved XPS (ARXPS) can be used for protein determination (Stavis et al. 2011). Time-of-flight secondary ion mass spectroscopy (TOF-SIMS) is another technique that can be used for analysis of immobilized biomolecules. The disulfide bond in TOF-SIMS signal is directly related to the activity of the protein adsorbed coatings (Awsiuk et al. 2013; Kosobrodova et al. 2015). The use of analytical techniques AFM, ARXPS, and TOF (SIMS) can provide a relevant characterization model structure vertically with respect to the original surface density of biomolecules (Vashist et al. 2014a). FTIR analysis can be useful for connecting to the probe method, surface coverage, and the efficiency of chemical functionalization of surfaces (Bhadra et al. 2015; Kengne-Momo et al. 2012).

## Magneto-elastic sensor detection of pathogens and associated toxins

Biosensors combine a biological recognition element with an integrated transducer (Turner, 2000). The use of magnetic materials in sensor applications has a long history (Ripka and Zaveta 2009; Kurlyandskaya et al. 2009; Garcia-Arribas et al. 2014). Magnetic sensors based upon the Hall effect, magnetoresistance, and fluxgate sensing have been in use for some time (Ripka and Zaveta 2009). Biosensors employing magnetic particle labels and magnetoresistance technology offer the possibility of studying interactions between single molecules (Baselt et al. 1998). The more recent use of magneto-impedance spectroscopy employs magnetic particle labels but uses magnetic-field-induced changes in the skin depth as a detection mechanism to construct very sensitive magnetic field sensors and, specifically, biosensors (Garcia-Arribas et al. 2014; Kurlyandskaya et al. 2015). The intimate coupling between magnetization and stress opens the possibility of a mechanical perturbation modifying the magnetic properties of a material to allow remote detection (Grimes et al. 2002, 2011) in label-free magneto-elastic biosensors.

The principles of detection of magneto-elastic biosensors are based on the Joule magnetostriction property of the magnetic material. When the material is subjected to a variable magnetic field, a longitudinal vibration occurs, with a characteristic frequency and an associated change in its dimensions (Li et al. 2012). The presence of mass adhering to the sensor surface also causes a variation in the frequency (Grimes et al. 2011). The advantage of using this method of detection is the fact that the sensing is label-free and can be promoted without using direct physical connections, that is, by *wireless* monitoring.

The principle of operation of a magneto-elastic immunosensor is shown in Fig. 5**.** The resonant frequency of the sensor is related to the vibration of the elastic material, and is closely linked to its dimensions, especially its length. The material thickness should be much smaller than its length and width to ensure vibrational modes in the longitudinal direction (Liang et al. 2007).

Several papers on magneto-elastic biosensors using antibodies as capture agents start from the principle that the mass variation of the sensor changes the resonance frequency (Berkenpas et al. 2006; Chen et al. 2014; Guntupalli et al. 2007a; Zourob et al. 2007). However, few studies characterize the modifications of the surface structure and investigate biological effects using that sensing platform. Modifications of the surface morphology of amorphous $Fe_5Co_{70}Si_{15}B_{10}$ ribbons subjected to human urine had previously been studied using magnetoimpedance measurements (Kurlyandskaya and Miyar, 2007). Recently, Possan et al. (2016) studied the effect of surface roughness of magneto-elastic sensors on the capture of *E. coli* using antibodies as recognition biomolecules. In this study, antibodies were immobilized on the surface coated with gold using a chemically modified thiol-SAM structure. It was found that a smooth surface captures more bacteria while rough surfaces captured them more quickly. The coating of ME sensors with a gold layer was also studied by Huang et al. (2008b), a thin gold layer was deposited on the surfaces without considerable changes in sensor responses. A gold cover provides advantages in the modification of biomolecular immobilization strategies due to the possibility of using SAM thiolates. Menti et al. (2016) showed that applying coating

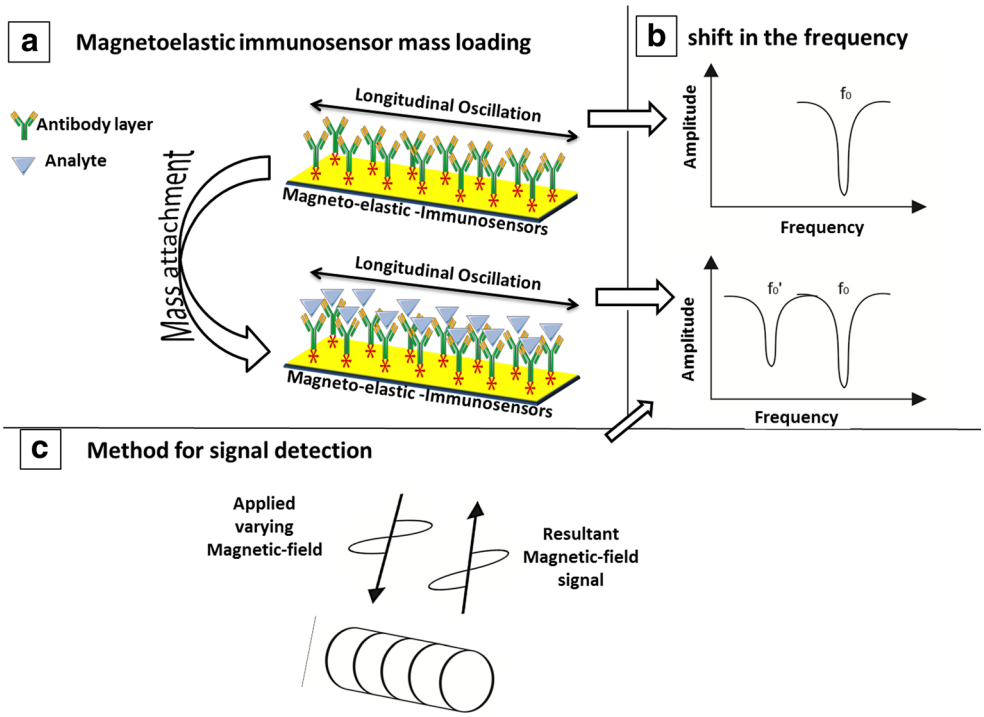


**Fig. 5** Operating diagram of the magnetoelastic-based immunosensor. **a** Mass loading by direct capture of antigen by antibodies, **b** generation of a shift in the frequency, **c** the longitudinal oscillation of sensor can be detecting by phototransistor, acoustic wave, and applied varying magnetic-field

materials like gold increases biocompatibility and the stability of the ME alloys, improving the final analysis.

Ruan et al. (2003) immobilized anti-*E. coli* antibodies on a magneto-elastic cantilever through the construction of a self-assembled monolayer (SAM). The principle of this assay was the conversion of a substrate, 5-bromo-4-chloro-3-indolyl phosphate (BCIP), to an oxidized and insoluble blue precipitate via an AP-catalyzed reaction

**Table 1** Performance of the magneto-elastic immunosensors for detecting different analytes using METGLAS® 2826 MB

| Ref. | Magneto-elastic platform and size | Antibody | Analyte | Immobilizationtechnique | Detection limit |
|---|---|---|---|---|---|
| (Chen et al. 2014) | METGLAS® 2826 MB Size: 18 mm | Anti-OCS | Octachlorostyrene (OCS) | Sensor surface: Bayhydrol 110 Gold nanoparticles interface by gold-thiol técnica by thio-ureia compound | 2.8 nM |
| (Guntupalli et al. 2007b) | METGLAS® 2826 MB Size: 2 × 0.4 × 0.015 $mm^3$ | Rabbit polyclonal antibody anti-*Salmonella sp.* | *Salmonella typhimurium* | Langmuir–Blodgett (LB) technique | 5 × $10^3$ CFU / mL |
| (Ruan et al. 2004) | METGLAS® 2826 MB Size: 6 mm × 2 mm × 28 μm | Anti-SEB | *Staphylococcal* enterotoxin B (SEB) | Thiol-gold surface funcionalization. Glutaric dialdehyde Antibody immobilization | 0.5 ng/mL |
| (Ruan et al. 2003) | METGLAS® 2826 MB Size 6 mm × 1 mm × 28 μm | Anti-*E. coli* O157:H7 | *Escherichia coli* O157:H7 | Thiol-gold surface funcionalization. Glutaric dialdehyde Antibody imobilization | 6 × $10^2$ cells/mL |
| (Guntupalli et al. 2007a) | METGLAS® 2826 MB Size: 2 mm × 0.4 mm × 15 μm; 5 mm × 1 mm × 15 μm and 25 mm × 5 mm × 15 μm | Anti-*S. typhimurium* | *Salmonella typhimurium* | Antibody imobilization Langmuir–Blodgett (LB) film technique | 5 × 103 CFU/ml, 105 CFU/ml and 107 CFU/ml respectively depending upon size of the sensor |
| ( Possan et al. 2016) | 5 mm × 1 mm × 15 μm | Anti-*E.coli* polyclonal antibody | *Escherichia coli* | Cystamine 20 mM SAM surface, antibody is connect on de surface by EDC reaction | 80–400 ng/mL |

(secondary antibody). This product accumulated on the sensor surface, and the resulting changes in resonance frequency were recorded, facilitating the detection of $1 \times 10^2$ cells/mL of *E. coli*. Several studies with magneto-elastic immunosensors based upon METGLAS® 2628 MB and their performance are listed in Table 1.

Bacteriophage-based probes have been combined with various analytical methods. Li et al. (2010) immobilized genetically modified E2 phage to express a peptide against *Salmonella typhimurium* onto the surface of ME resonators, using the modified ME biosensor to demonstrate the real-time ability to track in situ *S. typhimurium* levels on the surface of a tomato. The detection limit was of the order of $5 \times 10^2$ CFU/ml. In another study, Chai et al. (2012) detected *S. typhimurium* on eggshells by using wireless magnetoelastic biosensors METGLAS 2826 MB. The multiple E2 phage-coated biosensors were placed on eggshells spiked with *S. typhimurium*. The detection limit was $1.6 \times 10^2$ CFU/cm$^2$ in 30-min time. Shen et al. (2009) immobilized the JRB7 phage, modified to express a library against *Bacillus anthracis* spores onto a ME resonator, and used the system to take real-time measurements of in vitro biomarker concentrations from the solution. Park et al. (2013) demonstrated in their study the possibility of monitoring the growth of pathogens on the surfaces of food.

## Final considerations

Antibody-based sensors can provide robust, sensitive, and rapid analysis. In most cases the key element is the quality of the antibody used. Recombinant antibodies have many advantages, including the ability to be genetically modified to improve selectivity and sensitivity. The use of thin films, especially self-assembled and gold-thiol monolayers, provides a simple method for the functionalization of magnetoelastic surfaces using nanogram amounts of material. Different techniques can give either highly ordered or amorphous films, giving a high level of control of the environment. The great advantage of using this method of detection is the fact that the sensing can be promoted without using direct physical connections, that is, by *wireless* monitoring.

Magneto-elastic sensors provide a promising possibility in pathogen and toxin detection, generating much faster results than conventional approaches, typically around 20 min. Furthermore, literature reports advantages of detection limit approximate to the real-time PCR usual detection technique. Future directions and challenges for these systems should involve a better understanding of the effect of different molecules over the sensors and the consequent physical properties. Alternative arrangements with multiple sensors to monitor the presence of different pathogens in a real-time perspective are also being developed recently and should provide excellent results. Therefore, choosing an efficient immobilization method, which can produce binding to a specific target, is essential to achieve satisfactory diagnostic results.

