FEDERAL UNIVERSITY OF SANTA CATARINA

TECHNOLOGY CENTER

GRADUATE PROGRAM IN CHEMICAL ENGINEERING

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# DEVELOPMENT AND APPLICATION OF ANALYTICAL METHODOLOGY WITH THE COMBINED USE OF GOLD NANOPARTICLES AND APTAMERS FOR THE COLORIMETRIC DETECTION OF *PSEUDOMONAS AERUGINOSA*

Florianópolis

2022

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Doctorate thesis for the degree of Doctor in Chemical Engineering presented to the Graduate Program in Chemical Engineering at the Federal University of Santa Catarina.

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Florianópolis

2022

Ficha de identificação da obra elaborada pelo autor,
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Schmitz, Fernanda Raquel Wust
DEVELOPMENT AND APPLICATION OF ANALYTICAL METHODOLOGY WITH THE COMBINED USE OF GOLD NANOPARTICLES AND APTAMERS FOR THE COLORIMETRIC DETECTION OF PSEUDOMONAS AERUGINOSA / Fernanda Raquel Wust Schmitz ; orientador, Dachamir Hotza, coorientador, Débora de Oliveira, 2022.
73 p.

Tese (doutorado) - Universidade Federal de Santa Catarina, , Programa de Pós-Graduação em , Florianópolis, 2022.

Inclui referências.

1. . 2. Aptâmeros. 3. Nanoparticulas de ouro. 4. Pseudomonas aeruginosa. 5. Biossensor. I. Hotza, Dachamir . II. de Oliveira, Débora . III. Valério, Alexsandra IV. Universidade Federal de Santa Catarina. Programa de Pós Graduação em . V. Titulo.

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The present work at the doctoral level was evaluated and approved by an examining board composed of the following members:

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Florianópolis, March 2022

This work is dedicated to my parents Vidal Lauro Schmitz and Marieta Henning Wust Schmitz, and my sister Carolina Wust Schmitz who always supported me.

## ACKNOWLEDGEMENTS

First, I would like to thank the Federal University of Santa Catarina for the opportunity awarded to collaborate in the development of research in the Graduate Program in Chemical Engineering. I would like to thank the professors Dachamir Hotza and Débora de Oliveira for their support, motivation, patience, knowledge and for believing and trusting me for this research, and for always pushing me to new limits. I also thank Alexsandra Valério, co-advisor of this work.

Thanks to the Laboratory of Mass Transfer and Numerical Simulation of Chemical Systems (LABMASSA/LABSIN), and Biological Engineering Laboratory (LiEB) for all the infrastructure available to carry out this research.

Thanks to TNS Nano and FAPESC for the financial support.

I thank my family: Marieta, Vidal, and Carolina, for all the support, help, motivation, and for believing in my academic journey, without you I would not have gotten this far. Thanks to Ricardo for also believing in my steps and for the affection and patience on this journey.

I am immensely grateful to postdoctoral fellow Karina Cesca, for all the help, encouragement, dedication, and for passing on her knowledge, and for believing in my potential. Also, thank you for the moments of relaxation, coffees, laughter, and happy h. For sure the final walk was more peaceful.

Finally, I am really grateful to Ana Caroline Klemz, Sarah Mozzaquatro Pasini, Vanessa Kristine de Oliveira Schmidt, and Renato Matosinhos for the fun and helpful moments inside the laboratory also for the moments of relaxation, relief, and encouragement during the journey.

**“***Knowledge is not what you know,*
*but what do you do with what you know”.*

*Aldous Huxley*

## ABSTRACT

*Pseudomonas aeruginosa* is a pathogen responsible for causing disease worldwide. It is a Gram-negative bacterium frequently found in terrestrial, aquatic, and hospital environments. Due to the great contamination by this bacterium, the objective of this work was to study the pathogen colorimetric detection through the use of aptamers, which are single-stranded oligonucleotides of high affinity and specificity, capable of binding to the target, conjugated to gold nanoparticles. Due to their specificity and affinity, the gold nanoparticles are released and they aggregate, changing the dispersion color from red to blue, indicating the presence of the contaminant. In this work, the aptamer for *P. aeruginosa* detection was conjugated to gold nanoparticles and incubated with the target bacteria after assays to verify the ideal concentration of NaCl and aptamer. The aptamer specificity was verified with the bacteria *Staphylococcus aureus, Escherichia coli, Salmonella Typhimurium,* and *Enterobacteriaceae bacterium.* It was observed that aptamers conjugated to the gold nanoparticles changed between red and blue in the presence of NaCl at concentrations above 150 mM, destabilizing the gold nanoparticles, but in the presence of the adsorbed aptamers, the dispersion remained stable. In this study, it was observed that the technique developed was able to detect *P. aeruginosa* through the color change from red to blue at concentrations from $10^8$ CFU·mL$^{-1}$ to $10^5$ CFU·mL$^{-1}$ after 5 h of incubation. Thus, a biosensor was developed to recognize the target rapidly when compared to conventional methods. A visible identification was possible through the change in color of the dispersion, at concentrations of $10^8$ to $10^5$ CFU/mL, which may help the detection of *P. aeruginosa* in hospital sectors.



**Keywords:** *P. aeruginosa.* Biosensor. Aptamers. Gold nanoparticles.

## RESUMO

*Pseudomonas aeruginosa* é um patógeno responsável por causar doenças em todo o mundo. É uma bactéria Gram-negativa frequentemente encontrada em ambientes terrestres, aquáticos e hospitalar. Devido às grandes contaminações por essa bactéria, objetivou-se estudar a detecção colorimétrica deste patógeno através da utilização de aptâmeros, que são oligonucleotídeos de fita simples de alta afinidade e especificidade, capazes de ligar-se ao alvo, conjugados a nanopartículas de ouro. Devido à especificidade e afinidade destes, as nanopartículas de ouro são liberadas e as mesmas agregam-se, fazendo com que a solução passe da cor vermelha para roxo, indicando a presença do contaminante. Neste trabalho, os aptâmeros para detecção de *P. aeruginosa* foram conjugados a nanopartículas de ouro e incubados com a bactéria alvo após ensaios para verificar a concentração de NaCl e aptâmero ideal. Já a especificidade do aptâmero foi verificada com as bactérias *Staphylococcus aureus, Escherichia coli, Salmonella Typhimurium, Enterobacteriaceae bacterium*. Foi observado que os aptâmeros conjugados às nanopartículas de ouro alteraram entre vermelho e roxo na presença de NaCl em concentrações acima que 150 mM, desestabilizando as nanopartículas de ouro, mas na presença dos aptâmeros adsorvidos, a solução permaneceu estável. Neste estudo, foi observado que a técnica desenvolvida foi capaz de detectar a presença de *P. aeruginosa* através da mudança de cor de vermelho para roxo nas concentrações de $10^8$ UFC·mL$^{-1}$ a $10^5$ UFC·mL$^{-1}$ após 5 horas de incubação. Assim, foi desenvolvido um biossensor capaz de ligar-se ao alvo em estudo de forma rápida, comparado a métodos convencionais. A identificação visível foi possível devido à mudança de coloração da solução, em concentrações de $10^8$ a $10^5$ UFC·mL$^{-1}$, podendo auxiliar a detecção de *P. aeruginosa* em setores hospitalares.



**Palavras-chave:** *P. aeruginosa.* Biossensor. Aptâmeros. Nanopartículas de ouro.

## RESUMO EXPANDIDO

# Desenvolvimento e Aplicação de Metodologia Analítica com uso Combinado de Nanopartículas de Ouro e Aptâmeros para Detecção Colorimétrica de *Pseudomonas aeruginosa*

## Introdução

Bactérias patogênicas podem ser encontradas em diferentes ambientes e causar infecções fatais. Dentre elas está a *Pseudomonas aeruginosa*, muito encontrada em ambientes hospitalares e até mesmo em água e solo (KRITHIGA *et al.*, 2016; SANTOS *et al.*, 2012). A *P. aeruginosa* é uma bactéria Gram-negativa e devido a sua fisiologia e a seu metabolismo, possui capacidade de colonização em ambientes úmidos e com pouca disponibilidade de nutrientes como hospitalares, terrestres e aquáticos, sendo responsável por doenças infecciosas mesmo em baixas concentrações (SOUNDY; DAY, 2017). Clinicamente, essa bactéria é capaz de criar biofilmes em superfícies de equipamentos médicos, cateteres e outros dispositivos implantados no corpo humano (KRITHIGA *et al.*, 2016).

Diversos métodos são capazes de identificar a *P. aeruginosa*, como os métodos convencionais de cultura bacteriana, método de imunofluorescência, técnica de PCR (Polymerase Chain Reaction) (DAS *et al.*, 2019). No entanto, essas técnicas muitas vezes apresentam resultado demorado, não resultam em análises quantitativas ou possuem custo elevado. Tendo em vista as dificuldades na detecção rápida de focos contaminantes, muitos causados por bactérias, torna-se importante a pesquisa de alternativas mais rápidas e eficientes (SCHMITZ *et al.*, 2020). Dentre as formas analíticas de detecção de microrganismos, estão inseridos os aptâmeros, que são fitas simples de DNA ou RNA estáveis capazes de detectar moléculas que têm afinidade e especificidade com os mesmos e podem ser utilizados para detecção e captura de microrganismos. Os aptâmeros podem ser modificados, como a conjugação com metais, biotina, radioativos, dentre outros. Além disso, são estáveis à temperatura ambiente (SCHMITZ *et al.*, 2020).

A conjugação destes aptâmeros com nanopartículas de ouro torna possível a detecção por método colorimétrico, sendo a mudança de cor da solução contendo estas nanopartículas visível a olho nu (SCHMITZ *et al.*, 2020). A utilização de aptâmeros conjugados com nanopartículas de ouro possibilita a identificação da bactéria deste estudo em poucas horas através de alteração de cor, onde não são necessários outros equipamentos para confirmar a contaminação de amostras de alimentos.

Embora sejam encontrados estudos em literatura sobre a utilização de nanopartículas e aptâmeros para identificação de *P. aeruginosa*, ainda persiste o desafio de desenvolver um método colorimétrico que utilize aptâmeros não marcados e nanopartículas de ouro sem modificação, que são mais estáveis e de menor custo, visto a necessidade e dada a importância da rápida detecção de diferentes concentrações desse patógeno em amostras hospitalares.

**Objetivos**

O objetivo principal deste trabalho consistiu em desenvolver um método de detecção colorimétrica a olho nu, utilizando nanopartículas de ouro não modificadas e aptâmeros não marcados para identificação de *P. aeruginosa*.

**Metodologia**

A primeira parte do trabalho englobou a síntese de nanopartículas de ouro (AuNPs) por redução e estabilização por citrato de sódio. As mesmas foram caracterizadas por espectrofotometria em UV-Vis e por microscopia eletrônica de transmissão (MET).

Na segunda parte do estudo, verificaram-se as concentrações ideais de aptâmero, NaCl, além do tempo de incubação para detecção colorimétrica da bactéria alvo. Também foi realizado o controle negativo para confirmar a especificidade e afinidade do aptâmero, utilizando-se as bactérias *Staphylococcus aureus, Escherichia coli, Salmonella Typhimurium* e *Enterobacteriaceae bacterium.* Primeiramente, incubou-se 1 mL de nanopartícula de ouro com aptâmero 5 µM por 18 h a 37 °C. Após, 120 µL da solução foi inserida em microplaca de 96 poços e fez-se a adição de 20 µL de NaCl 200 mM em cada poço e incubou-se por 4 h a 25 °C. Posteriormente, 20 µL de diferentes concentrações de *P. aeruginosa* ($10^8$ a $10^0$ UFC·mL$^{-1}$) e 20 µL de BHI (branco) foram misturadas à solução e permaneceram a 25 °C por 1 h. Por fim, incubaram-se 20 µL de NaCl 200 mM por 12 h. Para o controle negativo, repetiu-se a mesma metodologia, porém com adição de *S. aureus, E. coli, S. Typhimurium* e *Enterobacteriaceae bacterium.* A cada etapa foi realizada análise em UV-Vis a 520 e 630 nm e varredura de 400 a 700 nm.

Por fim, foi estudada a otimização da concentração de aptâmeros (0, 0,25, 0,50, 075, 1, 5 e 10 µM) conjugados às nanopartículas de ouro seguindo o método anterior utilizando apenas *P. aeruginosa* na concentração de $10^8$ UFC·mL$^{-1}$. O tempo de incubação de NaCl após a conjugação também foi estudado. Os passos da descrição anteriores foram seguidos,

alterando-se o tempo em que o sal foi incubado (1, 2 e 4 h). As mudanças ocorridas foram analisadas em UV-Vis no comprimento de onda de 400 a 700 nm.

**Resultados e Discussão**

Na primeira etapa do estudo, foram obtidas nanopartículas de ouro esféricas com diâmetro médio de 25 nm e com pico de absorbância em 520 nm, indicando que estão dispersas. A concentração de AuNPs utilizada para as etapas posteriores foi de $8{,}29\times10^{14}$ mol/L.

Em seguida, a estabilidade das nanopartículas de ouro foi avaliada frente a adição de NaCl 200 mM, já que este é capaz de neutralizar as forças repulsivas entre citrato-AuNP. Nesse ensaio, observou-se que concentrações menores que 150 mM são capazes de manter as nanopartículas de ouro estáveis, enquanto que, a partir dessa concentração, as AuNPs agregam. Por outro lado, quando as nanopartículas são conjugadas aos aptâmeros, a concentração de NaCl 200 mM não é capaz de desestabilizar a solução, permanecendo estável. Posteriormente, diferentes concentrações de *P. aeruginosa* foram incubadas à solução AuNP-aptâmero-NaCl. Após a segunda adição de sal, confirmou-se a mudança de cor da solução de vermelho para roxo para as concentrações de $10^8$ a $10^5$ UFC·mL$^{-1}$ a partir de 5 h. Já no controle negativo, a solução permaneceu com coloração avermelhada após a adição de *S. aureus* e BHI (branco). Porém, para as bactérias *E. coli, S. Typhimurium* e *Enterobacteriaceae bacterium*, a solução alterou de vermelho para cinza, indicando que as AuNPs aglomeraram e que o aptâmero não é específico para *P. aeruginosa*. Entretanto, é possível utilizá-lo já que a mudança de cor para *P. aeruginosa* ocorre de vermelho para roxo e para as demais de vermelho para cinza.

