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POLYTECHNIQUE MONTRÉAL

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Development of an Electrochemical Immunosensor for Detection of Staphylococcus Aureus in Prosthetic Joint Infection

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Mémoire présenté en vue de l'obtention du diplôme de *Maîtrise ès sciences appliquées*

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Ce mémoire intitulé :

Development of an Electrochemical Immunosensor for Detection of Staphylococcus Aureus in Prosthetic Joint Infection

présenté par **Zahra MARVI**

en vue de l'obtention du diplôme de *Maîtrise ès sciences appliquées*

a été dûment accepté par le jury d'examen constitué de :

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RÉSUMÉ

L'infection des prothèses articulaires est la principale cause de défaillance des implants, ce qui représente un fardeau considérable pour les patients et le système de santé. Il est essentiel de diagnostiquer l'IPP à un stade précoce, car un diagnostic tardif entraîne une infection plus étendue et des dommages à l'implant. L'identification de la cause microbienne de l'IPJ est cruciale pour déterminer la décision appropriée en matière de traitement antimicrobien. *S. aureus* est considéré comme l'agent pathogène le plus fréquent, contribuant à 19-29% des IPJ. La détection des infections bactériennes repose actuellement sur des méthodes laborieuses, coûteuses, chronophages et exigeantes en termes de compétences, ce qui rend le diagnostic difficile pour les systèmes de soins de santé et les individus. Les biocapteurs électrochimiques constituent une méthode alternative permettant d'accélérer les résultats de la détection en quelques minutes. Il a été établi qu'ils sont plus rapides, très sensibles, fiables et faciles à miniaturiser, ce qui en fait une solution attrayante pour la détection en temps réel des agents pathogènes. Dans cette thèse, un immunocapteur ultra-sensible sans étiquette a été développé pour détecter *S. aureus* par la mesure d'une métalloprotéase extracellulaire de *S. aureus*, l'aureolysine, pour la première fois. L'immunocapteur a été fabriqué à partir d'une électrode en or sérigraphiée (SPGE) modifiée par des nanopointes d'or électrodéposées. En raison de leur conductivité élevée et de leur grande surface, les nanopointes d'or ont été utilisées pour augmenter le transfert d'électrons. Un auto-assemblage d'acide 11-mercaptoundécanoïque (MUA) a été ancré à la surface de l'électrode en utilisant la chimie or-soufre. Des anticorps anti-auréolysine ont été fixés de manière covalente aux MUA sur des nanopointes d'Au, via la chimie de couplage N-(3-diméthylaminopropyl)-N'-éthylcarbodiimide chlorhydrate (EDC)/N-hydroxysuccinimide (NHS). De l'albumine sérique bovine (BSA) a été adsorbée à la surface du SPGE pour bloquer les interactions non spécifiques. La microscopie électronique à balayage a été utilisée pour confirmer le dépôt électrochimique des nanopointes, et les techniques de voltampérométrie cyclique (CV) et de voltampérométrie différentielle (DPV) ont été employées pour caractériser le capteur tout au long du processus de fabrication. La présence d'auréolysine et de *S. aureus* a été détectée en analysant la variation du

courant de crête après la formation du complexe antigène-anticorps. La mesure de l'auréolysine dans une solution tampon a été effectuée en utilisant le DPV dans une solution redox de $[Fe(CN)_6]^{3-/4-}$. La mesure de *S. aureus* dans le milieu de culture a été effectuée en utilisant la même technique et la même solution. Les résultats montrent que l'immunocapteur est capable de détecter rapidement l'auréolysine et *S. aureus* en 1 heure, dans une gamme linéaire de 1 pg/mL à 100 pg/mL avec une limite de détection de 5 pg/mL pour l'auréolysine et une gamme linéaire de 10^2 à 10^8 CFU/mL avec une limite de détection de 28,5 CFU/mL pour *S. aureus*. En outre, un test de sélectivité utilisant des interférences, *Escherichia coli* et Pseudomonas Aerugonisa, a confirmé que l'immunocapteur d'auréolysine était spécifique. L'immunocapteur développé, peu coûteux et sans étiquette, pourrait servir de test au point d'intervention, fournissant des résultats rapides et précis pour les infections à *S. aureus* telles que l'IJP en milieu clinique.

ABSTRACT

Prosthetic Joint Infection (PJI) is the leading cause of implant failure, causing considerable burden on patients and the healthcare system. It is crucial to diagnose PJI in early stages, because a delayed diagnosis causes more extensive infection and damage to the implant. Identifying the microbial cause of PJI is crucial to determine the appropriate decision for antimicrobial treatment. *Staphylococcus aureus* (*S. aureus*) is considered the most frequent pathogens contributing to 19–29% of PJIs. The detection of bacterial infections currently relies on laborious, costly, time-consuming, and skill-intensive methods, making diagnosis challenging for health care system and individuals. Electrochemical biosensors are an alternative method to expedite detection results in order of minutes. They have been established to be more rapid, highly sensitive, reliable, and easy to miniaturize, thus making them an attractive solution for real-time pathogen detection. In this thesis, an ultra-sensitive label-free immunosensor was developed to detect *S. aureus* through the measurement of an extracellular metalloprotease of *S. aureus*, aureolysin, for the first time. The immunosensor was fabricated based on a screen-printed gold electrode (SPGE) which was modified by electrodeposited gold nanospikes. Due to high conductivity and large surface area, gold nanospikes were employed to increase electron transfer. A self assembly of 11-Mercaptoundecanoic acid (MUA) were anchored on the surface of the electrode using gold-sulfur chemistry. Anti-aureolysin antibodies were covalently attached to MUA on Au nanospikes, via N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS) coupling chemistry. Bovine serum albumin (BSA) was adsorbed on SPGE's surface to block nonspecific interaction. The scanning electron microscopy was used to confirm electrochemical deposition of nanospikes, and cyclic voltammetry (CV) and differential pulse voltammetry (DPV) techniques were employed to characterize the sensor through the fabrication process. The presence of aureolysin and *S. aureus* was detected by analyzing the change in peak current following antigen-antibody complex formation. The measurement of aureolysin in buffer solution was carried out using DPV in a redox solution of $[Fe(CN)_6]^{3-/4-}$. Subsequently, the measurement of *S. aureus* in culture medium was performed using the same technique and solution. The results exhibit that the immunosensor was capable of detecting aureolysin and *S. aureus* rapidly in 1h, in a linear range of 1 pg/mL to 100 pg/mL with a detection limit of 5 pg/mL for