**Compliance with ethical standards**

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**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** This article does not contain any studies with animals performed by any of the author.

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# 3.2 Capítulo II

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# Biocompatibility and degradation of gold-covered magneto-elastic biosensors exposed to cell culture

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ABSTRACT

Magneto-elastic materials (ME) have important advantages when applied as biosensors due to the possibility of wireless monitoring. Commercial Metglas 2826MB3™ (FeNiMoB) is widely used, however sensor stabilization is an important factor for biosensor performance. This study compared the effects of biocompatibility and degradation of the Metglas 2826MB3™ alloy, covered or not with a gold layer, when in contact with cell culture medium. Strips of amorphous Metglas 2826MB3™ were cut and coated with thin layers of Cr and Au, as verified by Rutherford Backscattering Spectroscopy (RBS). Using Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES), the presence of metals in the culture medium was quantitatively determined for up to seven days after alloy exposure. Biocompatibility of fibroblast Chinese Hamster Ovary (CHO) cultures was tested and cytotoxicity parameters were investigated by indirect means of reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) at 1, 2 and 7 days. Cell death was further evaluated through *in situ* analysis using Acridine Orange/Ethidium Bromide (AO/EB) staining and images were processed with ImageJ software. Ions from Metglas® 2826MB3™ induced a degradation process in living organisms. The cytotoxicity assay showed a decrease in the percentage of live cells compared to control for the ME strip not coated with gold. AO/EB *in situ* staining revealed that most of the cells grown on top of the gold-covered sensor presented a normal morphology (85.46%). Covering ME sensors with a gold coating improved their effectiveness by generating protection of the transducer by reducing the release of ions and promoting a significant cell survival.



## 1. Introduction

Magneto-elastic (ME) sensors [1,2] have attracted considerable interest within the sensor community as an excellent platform for measuring a wide range of chemical and biological parameters. From the detection of hydrocarbons [3] to the determination of glucose in urine sample [4] to the evaluation of contaminants in milk [5,6], ME sensors have proven their usefulness. ME materials have advantages when applied as biosensors in the biomedical area due to the possibility of wireless monitoring [7,8]. Amorphous ME ribbons have been widely used in various studies, including determined efforts to detect food contaminants [9,10]. They have also been used in several applications, like monitoring growth in cell cultures [11], evaluating cell behavior such as adhesion [12] and controlling restenosis in peripheral artery stents [13].

The application of magnetic materials in biological problems naturally leads to the question of the compatibility of the magnetic material with the biological system under consideration. Magnetic materials in the form of nanoparticles [14,15] or thin films [16] have recently been examined from the point of view of possible toxic or injurious effects which might be caused. This is a more restricted form of biocompatibility and does not contemplate any positive interactions between biological tissues and the magnetic materials.

The ME amorphous alloy Metglas® 2826MB™, for example, is readily available for use in the development of biomedical devices, but requires special care, since ion release can promote cell cytotoxicity [12,17]. That alloy has approximate composition $Fe_{45}Ni_{45}Mo_3B_7$ and molybdenum and nickel are reported to have harmful effects on organisms. Molybdenum has been associated with various toxic effects on the body, causing damage to various

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organs such as kidney, liver and spleen [18,19]. Nickel is known to be carcinogenic and toxic [20]. The dose-dependent cytotoxic effect of nickel has been reported in cultured fibroblasts [21]. A recent study showed that molybdenum nanoparticles (Mo-NPs) induced cytotoxicity in mouse skin fibroblast cells (L929), with decrease in cell viability and alterations in cell morphology [22]. The search for coatings that promote biocompatibility and stability in ME alloys for biological systems has been recently reported [17]. However, the performance of a biosensor for the wireless monitoring of biological samples can be affected by the application of these coatings. Different types of analyte solutions, such as the ones based on buffered salt solutions, which preserve living organisms, can induce accelerated corrosion of the magneto-elastic material and the release of toxic ions. The coating of these materials with nanometric chrome and gold films provides a surface capable of immobilizing the bioactive component. In addition, the preservation of the physical and mechanical properties of these sensors [23] has been attributed to the presence of a gold coating.

In order to investigate cytotoxic effects of ions in cell cultures, this study considered the effects of biocompatibility and degradation of the Metglas® 2826MB3™ alloy, as well as the effects of a gold covering layer, when in contact with cell culture medium. Cell cytotoxicity was evaluated through MTT, which measures the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) according to the methodology described by Denizolt and Lang [24]. Cell death induction was further evaluated through Acridine Orange/Ethidium Bromide (AO/EB) staining. Finally a quantitative determination of the presence of metal ions in the cell culture medium was made. This allowed an evaluation of the beneficial effects of the presence of a gold covering layer on the ME material. It also allows us to determine the time scale for these effects.

## 2. Material and methods

### 2.1. Substrate preparation

The amorphous alloy Metglas® 2826MB3™ was supplied by the Metglas Corp. of Conway SC, with approximate composition in wt.% of $Fe_{45}Ni_{45}Mo_3B_7$. The alloy was supplied in the form of 2 in. wide ribbons. The ribbons were first mechanically polished on both sides using a Struers Tegramin 20 polishing system with 0.05 μm alumina and water. After 1.5 h of polishing, their thickness was reduced to about ~15 μm. The debris or grease retained from the polishing process was removed by cleaning the strips ultrasonically in 100% methanol for 30 min. After the cleaning process, tapes were sputtered-coated (AJA, model ATC 2000) on both sides with a protective layer of chromium and then with gold. The Au-coated strips were cut to dimensions 5 mm × 1 mm × 15 μm. Bare alloy strips were cut to dimensions 5 mm × 1 mm × 30 μm with a microdicing saw. ME ribbons were sterilized at 200 °C for 120 min before exposure to cell culture medium.

### 2.2. Substrate characterization

The thicknesses of the chromium and gold layers were evaluated by Rutherford Backscattering Spectroscopy (RBS). Simulations of the spectra associated with deposited layers were made with the usual RUMP software routines [25,26]. The covered sensor surfaces were examined using Scanning Electron Microscopy (SEM).

### 2.3. Cell culture

Chinese Hamster Ovary cell lines were purchased from American Type Culture Collection (CCL-61, ATCC). Cells were cultured in Dulbeccoís Modified Eagleís Medium (DMEM), supplemented with 10% of Fetal Bovine Serum (FBS) and 1% of penicillin-streptomycin BRL; Life Technologies (Van Allen Way, Carlsbad, CA, USA). Cell lines were kept in a humidified atmosphere at 37 °C and 5% of $CO_2$.

### 2.4. Cytotoxicity assay

Cell cytotoxicity was assessed through MTT, an indirect cytotoxic test based on the formation and colorimetric quantification of an enzyme reaction product, which evaluates the reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) according to the methodology described by Denizolt and Lang [24]. Briefly, $8 \times 10^4$ cells were inoculated for analysis after one day, $10^3$ cells for analysis after two days, and $5 \times 10^2$ cells after seven days exposure to the strip surface. Afterward cells were seeded with 1 mL of supplemented culture medium in a 24-well plate and incubated for 24 h at 37 °C. The ME alloy was placed in the well for different incubation times (1, 2 and 7 days), while the solution of MTT (1 mg/mL in serum-free medium) was added for two hours after the incubation period. The formazan crystals were dissolved with dimethyl sulfoxide (DMSO) for 30 min, and the absorbance was measured using a microplate reader (Spectra Max M2e, Molecular Devices) at 570 nm. The absorbance of the negative control (cells without ME alloy) represents 100%. The percentage of growth inhibition was calculated as: cell viability (%) = (absorbance of experimental wells/absorbance control wells) × 100. Each experiment was performed in triplicate.

### 2.5. Acridine Orange/Ethidium Bromide (AO/EB) staining and image processing

The AO/EB technique is suitable to visualize viable cells and cell death induction. Initially, $8 \times 10^4$ cells/well were seeded in 24 well plates and incubated for 24 h with bare or Au-covered magneto-elastic strips. After incubation, the strips were washed twice with phosphate buffer saline (PBS). Cell death was evaluated through a direct *in situ* analysis on the surface of the sensor using Ethidium Bromide and Acridine Orange (Sigma-Aldrich). Each surface received 2 μL of AO and EB (100 μg/mL). Images were taken with a fluorescent light microscope (BX43—Olympus) with 10× and 40× objective magnification. The correlation between the intensity of green and red pixels were analyzed using ImageJ software v. 1.50 (http://rsb.info.nih.gov) and processed as described by Mironova et al. [27]. Briefly, AO/EB images were divided into three channels (R-red, G-green, B-black). Images of the green and red channels were quantified and the correlation plot of co-localized and non colocalized fluorescence was processed. Non-correlated pixels looked green and red and were attributed to living and dead cells, respectively. Correlated pixels looked yellow-orange and were also attributed to dead cells. The percentage of live cells was determined by dividing the number of green pixels by the total number of red and green pixels. The threshold was fixed at an intensity of 75, determined by the 8 bits extraction of channel images.

### 2.6. Ions concentration in media culture

To evaluate ion detachment into the medium from the surface of ME strips not covered with Au, the strips were placed in 160 mL of DMEM. After 7 days, the strips were removed and weighed dry. The incubation medium was sent for analysis by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) method. Analysis was performed by the laboratory *Greenlab® Análises químicas e toxicológicas Ltda.*

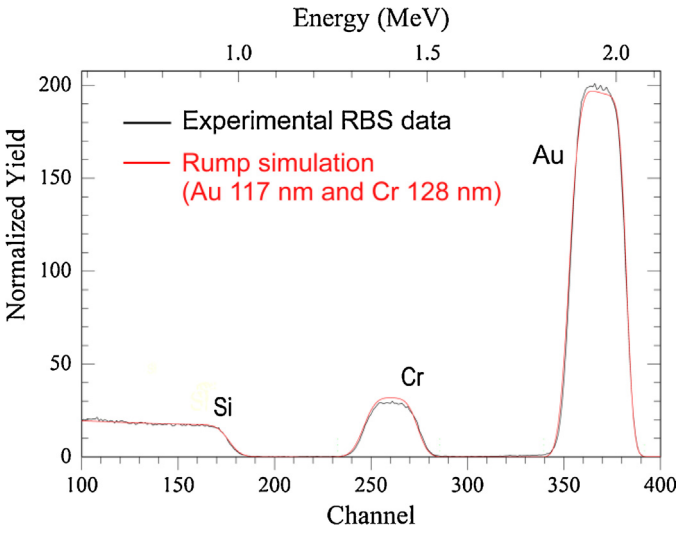


**Fig. 1.** Rutherford backscattering spectrum (RBS) for layers of Cr and Au on a Si substrate, with the simulated spectrum, taken from Ref. [30]. The simulation assumed a Cr layer of 128 nm as well as a Au layer of 117 nm.