Por fim, os ensaios de otimização do tempo de incubação de NaCl mostraram que, depois da conjugação, o tempo mínimo de incubação de NaCl deve ser de 2 h. Sendo assim, pode-se diminuir o tempo do método, obtendo-se o mesmo resultado de detecção. Já para a otimização de concentrações de aptâmero estudadas, na análise em UV-Vis, foi verificada mudança no comprimento de onda, porém visualmente não houve alteração. Assim, a melhor concentração de aptâmero foi de 5 μM, pois apesar da alta concentração de aptâmero gerar estabilidade das AuNPs, os mesmos podem reduzir a sensibilidade de detecção e causar falhas na detecção.

**Considerações Finais**

A revisão de literatura mostrou que o uso de nanopartículas de ouro e aptâmeros vem sendo muito estudado nos últimos anos e diversas metodologias são empregadas para o reconhecimento de bactérias, vírus, entre outros microrganismos patogênicos.

Através dos estudos, foi confirmado que as AuNPs sintetizadas pelo método de redução por citrato são estáveis. As mesmas podem ser utilizadas como agente identificador e o aptâmero como agente de ligação com o alvo. Dessa forma, foi possível detectar *P. aeruginosa* por diferença colorimétrica (vermelho para roxo) em diferentes concentrações, sendo $10^5$ UFC·mL$^{-1}$ o limite de detecção a olho nu e $10^4$ UFC·mL$^{-1}$ quando analisado em espectrofotômetro UV-Vis. Entretanto, quando as bactérias de controle negativo foram incubadas, o sistema mudou de cor de vermelho para cinza frente a *S. Typhimurium, E. coli* e *Enterobacteriaceae bacterium.*

Ainda que a metodologia não se mostrou específica, pode ser utilizada em ambientes hospitalares, pois infecções por *P. aeruginosa* são mais comuns que as demais bactérias reconhecidas. Além disso, a mudança de cor para o patógeno em estudo foi de vermelho para roxo e para as demais bactérias de vermelho para cinza, sendo possível diferenciar a detecção.

**Palavras-chave:** Aptâmeros de DNA. Detecção colorimétrica. *P. aeruginosa*. Nanopartículas de ouro.

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**SUPPLEMENTARY MATERIAL**

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## LIST OF SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| Aw | Water activity |
| AuNPs | Gold nanoparticles |
| Cell-SELEX | *Systematic Evolution of Ligands by Exponential Enrichment* for whole cells |
| DLS | Dynamic Light Scattering |
| DNA | Deoxyribonucleic acid |
| dsDNA | Double strand DNA |
| Dp | Particle diameter (nm) |
| *E. coli* | *Escherichia coli* |
| EDTA | Ethylenediamine tetraacetic acid |
| ELISA | Enzyme-linked immunosorbent assay |
| FISH | Fluorescence in situ hybridization |
| Kd | Dissociation constant |
| NPs | Nanoparticles |
| *P. aeruginosa* | *Pseudomonas aeruginosa* |
| PCR | Polymerase chain reaction |
| qPCR | Polymerase chain reaction |
| RNA | Ribonucleic acid |
| *S. aureus* | *Staphylococcus aureus* |
| *S. enterica* | *Salmonella enterica* |
| *S. Typhimurium* | *Salmonella Typhimurium* |
| SDS | Sodium dodecyl sulfate |
| SELEX | *Systematic Evolution of Ligands by Exponential Enrichment* |
| ssDNA | Single strand DNA |
| ZP | Zeta potential |

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# 1 INTRODUCTION

Currently, there is a growing demand to develop rapid detection methods for different molecules, among them are the pathogenic bacteria that are responsible for several infections worldwide even in low concentrations (SCHMITZ *et al.*, 2020). Bacteria can develop in plants, water, soil, hospital environment, in the body of humans and animals, and the best way to prevent this growth so as not to cause further damage is prevention and rapid detection even at low concentrations. (VERMA *et al.*, 2015) and conventional methods often take time to identify the bacteria or are more expensive (LAVU *et al.*, 2016). An example is *P. aeruginosa*, a Gram-negative bacterium, frequently present in hospital infections (DAS *et al.*, 2019), which attracts much research and is the target of this study.

Recent studies for rapid identification use aptamers, which are nucleic acids applied for molecular recognition and can be DNA or RNA. (QIAN *et al.*, 2022). These molecules were discovered by two separate work teams in the 1990s: an RNA aptamer capable of binding to organic dyes (ELLINGTON; SZOSTAK, 1990) and another one that binds to T4 DNA polymerase (TUERK; GOLD, 1990) through a technique called SELEX (Systematic Evolution of Ligands by Exponential Enrichment). After, DNA aptamers were discovered (BOCK *et al.*, 1992; ELLINGTON; SZOSTAK, 1992) and since then the technique has been widely applied to all types of targets (bacteria, viruses, metals, drugs, proteins, organic and inorganic compounds) (DAS *et al.*, 2019).

However, detection methodologies do not use only aptamers, but their conjugation with nanomaterials including silver, gold, carbon, and silica nanoparticles due to their biocompatibility, stability, and controllable physical and chemical properties (SCHMITZ *et al.*, 2020). Gold nanoparticles (AuNPs) are widely used due to their optical properties, which allow the target detection by colorimetric difference seen with the naked eye, as they change color from red to blue/purple when dispersed and agglomerated, respectively (SCHMITZ *et al.*, 2020; XU *et al.*, 2018). Basically, this method consists in conjugating the aptamer with AuNPs by adsorption or using some ligand and after incubation with the target the aptamer binds to it, leaving the nanoparticles free, and with the salt addition the AuNPs aggregate changing color (KIM *et al.*, 2021; XIE *et al.*, 2021).

Although many studies mentioned the use of AuNPs and aptamers to identify *P. aeruginosa* (the target bacteria of this study), the challenge of developing colorimetric detection is important, since simpler and more efficient techniques can bring benefits and agility.

## 1.1 HYPOTHESES

In this section, the hypotheses within this work are presented:

- Are the synthesized gold nanoparticles stable for colorimetric detection?
- Are the selected aptamers specific to *P. aeruginosa*?
- Is the conjugation of aptamers to gold nanoparticles capable of revealing the presence of *P. aeruginosa* by color change?
- Are the concentrations of NaCl and aptamer optimal for the method?
- Does detection occur at different concentrations of *P. aeruginosa* ($10^0$ a $10^8$ $CFU \cdot mL^{-1}$)?

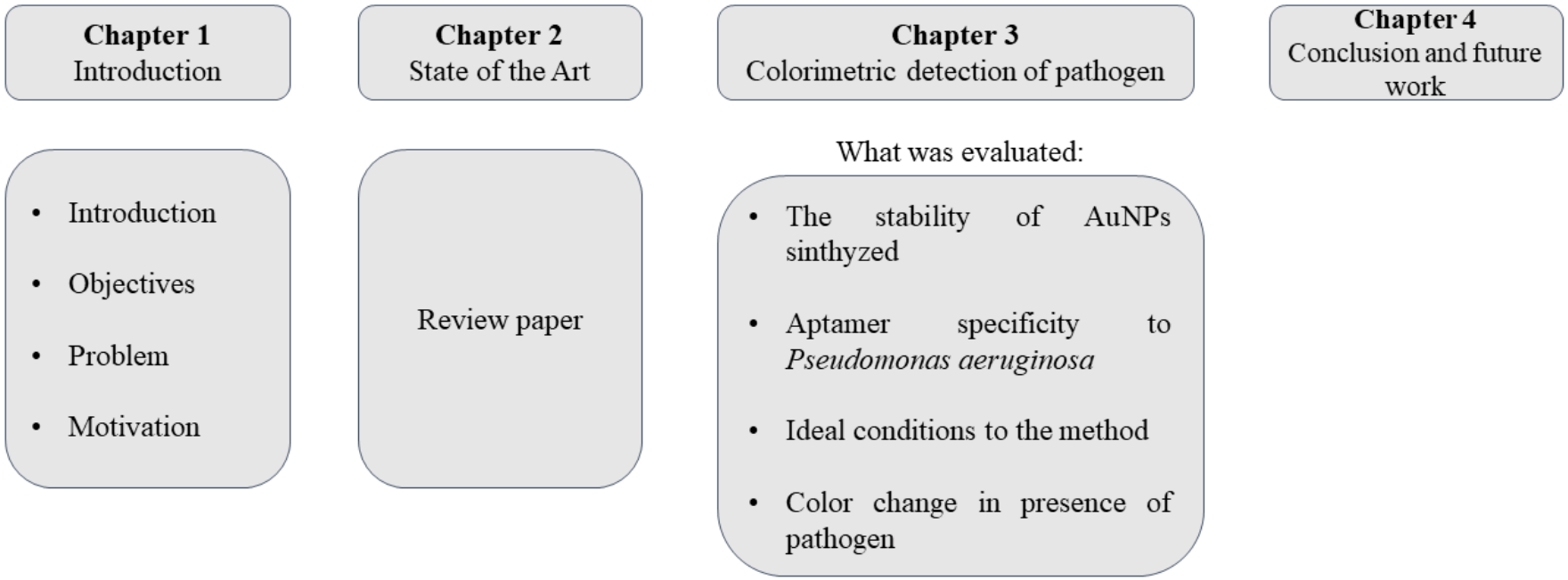
## 1.2 OBJECTIVES

The general goal of this work is to prepare a colorimetric method to detect *P. aeruginosa* using unmodified gold nanoparticles conjugated with aptamer without markers.

The specific objectives selected to fulfill the main objective are:

- Verify that synthesized AuNPs are stable for colorimetric detection;
- Obtain the best concentration of salt and aptamers for the detection methodology;
- Investigate the affinity and specificity of the unlabeled aptamers for *P. aeruginosa*;
- Identify *P. aeruginosa* at different concentrations through the conjugation of aptamers to gold nanoparticles.

## 1.3 CONCEPTUAL DIAGRAM



## 1.4 STRUCTURE OF THE THESIS

This thesis is divided into four chapters, as follows:

In Chapter 1, Introduction, a general approach is made to contextualize the objective of the work.

Chapter 2, State of the Art, helps the reader to visualize where this thesis fits and is composed of a published literature review paper on rapid detection methods using aptamers. In this review, obtaining aptamers and their applications in colorimetric methodologies are discussed. Subsequently, the reader will find information about materials to be conjugated to aptamers and molecules that have already been identified through these new techniques.

Chapter 3, Colorimetric Detection of Pathogens, comprises the experimental results obtained during this work, which consisted of synthesizing spherical gold nanoparticles of 20 nm on average, conjugating them with aptamers, optimizing the concentrations and time of the steps of the colorimetric detection methodology, verifying the detection at the naked eye of *P. aeruginosa,* and confirming the specificity of the method against negative control bacteria.

In Chapter 4, Conclusions and Future Work, a summary of the main conclusions of this work is presented with the respective contributions. In addition, future work is suggested for studies related to this topic with remaining perspectives and gaps on the subject.

# 2 STATE OF THE ART

Chapter 2 aims to provide the reader with an overview of rapid detection methods of different molecules such as bacteria, viruses, organic compounds, metals, and drugs. This chapter corresponds to a published review article, which describes the method of obtaining aptamers, which are used in different detection methodologies mentioned, their advantages, and applications.[1]

Many bacteria are responsible for infections in humans and plants, being found in vegetables, water, and medical devices. Most bacterial detection methods are time-consuming and take days to give the result. Aptamers are a promising alternative for a quick and reliable measurement technique to detect bacteria present in food products. Selected aptamers are DNA or RNA oligonucleotides that can bind with bacteria or other molecules with affinity and specificity for the target cells by the SELEX or cell-SELEX technique. This method is based on some rounds to remove the non-ligand oligonucleotides, leaving the aptamers specific to bind to the selected bacteria. Compared with conventional methodologies, the detection approach using aptamers is a rapid, low-cost form of analysis.

## 2.1 INTRODUCTION

The growth of epidemiologic diseases has been increasing due to food production and consumption trends, such as production processes, globalization of consumer goods, and high demand for raw or undercooked foods. Gram-positive and Gram-negative bacteria are responsible for infectious diseases even at low concentrations (SOUNDY; DAY, 2017). Many methods may be applied to identify these bacteria, such as conventional bacterial culture, immunofluorescence, and polymerase chain reaction (PCR). However, these techniques are often time-consuming, without quantitative response and often they are high-cost analyses (LAVU *et al.*, 2016). In the last decades, rapid and efficient methods of detection are being developed to decrease food contamination and improve human health (AMAYA-GONZÁLEZ *et al.*, 2013).

Alternative methods to the detection of microorganisms are the use of single strands of DNA or RNA, called aptamers, which are capable to bind to non-nucleic acid molecules. The

[1] Published in 2020 in Applied Microbiology and Biotechnology (https://doi.org/10.1007/s00253-020-10747-0).

technique known as systematic evolution of ligands by exponential enrichment (SELEX) combines different steps such as incubation of oligonucleotides library, separation, amplification by polymerase chain reaction (PCR), and purification (LIU, XIAOFEI; ZHANG, 2015). This approach has been used since the 1990s when Tuerk and Gold (1990) discovered an RNA aptamer capable to bind to the T4 DNA polymerase, and Ellington and Szostak (1990) studied the binding of RNA aptamer to organic dyes. Over the years many publications were published on the use of aptamers in different ways, eventually conjugated with metallic, oxide, or polymeric nanoparticles and carbon nanotubes (LIU, XIAOFEI; ZHANG, 2015).

This review aims to address the techniques for obtaining aptamers, and their use, particularly for contaminants detection in food products. The structure and properties of aptamers, as well as nanomaterials commonly used to be conjugated with aptamers, are presented. Moreover, recent applications and market perspectives are also introduced.

## 2.2 APTAMER STRUCTURE AND PROPERTIES

Aptamers are single-stranded DNA or RNA molecules (oligonucleotides) with 50 to 100 nucleotides bases, 1 to 2 nm in size, and 7.5 to 32 kDa, with high affinity and specificity to bind to a target molecule (ELLINGTON; SZOSTAK, 1990; STOLTENBURG, R; REINEMANN; STREHLITZ, 2005). According to Radom *et al*. (2013), DNA aptamers are molecules more stable than RNA, however, no significant differences were found between their specificities and binding capacities.