aureolysin and a linear range of 10^2 CFU/mL to 10^8 CFU/mL and a limit of detection of 28.5 CFU/mL for *S. aureus*. Additionally, a selectivity test using interferences, *Escherichia coli* and *Pseudomonas aerugonisa* confirmed that the aureolysin immunosensor was specific. The low-cost and label-free developed immunosensor holds the potential for a point-of-care test, providing rapid and precise results for *S. aureus* infections such as PJI in clinical settings.

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LIST OF SYMBOLS AND ABBREVIATIONS

PJI	Prosthetic Joint Infection
Ab	Antibody
AuNPs	Gold nanoparticles
Aur	Aureolysin
BREs	Biological recognition elements
BSA	Bovine serum albumin
CDC	Centers for disease control
CHA	Catalytic hairpin assembly
CLIA	Chemiluminescent immunoassay
CNFs	Cellulose nanofibers
CNOs	Carbon nano-onions
CN-PJI	Culture-negative PJI
CNPs	Carbon nanoparticles
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CV	Cyclic Voltammetry
DMSNs	Dendritic mesoporous silica nanospheres
DNA	Deoxyribonucleic acid
DPV	Differential pulse voltammetry
E	Potential

E. Coli	*Escherichia coli*
ECSA	Electrochemically active surface area
EDC	N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
EIS	Electrochemical impedance spectroscopy
ELISA	Enzyme-linked immunosorbent assay
E_p	Peak potential
FSCV	Fast scan cyclic voltammetry
GCE	Glassy carbon electrode
GNDs	Graphene nano dots
GQDs	Graphene quantum dots
HRP	Horseradish peroxidase
I	Current
I_{ap}	Anodic peaks
I_{cp}	Cathpodic peaks
J	Current density
LAMP	Loop-mediated isothermal amplification
LOD	Limit of detection
MALDI-TOF MS	Matrix-assisted laser desorption/ionization time of flight mass spectrometry
MES	4-Morpholineethanesulfonic acid
MPCR	Multiplex polymerase chain reaction
MUA	11-Mercaptoundecanoic acid
MWCNT	Multiwall carbon nanotubes

NHS	N-hydroxysuccinimide
NP	Nanoparticles
P. aerugino	*Pseudomonas aeruginosa*
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PEI	Polyethyleneimine
qPCR	Quantitative polymerase chain reaction
Rf	Roughness factor
S	Slope
S. aureus	*Staphylococcus aureus*
SAM	self assembly monolayer
SD	Standard deviation
SDA	Strand displacement amplification
SELEX	Systematic evolution of ligands by exponential enrichment
SPE	Screen printed electrodes
SPGE	Screen printed gold electrode
SPQC	Series electrode piezoelectric quartz crystal
SPR	Surface plasmon resonance
SRCA	Coupling saltatory rolling circle amplification
ssDNA	Single-stranded DNA
SWV	Square-wave voltammetry
THA	Total hip arthroplasty

Z	Impedance
Zeo	Zeolite

CHAPTER 1 INTRODUCTION

Prosthetic Joint Infection (PJI) is a severe infection that affects the tissue surrounding a prosthetic joint, leading to inflammation and infection, causing pain, swelling, and redness. It is the leading cause of implant failure and revision surgery following total knee arthroplasty (TKA) and total hip arthroplasty (THA), with an incident rate of 1.03% and 0.97% respectively [1, 2]. In severe cases, it can cause bone and tissue damage, leading to the need for prolonged antibiotic treatment, hospitalization, and revision surgery. According to the 2021 Annual Report of the American Joint Replacement Registry, more than two million primary total hip (54.5%) and knee (38.6%) replacement surgeries were performed in the United States from 2012 to 2020, with an increase of 82% in the number of recorded procedures by ambulatory surgery centers from 2012 to 2020 [3]. This increase is due to an aging population, modern lifestyle, and an increase in the prevalence of arthritis. With an increasing number of joint replacement surgeries, the risk of developing a PJI is higher and unfortunately current treatments are not always conclusive causing a considerable burden on patients and the healthcare system [4].

The treatment for PJI is challenging because of biofilm formation, antibiotic resistance surgical consideration, patient factors (i.e. comorbidities), and most of all a delayed diagnosis causing more extensive infection and damage to the implant which may require more aggressive treatment. The surgical treatment is the standard revision surgery for aseptic loosening, or a one or two-stage revision for infection, depending on the presence and identification of an infecting organism [4]. Figure 1.1 exhibits the rapid increase in total cost of patients with THA and TKA in the US [5, 6]. By 2030, the total annual hospital expenses associated with PJI in the hip and knee will reach $1.85 billion. It is crucial to diagnose PJI in early stages to prevent revision THA and TKA procedures [6].