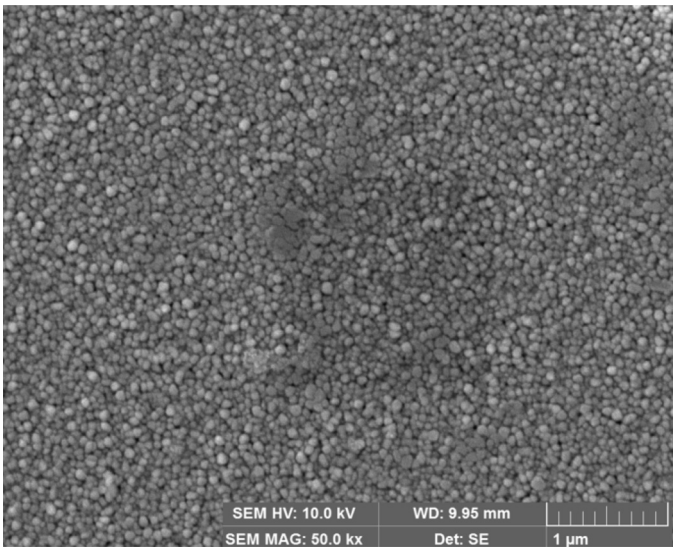


**Fig. 2.** SEM image showing grain structure of Au-covered surface.

### 2.7. Resonant frequency analysis

The resonant frequencies of the sensors were measured using a single pickup coil wound around a 250 μL Eppendorf tube, together with an Agilent® E5061B impedance analyzer. Sensors with dimensions 5 mm × 1 mm × 15 μm, covered or not with gold, were inserted into the tube with tube 200 μL of DMEM. The DC current furnished to the pickup coil was varied to maximize the signal received by the impedance analyzer. A measurement of the $S_{11}$ parameter allowed the resonant frequency of the sensor to be determined. The resonant frequencies were measured for up to 70 h.

## 3. Results and discussion

The amorphous alloy Metglas® 2826MB3™ is an interesting sensor candidate given its large saturation magnetostriction, high magnetization, low anisotropy energy and low coercivity [28]. Recently, Holmes et al. studied the biocompatibility of a Metglas® 2826MB™ alloy and observed low compatibility in cell cultures [17]. Biological compatibility has been under study for magneto-elastic strips coated with materials that provide stability to the sensor system [12,23].

The thickness of the coating layer on a Si wafer placed next to the ME alloy surface during coating was evaluated by RBS analysis. RBS revealed values of Cr and Au of 128 nm and 117 nm, respectively (Fig. 1). In order to gain a perspective on these numbers, we note that the diffusion of Ni through Au films is a problem that has been studied in the area of microelectronics. The diffusion coefficient for Ni atoms through bulk Au is five orders of magnitude smaller than the grain boundary diffusion coefficient [29]. Abdul-Lettif [29] found that the grain boundary diffusion coefficient for Ni in Au is given by $D_b = (3 \times 10^{-4}\ cm^2/s)\ \exp(-0.94\ eV/kT)$. Therefore, even grain boundary diffusion is negligible at the temperatures of our experiments. Thus we do not expect atomic diffusion through grain boundaries in the Cr and Au films to occur in our experiments.

A typical spectrum is shown in Fig. 1 along with a simulation of that spectrum. By means of the simulation, RBS provides a non-destructive assessment of the quantitative concentration profiles of the elements down from the surface. The simulation shown in Fig. 1 assumed a Cr layer of 128 nm as well as an Au layer of 117 nm. The layers are seen to be well-defined, with little or no mixing. The determined thicknesses correspond to about 400–500 atomic diameters. Au-covered surfaces were further analyzed using SEM (Fig. 2), which showed a regular distribution of Au over the ribbon surface.

If the Mo and Ni ions cannot pass through the Au coating on the ME strips, then it is easy to understand the cytotoxicity results. Cytotoxicity assay with CHO cell lines was performed at different stages (1, 2 and 7 days) using the MTT method and showed significant reduction in cell survival after exposure to the strip not covered with gold (Fig. 3). This was not observed with the Au-coated strip compared to the control sample. Fig. 3 shows a decrease in the percentage of live cells compared to control over time for the uncoated strip, with a greater reduction after 7 days. However, strips covered with Au presented almost no statistical difference when compared with the control.

Degradation products present in the culture medium during cytotoxic analyses were determined over the course of one week (Table 1). The ion concentration of elements from the uncoated magneto-elastic alloy, after 7 days exposure, caused a ~50% reduction in cell survival. A similar time exposure of the Au-coated magneto-elastic alloy presented no reduction in cell survival.

In the present study, cyto-incompatibility of Metglas® 2826MB3™ induced a degradation process in living organ-

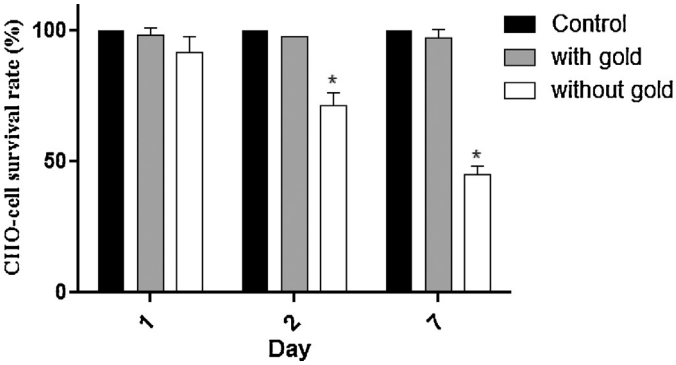


**Fig. 3.** Analysis of cell survival by MTT. Gold covered ME strip showed no significant difference in cell survival when compared to controls. However, a significant reduction in cell survival was observed in the uncoated strip from day 2. Results were obtained from three independent experiments. Bars with * correspond to statistically significant differences using ANOVA-Tukey test ($p \leq 0.05$) in comparison to control.

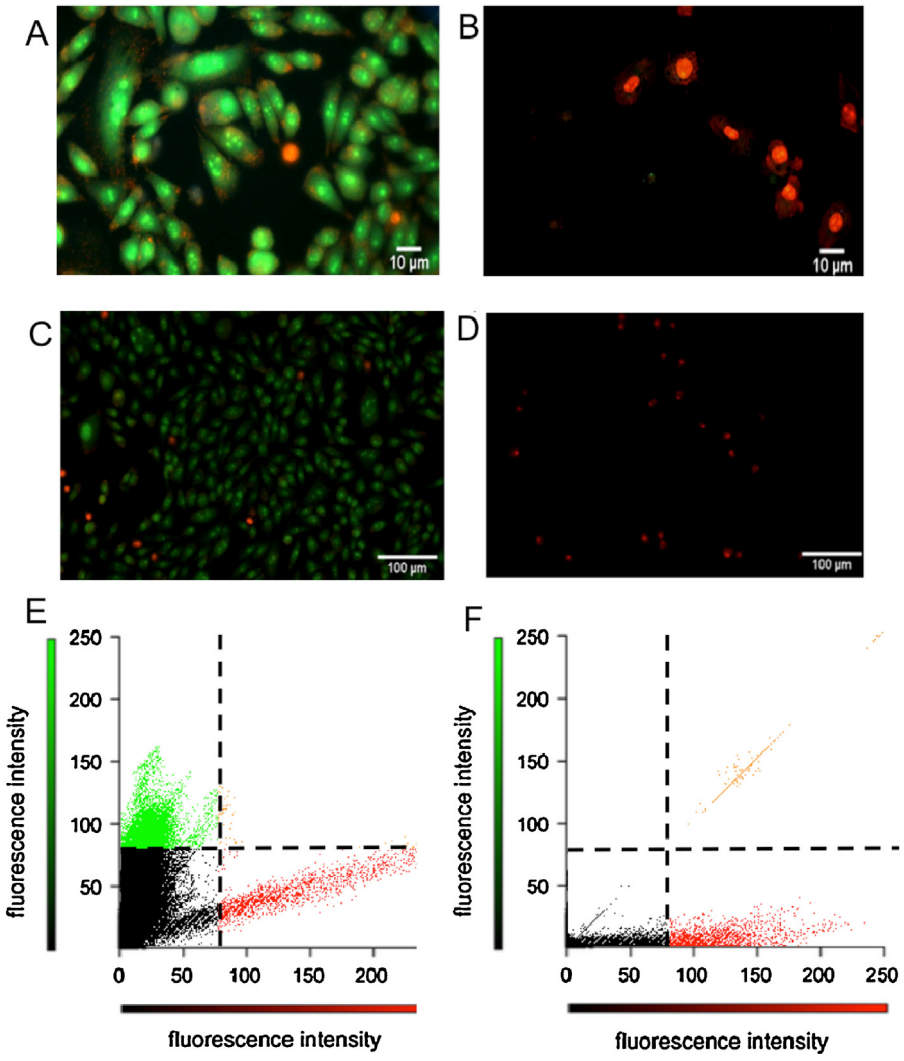


**Fig. 4.** *In situ* analysis of cell death through AO/EB staining: A and C—Au-covered strips with few cells stained by EB (red). Most of the cells presented normal morphology and were stained by AO (green). B and D—uncoated strips showing EB (red) staining indicating membrane permeability in dead cells. E—Correlation plot for the image presented in panel C. F—Correlation plot for the image presented in panel D. Dashed lines indicate thresholds of fluorescence at 75, taken from 8 bits extraction channel images chosen empirically, that separates visible fluorescence from dark pixels. No correlation was observed between images obtained in green and red spectral regions. Above these threshold pixel values, points were counted to evaluate the cell viability. The percentage of live cells was determined by dividing the number of green pixels by the total number of red and green pixels. Cell viability for image E presented 85.46% green in comparison to death morphology with 13.97% red and 0.57% yellow-orange fluorescence. No viable cells were observed in image F, corresponding to cells seen in panel D. Intensities from both green and red channels from below the threshold were not quantified and are represented in black. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Table 1**
Degradation products present during cytotoxicity analyses were determined over the course of one week. The ion concentration in the culture medium after magneto-elastic alloy exposure was evaluated by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) and is expressed in μg/mL.

| Sample | Ion Concentrations (μg/mL) | | | |
|---|---|---|---|---|
| | Day 7 | | | |
| | Fe | Ni | Mo | B |
| without Au | 0.053 | 0.050 | 0.006 | 0.0066 |
| with Au | 0.000048 | 0.00005 | 0.0006 | 0.00026 |

isms, with the release of some of their atoms into the surrounding medium. This effect is related to degradation of the bare strip and the presence of metal atoms from the alloy, freely distributed in the culture medium [31–33]. The toxicity of these metal ions has been reported in different studies. Molybdenum has been associated with various toxic effects on the body, causing damage to various organs such as kidney, liver and spleen [18,19]. Furthermore, nickel was shown to be carcinogenic and toxic [20]. Taira et al. [21] demonstrated that nickel cytotoxicity shows a dose dependent pattern as released in media culture.