Aptamers can assume stable three-dimensional configurations in the aqueous phase, such as lops, triplexes, pseudoknots, G-quadruplexes, and staples (BING *et al.*, 2017). Due to the ability to change their shape, they can bind to different targets such as amino acids, vitamins, nucleotides, proteins, pesticides, drugs, bacteria, and inorganic or organic compounds. Moreover, they can be used as enzyme inhibitors to recognize proteins and nucleic acids, as well as inhibitors of toxins and hormones, applied in the detection of molecules in complex mixtures, purification, and biosensors (LIU, XIAOFEI; ZHANG, 2015; PETERSON; JAHNKE; HEEMSTRA, 2015).

The affinity with the target molecule is associated with the dissociation constant that ranges from picomoles per liter to nanomoles per liter, being calculated through thermodynamic stability (TAKENAKA *et al.*, 2017). Besides, aptamers may be stored and transported at room temperature due to the stability in the environmental conditions (MISSAILIDIS; PERKINS,

2007). Also, modifications in the aptamers can make them more resistant, and stable, and improve the targeting ability, as shown by Ni *et al*. (2017). According to the authors, aptamers for the therapeutic area are susceptible to nuclease degradation and can be excreted by renal filtration even before they bind to the target. Thus, to increase the resistance to nuclease degradation, aptamers can be modified in the 3' end with inverted thymidine or conjugated with biotin or biotin-streptavidin. Another modification is at the 5' position, which consists of the addition of cholesterol to increase the aptamer stability in plasma (NI *et al.*, 2017).

In the same way, it is possible to combine aptamers with nanostructures, such as gold nanoparticles or carbon nanotubes (RADOM *et al.*, 2013). They can be labeled with compounds such as dye or biotin. This process is widely used as systematic evolution of ligands by exponential enrichment (SELEX) to control the binding properties (MISSAILIDIS; PERKINS, 2007; RADOM *et al.*, 2013).

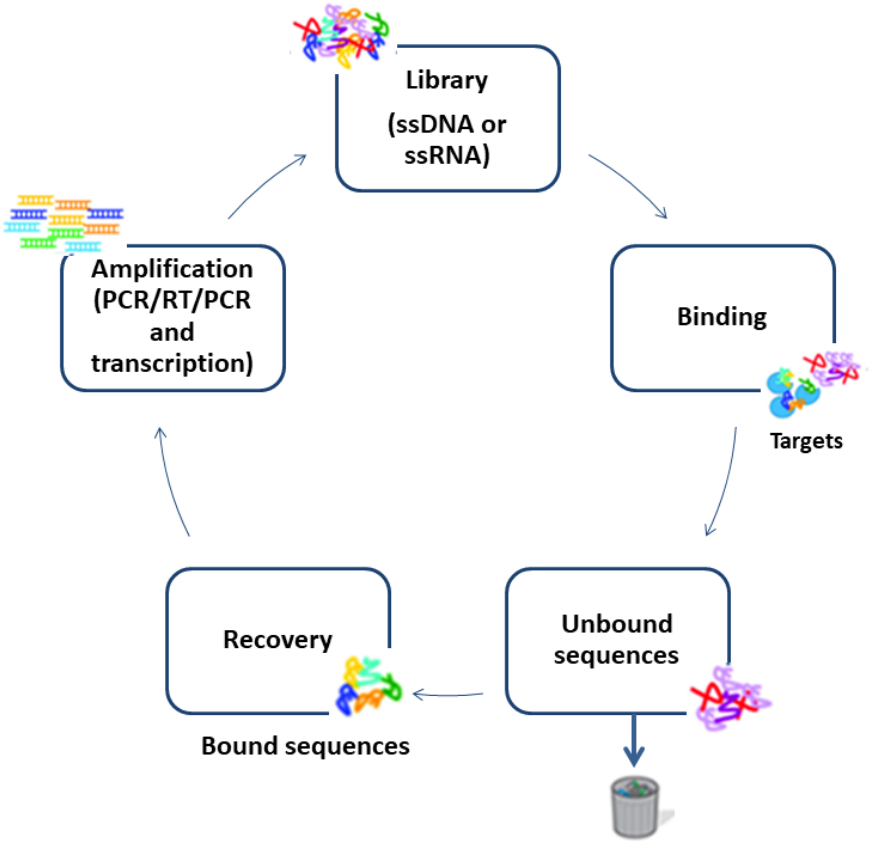
## 2.3 SYSTEMATIC EVOLUTION OF LIGANDS BY EXPONENTIAL ENRICHMENT

SELEX is a technique used to obtain aptamers through the *in vitro* selection of oligonucleotides over many rounds (RADOM *et al.*, 2013). In the 1990s, the first reports using the SELEX technique were published with RNA aptamers bounded to T4 DNA polymerase with high affinity (TUERK; GOLD, 1990). In addition, Ellington and Szostak (1990) studied *in vitro* selection of organic dyes-binding RNA, and Robertson and Joyce (1990) selected the tetrahymena ribozyme and through amplifications obtained an RNA sequence able to cleave a specific DNA sequence.

This technique allows the selection of specific oligonucleotides that can be chemically synthesized, without the need to be produced or selected in a living organism (STOLTENBURG, REGINA; REINEMANN; STREHLITZ, 2007). As shown in Figure 1, SELEX's first step is to obtain a library of random oligonucleotides, which can be single strands of DNA or RNA, with $10^{14}$ to $10^{15}$ different sequences. This library, also called a bank, has a fixed region and a random region of 20 to 80 nucleotides, which allows the molecules to form different structures as already mentioned, ready to bind the desired molecules (STOLTENBURG, R; REINEMANN; STREHLITZ, 2005). The fixed region, which is the library extremities, allows the amplification (STOLTENBURG, R; REINEMANN; STREHLITZ, 2005).

The binding step occurs by the incubation of the bank of ligands (aptamer candidates) and the target molecule in a buffer solution. After this, the bounded oligonucleotides are separated from non-bounded by physical separation and PCR amplification is carried out on the recovered bounded oligonucleotides, thus the first SELEX cycle started (RADOM *et al.*, 2013). Different techniques can be used in the separation process, such as centrifugation (RAMLAL *et al.*, 2018; SOUNDY; DAY, 2017), capillary electrophoresis (TANG, JIJUN *et al.*, 2006), ultrafiltration with nitrocellulose filter (JOSHI *et al.*, 2009), flow cytometry (DAVIS *et al.*, 1996), affinity chromatography using agarose, sepharose, magnetic beads and microwell plate or sol-gel channels to immobilize the target (BAE *et al.*, 2013; KIM *et al.*, 2014; MCKEAGUE; GIAMBERARDINO; DEROSA, 2009).

Figure 1 – Illustration of DNA aptamers selection by SELEX.




Source: Adapted from Liu and Zhang (2015)

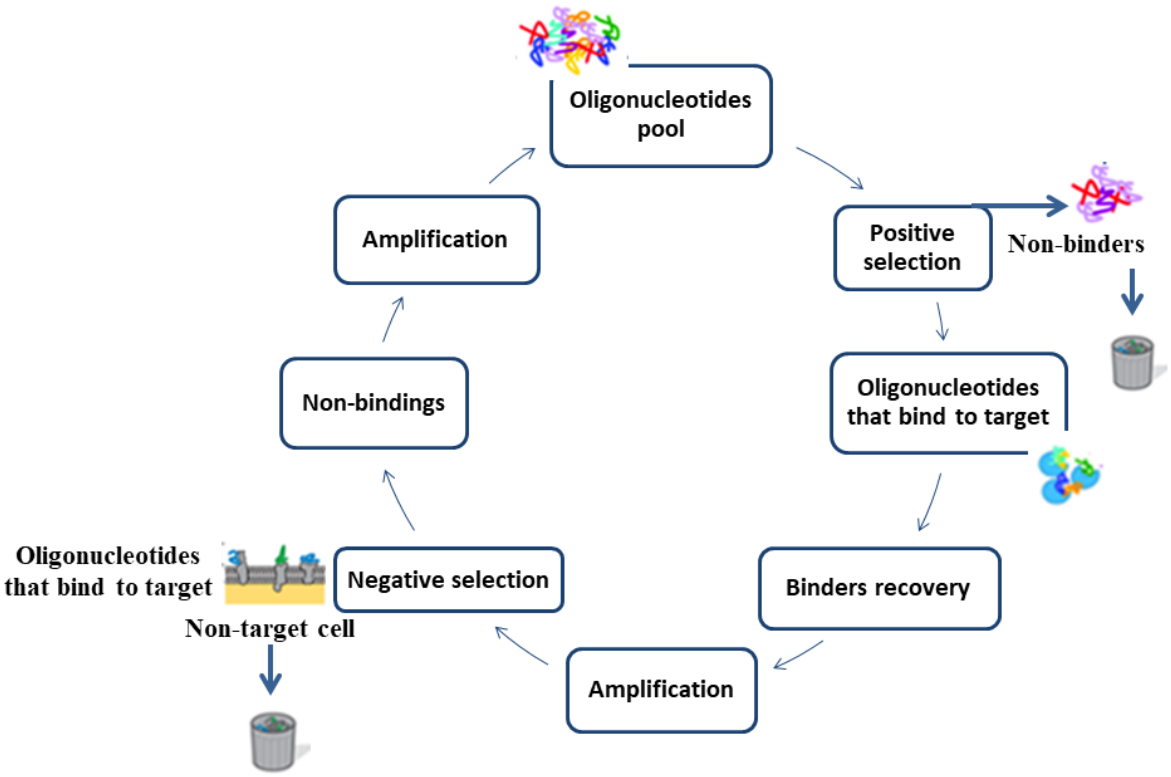
The separation of aptamer candidates from the target molecule can be carried out by heat treatment, competing for ligand elution, urea addition, EDTA or SDS techniques (BIANCHINI *et al.*, 2001; STOLTENBURG, R; REINEMANN; STREHLITZ, 2005; THEIS

*et al.*, 2004; WEISS *et al.*, 1997). According to Ellington and Szostak (1990), the affinity chromatography technique is used when it is desired to obtain aptamers for small targets.

For the second SELEX cycle, the amplified product (double-stranded DNA or RNA) should be converted by PCR to a single-stranded. According to Stoltenburg; Reinemann; Strehlitz (2005), if the library is DNA, the most used methods are biotin/streptavidin-added electrophoresis for strand distinction (desired and unwanted), size-difference primers where the unwanted strand is modified, modified primers at tip 3' (addition of a ribose) or hexamethylene glycol spacer primers. For the last two methods, electrophoresis is used to check the size difference and separate the unwanted tape. If the double strand is RNA, dsDNA is transcribed into RNA with T7 RNA polymerase. After the simple tapes are incubated again with the target, one more SELEX cycle occurs (STOLTENBURG, R; REINEMANN; STREHLITZ, 2005).

The cycles are performed until a binder with high affinity and specificity is obtained (Figure 2), and in this case, 5 to 20 cycles can be performed (LIU, XIAOFEI; ZHANG, 2015; MISSAILIDIS; PERKINS, 2007; TUERK; GOLD, 1990). This number depends on some factors such as the selected library, selection conditions of oligonucleotides, the concentration of incubated target, as well as the concentration of aptamer candidates (RADOM *et al.*, 2013). Once obtained, the high affinity and specificity aptamers are cloned into bacterial vectors, usually, *Escherichia coli*, sequenced and characterized (MISSAILIDIS; PERKINS, 2007; STOLTENBURG, R; REINEMANN; STREHLITZ, 2005; TUERK; GOLD, 1990).

Figure 2 – cell-SELEX steps to obtain a DNA aptamer.




Source: Adapted from Ye *et al.* (2012)

Several aptamers have already been selected for a range of targets as inorganic ions ($Na^+$) (ZHOU; DING; LIU, 2016), dopamine, and other organic compounds such as amino acids like L-tryptophan (IDILI *et al.*, 2019), proteins (DENG *et al.*, 2014), antibiotics (SMART *et al.*, 2020), and microorganisms (bacteria, virus, and fungi) (SMART *et al.*, 2020; SOUNDY; DAY, 2017). When the aptamers are obtained by whole cells, the technique is called cell-SELEX (RADOM *et al.*, 2013).

Contrariwise, to the SELEX approach, which uses purified targets, the cell-SELEX technique allows the use of whole cells where no knowledge of target conformation or protein purification is required, and whole cells remain in their natural state throughout the selection process. This technique is used because in some cases, when the target is purified, the native configuration can be lost, and the target is masked. So, the candidate aptamers may not bind since the natural structure of the targets is not recognized (YE, MAO *et al.*, 2012). The cell-SELEX cycle follows the same structure as the SELEX technique, but with the addition of negative selection. This approach uses different cells that are non-target for reducing the

number of aptamers, which bind with non-specific cells, thus increasing aptamer specificity (Figure 2). In the negative selection, Ye *et al*. (2012) described those non-binding aptamers are discarded and those targeting cell-binding are eluted and amplified by PCR. On the other hand, in the negative control, non-target cells are incubated with an amplified library and non-binding cells are separated and amplified by PCR and so on until high affinity and specificity aptamers are obtained (YE, MAO *et al.*, 2012). In the cell-SELEX, the candidate aptamers can bind to the three-dimensional configuration of the target (YE, MAO *et al.*, 2012).

Cell-SELEX is reported, in the literature, to select aptamers i.e. *S. enterica* serovar T*yphimurium* (DUAN *et al.*, 2013; LAVU *et al.*, 2016), *P. aeruginosa* (SOUNDY; DAY, 2017), *Neisseria meningitides* (MIRZAKHANI *et al.*, 2018), *E. coli* O157:H7 (AMRAEE *et al.*, 2017), *Streptococcus pyogenes* (HAMULA *et al.*, 2011), *S. aureus* (RAMLAL *et al.*, 2018), *Haemophilus influenzae* (BITARAF; RASOOLI; MOUSAVI GARGARI, 2016), *Trypanosoma cruzi* (ULRICH *et al.*, 2002), tumor liver cells (MI *et al.*, 2009) and mouse stem cells (GUO *et al.*, 2007).

In this way, the chosen method depends on the target. Many works employed the cell-SELEX technique to obtain aptamers for bacteria detection, providing high affinity and selectivity. On the other hand, the SELEX technique can be used to identify bacteria and other compounds such as organic and inorganic molecules, viruses, and tumors.