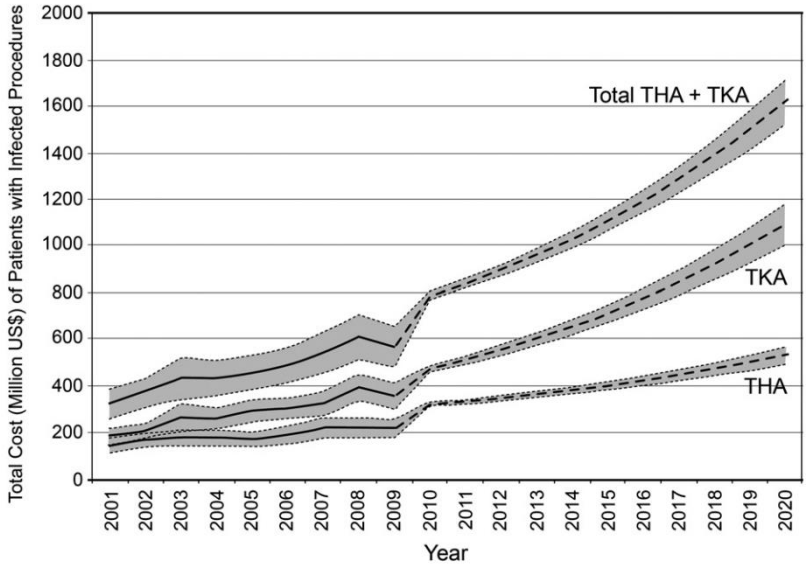

Figure 1.1 Total cost of patients with THA and TKA in the US between 2001 to 2020 [5]

Identifying the microbial cause of PJI is crucial to determine the appropriate decision for antimicrobial treatment. A previous comprehensive study reported that the majority of hip and knee PJIs involve gram-positive cocci, mainly attributed to infections caused by *S. aureus* and coagulase-negative staphylococci, which collectively are responsible for 50-60% of PJIs [7, 8]. In contrast, streptococci and enterococci contribute only to around 10% of PJI cases [7]. Given that *Staphylococcus aureus* is the leading cause of many invasive infections, notably PJI, its detection is the focus of this work.

Although current methods for detecting pathogenic infections, including plating and culturing, polymerase chain reaction (PCR) [9], and enzyme-linked immunosorbent assay (ELISA) [10], are accurate for detecting pathogens, they are not ideal for point-of-care diagnostics due to their complexity, high cost and long process when applied to clinical infections [11]. For instance, culturing and plating can take an extended period, ranging from hours to weeks, and PCR is a multi-step technique that requires additional reagents. All three methods require trained operators, which increases the cost of labor [12]. As a result, these methods are usually reserved for severe or chronic cases, and the diagnosis of clinical infections is primarily based on seasonal trends and patient symptoms, which are prone to errors due to shared symptoms. In diagnosing PJI, a combination of clinical findings such as redness, pain and swelling, laboratory results, microbiological data, histological results, intraoperative inspection, and sometimes radiographic

results are required [13]. However, there is no single test or techniques that can accurately diagnose PJI, and clinicians must choose the most appropriate approach of diagnosis for each patient based on test characteristics and relative costs. The general PJI diagnosis approach involves first determining whether the joint is infected and secondly identifying the causative microorganisms and determining their antimicrobial susceptibility [14]. It is important to note that type of joint and post-arthroplasty implantation timing may change the test performance [15].

Culture-negative PJI (CN-PJI) is as the microbiological culture results are negative with existing symptoms in patient such as periprosthetic purulence, rapid-onset inflammation or a sinus tract communicating with the prosthesis [9]. CN-PJI is an increasingly difficult condition to manage which accounts for approximately 6% of hip and knee PJIs [16]. Conventional methods for identifying the causative bacterial agent often fall short in culture-negative patients. Newer technologies, including advanced culturing methods [17] and next-generation sequencing [18], are essential for accurately identifying the infectious agent and prescribing appropriate antibiotic treatment. The high incidence rate and unfavorable treatment outcomes associated with culture negative PJI highlight the critical need to isolate the infectious agent before surgical intervention. Approaches such as prolonging the incubation period for culture samples [19], refraining from administering antibiotics until samples have been obtained, and utilizing molecular techniques can aid in identifying the infectious agent, leading to better outcomes for patients with culture-negative PJI [7].

The development of PJI is strongly associated with the formation of biofilms [20], which are comprised of a community of microorganisms embedded within an extracellular polymeric substances (EPSs) and growing on a surface [21]. The extracellular matrix consists of polysaccharides, polypeptides, and/or extracellular DNA, which vary in composition depending on the type of organism present [22]. Biofilms can be either monomicrobial or polymicrobial and exhibit a greater resistance to antibiotics and the host immune system than free-floating bacteria [23]. Consequently, treatment of infections involving biofilms can be challenging and requires a biofilm-directed treatment strategy. Apart from impacting treatment outcomes, biofilm formation also affects the diagnosis of PJI, particularly in cases with delayed or late-onset presentation. In such cases, the bacteria responsible for PJI tend to concentrate on the surface of the prosthesis,

which makes them difficult to detect in fluid or tissue samples taken from the surrounding area. To address this issue, sampling of the biofilm on the prosthesis surface, followed by using techniques such as vortexing-sonication, has been proposed as a method of improving diagnostic accuracy [24]. Challenges in identifying the causative bacteria of PJI often result in unnecessary prescriptions of antibiotics and the emergence of antibiotic-resistant bacterial strains. The Centers for Disease Control and Prevention (CDC) has reported over 2.8 million cases of antibiotic-resistant bacterial infections in the United States in 2019, causing the death of more than 35,000 individuals [25]. Therefore, there is a critical need to develop point-of-care diagnostic tools that can detect and monitor PJI in its early stages, reducing economic burden and the morbidity and mortality rates associated with the disease. In order to design an effective point-of-care diagnostic tool for an infectious disease, it is necessary to take into account a variety of factors. The diagnostic tool must possess high levels of sensitivity and specificity, enabling it to produce accurate and prompt results at a reasonable cost. Moreover, it should be user-friendly, easily transportable, and dependable, withstanding a range of environmental conditions while delivering consistent outcomes. Ensuring compatibility with the current healthcare infrastructure and complying with regulatory standards are also crucial aspects to consider during the design process.