Induction of cell death was also evaluated through AO/EB *in situ* staining and showed significant reduction in cell survival and consequent low cell adhesion on top of ME strips not covered with Au, when compared to the Au-coated strip (Fig. 4). These results demonstrated the efficacy of working with ME sensors in conjunction with a gold coating to protect living cells. *In situ* analysis of samples not covered with gold showed a cell death pattern compatible with harmful cytotoxic effect (Fig. 4B and D) compared to the surface covered with gold (Fig. 4A and C). Gold coating protected cells, maintaining morphologically normal characteristics, with only a few cells presenting a pattern of cell death. Morphological cell patterns from the Au-coated surface were further analyzed through the ImageJ software in order to determine the number of normal cells and cells presenting death patterns. The dual AO/EB staining method generates color images, which were initially handled through channels. Normal cells presented a green fluorescence

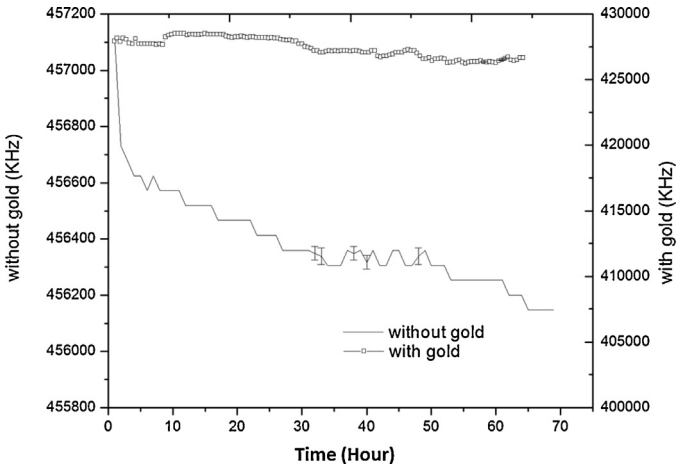


**Fig. 5.** Effect on the resonant frequency of the bare Metglas® 2826MB3™ ribbon compared to the Au-covered ribbon after 70 h exposure to the culture medium. Note frequency decrease in the first hours of experiment for bare ribbon.

(OA) in contrast with dead cells that present a red fluorescence (EB). The analysis of these result is presented in Fig. 4E. Most of the cells grown on top of the gold-covered sensor presented normal-like morphology represented by a green fluorescence (85.46%). The remaining cells show a death pattern and exhibit red and yellow-orange fluorescence (14.54%), with 13.97% red and 0.57% yellow-orange. No viable cells were observed in Fig. 4F, corresponding to cells seen in panel 4D.

Cytotoxicity of a biomaterial can be investigated using different types of cells for the MTT assay [34,35]. The MTT analysis as described by Denizot and Lang [24], measures mitochondrial activity of living cells and indirectly determines alterations in metabolic activity. The LIVE/DEAD assay is used to determine cellular membrane integrity since membrane-compromised cells are permeable to ethidium homodimer-1, which binds to nucleic acids resulting in nuclear-localized red fluorescence [36,37]. Several studies investigate biocompatibility of material surfaces in biomedical application by indirect analysis using the LIVE/DEAD assay [38–40]. Holmes et al. [36] studied the effect of cell cytotocixity on 2 types of ME materials, demonstrating qualitatively a lack of biocompatibility for the Metglas® 2826MB™ material.

Cell death can be classified according to its morphological appearance [41]. Two major cell death processes were considered here. Necrosis is morphologically characterized by a gain in cell volume, swelling of organelles, plasma membrane rupture and subsequent loss of intracellular contents. For a long time, necrosis has been considered merely as an accidental uncontrolled form of cell death. Apoptosis or programed cell death is characterized as a death pattern accompanied by rounding-up of the cell, with a progressive condensation of chromatin that induces nuclei to shrink into little single balls. In advanced stages of apoptosis, morphological changes characterized by nuclear and cytoplasmic condensation and cell fragmentation into membrane bound apoptotic bodies are observed [42,43]. Here, cells were grown on the top of the ME strips and analyzed trough AO/EB dual staining with discrimination of live from dead cells on the basis of membrane integrity. Degradation of the bare strip caused release of metal atoms from the alloy and induced cell death. Morphological cell changes could be easily observed from the monolayer of residual cells placed on sensor surfaces without gold protection. *In situ* analysis through AO/EB staining revealed red fluorescence from cells on top of the uncoated sensor, indicating that ethidium bromide (red) staining entered the cell because of membrane permeability. Cells presenting normal morphology, corresponding to most of those observed on the Au-covered strips, are stained only for acridine orange (green) staining, maintaining their membrane integrity. ImageJ analysis was performed and could find few cells with correlated pixels that looked yellow-orange, a condition also attributed to cell death. Cell viability for the Au-covered ME alloy presented 85.46% green fluorescence in comparison to the death morphologies on bare ME alloy with 13.97% red and 0.57% yellow-orange fluorescence.

Degradation of strips not covered with gold can be visualized by the instability of the resonance frequency, which is not seen on the alloy covered with gold, as reported in the present study. Effects on the resonant frequency of the bare Metglas® 2826MB3™ sensor are compared to those on the Au-covered sensor after 70 h exposure to the culture medium in Fig. 5. The resonant frequency of the bare sensor showed a much more expressive decrease indicating a net increase in mass, probably due to corrosion.

Exposing magneto-elastic material to a liquid medium may generate intermediate chemical reactions such as oxidation [44]. Iron corrosion may occur along with the leaching of other alloy components. The process of oxidation of metals has the characteristic formation of products such as oxides, which can adhere to the substrate base (magneto-elastic material) thereby increasing its mass. Another process that should be considered is the loss of alloy elements into the medium through leaching processes, causing changes in the characteristics and properties of the magneto-elastic material. The sum of these effects can be observed in the resonant frequency, which presents a marked decrease over time for the bare sensors, along with the amplitude of the signal response [2].

Coating surfaces of biosensors with gold layers is widely applicable because of their ability to form covalent bonds with spontaneous thiolates, facilitating bio functionalization via self-assembled monolayers (SAM) [45,46]. Huang et al. [23] observed qualitatively that coating Metglas® 2826MB™ ribbons with Au (thickness ~125 nm) maintains their physical properties and increases their stability. The gold and chromium coatings used in this study were found to have thicknesses of 117 nm and 128 nm through RBS. The biocompatibility of Metglas® 2826MB3™ strips was investigated here *quantitatively* through a survival assay in a cell culture. The results suggested that strips covered with gold presented an expressive reduction in cell death with an increase of cell surface adhesion. The percentage of living cells remained stable for different exposure times compared to the non-covered strip, where a decreased survival over time and a decreased adhesion of cells on the surface were observed.

Despite the good availability of Metglas® 2826MB3™, the resultant cytotoxicity from ion release should be carefully evaluated in each study design. Protection of alloys with appropriate coatings to ensure biocompatibility and avoid cytotoxicity has been explored [12]. The fact that the sensor is produced on a magneto-elastic substrate means that they have potential use in remote measurements for medical applications [47] and monitoring of different parameters in biological assays [11]. Gold is one of the most commonly used noble metal coating materials because of its high corrosion resistance, besides being very stable and inert. Another advantage of the gold coverage is the possibility of chemical functionalization with probes to recognize biomolecules. The present work has shown that the gold coating has the additional benefit of reducing cytotoxic effects due to ion release.

## 4. Conclusion

Wireless monitoring is an important plus when analyzing different biological processes. However, biological incompatibility of the magneto-elastic substrate alloy may induce cytotoxic processes due to the dissolution of Mo and Ni. Beside this, the ME sensor itself degrades in contact with the solutions, causing the experimental

results to be incorrectly interpreted. Applying coating materials like gold increases biocompatibility and the stability of magneto-elastic alloys, improving the final analysis. In this work, we have quantified the biological incompatibility in a particular case and have suggested a time scale for these effects. Experiments processed during seven days with the Cr/Au protective layer have shown very good signal stability of the sensor and high stability of the concentration of live cells, while, without the protective layer, during the same period, a reduction of 50% in the live cells was registered. Considering the huge variety of biological materials on numerous substrates that may interact and generate different biocompatibility issues, further studies are required to elucidate the effect of material degradation and potential effects generated after incubation in biological media.

## Contributors

CM and MRE planned the experiments. ALP assisted with sample preparation. CM did the bulk of the data collection and MB assisted with data collection. ADS assisted with sample preparation and RBS analyses. STM analyzed the live/dead photographs. JAPH was responsible for MTT analyses. The paper was written by CM, MRE, and FPM, with input from all authors.

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# 3.3 Capítulo III

**Effect of distinct antibody immobilization strategies on the analytical performance of a magneto-elastic immunosensor for *Staphylococcus aureus* detection**

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**Abstract**

Magneto-elastic (ME) sensors have a great advantage in microbiology due to their ability to be queried wirelessly. Immunosensors are affinity-based assays where the analyte is highly selective. The immobilization of antibodies (Ab) is an important step in the development of such devices. This study compared the effects of two antibody immobilization strategies on the analytical performance of a magneto-elastic immunosensor: (1) random antibody covalent immobilization (CysAb) and (2) specific-oriented antibody covalent immobilization (PrGAb). Immunosensors were exposed to solutions containing *S. aureus* at different concentrations ($10^4$ to $10^8$ CFU/ml) and sensor resonant frequencies were measured. In order to confirm that the frequency shifts were mainly caused by the binding of *S. aureus* to the sensor's surface, scanning electron microscope (SEM) and indirect immunofluorescence (IIF) images were taken after bacteria exposure at $10^8$ CFU/ml. Sensor surfaces were further monitored by non-contact topographic atomic force microscopy (AFM) images. In the covalent-oriented strategy, PrG was first bound covalently, to the surface, which in turn, then binds the anti-*S. aureus* antibody in an oriented manner. Topographic AFM image indicate the orientation of the antibody on the surface leading to an enhancement in the sensitivity of the immunosensor. This immobilization strategy gave the highest anti-*S. aureus*

antibody immobilization density. Therefore, the covalent-oriented strategy presented the best performance for *S. aureus* capture, detecting $10^4$ CFU/ml.

## 1. Introduction

Magneto-elastic (ME) sensors (Grimes *et al.* 2011) have been widely studied as biosensors (Gao et al. 2009; Huang et al. 2008a; Zourob et al. 2007) due to their ability to be queried wirelessly, which brings a great advantage in the microbiology area (Gao *et al.* 2009, Chin *et al.* 2014, Li *et al.* 2015). Upon contact with the specific target bacteria, the biorecognition element captures cells, increasing the sensor mass and decreasing the resonant frequency, which is remotely and wirelessly measured using a pick-up coil (Chin *et al.* 2014, Rodriguez-Rodriguez *et al.* 2016). Sensitive and selective sensors capable of rapidly and accurately detecting minute quantities of pathogens are urgently required. The need for real-time detection of biological and biochemical analytes is a major driving force behind the development of novel biosensor technology (Chin *et al.* 2014, Li *et al.* 2015).

*Staphylococcus aureus* is one of the most common bacteria widespread in the environment, and a major human pathogen related to numerous pathological processes (Kluytmans *et al.* 1995). *S*ome strains of *S. aureus* produce potent toxins and cause food poisoning (Jamison 2001). So far, a large number of approaches for pathogen detection have been proposed using traditional methods and most are time consuming, expensive and fail to differentiate species. Polymerase chain reaction (PCR) is an alternative created to amplify the target genes. However, PCR requires comparatively strict template DNA preparation and hours of operating time to have enough end product for detection (Xu *et al.* 2012). The development of a rapid method for detecting *S. aureus* is critical for early diagnosis of infection and bacteria detection in several substrates.