## 2.4 APTAMERS CONJUGATION

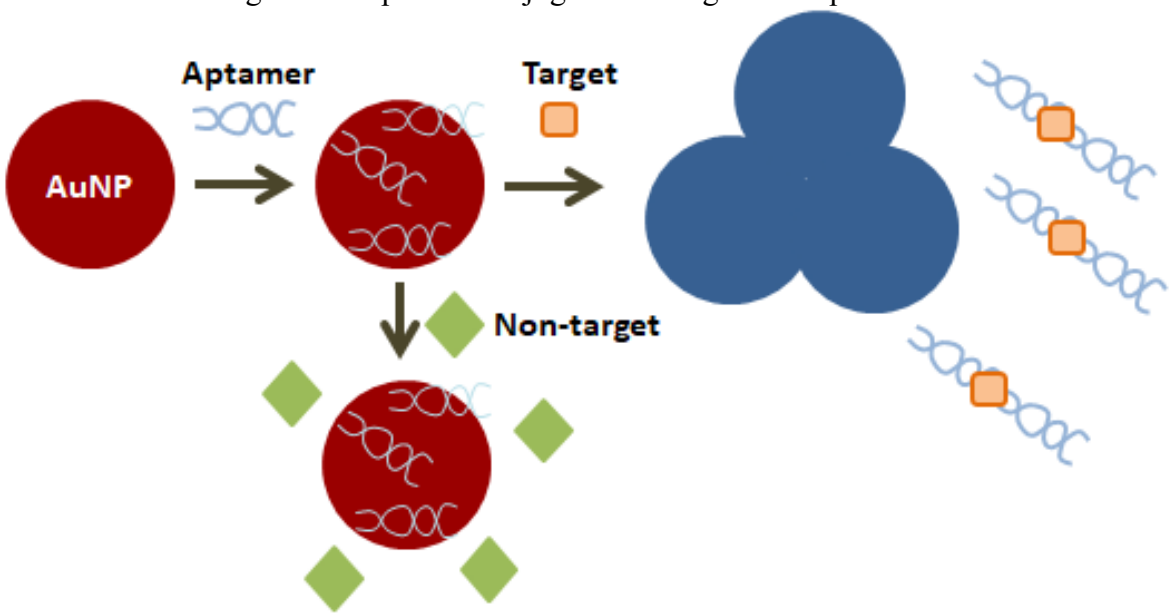
Aptamers can have diverse applications, from basic research in medicine, pharmaceuticals, diagnostics, therapy, and drug development to pathogen detection, which encompasses the medical field and the food industry (LAVU *et al.*, 2016; TUERK; GOLD, 1990). In therapy, aptamers act as inhibitors of targets, such as nucleolin inhibition (RADOM *et al.*, 2013), while for food safety aptamers are used to detect contaminants (AMAYA-GONZÁLEZ *et al.*, 2013).

To improve the application range, aptamers may be conjugated to nanostructures, which assist in the identification of the target compounds. Common conjugates for aptamers are metal or silica nanoparticles, hydrogels, and even carbon nanomaterials, due to their biocompatibility, controllable chemical and physical properties, and stability (LIU, XIAOFEI; ZHANG, 2015; YANG, XIAOJUAN *et al.*, 2015).

Among the conjugation applications, one can be the aptamer conjugation for colorimetric detection. This type of detection is the most attractive and widely used since the target is detected through visual observation with the aid of a colored reactant without the use of analytical instruments as a spectrophotometer. For this kind of application, gold, magnetic, or cerium oxide nanoparticles, carbon nanotubes, graphene oxide or even polymers may be conjugated to the aptamers (SHARMA *et al.*, 2015). These nanostructured supports have been commonly synthesized and applied (ALMEIDA; DE OLIVEIRA; HOTZA, 2017; CHIARADIA *et al.*, 2018; HOELSCHER *et al.*, 2018; MAASS *et al.*, 2019; VALÉRIO *et al.*, 2017).

Gold nanoparticles are widely used because they decrease the distance between dispersed particles and increase the size after aggregation resulting in red to blue colors, as shown in Figure 3 (SHARMA *et al.*, 2015). Some authors reported the use of silver ions to improve detection sensitivity. In this case, silver ions adhere to the surface of AuNPs reducing silver atoms by electrons released from the reducing agent around the gold nanoparticles. Thus, the nucleation reaction increases the size of the gold nanoparticles changing the color and making possible a visible identification (LIU, RUI *et al.*, 2014).

Figure 3 – Aptamer conjugation with gold nanoparticles.




Source: Adapted from Sharma *et al.* (2015)

Fluorescence is the emission of light from an excited molecule, a dye, or even a nanomaterial that then returns to its initial state (SHARMA *et al.*, 2015). Both colorimetric and

fluorescence assays are widely used in aptamer studies, as they present high sensitivity, high efficiency, and easy operation. Those techniques require the use of fluorophore and chromophore dyes for measurable signal emission (SHARMA *et al.*, 2015).

According to Sharma *et al*. (2015), besides dyes, some nanomaterials provide fluorescence emissions, which are economically viable but have a time-consuming laborious process that can affect the selectivity of the aptamer to the target binding. Assays without fluorescent markers consist of the use of DNA intercalators, base site binding dyes, and metallic nanomaterials with fluorescence emission.

Organic dyes such as FAM (fluorescein amidite) are commonly bounded to oligonucleotides but have some limitations such as broad emission range, low photostability, low absorption, and photodegradation (LI *et al*., 2008). To improve these limitations, inorganic fluorescents like quantum dots can be used, since their optical characteristics depend on the size (1 to 10 nm). They have a broad absorption spectrum, narrow emission spectrum, and long-life fluorescence. However, their drawbacks are the high-cost synthesis and toxicity associated (SHARMA *et al.*, 2015). An example of quantum dots is cadmium selenide, which has high luminescence and good quantum yield (XU, LIZHOU *et al*., 2015) as already reported to detect the presence of *E. coli* O157: H7 by Xu *et al*. (2015).

Yang *et al*. (2011) reported the use of carbon nanoparticles for fluorescence testing, highlighting material advantages such as low cost, high quantum productivity, simple preparation, low toxicity, good biocompatibility, good aqueous solubility, and superior photoluminescence properties. In addition to fluorescent dyes, nucleic acid dyes may be used (SYBR green I, AccuBlue, and PicoGreen). The drawbacks of the nucleic acid dyes are the low fluorescence intensity and the requirement to interleave with the aptamer DNA sequence (DUAN, NUO *et al.*, 2014).

The chemiluminescence technique also uses optical detection and is mainly applied in food safety since it produces energy through chemical reactions without the need for an excitation source as in the fluorescence technique (SHARMA *et al.*, 2015). Chemiluminescence signals can be increased using AuNPs as catalysts enhancing biocompatibility and stability (YANG, LIU *et al.*, 2011). DNA aptamers that are rich in guanine (G) can react with 3,4,5-trimethoxylphenylglyoxal (TMPG), which forms an energy-rich compound that emits light or transfers energy to some aptamer-coupled dye, such as 6-FAM (6-carboxyfluorescein) with green light emission and can be employed in target detection studies (KWON; PARK; LEE,

2015). Thus, aptamers can be conjugated to different nanoparticles to increase the selectivity of the target. Besides increasing the selectivity, these nanomaterials allow visual detection.

## 2.5 APTAMERS APPLICATIONS

Several aptamers have been developed for different applications, as reported by Chan *et al*. (2008) that employed PEG-conjugated aptamers RB006 against coagulation. Wu *et al*. (2008) developed a PO RO10-60 aptamer to stimulate the immune response against pathogens by delaying symptoms and allowing the use of antibiotics. There are also electrochemical sensors, which are aptamers that act in real-time detection of cocaine in fetal bovine serum (SWENSEN *et al.*, 2009).

Aptamer-based nanostructures have also been widely used in medicine, biology, and nanoelectronics due to their high stability, as shown in Table 1. The AS1411 aptamer conjugated with gold nanoclusters was tested in mice cancer cells and demonstrated to be a good radiosensitizer (GHAHREMANI *et al.*, 2018). Through cell-SELEX the JHIT2 aptamer was selected and labeled with FAM and iodine-131 to detect human hepatoma cell line HepG2 by a fluorescent signal (ZHANG *et al.*, 2020). All these studies show that aptamers can assist in the rapid detection of different targets, shortening the time to start treatments that are important for human health.

Lavu *et al*. (2016) studied the use of gold nanoparticles with aptamers for the detection of *Salmonella enterica.* The aptamer SAL 26 was conjugated with gold nanoparticles at room temperature and in the presence of NaCl keeping the solution red and when in the presence of *Salmonella enterica* ($10^2$ to $10^6$ $CFU.mL^{-1}$) the solution turned blue after 30 min. According to the authors, the color change is associated with the formation of a tertiary structure with the target cell that has no affinity for gold nanoparticles, resulting in salt-induced aggregation.

Table 1 – Examples of aptamers application to different targets in different areas.

| **Target** | **Method** | **Sample** | **Reference** |
|---|---|---|---|
| *Streptococcus pyogenes* | Cell-SELEX | Cooked chicken | (HUANG *et al.*, 2018) |
| *Salmonella Typhimurium* | Cell-SELEX | Pasteurized milk | (DUAN, YING FEN *et al.*, 2014) |
| *Salmonella Typhimurium* and *Vibrio parahemolyticus* | Cell-SELEX | Frozen shrimp, chicken breasts | (DUAN, NUO *et al.*, 2014) |
| *Salmonella* | - | Pork | (MA *et al.*, 2014) |
| *Escherichia coli* | Cell-SELEX | Milk and tap water and pond | (JIN *et al.*, 2017; KIM, YEON SEOK *et al.*, 2013) |
| *Staphylococcus aureus* | - | Fresh fish | (JIA *et al.*, 2014) |
| *Staphylococcus aureus* | Cell-SELEX | Pork meat | (HAO *et al.*, 2017) |
| *Staphylococcus aureus* | Cell-SELEX | Milk | (YUAN *et al.*, 2014) |
| *Listeria monocytogenes* | SELEX | Liced beef, chicken, turkey | (OHK *et al.*, 2010) |
| *Campylobacter jejuni* | SELEX | Live cell | (BRUNO *et al.*, 2009) |
| *Lactobacillus acidophilus* | Cell-SELEX | Oxidized PSi Fabry-Pérot thin films | (URMANN *et al.*, 2016) |
| *Francisella tularensis* | SELEX | Bacterial antigen | (VIVEKANANDA; KIEL, 2006) |
| *Mycobacterium tuberculosis* | SELEX | Live cell | (CHEN, FAN *et al.*, 2012) |
| *Vibrio parahemolyticus* | Cell-SELEX | Live cell | (DUAN, NUO *et al.*, 2012) |
| *Shigella sonnei* | Cell-SELEX | Live cell | (SONG *et al.*, 2017) |
| *C. jejuni* | Cell-SELEX | Live cell | (DWIVEDI; SMILEY; JAYKUS, 2010) |

| | | | |
|---|---|---|---|
| Vaccinia virus | SELEX | Vaccinia intacto | (LABIB *et al.*, 2012) |
| herpes simplex virus | SELEX | Gd protein of HSV-1 | (GOPINATH; HAYASHI; KUMAR, 2012) |
| Hepatitis C and hepatitis B virus | SELEX | Hepatitis C virus | (KUMAR *et al.*, 1997) |
| Human immunodeficiency virus | *In vitro* selection | Human immunodeficiency virus type-1 | (BOIZIAU *et al.*, 1999) |
| Influenza virus | SELEX | Hemagglutinin protein of human influenza virus B | (GOPINATH; KAWASAKI; KUMAR, 2005) |
| Severe Acute Respiratory Syndrome (SARS) coronavirus | SELEX | Live cell | (JANG *et al.*, 2008) |
| *Trypanosoma* spp. | SELEX | Plasma of *T. cruzi* infected mice | (NAGARKATTI *et al.*, 2014) |
| *Leishmania* spp. | SELEX | Live cell | (GUERRA-PÉREZ *et al.*, 2015) |
| *Plasmodium* spp. | - | *P. falciparum* parasites | (CHEUNG *et al.*, 2018) |
| *Cryptosporidium parvum* | SELEX | Fresh fruits | (IQBAL *et al.*, 2015) |
| *Entamoeba histolytica* | SELEX | Live cell | (OSPINA-VILLA *et al.*, 2015) |
| MCF-7 breast cancer cells | - | Target cancer cells | (WANG, KUN *et al.*, 2015) |
| Leukemia CCRF-CEM cells | - | Human leukemia CCRF-CEM cells | (YE, XIAOSHENG *et al.*, 2015) |
| Metastatic tumor tissues | Cell-SELEX | Colon cancer cell SW620 | (LI, XILAN *et al.*, 2015) |
| Ochratoxin A (OTA) | SELEX | Immobilized OTA | (CRUZ-AGUADO; PENNER, 2008) |
| Bacterial endotoxins | SELEX | Lipopolysaccharide | (KIM, SUNG-EUN *et al.*, 2012) |
| Copper | - | Lake samples | (CHEN, ZHENGBO *et al.*, 2011) |

| | | | |
|---|---|---|---|
| Arsenic | - | Aqueous solution | (OROVAL *et al.*, 2017) |
| Acetamiprid | - | Wastewater and tomatoes | (FAN *et al.*, 2013) |
| Herbicides | SELEX | Atrazine | (SINHA; REYES; GALLIVAN, 2010) |
| Milk allergen | SELEX | β-LG variants A and B | (EISSA; ZOUROB, 2017) |
| Bisphenol A | - | Aqueous solution | (CHEN, MAO-LONG *et al.*, 2017) |
| Beta1-adrenoreceptor autoantibodies | - | Serum of patients | (WALLUKAT *et al.*, 2016) |
| Lung cancer | SELEX | Cells | (BATES *et al.*, 2009) |
| Colorectal cancer | - | Camptothecin loaded-pegylated dendrimer | (ALIBOLANDI *et al.*, 2017) |
| Breast cancer | - | Breast cancer tissues | (WANG, YAYU *et al.*, 2017) |

Source: SCHMITZ *et al.* (2020)

De Girolamo *et al*. (2011) developed a DNA aptamer to detect OTA (Ochratoxin A) mycotoxin produced by *Aspergillus ochraceus* and *Penicillium verrucosum*, found in wheat. They showed a system able to detect OTA in a range from 0.4 to 500 ng. Chen *et al*. (2015) reported the direct detection of FB1 (fumonisin B1) in maize samples by using gold nanoparticles conjugated to modified aptamers (5′-SH-$(CH_2)$6-AGCAGCACAGAGGTCAGATGCGATCTGGATATTATTTTGATACCCCTTTGGGGA GACATCCTATGCGTGCTACCGTGAA-3′). The authors reported an accrued detection after 40 min at room temperature for FB1 concentrations above 2 pM. Another mycotoxin that is toxic to humans is zearalenone (ZEN). It is found in cereal crops and produced by *Fusarium graminiarum*. To detect ZEN, the mycotoxin was extracted from cereal crops and different solutions were prepared and analyzed by the aptamer conjugated with zinc oxide-nitrogen doped graphene quantum dots (ZnO-NGQDs), which was capable to detect $3.3\times10^{-14}$ g.mL$^{-1}$ (LUO *et al.*, 2020).