Biosensors have gained significant attention in pathogen diagnosis in recent years, owing to their capability to rapidly, sensitively, and specifically detect pathogens. The development of new recognition elements and detection platforms in biosensor technology has facilitated the creation of highly sensitive and specific biosensors for pathogen diagnosis. Additionally, the emergence of portable and user-friendly biosensors has broadened their potential utilization in remote or resource-constrained areas where infectious diseases are prevalent. Bacteria release a wide range of compounds, varying from secondary metabolites to extracellular enzymes, which can be utilized for detecting and recognizing the existence of bacteria [26]. Aureolysin is a metalloprotease enzyme produced by *S. aureus* that plays a role in breaking down host tissues and aiding in the spread of the bacteria within the host [27].

This research aims to design and develop an electrochemical biosensing device for the rapid, accurate, and easy detection of *Staphylococcus aureus* through the detection of Aureolysin. The device will meet the criteria of low limit of detection and clinically relevant detection range, allowing for the early detection of PJI and other *S. aureus* infections at a low cost. The device has

the potential to be used as a point-of-care diagnostic tool for *Staphylococcus aureus* and other *S. aureus* infections, ensuring safety and effectiveness in clinical settings. Chapter two consists of a literature review on biosensors, with a specific focus on electrochemical biosensors for detecting pathogens. This will be followed by an outline of the research objectives in chapter three. Chapter four presents the submitted manuscript to the journal of biosensors and bioelectronics, and the thesis concludes with an overall perspective and a discussion on limitation and recommendation.

CHAPTER 2 LITERATURE REVIEW

2.1 Gram-positive *Staphylococcus aureus*

Staphylococcus aureus, commonly known as *S. aureus*, is a gram-positive, non-spore-forming, facultative, anaerobic, and round shape bacterium which was firstly discover by Dr. Alexander Ogston in 1880 (Figure 2.1) [28]. According to Argudín et al. [29], *S. aureus* is a bacterium that occurs naturally in the skin, mucous membranes, and nasal flora of humans. Research indicates that approximately 30-50% of the overall population are carriers of this bacterium. *S. aureus* is considered the one of the most common causes for wide range of hospital-acquired infections and foodborne illnesses and it is the most frequent pathogens contributing to 19–29% of PJIs [30]. Despite numerous attempts, there has been no successful development of a vaccine specific to *S. aureus*. As a result, the only approach to reduce the morbidity and mortality associated with staph infections is through early diagnosis to guide appropriate treatment decisions [31].

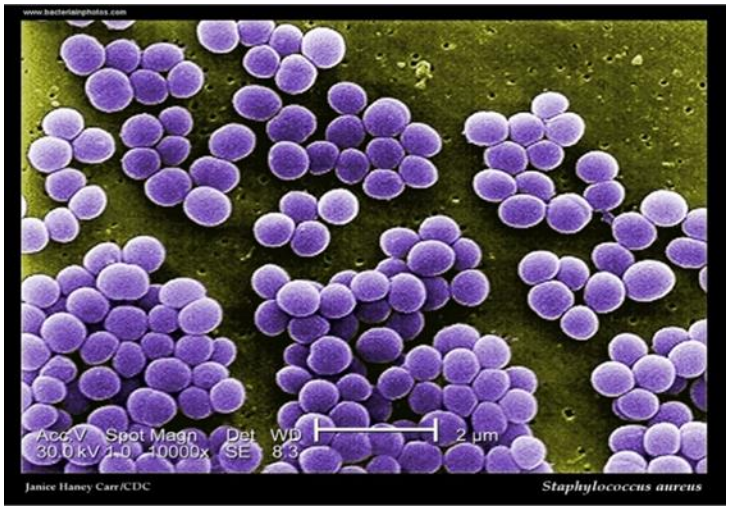

Figure 2.1 Scanning electron micrograph of *S. aureus* provided by Centers for Disease Control and Prevention

2.2 Conventional diagnosis approaches for *S. aureus* infection`

2.2.1 Conventional bacterial culture

The diagnosis of *S. aureus* infectious diseases typically involves the extraction and identification of the infectious agent through culture-based methods including enrichment cultures and the use

of selective and differential media [32]. Briefly, after collection of biological specimens (sputum, bodily fluids or tissues, samples are cultured and incubated under appropriate conditions to allow bacterial growth. Identification of bacteria is carried out using standard biochemical tests and/or molecular techniques. These diagnostic procedures are essential for differentiating and isolating *S. aureus* from other bacterial pathogens using wound, tegument, and soft tissue specimens. However, despite their high accuracy, these methods are labor-intensive, laboratory-sensitive and time-consuming, often taking from 48 hours to 7 days for turnaround time [33]. To expedite the detection of the pathological agent, biochemical and molecular assays are often used in conjunction with culture-based methods [34].

2.2.2 Immunological assays

The principle of immunoassay technique is based on the specific affinity between antigens and antibodies [35]. The most commonly used immunoassay method is the enzyme-linked immunosorbent assay (ELISA), which is widely used to detect *S. aureus*, other bacteria, fungus and their toxins [10]. A modified version of ELISA, known as sandwich ELISA, utilizes two antibodies for a single antigen and amplifies the signal, resulting in higher sensitivity and specificity [36]. ELISAs have become one of the main tools for detection of toxigenic bacteria rapidly [10]. Researchers have developed new kits, such as the SEA detection kit for milk, which has a detection time of about 15 minutes and a sensitivity of 15.6 ng of toxin [37], and a sandwich chemiluminescent immunoassay (CLIA) to detect SEB, which has a detection limit of 1.44 ng/ml [38]. Although ELISA has high sensitivity and short detection times compared to traditional culture-based methods, it falls short of real-time pathogen detection. Other limitations are the risk of cross-contamination which leads to false positive and their low stability. They often need a large amount of sample to effectively detect bacteria and their toxins [10].