Immunosensors for pathogen detection are affinity-based assays that present high specificity between the analyte and the surface of the functionalized device. The way in which the recognition element and the interface are configured will play a large role in the performance of the final device. Immobilization of antibodies (Ab) is an important step in the development of an immunosensor. Properly oriented antibodies exhibit better antigen binding (Cecchet *et al.* 2007, Trilling *et al.* 2013) and, subsequently, improved assay performance. Different methods of immobilization can result in random or specific orientations of Abs, and are dependent on the capacity for self-organization of immunoglobulins, which can be controlled for specific reactive

groups on the surface. Strategies for oriented immobilization using molecules that enable the capture through specific locations on the Ab, such as Protein A (PrA) or Protein G (PrG), are interesting alternatives to bind the Fc immunoglobulin region (Bjorck and Kronvall 1984, Gronenborn and Clore 1993).

Different techniques can be used to evaluate the surface before and after bioconjugation and signaling (R. P. Kengne-Momo 2012, Bhadra *et al.* 2015). These distinct experimental approaches also allow an estimate of the immobilization density and morphology of the target organism. Atomic force microscopy (AFM) has been used to surmise the physical characteristics of the antibody layers (Preiner *et al.* 2014, De Thier *et al.* 2015). Non-contact atomic force microscopy is a logical method for biological samples (Allison *et al.* 2010) and can be examined directly under ambient conditions, with the resolution necessary to identify the position of the antibody on the surface (Farris and Mcdonald 2011). Scanning electron microscope (SEM) is also an important tool to evaluate differences in topographic distribution of bacteria captured on biosensor-functionalized surfaces (Wang and Alocilja 2015)

Here, the effects of different immobilization techniques on the immunosensor surface were investigated. Random antibody immobilization (CysAb) and oriented covalent antibody immobilization (PrGAb) strategies were considered and evaluated through variations of sensor resonant frequencies and topographical images of surfaces. Furthermore, quality control of the selected antibody applied over the sensor surface was investigated through immunodetection techniques and protein expression was monitored in different growth phases of the bacteria though antigen-antibody specific binding.

## 2. Material and Methods

### 2.1 *Substrate preparation*

The amorphous alloy Metglas® 2826MB3, with approximate composition in wt.% of $Fe_{45}Ni_{45}Mo_3B_7$, was used as the sensor platform. The alloy was supplied in the form of 2 inch wide ribbons. The ribbons were first mechanically polished on both sides using a Struers Tegramin 20 polishing system with 0.05 µm alumina and water. After 1.5 hours of polishing, the thickness was reduced to about ~ 15 µm. The debris or grease retained from the polishing process was removed by cleaning the strips ultrasonically in 100% methanol for 30 min. After the cleaning process, tapes were sputtered-coated (AJA, model ATC 2000) on both sides with a protective layer of

chromium and then with gold. The Au-coated strips were cut to dimensions 5 mm x 1 mm x 15 µm.

### *2.2 Growth condition, bacterial identification and quantification*

*Staphylococcus aureus* (ATCC 25923) was obtained from the Laboratory of Medical and Human Microbiology at the University of Caxias do Sul. This stock strain was frozen at -80º C. The cultures were grown for experimental analysis on Luria-Bertan (LB) and samples were collected at different stages of growth. The quantification and determination of the parameters at different stages of growth were evaluated by optical density (OD) and colony counting, according to Aneja (2005) and Rolfe *et al.* (2012), with modifications. The bacterial concentration was determined by OD and colony count for magneto-elastic imunossensor analysis. The identification and control of bacteria was performed by the Gram staining method and coagulase tests.

### *2.3 Antibody validation*

#### *2.3.1 Extraction of proteins from the membrane*

Protein extraction of the membrane of the *S. aureus* (target sample) and *S. epidermids* (negative control sample) was performed using 8M Urea, Tio Urea 2M, 40 mM Tris pH8, 5, 20 mM DTT, 4% Chaps, and 1% PMSF, 1 g of Glass beads® (Sigma) and 0,01g/ml Lysozime. Here, 250 mL of sample was centrifuged and the pellet obtained was resuspended in 500 ul of lysis buffer and incubated for 30 minutes at $4^0$C, according to a modification of the methodology described by Nandakumar *et al.* (2005). Protein quantification was performed by the method of Bradford (Bradford 1976).

#### *2.3.2 Western blot*

50 µg of total protein was determined by Bradford, and separated by electrophoresis with polyacrylamide SDS-PAGE 12% during one hour, at 150 V. The proteins were transferred to a PVDF membrane (Millipore®), for 1h , at 300 mA. For immunological analysis, the membrane was blocked for 1h in 5% milk powder diluted in TTBS, further incubated with monoclonal primary anti *Staphylococcus aureus* (antibody 37644,1:2500 v/v), diluted in TTBS, containing 5% powdered milk. The membranes were washed three times with TTBS followed by 1h incubation with secondary anti mouse anti body IgG in, diluted in 1:5000 v/v in TTBS, with 5% powdered milk, as described by Nguyen 2010. After washing, a chemiluminescent

substrate was added (Luminta-Millipore®) according to the manufacturer's instructions. The bands were revealed by LAS500 ImageQuant (GE Healthcare®) and analyzed by the program ImageQuantTL (GE Healthcare®).

### *2.3.3 Immunofluorescence assay*

Cells at different concentrations and growth phases were fixed with 4% paraformaldehyde in 10-well slides, and blocked with 2% serum for 1h. The cells were incubated with the primary monoclonal antibody, anti-*Staphylococcus aureus* (ab37644, 1:250 v/v) for 1h, followed by incubation with secondary anti-mouse fluorescein isothiocyanate (FITC) (Anti-mouse IgG, Sigma, 1:150 v/v) for 1h. The slides were analyzed with a fluorescence light microscope (BX43 – Olympus). The controls were isolated from *S. epidermidis* and submitted to the same experimental conditions.

## *2.4 Synthesis of self assembled monolayer (SAM) on magneto-elastic sensor surface*

Strips of size 5 mm x 1 mm x 15 µm were washed in an ultrasonic cleaning bath in a 1:1 solution of ethanol and methanol. After the rinsing, the substrates were dried under a stream of dry nitrogen and then stored in a desiccator. The application of cystamine hydrochloride (CYS) (Sigma) for the formation of the SAM was processed using a 9:1 ethanol/water solution with concentration of 20 mM during 12 hours in orbited shaking at room temperature. Afterward, the sensor was washed with a 9:1 ethanol/water solution to remove the CYS, unbound to the Au surface. Finally, the ribbons were dried with streaming nitrogen and stored in a desiccator.

## *2.5* Anti-*Staphylococcus aureus* immobilization

Two different immobilization methods were applied and compared on the magneto-elastic sensor: 1) random antibody non-covalent immobilization strategy (CysAb): Amine groups on the SAM-coated sensor and antibody carboxyl groups were conjugated by a bifunctional cross-linking reagent 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Sigma), with 0.4 mg dissolved in 100 µl of phosphate buffered solution (pH 7.4). The antibody (0.02 mg/mL) was activated with EDC (4mg/mL) in 1:100 EDC/Ab solution for 15 min at room temperature (RT). Afterward, the activated antibody was incubated on the SAM-modified sensor surface for 1 h at 37 °C and washed five times with 0.1 M PBS (pH 7.4). Blocking of the non-

specific binding surface was performed with 300 µl of 1% (vol/vol) BSA for 30 min at RT; followed by five washings with PBS according to a modification of the methodology described by Dixit *et al. (Dixit et al. 2011).* 2) specific-oriented antibody covalent immobilization strategy (PrGAb): EDC was prepared as previously described. The protein G (1 mg/mL) was activated with EDC (4mg/mL) at the ratio of 1:100 in an EDC/PrG solution for 15 min at RT. Afterward the activated antibody in the SAM-modified sensor surface was incubated for 1 h at 37 °C and washed five times with 0.1 M PBS (pH 7.4), followed by the antibody-immobilized surface block as described above. Non-specific binding was blocked.

### *2.6 Immobilization detection*

#### 2.6.1 *Magneto-elastic S.aureus sensor resonant frequencies detection*

The resonant frequencies of the sensors were measured using a single pickup coil wound around a 200 µL Eppendorf tube, together with an Agilent E5061B impedance analyzer. Solutions containing different concentrations of bacteria were in contact with the sensor. Results were analyzed in triplicate at the controlled temperature of 22°C. Dc current furnished to the pickup coil was adjusted to maximize the signal received by the impedance analyzer. A measurement of the S11 parameter allowed the resonant frequency of the sensor to be determined. During the testing process, the temperature was controlled.

#### *2.6.2 Electron and optical surface microscopy analysis*

Confirmation of antibody–bacteria binding at the sensor surface was conducted by SEM. Sensors were mounted for the SpGAb and CysAb interface and SEM images were taken for the sensor after exposure to *S. aureus* at $10^8$ CFU/ml. The sensors were examined using a MIRA3TESCAN SEM, operating at 10 kV. Fluorescence analysis with an optical microscope was performed on the bacteria-covered sensor with indirect immunofluorescence staining according to the protocol described in section 2.3.3.

#### 2.6.3 Non-contact topographic AFM images analysis

AFM analysis was performed in a Shimadzu Scanning Probe Microscopy (SPM) 9700 microscope. The magneto-elastic immunosensors were imaged in the *tapping* mode using silicon cantilevers model NCHR10 under ambient conditions. Software from the Shimadzu instrument SPM 9700 was used for data capture, and image

modification was processed by Gwyddion 2.42 software. The substrate was probed on three random 250nm spots.

## 3. Results and discussion

Before investigating the direct effects of different immobilization techniques on the immunosensor surface, quality control of the selected antibody applied over the sensor surface was evaluated through immunodetection techniques. Also, protein A (SpA) expression was further monitored in different bacteria growth phases though antigen-antibody specific binding. Therefore, the first step of this study was to validate the antibody to be used throughout the project. When selecting monoclonal, polyclonal or recombinant antibodies for the detection of pathogens, certain characteristics are of great importance. Firstly, the antibody should be able to detect and quantify very low concentrations of cells (sensitivity). It should be also able to differentiate specific strains of interest (specificity). It is recommended that a constitutively expressed antigen, which is specific for the species is targeted (Byrne *et al.* 2009). The antibody was tested for its specificity and affinity by indirect immunofluorescence and Western blot assay for *S. aureus*, and *S. epidermidis* was used as a negative control (**Fig. 1E**). In this case, no FITC staining was visualized under the microscope, indicating the absence of this microorganism and confirming the specificity of the antibody.

The different growth phases of *S. aureus* were determined by optical density time experiments. For the first four hours it remained at the lag phase and after six hours entered the exponential phase (**Fig. 1 A)**. For analysis of SpA expression in the different growth phases, Western blot technique was performed and samples were collected in the lag and stationary phases (**Fig. 1F and 1G).** Western blot is a suitable method to evaluate the expression of proteins in bacteria by a semi quantitative scale technique. This has been reported in other studies (Jones *et al.* 2008, Lijek *et al.* 2012).

To date, some studies have presented a relation between the variation of protein A and bacterial growth and function (Morrison *et al.* 2012). When assessing the different stages of growth by optical density and colony counting, the bacteria showed a higher expression of protein A during the lag phase. The level of this protein decreased before the exponential phase was reached after 5 hours under the experimental conditions. Similar behavior was also described by Soong *et al.* (2011) and Jones *et al.* (2008). The identification of molecules that meet these requirements can be facilitated through screening by immunologic analysis.