Heavy metals present in milk and dairy products, fish, eggs, oils, and seeds can be also detected by aptamers. Hazardous metals, such as arsenic and mercury, can affect human health by interfering with the central nervous system and endocrine system. Thereby, colorimetric detection by DNA aptamers has been reported by Li *et al*. (2009), who used aptamer (5′-TTTTTTTTTT-3′) conjugated with AuNPs (13 nm) incubated with mercury ($1 \times 10^{-4}$ mol.L$^{-1}$) at room temperature. After the addition of 50 μL of 0.5 M NaCl, it was observed that the dispersion turned to blue confirming the presence of metal. Wu *et al*. (2014) investigated DNA aptamer conjugated with gold nanoparticles to cadmium and reported a high-affinity detection for an aqueous dispersion containing cadmium at a lower concentration (4.6 nM).

The green malachite fungicide is widely used in aquaculture and can contaminate fish and their eggs, posing a risk to those who consume them (STEAD *et al.*, 2010). In 2010 it was reported the first malachite green (MG) detection by RNA aptamer (5'-GGAUCCCGACUGGCGAGAGCCAGGUAACGAAUGGAUCC-3') in fish skin samples, and the developed approach was able to quickly confirm the contamination after 15-20 min at 2 μg.kg$^{-1}$ of salmon tissue (STEAD *et al.*, 2010).

Many types of pesticides are used to prevent contamination by bacteria, fungi, and viruses and the detection methods should be efficient even at low pesticide concentrations. However, detection by liquid and gas chromatography is expensive and time-consuming (FAN *et al.*, 2013). To reduce the costs and analysis time, new technologies are employed, such as the use of biosensors. Fan *et al*. (2013) developed a conjugated aptamer with a gold nanoparticle

that generates a signal of impedance to identify acetamiprid with a detection limit of 1 nM in wastewater and tomatoes and the process takes up to 3 h.

For herbicides, widely used in the cultivation of corn and oilseed rape, with the consequent environment and human life issues, different DNA aptamers with affinity to atrazine were studied by Williams *et al*. (2014). The double-stranded DNA aptamer for fluorescence detection was drawn to detect fipronil insecticide in river water samples and showed high sensitivity (HONG; CHEON; LEE, 2018).

Moreover, the C07 aptamer was developed to detect Sudan dye III in chili sauce. From the study, it was reported a fast and accurate binding to the target, and according to the authors, 100 nM of aptamer was enough to detect 400 ng of Sudan dye III (WANG, YING *et al.*, 2018). Besides, organic molecules, such as bisphenol A (BPA), present in some food products are harmful to the human endocrine system, and in 2010 the US and Canadian governments banned their use (MCKEAGUE; GIAMBERARDINO; DEROSA, 2009). In this sense, Lee *et al*. (2011) studied the detection of BPA by aptamers conjugated with carbon nanotube as a biosensor and showed a detection limit at low concentrations (10 fM).

In this context, Smart *et al.* (2020) reported several promising biosensors for agribusiness. In some cases, aptamers were conjugated to nanoparticles forming carbon electrodes for the detection of pesticides, toxins, antibiotics, microorganisms, vitamins, fructose, and lactate. In addition, Yan *et al*. (2020) showed different photoelectrochemical and electrochemiluminescent apta-sensors capable to detect food contaminants and pollutants.

## 2.6 CONCLUSIONS AND MARKET PERSPECTIVES

There are several studies in the literature related to the development of aptamers for different targets. They are widely studied in the therapeutic area, to identify cancer cells, bacterial contamination, and viruses. Moreover, aptamers are being developed for food safety since there is a high diversity of pathogens in food products from different origins (animal, vegetable, processed), as well as contamination by packaging or transportation.

Despite being basic, inexpensive, and selective, most existing aptamers are still not currently used in the industries and agribusiness routine. Through the aptamer applications, a quick analysis system can be launched as a biosensor, bringing advantages to the market as visual detection is low cost compared to conventional techniques, as well as delivering robustness and selectivity.

Chemiluminescence detection is one of the most studied technics since it does not require equipment for signal detection. However, for many targets, studies are needed to ensure that sensitivity and specificity are enhanced using nanoparticles. Further investigation should be directed to sample preparation methods.

Many of the aptamers are not able to recognize samples in the raw phase and need to be prepared in aqueous solutions. To reduce the gap between lab-scale and industrial large-scale applications, advances in fast and efficient detection for food safety are increasing, but some aspects still need to be improved, such as sample preparation, concentration, and the presence of contaminants from raw materials.

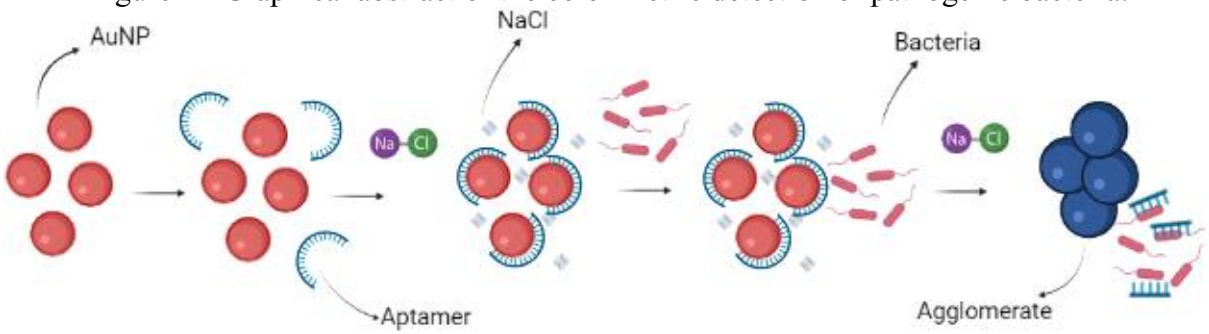
Due to the difficulties related to food safety, companies are looking for cheaper and faster alternatives. Whereas the world population is expected to reach 8.5 billion people by 2030 (UNITED NATIONS, 2019), the food industry faces problems with changes in food production and supply, increased imports, changes in the environment that lead to contamination, development of outbreaks or pests on crops in different locations. Herewith, the market is turning to faster and more effective sensors for detecting contamination. In this context, aptamers come to the market as a promssing alternative (LIU, XIAOFEI; ZHANG, 2015).

According to their advantages, aptamers can supply the agribusiness as well as food industrialization needs, being increasingly used worldwide to speed up the food safety analysis, to avoid products recall and unnecessary business expenses around the world (AMAYA-GONZÁLEZ *et al.*, 2013; APTAMER GROUP, 2016).

# 3 COLORIMETRIC DETECTION OF PATOGHENS

This chapter presents the study of the development of colorimetric detection methodology for pathogens as shown in Figure 4, corresponding to an experimental article to be published. Also, the results of ideal salt concentration are presented to keep the gold nanoparticles stable, as well as the optimization of the salt incubation time and aptamer concentration, in addition to the affinity and specificity of the method.

Figure 4 - Graphical abstract of the colorimetric detection of pathogenic bacteria.




Novel rapid methodologies for the detection of bacteria have been recently investigated and applied. In hospital environments, infections by pathogens are very common and can cause serious health problems. *P. aeruginosa* is one of the most common bacteria, which can grow in hospital equipment such as catheters and respirators. Even at low concentrations, it can cause serious infections as it is resistant to antibiotics and other treatments. The objective of this work was to develop a colorimetric biosensor using aptamer-functionalized gold nanoparticles for identifying *P. aeruginosa*. The detection mechanism is based on the color change of gold nanoparticles (AuNPs) from red to blue-purple through salt induction after the bacterium is incubated and the aptamer-target binding occurs. AuNPs were synthesized and characterized. The influence of aptamer and sodium chloride concentration on the agglomeration of AuNPs was investigated. Optimizations of aptamer concentration and incubation time of the first salt addition were performed. The best condition for detection were 5 µM aptamers and 200 mM of NaCl, thus, *P. aeruginosa* detection occurred after 5 h for concentrations from $10^8$ to $10^5$ CFU·mL$^{-1}$, being $10^5$ and $10^4$ CFU·mL$^{-1}$ the detection limit for color change by the naked eye and UV-Vis spectrometry, respectively. In addition, other bacteria were also detected with color changes from reddish to gray, such as *E. coli, S. Typhimurium* e *Enterobacteriaceae bacterium.* With the optimization study, it was confirmed

that the salt incubation time can be 2 h, and that the ideal concentration of aptamer is 5 µM. On the optimized conditions, the colorimetric analysis can be a faster alternative for the detection of *P. aeruginosa*, in the range of $10^8$ to $10^5$ CFU·mL$^{-1}$ at the naked eye.

## 3.1 INTRODUCTION

Some bacteria can be easily found in water, food, soil, animal feces, and even in hospital environments (KRITHIGA *et al.*, 2016). Gram-negative pathogenic bacteria such as *P. aeruginosa* can colonize in terrestrial and aquatic environments (BRAZ *et al.*, 2018). It can be found in water, soil, and air. Even at low concentrations, it is responsible for severe infectious like metabolic disease and cancer (SARABAEGI; ROUSHANI, 2021; SOUNDY; DAY, 2017). *P. aeruginosa* can grow in environments with low nutrients and humidity, such as in medical and hospital equipment (KRITHIGA *et al.*, 2016). In addition, it is resistant to the action of antibiotics and antimicrobials due to biofilm formation and low permeability of the outer membrane, respectively (SISMAET; PINTO; GOLUCH, 2017). This makes it the object of study in several areas of research, including the area of rapid detection or identification in hospital environments.

Several methods can identify *P. aeruginosa*, such as conventional bacterial culture, fluorescence in situ hybridization (FISH) (SØGAARD; STENDER; SCHØNHEYDER, 2005), PCR (Polymerase Chain Reaction) (DESCHAGHT *et al.*, 2011), immunological technique (ELISA) (TANG, YONGJUN *et al.*, 2017), colorimetry, mass spectrometry and electrochemical techniques (TANG, YONGJUN *et al.*, 2017). However, these methods are often time-consuming and costly, and do not give quantitative results (LAVU *et al.*, 2016; SARABAEGI; ROUSHANI, 2021; TANG, YONGJUN *et al.*, 2017). Naturally, rapid, low-cost diagnosis is very important to human health and food safety (SHI; ZHANG; HE, 2019).

Only a few, recent studies for rapid detection of *P. aeruginosa* were found (ROUSHANI; SARABAEGI; POURAHMAD, 2019; SARABAEGI; ROUSHANI, 2021; ZHONG *et al.*, 2018, 2020). Within this universe, aptamers have been standing out for their advantages such as specificity with the target, and ease of conjugation with nanomaterials (FAMULOK; MAYER, 2011). However, most studies use instrument-based detection, and it is known that label-free aptamers are also capable of detecting the target under study with the same quality (Wang *et al*., 2006).

Thus, the development of aptamers capable of identifying the target *P. aeruginosa* in hospitals, water, or soil environments is necessary, particularly for the rapid detection of this pathogen. Unlike the studies found in the literature, this work uses unlabeled aptamers for the detection of the target bacterium. Moreover, no previous treatment of gold nanoparticles is required, i.e. only a single step functionalization directly with the aptamers is needed. In this case, when citrate is used as a reduction agent, this organic and non-toxic molecule also acts as a stabilizing agent. In other cases, a former step, e.g. a surface modification with thiol, is necessary for the binding between the aptamer and the AuNPs (VERMA *et al.*, 2015). Aptamers functionalization with fluorescent labels can also be used, but they require detection instruments (WANG *et al*., 2006) differently from this work, in which the detection is with the naked eye.

Moreover, the proposed method can recognize pathogenic bacteria with specificity and without the use of biotinylated ligands, which can cause affinity loss and increase the time and cost of the method (STOLTENBURG *et al*., 2007; WANG *et al.*, 2006). In this work, conditions of temperature, incubation time, and NaCl influence are optimized, and the specificity of the method is verified with non-target bacteria.

## 3.2 MATERIAL AND METHODS

### 3.2.1 Materials

Chemicals were purchased with high purity and used without further treatment: Tetrachloroauric (III) acid ($HAuCl_4.3H_2O$, (Sigma-Aldrich), sodium chloride (NaCl, Êxodo), trisodium citrate dihydrate ($C_6H_5Na_3O_7.2H_2O$, Êxodo). Brain-Heart Infusion (BHI, Kasvi) and agar (Kasvi). Ultrapure water was produced in the laboratory by a purifier (Direct-Q 3 UV).

Single-stranded DNA (ssDNA) aptamers (CCCCCGTTGCTTTCGCTTTTCCTTTC GCTTTTGTTCGTTTCGTCCCTGCTTCCTTTCTTG) (WANG *et al.*, 2011) for *P. aeruginosa* were synthesized (Exxtend, Brazil) and a DNA stock solution was prepared with sterilized ultrapure water with a concentration of 100 μM, according to the manufacturer's protocol.

### 3.2.2 Bacterial strains

The bacterial strains of *P. aeruginosa* ATCC 27853 were provided from a local laboratory (LabCal, Federal University of Santa Catarina). The strains were grown at 37 °C for 12 h in BHI broth until optical density (OD) of 0.7 ($10^8$ CFU·mL$^{-1}$). *E. coli* ATCC 10536, *S. aureus*, *S. Typhimurium* and *Enterobacteriaceae bacterium* were used as negative control and grown for 12 h in BHI broth at 37 °C until the optical density of 0.8 ($10^7$ CFU·mL$^{-1}$). All strains were first cultured in BHI agar to verify the purity, and then the concentration was determined by serial dilution and plating on agar plates and measurement of CFU·mL$^{-1}$. Cultures were cryopreserved in glycerol and stored at -20 °C.

*Enterobacteriaceae bacterium* was obtained by isolation in laboratory and the identification was performed by Neoprospecta Microbiome Technologies (Florianópolis, Brazil) by high-throughput sequencing technologies using MiSeq Sequencing System (Illumina Inc., USA).