2.2.3 Biomolecular assays

Nucleic acid-based techniques utilize the DNA or RNA of a pathogen target to achieve specificity. Over years, nucleic acid-based methods have been divided into various categories, such as polymerase chain reaction (PCR), multiplex polymerase chain reaction (MPCR), quantitative polymerase chain reaction (qPCR), and loop-mediated isothermal amplification (LAMP) where PCR is the most commonly employed method for detecting pathogens by amplifying nucleic acids through thermocycling. For instance, Martinon et al. developed a dual-target real-time PCR assay

using SYBR Green to simultaneously detect *Listeria monocytogenes* and *S. aureus* in food [39]. The detection limit for *S. aureus* in raw meat was 2 CFU/g. However, PCR-based assays alone cannot distinguish between living and dead cells since they do not differentiate between DNA from both. The specificity of PCR is dependent on primer and template DNA binding; thus, it can directly detect *S. aureus* at the gene level without cultivating the fungus. This technique has successfully detected a variety of pathogens in a more precise, quicker, and more sensitive manner, but is more costly than culture-based technique [40, 41].

2.3 Emerging technologies

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) is a new emerging technology to identify biomolecules in clinical microbiology [42]. MALDI-TOF MS is found to be very effective in detection and identifying bacteria such as *S. aureus* [43].The technique is based on utilizing an ionizing laser to strike a matrix of organic molecules and ionize an analyte. The gaseous ions formed by the ionizing laser will be sorted with mass spectrometry based on their mass-to-charge ratio [41]. Although the initial investment in a mass spectrometer is relatively high, MALDI-TOF MS offers rapid, less than a day, and accurate diagnostics that are less expensive than previously used molecular and immunological assays and do not require trained laboratory personnel. Due to its high initial cost, MALDI-TOF MS can not serve as a point of care tool to detect *S. aureus* in real-time [44]. Therefore, there is a continual need for a rapid, highly sensitive, and cost-effective technique to fill the current gap in point-of-care diagnosis of *S. aureus*. One promising alternative is biosensors, specifically electrochemical biosensors, which offer rapid response, ease of fabrication and ease of use, flexibility, affordability, sensitivity, and selectivity, in contrast to conventionally used methods. Thus, electrochemical biosensors have the prospect of serving as effective point-of-care diagnosis tests for *S. aureus* infections. Following is a comprehensive review of biosensors' classification and working principles, application of electrochemical techniques in bacterial detection and different types of electrochemical detection of *S. aureus*.

2.4 Biosensors

Biosensors are analytical devices that employ a biological sensing element and a detector element to convert chemical and biological information into a readable electrical signal (Figure 2.2). There is a wide range of biological recognition elements (BREs) that can be used to capture the target,

including enzymes, antibodies, amino acids, nucleic acids, cells, and more. The conversion of the signal to a readable electrical signal output is carried out by a transducer. The detector element works based on change in physicochemical properties such as optical, piezoelectric, and electrochemical principles. Usually, the biological element is attached to the transducer to effectively transmit the biological information between the target and BRE in a measurable output [45].

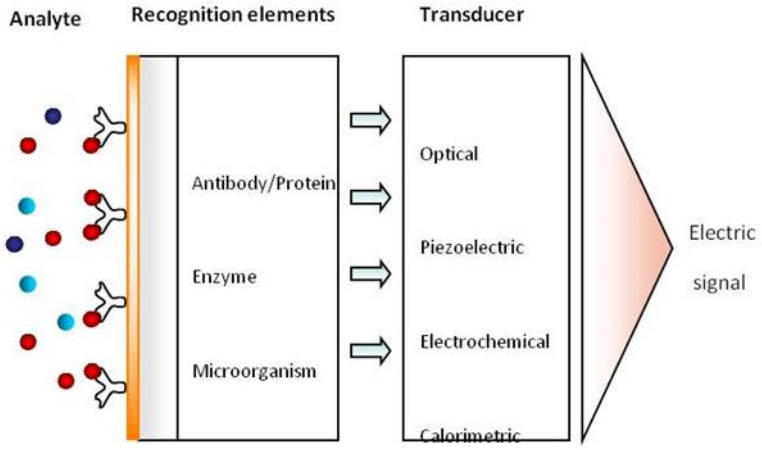

Figure 2.2 General schematic of a biosensor, its components and its different types [46].

Biosensors' sensitivity, portability, rapidity, real-time monitoring, and cost-effectiveness make them a valuable option for a wide range of applications, including but not limited to medical diagnosis, environmental monitoring, and biotechnology [47].

2.4.1 Design specifications of point-of-care biosensors

To develop a effective biosensor for point-of-care use in monitoring and detecting disease biomarkers and bacteria, it is necessary to satisfy the following specifications and requirements [48]:

1. Specificity: The ability to selectively react to a particular target, while disregarding the presence of other interfering substances in the the environment, is one of the key characteristics of

a biosensor. It is crucial to ensure that a biosensor is not susceptible to false-positive outcomes or cross-reactivity. Therefore, selecting a highly specific antibody or other appropriate biorecognition element is vital for achieving the desired level of selectivity in the biosensor platform.

2. Sensitivity: The sensitivity of a biosensor is determined by its ability to detect the minimum amount of analyte, known as the limit of detection (LOD). In medical monitoring applications, concentrations of biomarkers in clinical samples can be as low as ng/mL or fg/mL. An example is the detection of breast cancer biomarker MMP-1, where the concentrations are in order of ng/mL [49].

3. Reproducibility: The ability to maintain low variations in duplicated assays is reproducibility. A reproducible biosensor is accurate when compared to real values and precise when multiple tests are carried out on the same sample.