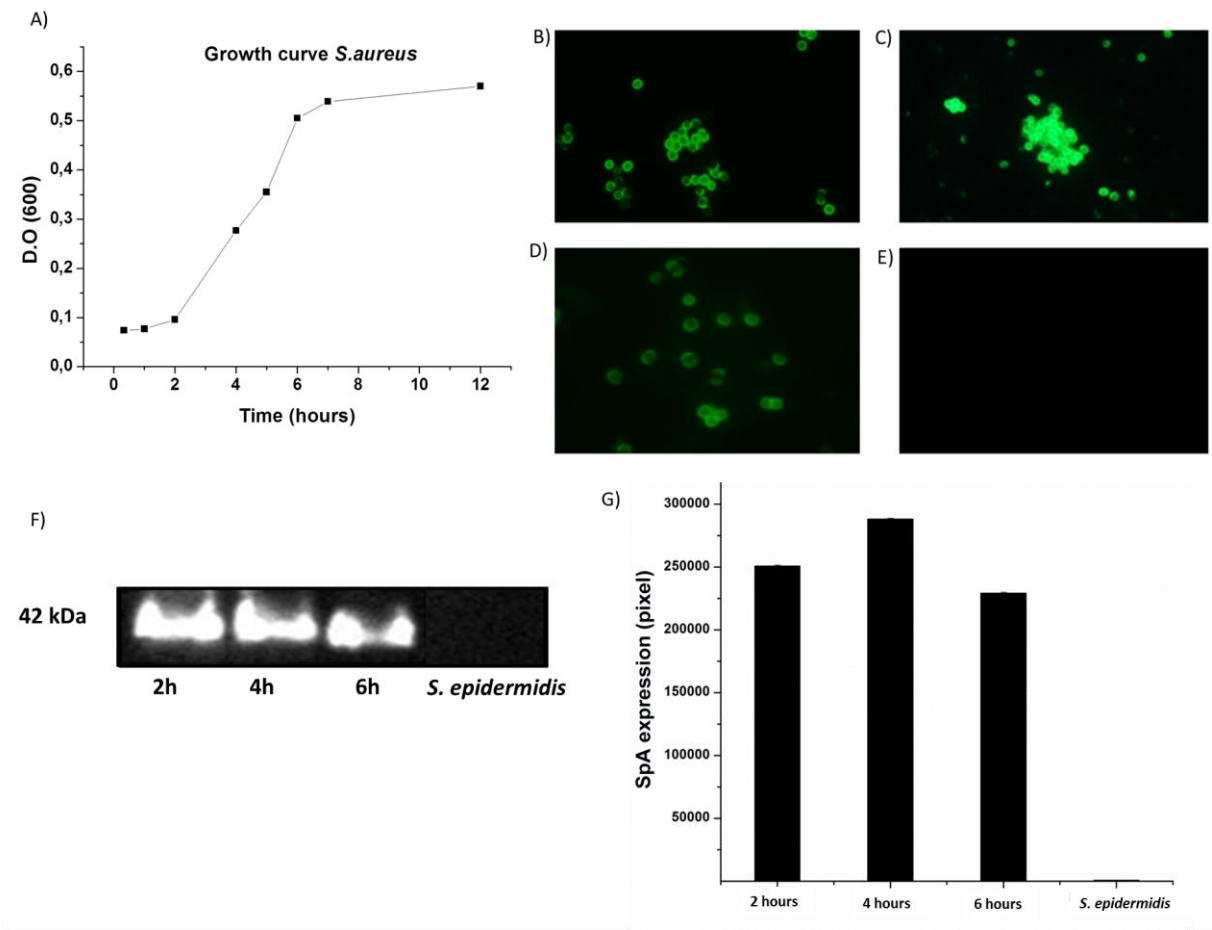


**Figure 1**: Growth conditions and SpA expression: antigen expression in samples was collected at different times of *S. aureus* growth and samples were processed through immunofluorescence and Western blot techniques. (A) growth curve of *S. aureus* ATCC 25923 at 37°C in LB medium; immunofluorescence of *S. aureus* cells at different times of growth: two hours (B), four hours (C), six hours (D) and negative control using *S. epidermidis* (E); Western blot showing a decrease of SpA expression in samples collected at different growth times for *S. aureus* (F); data acquisition was taken by LAS500 ImageQuant and analysis by ImageQuantTL. Note that negative control using *S. epidermidis* revealed no expression on panels E, F and G.

Here, the target SpA protein showed differential expression in all phases of *S. aureus* growth evaluated, most importantly with expression in early stages of growth. Based on this result, the monoclonal anti-*S. aureus* antibody here analyzed was shown to be cost–effective and rapidly screened, therefore was selected as a candidate to be applied throughout the investigation.

The second step of the study was to determine analytical performance of the magneto-elastic sensor for the two different methods of antibody immobilization on the sensor surface. In the development of biosensors some parameters are of great importance to be evaluated. The direction, shape and detection of molecules coupled to the solid phase are important to improve the sensitivity and binding capability to the

analyte. The resonant frequency analysis was monitored to evaluate immobilization efficiency on the surface of sensors. **Fig. 2** shows the resonant frequency shift for the CysAb and PrGAb immobilization processes and compared to control sample (sensor covered exclusively with gold) after exposure to solutions containing *S. aureus*, with different concentrations ranging from $10^4$ to $10^8$ CFU/ml. In order to confirm that the frequency shift was a result of *S. aureus* binding to the sensor's surface, SEM and indirect immunofluorescence images were taken after exposure to *S. aureus* at $10^8$ CFU/ml.

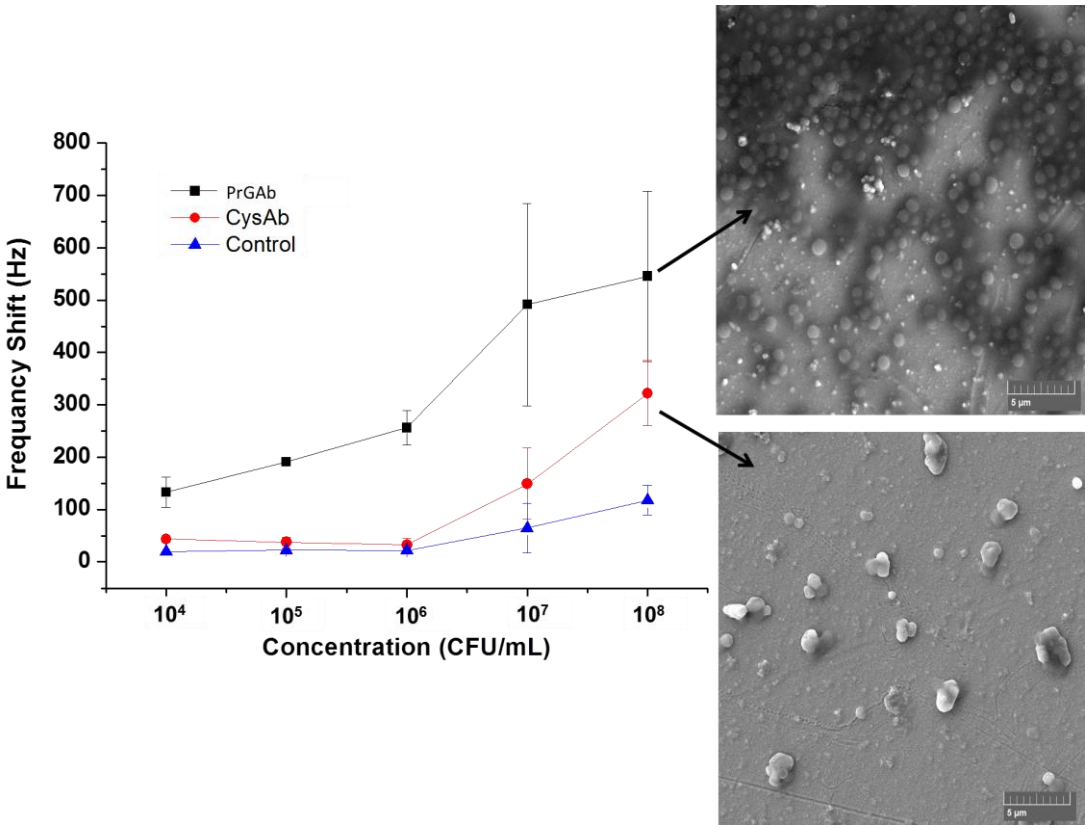


**Figure 2:** The resonant frequency shift upon exposure to solutions containing *S. aureus* with different concentrations ranging from $10^4$ to $10^8$ CFU/ml, using 5mm x 1mm x 15µm sensors. On the right a typical representation of the SEM images of *S. aureus* bound to an antibody immobilized by two different methods (top panel – PrGAb; and bottom panel - CysAb). Note that specific-oriented antibody covalent immobilization strategy (top right panel - PrGAb) reveals an uniform and more densely populated distribution of *S. aureus* over the sensor surface compared to the random antibody non-covalent immobilization strategy (bottom right panel – CysAb). The variation in the resonance frequency was obtained during 1 hour incubation

Oriented immobilization PrGAb presented a larger frequency shift compared to the CysAb random immobilization (**Fig. 2**). The lower antibody immobilization density

for the random strategy was attributed to the irregular and uncontrolled binding of the antibody over the surface, mainly by electrostatic and hydrophobic interactions (Rusmini *et al.* 2007). However, the introduction of PrG molecules enabled the oriented immobilization of antibodies in a site-directed fashion. The anti-*S. aureus* immobilization density using PrG was greater than that of cystamine, since protein G binds very strongly to the Au surface. A gold surface cover is used extensively for biosensing applications in various biosensor formats (Cecchet *et al.* 2007, Trilling *et al.* 2013) and increases biocompatibility and the stability of the ME alloys, improving the final analysis (Menti *et al.* 2016). The higher antibody immobilization performance of the covalent-oriented strategy may be due to the fact that covalently bound PrG molecules could resist leaching while maintaining their active conformation for binding (Makaraviciute and Ramanaviciene 2013).

The control samples covered exclusively with gold (**Fig. 2**), without antibody loading, showed some slight frequency effects at higher bacteria concentrations ($>10^6$). These effects might be due to the viscosity enhancement caused by the high bacterial concentration in the media, since the sensor frequency is sensitive to viscosity variations. Sensor viscosity sensitivity has been described in previous studies by Grimes *et al* (2011). In order to complement and compare the results generated by SEM, analysis of fluorescence on the sensor surface was performed using an optical microscope (**Fig. 3**). The control samples showed no bacteria on its surface (**Fig. 3A**), indicating that the variation in frequency at high concentrations should be related to environmental factors, since sensors were exposed to bacteria at a concentration of $10^8$ CFU / mL. Both immobilization strategies used here (CysAb and PrGAb) were also evaluated through indirect immunofluorescence. Corroborating the images obtained by SEM, the specific-oriented antibody covalent immobilization strategy (PrGAb) revealed a more populated distribution of *S. aureus* over the sensor surface (**Fig. 3B**) compared to the random antibody non-covalent immobilization strategy (CysAb), capturing few copies of bacteria over its functionalized surface (**Fig. 3C**).