### 3.2.3 Synthesis and characterization of gold nanoparticles

The gold nanoparticles (AuNPs) were prepared by the trisodium citrate method (XU *et al.*, 2018). First, a balloon used in the synthesis was cleaned with a $HNO_3$/HCl (3:1 v/v) solution, rinsed thoroughly in ultrapure water, and dried. Then, 10 mL of chloroauric acid ($HAuCl_4$) 10 mM and 90 mL of ultrapure water were heated to boiling point for 20 min with vigorously stirring. Then, 10 mL of trisodium citrate solution were added for AuNPs production. The solution showed a color change of yellow to red and was cooled to room temperature and stored in a dark glass at 4 °C. The AuNPs dispersion was analyzed by UV-Vis spectrophotometry (SpectraMax Plus 384, Molecular Devices, USA) in glass cuvette at wavelength range from 400 to 700 nm. Morphology, size, and homogeneity of AuNPs were analyzed by transmission electron microscopy (TEM, JEM–2100). Dynamic Light Scattering (DLS - MALVERN Zetasizer Nanosizer) was also used to estimate the size of AuNPs. The hydrodynamic radius of AuNPs (BASSO, 2019) and concentration of AuNPs (LIU *et al*., 2007) were estimated according to the respective procedures described in the literature.

### 3.2.4 Salt and aptamer effect on AuNPs

The effects of salt and aptamers concentration on AuNPs stabilization was studied for developing the colorimetric detection method. A volume of 40 µL of NaCl in different concentrations (0, 50, 100, 150, 200, 250 mM) was mixed with 120 µL of AuNPs in a 96-well microplate. The data were collected after 30 min in a UV–visible spectrophotometer (UV-Vis, SpectraMax Plus 384, Molecular Devices, USA) in the wavelength range of 400 to 700 nm.

A light-protected pre-incubation of aptamers (0, 5, 10, 25, 50, 75, and 100 µM) with AuNPs was carried out for 18 h at 37 °C. Then, 120 µL of conjugated AuNPs-aptamers were mixed with 40 µL of salt (200 mM), transferred to a 96-well microplate, and incubated for 1 h at room temperature. The changes were analyzed by UV-Vis at 520 and 630 nm.

### 3.2.5 AuNP aptamer conjugation

First, 1 mL of AuNPs ($8.28 \times 10^{14}$ mol·L$^{-1}$) with DNA aptamers (5 µM) were added in a dark glass for 18 h at 37 °C. After incubation, the dispersion was observed by UV-Vis and then, 120 µL of AuNPs-aptamers were transferred to a 96-well microplate and 20 µL of NaCl (200 mM) was added to the dispersion and incubated for 4 h at 25 °C (XU *et al.*, 2018). After each step, the sensitivity was monitored by changes in the UV-Vis spectra (400 to 700 nm).

### 3.2.6 *Pathogen* colorimetric detection

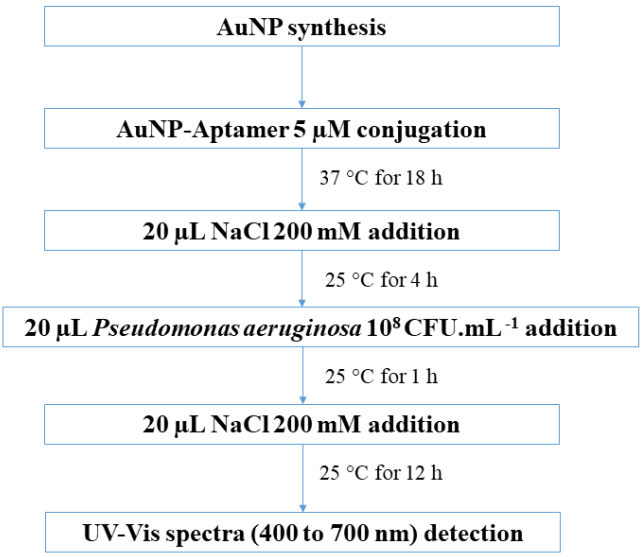
The detection of *P. aeruginosa* by the colorimetric method was performed in 96-well microplates using different bacteria concentrations. *P. aeruginosa* was cultured at $10^8$ CFU·mL$^{-1}$ (OD = 0.7) in BHI medium and serially diluted 10-fold down to $10^0$ CFU·mL$^{-1}$. A volume of 20 µL of each concentration were mixed with 120 µL AuNPs-aptamers (5 µM) and incubated for 60 min at room temperature. Then, 20 µL of NaCl (200 mM) were added to the mixture. The dispersion remained at room temperature for 12 h and was analyzed by UV-Vis in the range of 400-700 nm with kinetics followed at 630 nm.

### 3.2.7 Specificity of the colorimetric method

To evaluate the specificity of the colorimetric method, 20 µL of each bacterium in $10^7$ CFU·mL$^{-1}$ (*E. coli*, *S. aureus*, *S. Typhimurium*) were mixed with 120 µL of conjugated AuNPs (5 µM) for 60 min at room temperature. Then, 20 µL of NaCl (200 mM) were added to the mixture. The dispersion remained at room temperature for 12 h and was analyzed by UV-Vis with wavelength of 400 to 700 nm, and particularly for kinetics at 630 nm.

The steps of the method are shown in Figure 5.

Figure 5 - Colorimetric system for detection of *P. aeruginosa.*




### 3.2.8 Optimization of salt incubation time and aptamer concentration

Other conditions such as salt incubation time and aptamer concentration were also investigated to decrease the time of first NaCl incubation and to verify the minimum aptamer concentration. Briefly, 120 µL of AuNPs-aptamers (5 µM) were incubated with 20 µL of NaCl 200 mM for 4, 2 and 1 h at 25 °C.

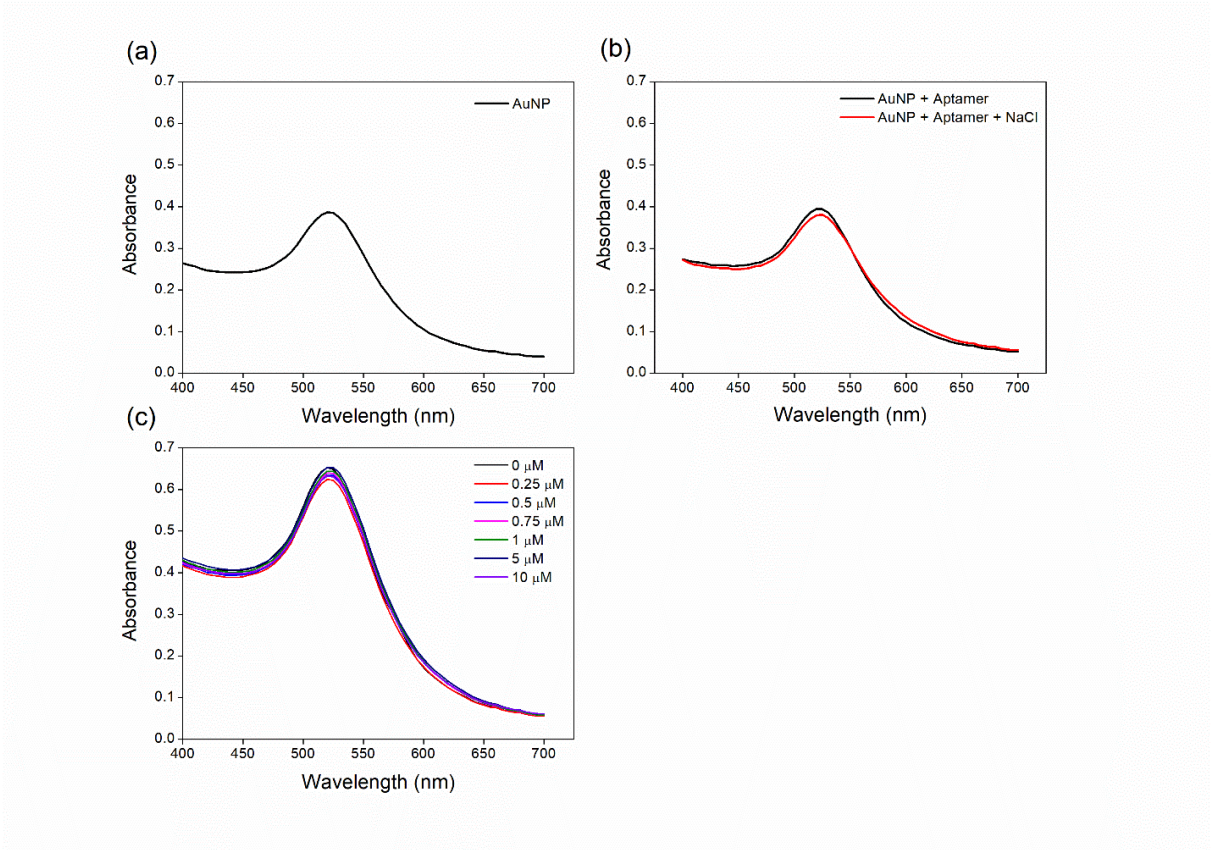
The other analysis occurred in the same way as the methodology described in items 2.5 and 2.6. However, the bacteria detection was studied against the following concentrations of aptamers: 0, 0.25, 0.50, 0.75, 1, 5 and 10 µM. Spectra were measured before and after each conjugation step at 400 to 700 nm to confirm the detection.

## 3.3 RESULTS AND DISCUSSION

### 3.3.1 Characterization of AuNPs and AuNPs-aptamers

Following the AuNPs synthesis method developed by (XU *et al.*, 2018), it was possible to obtain spherical gold nanoparticles in monodispersed suspension. AuNPs were stable due to citrate anions adsorbed (SPR absorption located in 520 nm), as shown in UV-Vis spectra and TEM images (Figure 6a and Figure 8a, respectively), with an average diameter of 25 nm. Spherical AuNPs with a size of 10 to 50 nm present red/pink color and an absorbance peak at 520 nm (LAVU *et al.*, 2016; VERMA *et al.*, 2015; ZHAO; BROOK; LI, 2008).

Figure 6 - UV-Vis spectrum in the wavelength range of 400 to 700 nm for (a) AuNPs, (b) AuNPs functionalized with aptamer 5 µM, AuNP-aptamer with salt addition, and (c) AuNP functionalized with different aptamer concentration.

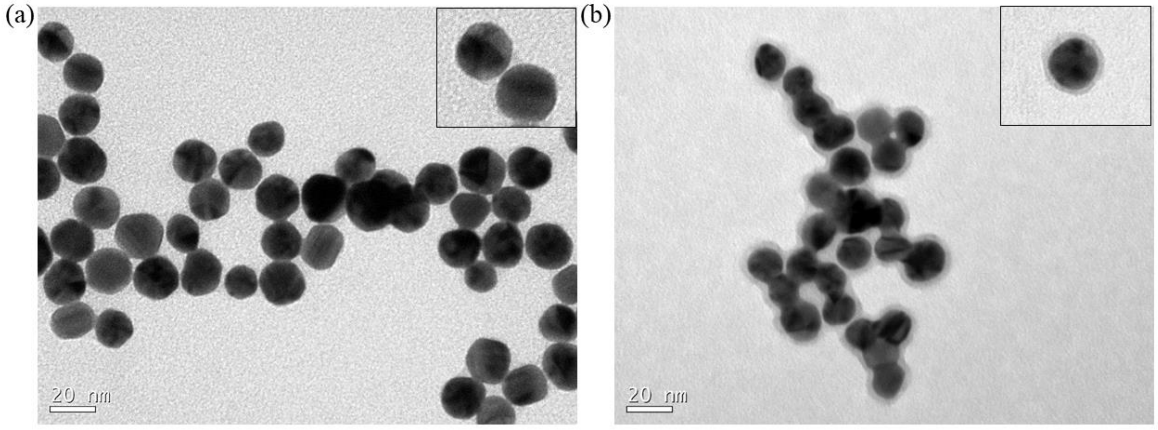



Gold nanoparticles have been widely used in new detection methods due to their ease of being synthesized and also due to their optical properties that change when they are dispersed or agglomerated) (SCHMITZ *et al.*, 2020; VERMA *et al.*, 2015). Usually AuNPs are previously

modified for target identification (VERMA *et al.*, 2015). In this case, more processing steps are needed to immobilize silver markers, thiol probes or antibodies, which consume time and resources. However, other studies for bacteria detection conjugate aptamers to non-modified AuNPs , which still present suitable results in detection, as the case of the studies of Kim *et al.* (2021); Mondal *et al.* (2018); Xu *et al.* (2018).

Most frequently, the conjugation of aptamers to AuNPs is carried out by either adsorption on gold nanoparticles or reaction of thiol groups bound to the aptamers. Since the adsorption is commonly applied for detection of viruses and bacteria (VERMA *et al.*, 2015), this method was used here to identify *P. aeruginosa*.

Figure 7 - TEM images of (a) AuNPs and (b) AuNPs conjugated with 5 μM aptamers.




The AuNPs conjugation with the aptamer was studied by UV-Vis, DLS (Dynamic Light Scattering) and TEM. No changes were seen in the peak of AuNPs when aptamer were added (Fig. 2a and 2b), such as in the work of Das *et al.* (2019). However, a modification on the AuNPs surface was confirmed by TEM as shown in Figure 7a and Figure 7b. According to DLS measurements (Figure S1), the AuNPs presented an average size of 27 ± 1.64 nm (withouth aptamer) and 47 ± 1.94 nm (conjugated with aptamer). The corresponding zeta potential (ZP) in pH 6 was -11.9 and -16.2 mV , respectively before and after aptamers conjugation. This increase in ZP indicates that there was adsorption of aptamers on the AuNPs surface. The results obtained in these characterizations are similar to the work of Das *et al.* (2019), that obtained AuNPs sizes of 17 nm and ZP of -21 mV, and 23 nm and ZP of -31 mV, respectively before and after conjugation.

Table 2 – Zeta Potential of AuNPs and AuNPs conjugated with aptamers in pH 6.