4. Stability: A biosensor is required to be independent of ambient disturbance including temperature and pH in a biosensing environment such as human blood. This is important, when the bioreceptor degrades or loses its affinity by change of temperature or over time. Stability is a design factor that needs to be considered based on estimated storage time and lifespan of the device.

5. Detection time: The period of time between receiving a new input parameter and the final stable output is biosensor response time. A point of care tool should be able to analyze human samples in real-time with response time as low as less than an hour to compete with other currently diagnosis techniques.

6. Ease of use: One of the considerations in designing biosensors is that the device should be easy to use for nonprofessional and nonskilled users. Therefore, miniaturized devices with simple instruction and no pre-treatment steps are desirable as a commercially viable assay.

7. Cost effectiveness: Cost-effectiveness: The cost of a biosensor device is influenced by factors such as the type of bioreceptor and transducer, the device's size, and the sample. Cheaper biosensing platforms with no need for bulky and expensive equipment are more attractive for commercialization, especially in medical diagnosis where point of care tests are mostly disposable.

These are some essential features, but not the only ones. Other factors such as linearity, signal-to-noise ratio, ease of fabrication, and portability are also important considerations.

2.4.2 Classification of biosensors

Biosensors can be classified based on either their biological components or transducers. An overview of the different biorecognition and detection elements commonly utilized in biosensors can be seen in Figure 2.3. The figure depicts a biosensor design as a versatile tool for rapid detection of analytes. To capture a specific target, different choices of bioreceptor such as enzymes, aptamers, DNA and peptides can be employed and combined with different transducers including optical, piezoelectric, calorimetric, and electrochemical. The choice of transducer and bioreceptor depends on the specific reaction or biological target being analyzed. Nanomaterials of different kinds such as metal nanoparticles are used to enhance biosensors' performance (Figure 2.3).

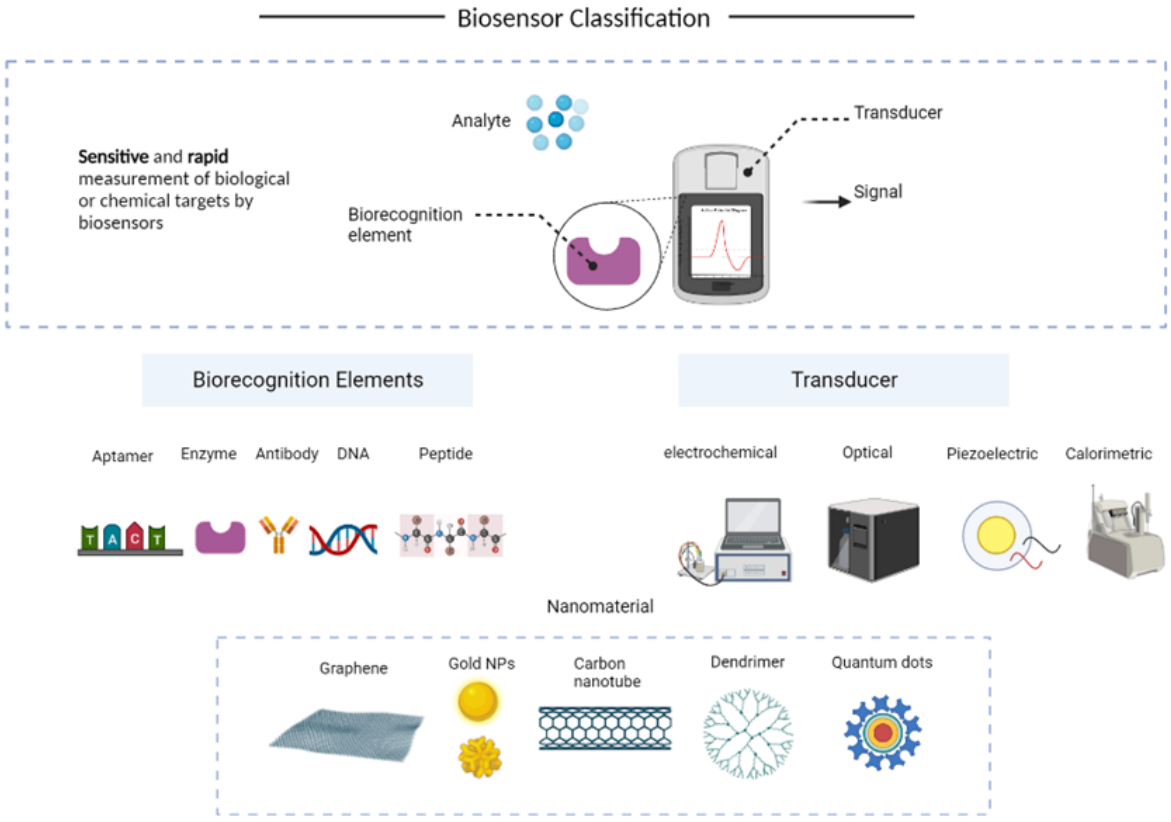

Figure 2.3 Components and classification of biosensors.

2.4.3 Optical biosensors

One particular type of biosensor is the optical sensors, which utilize surface plasmon resonance (SPR), fluorescence, UV-vis absorption, or chemiluminescence to convert biological information into changes in optical properties, including frequency, amplitude, phase, or polarization of input light [45]. The different optical sensors are presented in Figure 2.5 including SPR and OWLS. Several optical biosensors have been developed and applied for detection of pathogens including *S. aureus*. Development of a quantum dots immunofluorescence sensor was reported by Cui et al (Figure 2.4). This optical biosensor can identify *S. aureus* from other pathogenic bacteria and detect its presence at concentrations as low as 1×10^3 CFU/ml [50]. Optical sensors provide advantages such as high accuracy and fast measurement [51]. However, their miniaturization can be challenging due to the need for an optical detection system, transmission system, and light source in the device.