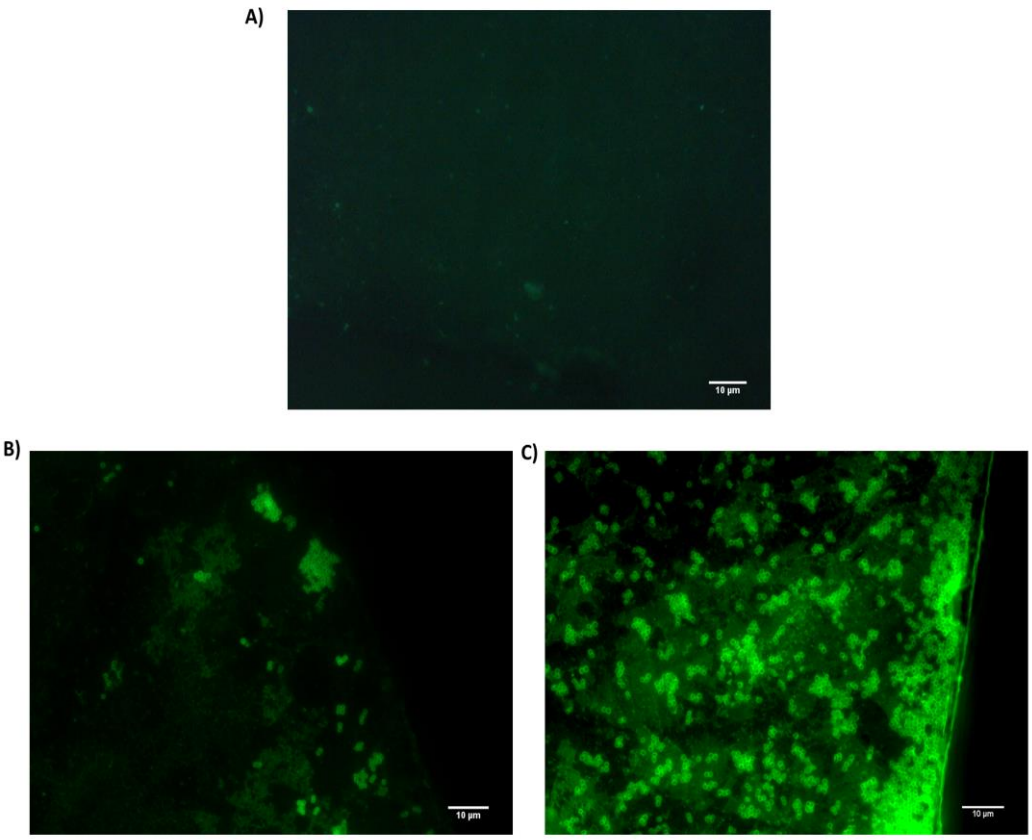


**Figure 3**: Fluorescence optical microscopy images of the bacterial capture on magneto-elastic sensor surface: A) control samples; B) CysAb and C) PrGAb immobilization strategy. Note that PrGAb immobilization presents an increased density of bacteria over the sensor surface. The variation in the resonance frequency was obtained during 1 hour incubation.

The intermittent contact mode of AFM in air was performed to visualize the bound antibodies on the modified cystamine and protein G surfaces, according to the procedures described in the experimental section (**Fig. 4**). Therefore, different immobilizations were processed and evidenced as topography and 3D image of: gold covered surfaces **(Fig. 4A)**, modified with mixed cystamine SAM **(Fig. 4B),** with Anti-SpA on the Cys-activated surface **(Fig. 4C)**, with SAMs after soaking in the SpG solutions **(Fig. 4D)** and with a complex of Cys-protein G and capture antibody **(Fig. 4E)**. The 3D AFM image shows the surface of the gold-coated ME sensor with two distinctive types of hills: first, homogenous, dense and low hills resulting from amine groups and second, sporadic higher hills created by immobilized antibody (**Fig. 4E)**.

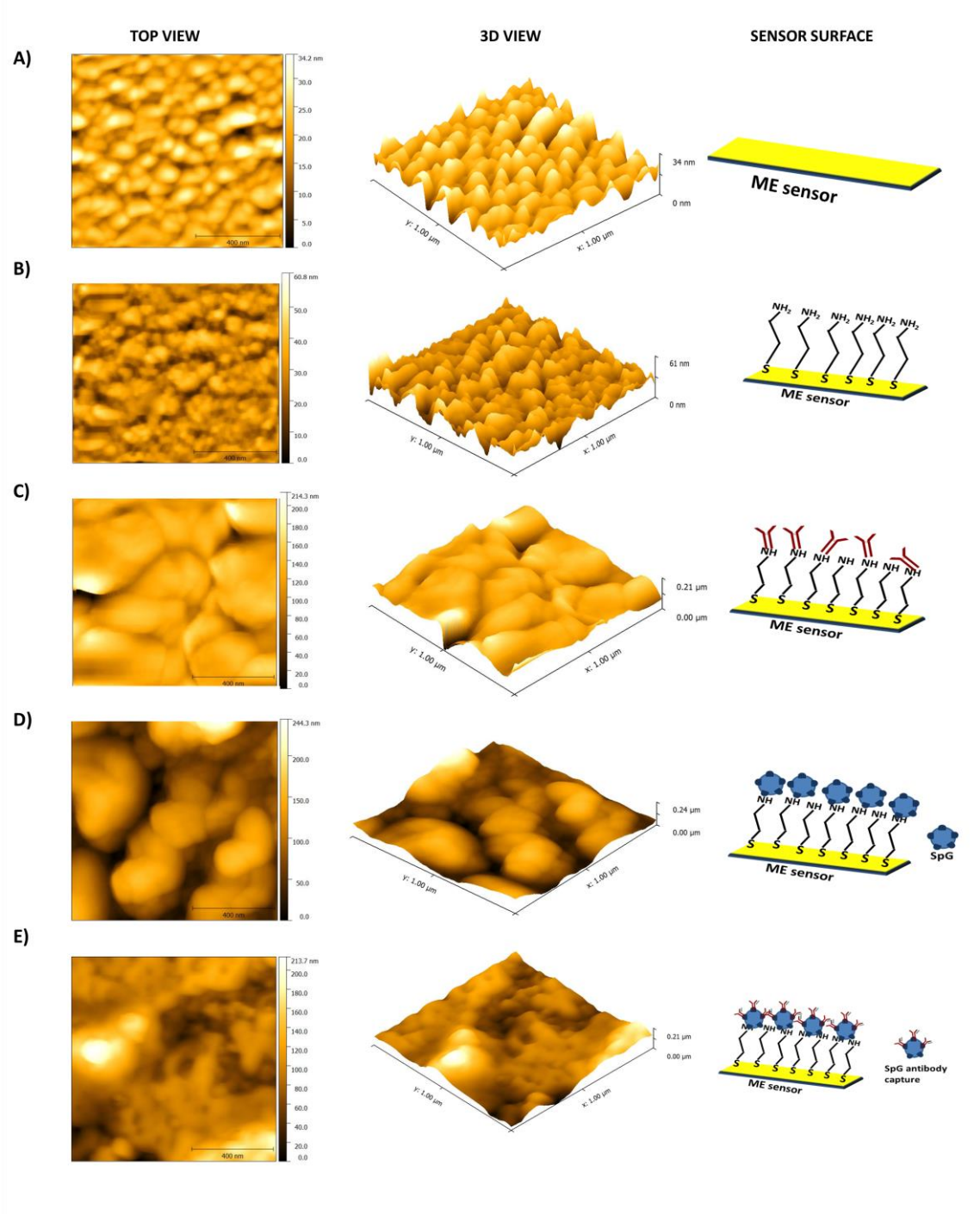


**Figure 4:** Tapping mode AFM images of Cys and Cys-Ab anti-SpA on the gold surface. A) Gold covered surface; B) Cys on the gold surface; C) Anti-SpA on the Cys-activated surface; D) Cys-protein G on the gold surface; E) Complex of Cys-protein G and capture antibody.

Root mean square (RMS) values were extracted from topographic AFM images, showing differences in surface roughness values from the interface formed (**Table 1**). The RMS parameter represents the mean and standard deviation of surface heights distribution. The mean line is the line that divides the profiles, so that the sum of the

squares of the deviations of the profile height from it is equal to zero (Gadelmawla *et al.* 2002). The RMS parameter drawn by AFM topographic image (**Table 1**) shows different patterns of roughness for the random and oriented immobilization strategies, increasing with the addition of human IgG. This discrepancy may be due to both the irregularity of the surface and to the difference in binding characteristics of the PrG , Cys and IgG compared to the gold surface of the magnetic elastic sensor. Similar results are observed for the other studies (Farris and Mcdonald 2011, Wang *et al.* 2012).

**Table 1:** Root mean square (RMS) mean and standard deviation taken from topographic AFM images.

| | **Root mean Square (nm)** | |
|---|---|---|
| | Mean | SD |
| Cys | 27 | 8 |
| CysAb | 57 | 17 |
| CysPrG | 32 | 3 |
| PrGAb | 36 | 4 |

Antibodies can become attached to the sensor surface in different orientations. Some possibilities are (1) binding laterally to the surface, (2) coupling exclusively to a heavy chain carboxyl terminal, or Fab antigen recognition region, and (3) lying over the surface in any possible position (Menti *et al.* 2016). Immobilization of the antibodies on surfaces functionalized with SAM-thiol amino terminals such as cystamine has been exploited for long time (Sharma *et al.* 2010, Chauhan and Basu 2015) . The coupling of antibodies on gold surfaces using cystamine provides a random distribution of molecules, that may generate low rate (e.g., 5 to 10%) of the active antibody density compared to oriented antibody immobilization strategies (Schramm and Paek 1992). From a quantitative and qualitative perspective, the results obtained in this report by SEM, IIF and AFM showed that immobilization with oriented antibodies are most favorable in capturing *S. aureus* and provide better sensor performance.

## Conclusion

We compared two antibody immobilization methods for convenient and effective application in a ME sensor: direct antibody thiol-conjugated random immobilization and protein G thiol-conjugated antibody-mediated methods for oriented immobilizations. *Staphylococcus aureus* was used as a model target antigen and anti-*S. aureus* IgG as a target antibody. Bacterial concentration ratios for antigen binding were considered for analysis of frequency. The PrGAb oriented immobilization presented a larger frequency shift compared to the CysAb random immobilization. AFM topographical analyses of the two immobilization methods supports these observations. We confirmed that protein G, which favors an orientation of the target antibody during immobilization, showed a significantly higher antigen binding efficiency than that of the direct thiol-conjugated strategy. Notably, an oriented immobilization strategy showed the best target antigen binding efficiency based on same target antibody amount. Thus, the oriented antibody immobilization methods using materials such as protein G could be useful in different antibody-antigen interaction problems.

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## 4. DISCUSSÃO GERAL

O presente estudo visa um melhor entendimento relacionado às técnicas de modificação de superfícies de fitas magneto-elásticas e o desenvolvimento de um imunossensor para detecção de *S. aureus*. Assim, este trabalho está dividido em capítulos, os quais tratam de questões relacionadas à sensibilidade, estabilidade e desempenho de sensores magneto-elásticos.

Diferentes técnicas estão disponíveis na literatura para a funcionalização da superfície de sensoriamento (Escamilla-Gomez *et al.* 2008, Makaraviciute and Ramanaviciene 2013). Foi elaborada inicialmente uma revisão da literatura que está apresentada no **Capitulo I**, com objetivo de melhor elucidar mecanismos que levem ao aprimoramento de propriedades físicas e químicas na formação da interface de reconhecimento. A partir desta revisão foi possível desenvolver estratégias experimentais para montagem do biossensor.