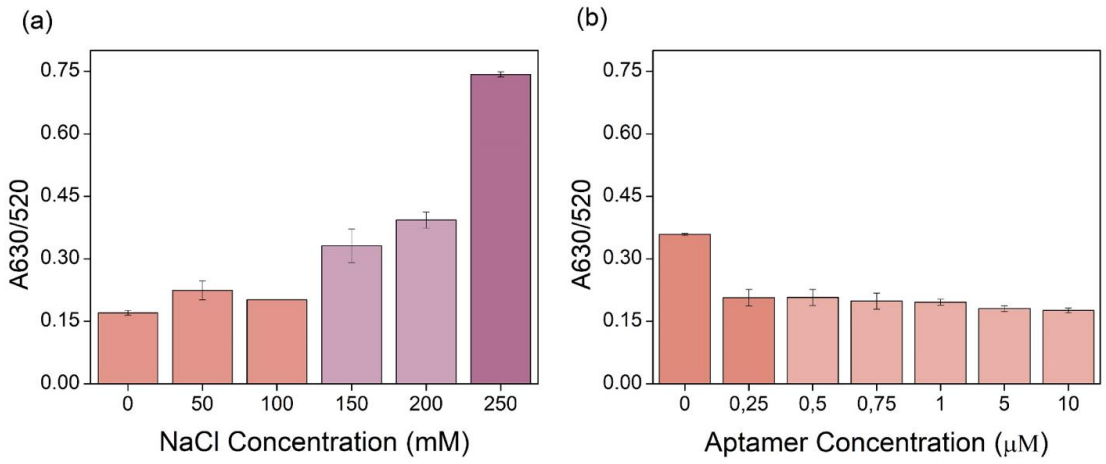
| **Zeta Potential** | **AuNPs** | **AuNPs-Aptamers** |
|---|---|---|
| Zeta Potential (mV) | -11.90 ± 0.43 | -16.20 ± 0.52 |
| PdI | 0.32 ± 0.10 | 0.41 ± 0.10 |

After adding the negatively charged aptamers, they are adsorbed nonspecifically on gold nanoparticles by nitrogenous bases found in DNA, resulting in a negatively charged AuNPs-aptamers dispersion. In addition, it is generally assumed that all aptamer bases bind to AuNPs if they have an available structure (ALSAGER *et al.*, 2018).

The influence of salt concentration was investigated to optimize the conjugation process, since NaCl can shield the electrostatic repulsion of citrate synthesized AuNPs, increasing AuNPs facility to aggregated, changing color of red/pink to purple/blue and a longer wavelength in spectra (630 nm) (ALSAGER *et al.*, 2018; KIM *et al.*, 2021; MONDAL *et al.*, 2018; VASEGHI *et al.*, 2013). A range of NaCl concentrations of 0 to 250 mM (40 µL) was tested. The salt was added to 120 µL AuNPs dispersion with or without aptamers. When the salt addition was performed in the absence of aptamers, a color change of reddish to purple was observed as the salt concentration increases, with absorption ratios (A630/A520) of 0.33 and 0.39, respectively for 150 and 200 mM NaCl in the dispersion with similar absorbance ratio (Figure 8a). The absorbance ratio increased due to nanoparticle aggregation, reducing the surface area that interacts with laser, indicating their destabilization (LAVU *et al.*, 2016). AuNPs color change occurred with 150 mM or more, while the dispersion was still reddish with a concentration equal to or above 100 mM. Therefore, 200 mM was chosen for the following experiments.

After the addition of different aptamer concentrations (0, 0.25, 0.50, 0,75, 1, 5, and 10 µM) the AuNPs showed more stability against the salt addition, because aptamers protect them, increasing the resistance to salt induce aggregation (VERMA *et al.*, 2015; WANG *et al.*, 2006). AuNPs maintained the reddish color above 0.5 µM aptamer and showed low A630/A520 values with 1, 5, and 10 µM aptamers (Figure 8b). High aptamers concentration makes gold nanoparticles more stable but may reduce detection sensitivity due to residual aptamer concentration or cause interference from the background (KIM *et al.*, 2021; LAVU *et al.*, 2016; MONDAL *et al.*, 2018). Thereby, 5 µM aptamer was selected as the optimal concentration for further experiments.

Figure 8 - Evaluation of NaCl concentrations (a) and aptamer concentration with 200 mM of NaCl (b) in the stability of AuNPs. Red color represents the stability of AuNPs and purple represents the aggregation of AuNPs by salt addition.



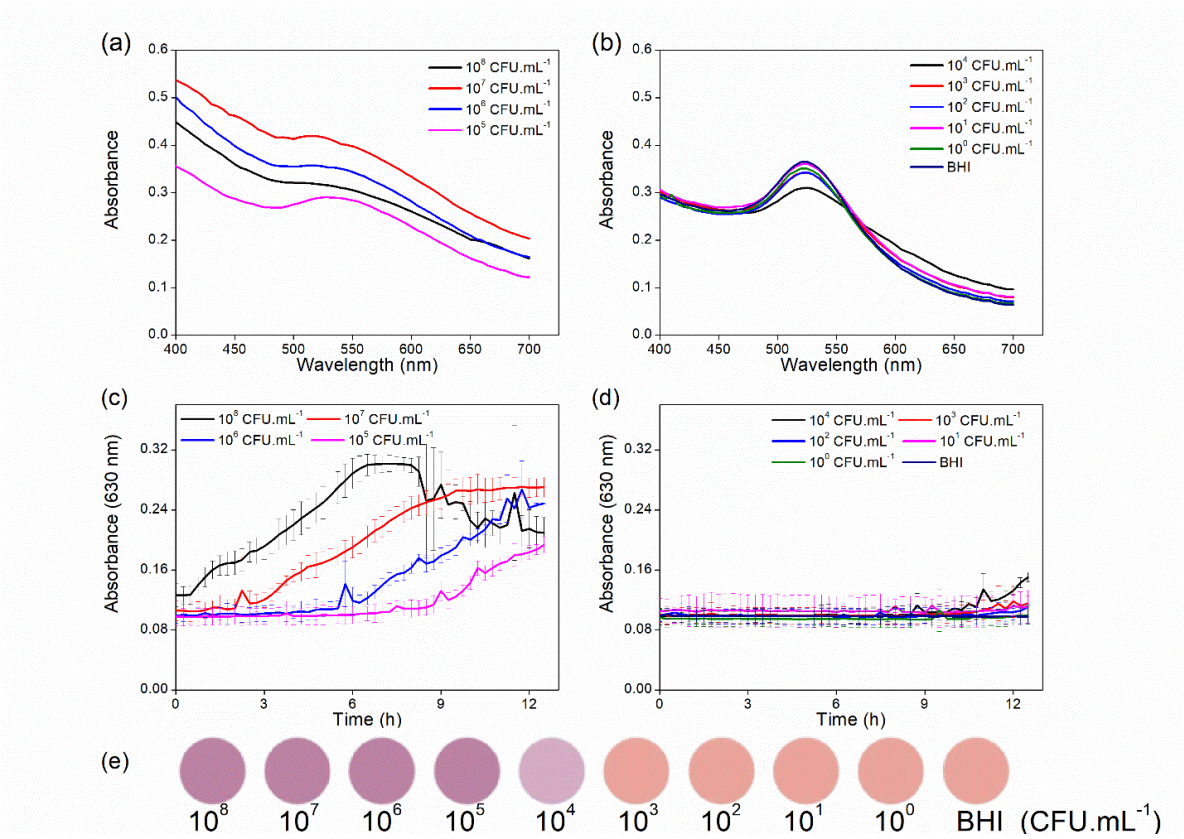

Different aptamer concentrations are used in colorimetric detection methods of *P. aeruginosa*, because each DNA sequence has a different size, structure and composition that interferes in adsorption affinity for AuNPs (ALSAGER *et al.*, 2018). As an example: for an impedimetric sensor, 2 μM of the selected aptamer is required (ROUSHANI; SARABAEGI; POURAHMAD, 2019). The same concentration was used to this target in an aptasensor with gold nanoparticles and carbon electrode (SARABAEGI; ROUSHANI, 2021). Zhong *et al.* (2020) developed a fluorescence biosensor with the aid of two aptamers at concentration of 10 μM, while Amini *et al.* (2017) used 2 μM of the probe to the method.

After confirming the appropriated salt and aptamer concentration, the *P. aeruginosa* detection was evaluated using different concentrations of the bacteria (Figure 9a,b). The limit of detection (LOD) with the naked eye was $10^4$ CFU·mL$^{-1}$, with a light purple color compared to the blank sample. By UV-Vis, the peak extension from 520 to 630 nm indicates the aggregation of AuNPs and aptamer binding with *P. aeruginosa*. In this case, when bacteria are added, the aptamers lose affinity with AuNPs and are desorbed from their surface. Then, AuNPs aggregate due to the addition of the target and salt and the dispersion changes of reddish-to-purple (ALSAGER *et al.*, 2018).

Figure 9c and 9d show the detection kinetics for all *P. aeruginosa* concentrations. After 5 h of bacterial incubation, the nanoparticles changed from reddish to purple (Figure 9e). BHI medium was used as a control test, and no color change and no difference in absorption spectrum were detected. The limit and time of detection of this work are higher when compared

to other studies. The electrochemical aptasensor for *P. aeruginosa* studied by Das *et al.* (2019) was capable of detecting 60 CFU·mL$^{-1}$ in 10 min, the biosensor with silver nanoparticles was able to detect $10^2$ CFU·mL$^{-1}$ (ROUSHANI; SARABAEGI; POURAHMAD, 2019), and the LOD of 9.89 ng·mL$^{-1}$ to AuNPs-aptamers-endonuclease enzyme (AMINI *et al.*, 2017). In this study, the detection limit is greater than the other techniques mentioned. However, the method of this work it is still a cheaper and faster technique than the conventional ones, since the nanoparticles and aptamer used are not modified and do not have ligands that can lead to affinity loss (MONDAL *et al.*, 2018; STOLTENBURG, REGINA; REINEMANN; STREHLITZ, 2007).

Figure 9 - UV-Vis spectra showing all absorption modification steps: (a) AuNPs-aptamers in the presence of *P. aeruginosa* $10^8$ to $10^5$ CFU·mL$^{-1}$, (b) AuNPs-aptamers in the presence of *P. aeruginosa* $10^4$ to $10^0$ CFU·mL$^{-1}$ and blank (BHI), (c) kinetics of detection *P. aeruginosa* $10^8$ to $10^5$ CFU·mL$^{-1}$, (d) kinetic of detection *P. aeruginosa* $10^4$ to $10^0$ CFU·mL$^{-1}$, blank (BHI), and (e) changes of colorimetric detection in presence of various concentrations of target ($10^8$ to $10^0$ CFU·mL$^{-1}$ and blank).

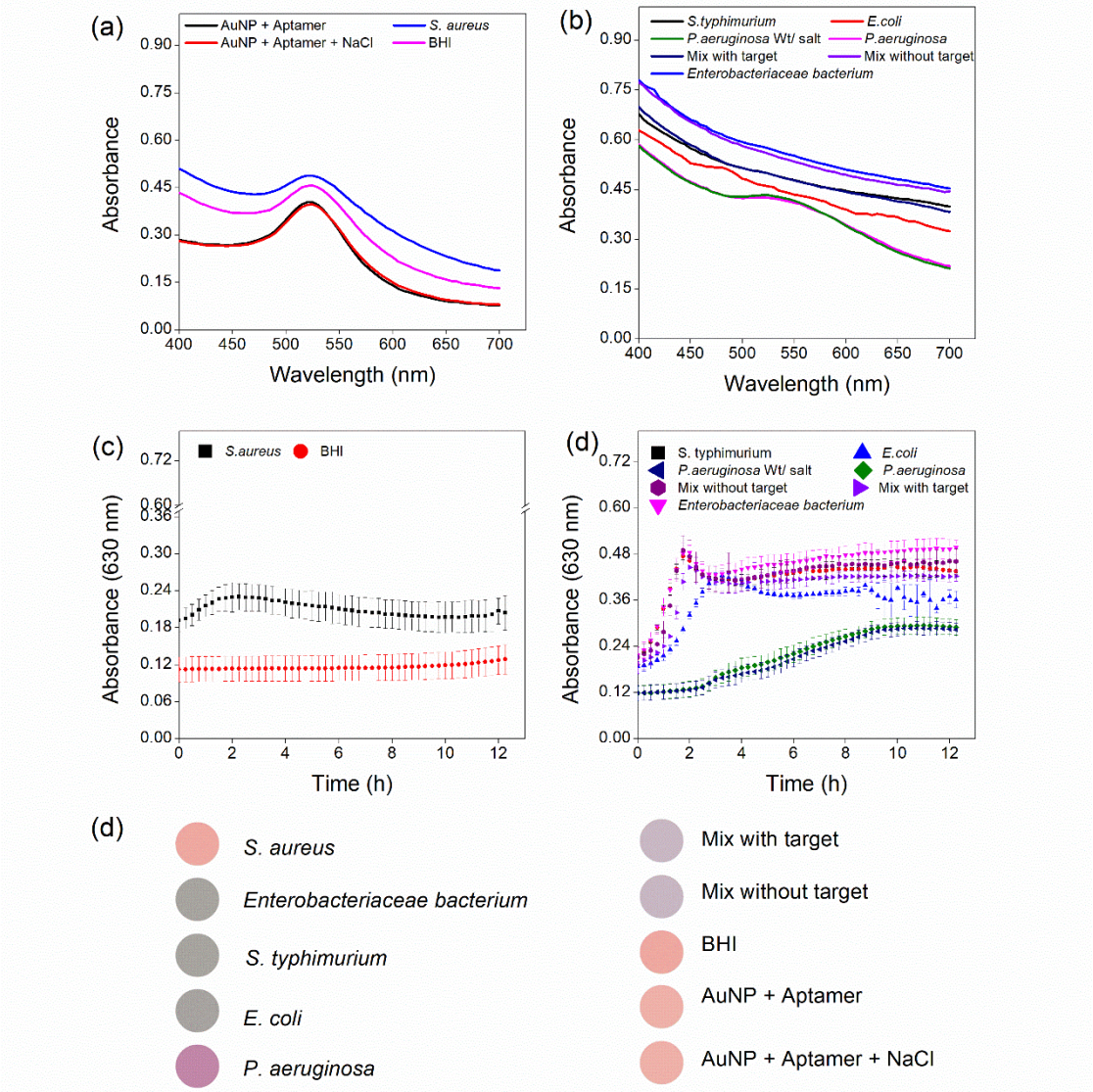


### 3.3.2 Specificity of the colorimetric detection

The specificity of the colorimetric method was evaluated by testing other gram-negative and gram-positive bacteria. The results show no difference in the absorbance peak when *S. aureus* was added to the AuNPs-aptamers salt dispersion, and the reddish color remained unchanged (Figure 10a, e). On the other hand, when *S. Typhimurium, Enterobacteriaceae bacterium* and *E. coli* were incubated, there was a decrease in absorbance at 520 nm. As observed, the band extended to 630 nm, indicating that AuNPs aggregation occurs (Figure 10b,e). However, the observed color change was different; for the detection of *P. aeruginosa* the AuNPs aggregated and changed to purple, while for the others, the color change was from reddish to gray. And these bacteria are less common in hospital infections than *P. aeruginosa* (CUSTODIO, 2022).