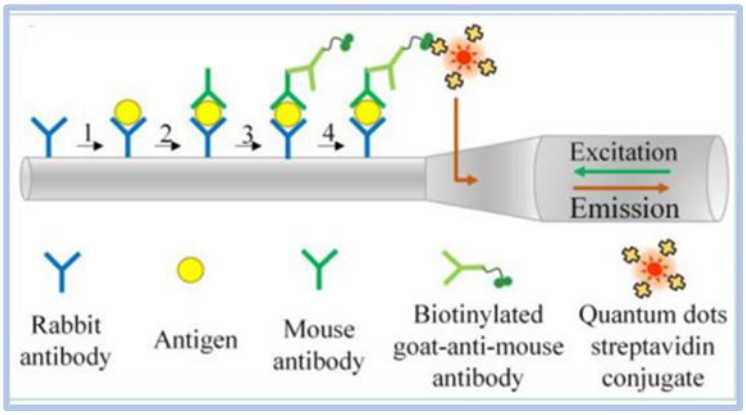

Figure 2.4 Working principle of a fiber probe to detect *S. aureus* [50].

2.4.4 Piezoelectric biosensors

The piezoelectric effect occurs when specific materials can generate an electrical charge upon mechanical stress. In a piezoelectric biosensor, a thin layer of piezoelectric material, such as quartz crystal or ceramic, is coated with a biorecognition element, such as an enzyme or antibody. When a target molecule interacts with the biorecognition element on the surface of the sensor, a mass change occurs, which causes a mechanical deformation of the piezoelectric layer. This deformation generates an electrical charge proportional to the mass change, which is detected by electrodes

attached to the surface of the piezoelectric material. By measuring the electrical response, the presence and concentration of the target molecule can be determined [52]. Piezoelectric biosensors are widely used due to their high sensitivity, low cost, and ease of use. However, the properties of piezoelectric material are highly sensitive to environmental factors such as change in pH, humidity, and temperature [53]. Use of a piezoelectric biosensor for detection of *S. aureus* was published in 2015. *S. aureus* aptamers were immobilized to graphene integrated gold electrodes connected to a series electrode piezoelectric quartz crystal (SPQC). The LOD and linear detection range were reported 41 CFU/mL and 4.1×10 - 4.1×10^5 CFU/mL, respectively [54].

2.4.5 Electrochemical biosensors

In nature, many biological information is transferred through electrochemical signals. The principle of an electrochemical biosensor involves a (bio)electrochemical redox reaction that generates electron. This electron transfer could be recorded as a measurable current, potential, or change in impedance of the medium. Based on these three different common output electrochemical sensors categorize into amperometric, potentiometric and impedimetric, respectively [55]. Figure 2.5 displays different electrochemical techniques such as voltammetric, amperometric, impedimetric and potentiometric. Electrochemical transducers are predominantly used in biosensing application, because they have higher potential to realize a non-invasive, label free and real-time measurement of biological analytes in a wide range of biological medium such as urine and human blood. As a prime example, glucometer is a commercially available hand-held medical device using amperometry for measuring the glucose level in blood which is an essential part of diabetes management [56]. Another electrochemical hand-held analyzer is Lactate Scout (Sports Resource Group, Inc.) that monitors lactate level in blood for performance improvement during physical activity [57]. In the past few years, advances in bioelectronic facilitate development of flexible electrochemical biosensors in different forms such as wearable skin patches, temporary tattoos, contact lenses, denture using sweat, tears and saliva for on-body monitoring [58].

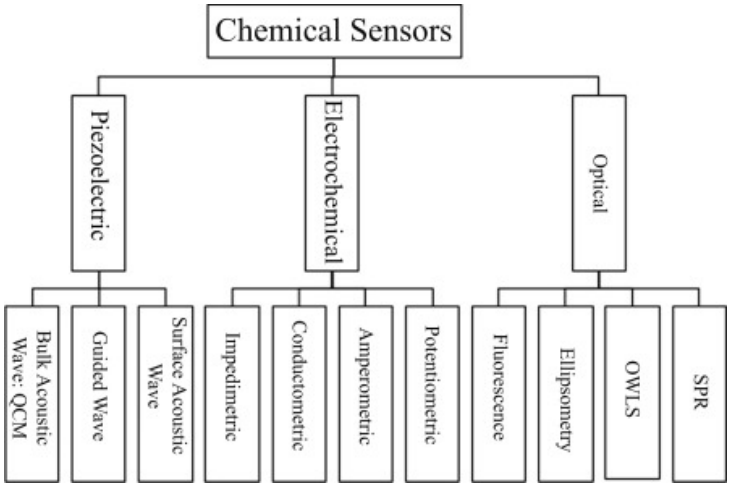

Figure 2.5 Classification of sensors based on their transducer [45].

2.5 Electrochemical detection of bacteria

Electrochemical biosensors offer versatility and flexibility, as they can be tailored and adapted to detect specific target pathogens. Extensive research efforts have been focused on developing electrochemical detection methods for bacterial detection, utilizing a wide range of bioreceptors such as antibodies, aptamers, DNA, and enzymes [59]. To achieve accurate and reliable detection, various strategies have been employed, including targeting the entire bacterial cell, bacterial DNA, toxins, metabolites, and secreted enzymes [60]. Recent advancements in nanotechnology have significantly improved the sensitivity of the electrochemical platforms by incorporating conductive nanostructures to increase the electron transfer and the surface area and consequently the number of immobilized biorecognition elements [61]. It includes metal nanoparticles like gold and silver [62], carbon nanostructures such as carbon nanotubes and graphene [63], and quantum dots [64] and conductive polymers such as polyaniline to increase the conductivity [65], electroactive surface area, and electrocatalytic activity. Ongoing investigations continue to push the boundaries of this technology, driving the development of novel biosensors with improved performance and expanded capabilities for bacterial detection. In the following section, a comprehensive discussion of the electrochemical systems and their different measurement techniques will be provided.