No **Capitulo II** foi avaliado a estabilidade e citotoxicidade de células eucarióticas da liga magneto-elástica. A detecção de analitos é obtida em meios líquidos, geralmente água ou solução salina, e sua estabilidade no meio é importante para utilização em monitoramento de contaminantes de amostras. Os transdutores magneto-elásticos possuem características físicas que proporcionam grandes vantagens no sensoriamento remoto (Grimes *et al.* 2011), estas propriedades impulsionam estudos para aplicações em uma diversidade de áreas. A citotoxicidade desta liga foi inicialmente demostrada por Holmes *et al*. (2014), e é uma característica que limita sua utilização em amostras que exijam segurança biológica. Estes sensores podem ser utilizados em contato com a amostra uma vez que não é necessária sua conexão com fios, desta forma a biocompatibilidade garante a segurança quanto possíveis interferências na amostra.

A eficiência de coberturas de ouro pelo método de *sputtering* na estabilização da degradação da tira e diminuição dos efeitos citotóxicos quando em contato com amostra biológica foram testadas (**Capítulo II**). Os resultados do **Capítulo II** referentes as análises de ICP-OES revelaram que ligas ME, quando expostas a meios líquidos, liberam íons metálicos, os quais, promovem efeitos citotóxicos. Além disso, foi observado que o contato direto das tiras com células induz mecanismos de morte celular e diminui significativamente a adesão celular na superfície desta liga. Em contrapartida, ligas com recobrimento bilateral de ouro não mostraram comprometimento na adesão celular nem tampouco apresentaram citotoxicidade no período de 7 dias. Estes resultados sugerem que a cobertura de ouro sobre o sensor foi eficiente para estabilização da fita quando em contato com a amostra biológica, contribuindo também para um melhor equilíbrio nas medidas de frequência aferidas pelo transdutor. Assim, fatores que possam levar a degradação da superfície do sensor, tendem a promover instabilidade nas medidas de frequência, o que pode ocasionar resultados falsos negativos ou positivos. A cobertura bilateral com ouro pelo método de *sputtering* apresentou melhoras satisfatórias quanto a estabilidade do dispositivo, com menor índice de degradação e liberação de íons.

Com propriedades mais estáveis alcançadas buscaram-se alternativas para captura de *S. aureus* sobre a superfície do imunossensor. A escolha de um método de imobilização eficiente, que promova maior especificidade, é fundamental para conseguir resultados satisfatórios de diagnóstico. O **Capítulo III** desta dissertação reporta o uso de moléculas de anticorpos na captura direta de *S. aureus.* Este capítulo foca majoritariamente nos efeitos de diferentes estratégias de imobilização quanto ao desempenho analítico do imunossensor. Anticorpos são moléculas de grande interesse no desenvolvimento de técnicas diagnósticas devido à sua elevada especificidade e

afinidade por um grupo de moléculas presentes em amostras biológicas. A aplicação de anticorpos é estudada em diferentes superfícies e abrange aplicações distintas em diferentes imunoensaios (S.K. Vashist, 2014; S. Johnson, 2011; A. Mahara, 2014). Algumas limitações na utilização de anticorpos estão associadas com a perda de atividade biológica. Uma das principais razões para esta característica é atribuída a fixação, e consequentemente, orientação aleatória das macromoléculas sobre as superfícies de suporte assimétricos (M. Fuentes, 2005).

É importante ressaltar que, quando imobilizados adequadamente anticorpos exibem melhor desempenho de ligação ao antígeno (Trilling AK 2013; Cecchet F). Diferentes métodos de imobilização podem resultar em orientações aleatórias ou específicas de anticorpos e são dependentes da capacidade de auto-organização de imunoglobulinas. A orientação do anticorpo de forma específica, não é facilmente conseguida, uma vez que os anticorpos possuem várias cópias de grupos reativos que podem reagir com os grupamentos químicos da superfície. Estratégias de imobilização que orientem a molécula de forma a proteger os sítios de ligação ao antígeno na região de reconhecimento são consideradas promissoras no desenvolvimento de imunossensores.

Desta forma, no **Capítulo III** deste documento, foi mostrado a importância de delinear estratégias que permitam a captura molecular por locais específicos no anticorpo, como o caso da Proteína A (SpA), ou Proteína G (SPG), que são alternativas interessantes para promover ligação a região Fc da imunoglobulinas (Barton *et al.* 2009). A sensibilidade do imunossensor em detectar *S. aureus* sobre a superfície do dispositivo foi investigada, utilizando dois métodos de imobilização: anticorpos ligados diretamente a molécula de cistamina (CysAb) e captura dos anticorpos pela proteína G (SpGAb) na superfície modificada.

Os resultados mostraram que o biossensor com a imobilização orientada PrGAb apresentou uma diminuição da frequência de 545 Hz quando exposto a uma concentração de $10^8$ UFC/mL. Na mesma concentração, a imobilização randômica CysAb mostrou uma variação de frequência de 272 Hz. Estes resultados indicam que a imobilização covalente orientada (PrGAb) aumentou consideravelmente a sensibilidade do sensor. Outra observação é que os sensores em alta concentração de bactérias apresentaram instabilidade, uma possível explicação é o aumento de viscosidade do meio. Grimes et. al; (2011) reporta que sensores ME podem ter amortecimento relacionados ao aumento da viscosidade.

Ao observar os resultados de controle e amostras descritos no Capítulo III, pode-se notar que as medidas de ressonância tornam-se mais estáveis em concentrações baixas, a partir de $10^6$ UFC/mL, aumentando a estabilidade das medidas de frequência do sensor. A imobilização com PrGAb apresentou resultados promissores por conseguir detectar bactéria em concentrações de $10^4$ UFC/mL, o que permite uma maior confiabilidade no sistema, uma vez que foi possível obter valores superiores a amostra controle. Por outro lado, a imobilização CysAb mostrou um limite de detecção de $10^6$ UFC/mL, o que inviabiliza a utilização desta técnica, uma vez que observamos interferências nas leituras em concentrações superiores a $10^6$ UFC/mL.

Imagens de microscopia eletrônica de varredura mostraram um incremento na captura de *S. aureus* utilizando a imobilização PrGAb. Somado a isso, foi utilizado um modo de contato intermitente com AFM que evidenciou alterações na imagem topográfica das diferentes modificações na superfície em estudo. Também foi possível, pela análise da rugosidade, estimar a orientação do anticorpo na superfície, o que corrobora com o aumento do desempenho do sensor na detecção do patógeno.

Os sensores à base de anticorpo podem fornecer uma análise robusta, sensível e rápida. Diferentes alternativas na utilização destas moléculas estão disponíveis, tais como os anticorpos recombinantes e os nano-anticorpos, que proporcionam alternativas interessantes no desenvolvimento de imunossensores.

A utilização de películas finas, especialmente monocamadas auto-montadas e camadas compostas de ouro-tiol, fornecem um método simples para a funcionalização química de superfícies magneto-elástica usando nanogramas de material. Diferentes técnicas podem obter películas, altamente ordenadas ou amorfas, gerando um elevado nível de controle do ambiente.

Os sensores magneto-elásticos, por fim, proporcionam uma possibilidade promissora para detecção de patógeno e toxinas, gerando resultados muito mais rápidos e sensíveis do que as abordagens convencionais. Os desafios futuros para construção destes sistemas devem envolver uma melhor compreensão do efeito de diferentes moléculas sobre os sensores na resposta físicas do mesmo.

Arranjos alternativos, os quais consistem na combinação de vários sensores com capacidade multiparamétrica são perspectivas promissoras. Monitorar em tempo real a presença de diferentes patógenos e toxinas podem proporcionar ferramentas que gerariam considerável impacto na área médica e de alimentos. Estas são perspectivas que vem sendo desenvolvidas por este grupo, e resultados promissores são esperados em um futuro próximo.

## 5. CONCLUSÃO

Este trabalho apresentou características relevantes de fitas magneto-elásticas para aplicação como biossensor. Sensores magneto-elásticos são ferramentas analíticas promissoras e muito pesquisadas para aplicação nas mais diversas áreas. Isso deriva de suas características vantajosas face aos dispositivos de sensoriamento disponíveis ou seja: respostas rápidas (minutos); possibilidade de miniaturização e aplicação sem conexão de fios; menor custo operacional de projeto e para o consumidor final, entre outras vantagens. A utilização destes dispositivos na área biológica é relativamente nova, dessa forma estudos que possibilitem o entendimento e melhorem as características físicas e biológicas são necessários. Os resultados obtidos neste estudo permitiram concluir que:

- ✓ A cobertura de ouro sobre o sensor foi eficiente para estabilização da fita quando em contato com a amostra biológica. A cobertura bilateral com ouro pelo método de *sputtering* apresentou melhoras satisfatórias quanto a estabilidade do dispositivo, com menor índice de degradação e liberação de íons. Ligas com recobrimento bilateral de ouro não mostraram comprometimento na adesão celular nem tampouco apresentaram citotoxicidade no período de 7 dias.

- ✓ A imobilização orientada com PrGAb apresentou resultados promissores por conseguir detectar bactéria em concentrações de até $10^4$ UFC/mL, o que permite uma maior confiabilidade no sistema, uma vez que foi possível obter valores superiores a amostra controle. Por outro lado, a imobilização randômica com CysAb mostrou um limite de detecção de $10^6$ UFC/mL, o que inviabiliza a utilização desta técnica, uma vez que observamos interferências nas leituras em concentrações superiores a $10^6$ UFC/mL.

- ✓ A análise topográfica da superfície por contato intermitente com AFM evidenciou alterações na imagem das diferentes modificações na superfície em estudo.

Também foi possível, pela análise da rugosidade, estimar a orientação do anticorpo na superfície, o que corrobora com o aumento do desempenho do sensor na detecção do patógeno.

A pesquisa em biossensores é multidisciplinar e, portanto, para o desenvolvimento de uma determinada aplicação, profissionais da área de Engenharia Elétrica, Química, Física e Biologia são necessárias. A utilização de transdutores magneto-elásticos como biossensores é um campo de pesquisa relativamente novo e com grandes prospecções, como a utilização para monitoramento de analitos convencionais que são questões de saúde pública, como é o caso da detecção rápida de bactérias e toxinas. A busca de métodos que sejam de baixo custo, descartáveis, portáteis e com aplicação on-line é de interesse de muitos pesquisadores em vários países. O grupo de pesquisa vem trabalhando para o melhoramento destes dispositivos visando sua aplicação em testes rápidos e de alta confiabilidade na detecção de patógenos. Também já estão sendo desenvolvidas possibilidades de monitoramento de múltiplos analitos.

## 6. PERSPECTIVAS

A partir dos resultados obtidos neste trabalho, propõem-se as seguintes perspectivas para continuidade deste estudo:

- Testes com sensores de dimensões reduzidas, os quais deverão apresentar melhor sensibilidade.
- Desenvolvimento de arranjos alternativos para utilização na identificação de diferentes patógenos.
- Desenvolvimento de sensor para detecção de diferentes parâmetros químicos e biológicos.
- Elaboração de sensores combinados para análises multiparamétricas.

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