AuNPs presented different colors, depending on their size. According to the results in Figure 10e, AuNPs and formed an aggregate with gray color, which corresponds to a size of 250 nm and the purple represents gold nanoparticles with less than 250 nm (FIGUEIRA; SANTOS, 2017). According to Li *et al.* (2017), AuNPs of different sizes have the ability to bind to proteins, bacteria and cells through the interaction of their charges, resulting in their color change. The authors used this property and obtained a biosensor for colorimetric detection of gram-positive and gram-negative bacteria. Elliott *et al.* (2021) developed a biosensor using AuNPs capable to identify *P. aeruginosa* and *E. coli* with different colorimetric patterns, resembling this work.

Figure 10 - Gold nanoparticle-based colorimetric detection assay (a) to AuNPs-aptamers, AuNPs-aptamers-salt, AuNPs-aptamers-salt in presence of *S. aureus* ($10^8$ CFU·mL$^{-1}$) and blank sample (BHI), (b) to *P. aeruginosa* ($10^8$ CFU·mL$^{-1}$) with and without salt addition and negative control bacteria: *S. Typhimurium*, *E. coli*, *Enterobacteriaceae bacterium* in $10^8$ CFU·mL$^{-1}$ (c) kinetic of *S. aureus* and BHI (blank) incubation, (d) incubation kinetic of *S. Typhimurium, E. coli, Enterobacteriaceae bacterium, P. aeruginosa* and mix with target and mix without target and (e) changes of colorimetric detection in presence of other bacteria.



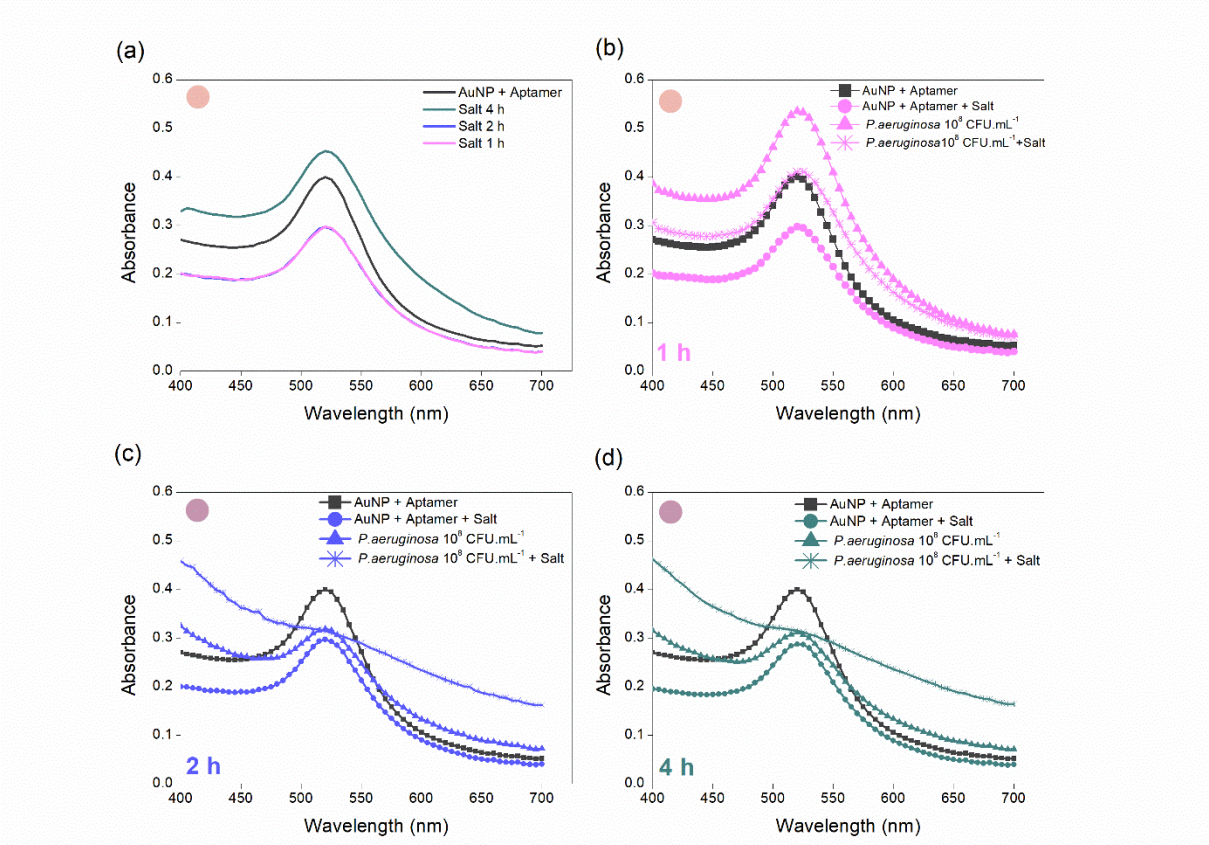

### 3.3.3 Optimization of time salt aging and aptamer concentration

To further optimize the first NaCl incubation, different times (1, 2, and 4 h) were tested. It was observed that the dispersion remained reddish color and that there was no change in the peak (520 nm) (Figure 11a). The same occurred when adding *P. aeruginosa*: there was

no change in the peak at 520 nm for the conditions studied, only in the absorbance, due to the bacteria addition. On the other hand, after the salt addition, the bacteria were detected for the incubation times of 2 and 4 h, confirmed by the extension of the curve up to 630 nm (Figure 11c,d).

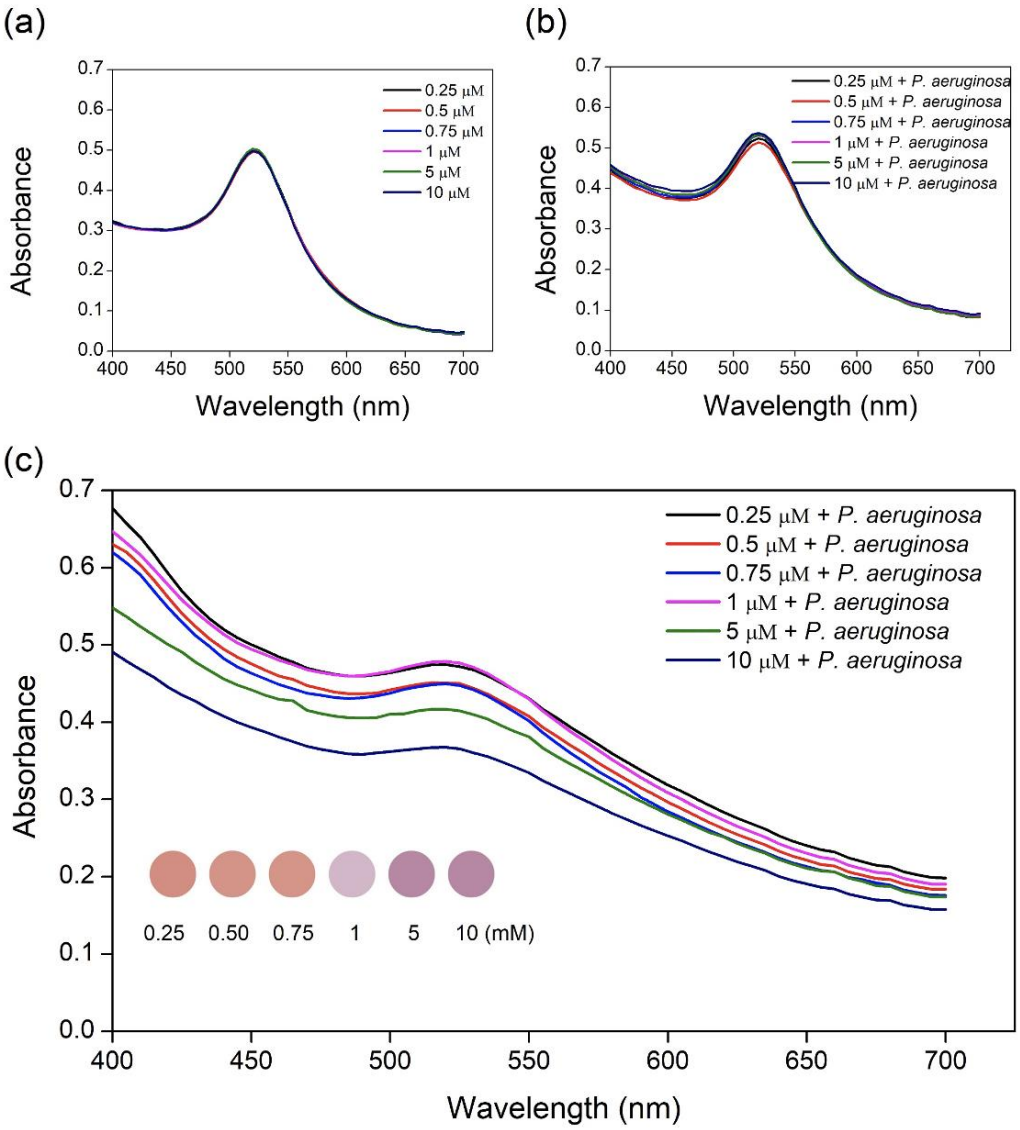
To validate the system using the concentration of 5 µM aptamers, a test with varying aptamers concentration was carried out with a subsequent addition of the target bacteria. The same procedure described before was followed, using 0.25, 0.50, 0.75, 1, 5 and 10 µM aptamers. It was seen that when incubating the target bacteria, there were no changes in absorbance (Figure 12b). Only after NaCl addition, the detection by UV-Vis occurred at all concentrations (Figure 12c). However, as shown in Figure 12d, with the naked eye, the detection occurred only at the highest concentrations of aptamers, 1, 5, and 10 µM, with a slight color change at the lowest concentration.

Figure 11 - UV-Vis spectra showing all absorption modification steps of: (a) AuNPs-aptamers with salt aging for 1, 2, and 4 h, AuNPs-aptamers-salt-*P. aeruginosa* with salt incubation of (b) 1 h, (c) 2 h, (d) 4 h. The colored balls represent the color change (reddish to purple) that occurred with the detection.



AuNPs and the target bacteria compete equally to bound with aptamers (ETEDALI *et al.*, 2022). In this way, the minimum aptamers concentration to detect the target must be investigated because aptamer is one of the three keys (NaCl, aptamers and AuNPs) to increase the method sensitivity (KIM *et al.*, 2021). High amounts of aptamers can decrease the detection sensitivity due to free aptamers in the system and due to the increase of background signal (ALSAGER *et al.*, 2018; MONDAL *et al.*, 2018). Basically, free aptamers can compete with aptamers that are adsorbed in AuNPs to the target binding (KIM *et al.*, 2021). Thus, specific optimizations are required to determine the conditions to observe colorimetric changes.

Figure 12 - UV-Vis spectra showing all absorption modification steps of: (a) AuNPs-aptamers in different concentration (b) AuNPs-aptamers-salt in presence of *P. aeruginosa* $10^8$ CFU·mL$^{-1}$, (c) AuNPs-aptamers-salt-*P. aeruginosa* ($10^8$ CFU·mL$^{-1}$) after second NaCl addition and (d) changes of colorimetric detection in presence of *P. aeruginosa* $10^8$ CFU·mL$^{-1}$.




## 3.4 CONCLUSION

To date, no studies were found using unmodified colorimetric sensors and markers to recognize *P. aeruginosa* using gold nanoparticles as identifiers and aptamers as target-binding agents. The method developed in this work is facile, rapid, and the result is detectable with the naked eye – without the need for expensive equipment such as a spectrophotometer. Moreover, pathogens can be detected without aptamer ligands or AuNPs previous treatments. Another important factor to be considered is the use of few reagents for detection, reducing the cost of

analysis, which can be accomplished in less than 5 h. The method could be optimized to decrease the time of NaCl incubation, and the three keys to detect the target was 5 μM of aptamers, 200 mM of NaCl and $8.28 \times 10^{14}$ mol·$L^{-1}$ AuNPs. In the present stage, the technique is not target specific, as the selected aptamer has affinity with the negative control bacteria (*E. coli, S. Typhimurium* and *Enterobacteriaceae bacterium*) presenting a color change from reddish to gray. Nevertheless, the developed biosensor can be used to identify hospital infections by *P. aeruginosa*, since the other detected bacteria are less common in such environments.

# 4 CONCLUSIONS AND FUTURE WORK

## 4.1 CONCLUSIONS

Regarding the experimental results, the objective was to develop a colorimetric detection method for pathogens using unmodified gold nanoparticles and unlabeled aptamers, unlike the methods found in the literature. Through the NaCl and aptamer concentration tests, the best optimization was obtained for *P. aeruginosa* detection with the naked eye by means of the color change from red to purple. The detection limit for the target was $10^5$ CFU·mL$^{-1}$ through color change, without the need to use any equipment, and $10^4$ CFU·mL$^{-1}$ when analyzed in a UV-Vis spectrophotometer. However, the method was not specific for *P. aeruginosa*, because in the specificity assays the dispersion changed from red to gray, with a difference in the UV-Vis scan, indicating the detection of *S. Typhimurium, E. coli* and *Enterobacteriaceae bacterium.* However, even with the identification of other bacteria, this method can be used to identify *P. aeruginosa* in a hospital environment, since the other recognized bacteria are less common in this environment. Moreover, those bacteria presented a different color change from red to gray, which can help differencing them from the target bacterium. In this way, it is expected that this work fills some gaps within this theme and that it can serve for other researchers to expand the possibilities to be unveiled.

## 4.2SUGGESTIONS FOR FUTURE STUDIES

Somme suggestions for future studies are given:

- Evaluate the stability of the kit developed in this methodology over time;
- Evaluate different sizes of AuNPs for *P. aeruginosa* detection;
- Verify if the methodology is able to identify contaminated samples from a hospital environment.

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# APPENDIX

Figure S1 - Dynamic Light Scattering of AuNPs and AuNPs conjugated with aptamer