2.5.1 Three-electrode systems

The electrochemical biosensors mostly function as a three-electrode cell consisting of working electrode or known as sensing electrode where the reaction of interest takes place, reference electrode and auxiliary or counter electrode. The roles of reference and counter electrodes are maintaining a known and stable potential in the system and establishing electrical circuit between the working electrode and the electrolyte, respectively (Figure 2.6). The electron transfer resulting from redox reaction occurs in proximity to the surface of the working electrode therefore electrode's material, surface geometry and dimension and surface modifications plays a significant role in sensitivity and performance of the device [66]. Screen printed electrodes (SPEs) are the class of three electrodes systems where all three working, reference and counter electrodes are integrated in a plastic or ceramic substrate using conductive ink [67]. They have been repeatedly used in biosensor and diagnosis research due to their low cost, portability, commercial availability, and flexibility to various modifications. They are produced in different materials based on the target application including gold, platinum and carbon [67].

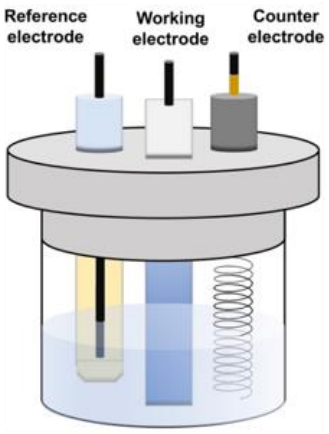

Figure 2.6 Schematic of a conventional 3-electrode cell in electrochemical biosensing [68].

2.5.2 Amperometric techniques

Amperometry is a widely used technique that measures the current (I) resulting from an oxidation or reduction reaction. The current value can be recorded in either a constant potential mode or a controlled potential mode, where potential varies in a controlled way, commonly referred to as

voltammetry. In the latter case, the bulk concentration of redox-active species can be determined by the peak current measured within a linear range of potential [66]. The biorecognition element specifically attaches to the targeted analyte, generating an electrochemical signal that can be measured using an amperometric electrode. The intensity of this signal is directly related to the concentration of the target analyte present in the sample. The next sections will focus on the most commonly employed voltammetric techniques in electrochemical detection of bacteria.

2.5.3 Cyclic voltammetry

Cyclic Voltammetry (CV) is a conventional electroanalytical technique in biosensing applications involving the measurement of the current response (I) in an electrochemical system as a potential (E) is scanned back and forth between two limits. Several factors influence the current response, including the scan rate (V/s), electrode properties, and solution properties [69]. Figure 2.7 A cyclic voltammogram for a reversible redox reaction with the linear change in potential by time (inset) [72].shows a typical cyclic voltammogram. CV is particularly useful for studying redox reactions involving changes in oxidation state. The resulting current response corresponds to the transfer of electrons during the electrochemical reaction at the working electrode. Analysis of the shape and magnitude of the cyclic voltammogram provides information about the electroactive species, such as its oxidation potential, diffusion coefficient, and concentration.

CV has been explored as a potential tool for detecting bacteria by functionalizing the electrolytic cell with a capture probe specific to the target bacteria [70]. Binding of the target bacteria to the capture probe induces a change in the redox properties of the system, which can be detected by CV. Studies have shown that CV-based detection of *S. aureus* has the potential to be rapid and sensitive, with a detection limit as low as 2 CFU/mL [71]. However, further research is needed to optimize the capture probe and electrode properties to improve sensitivity and specificity for bacterial detection using CV.

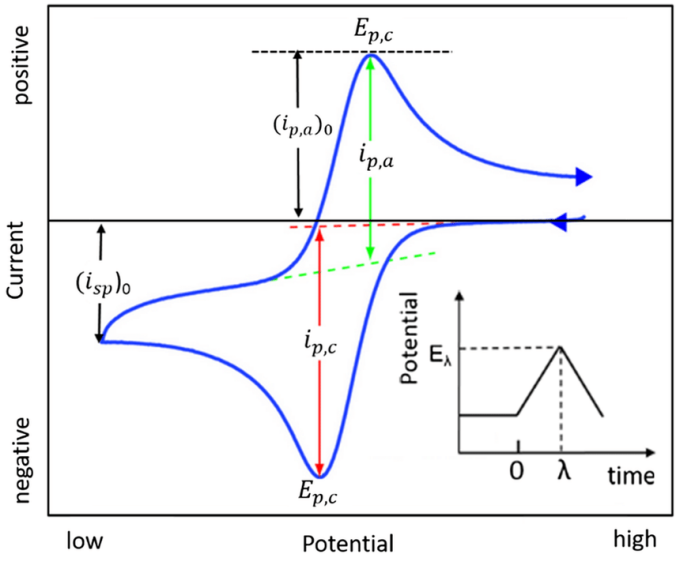

Figure 2.7 A cyclic voltammogram for a reversible redox reaction with the linear change in potential by time (inset) [72].

2.5.4 Square wave voltammetry

In Square-wave voltammetry (SWV) the working electrode's current is measured while a combined square wave and staircase potential applied to the working electrode [73]. It is usually used in conjunction with CV, offering higher sensitivity and wider linear detection range. For instance, in a study, ruthenium hexamine ($[Ru(NH_3)_6]^{3+}$) was used as a probe to enable real-time electrochemical monitoring of a LAMP amplicon of target genes of *S. aureus* and *E. Coli* using SWV. The technique successfully detected and quantified the amplicons in less than 30 minutes with a sensitivity of up to 30 copies μ/L for *S. aureus* and 20 copies μ/L for *E. Coli* [74].

2.5.5 Differential pulse voltammetry

Due to its ability to remove background charging current, resulting in higher sensitivity and a lower limit of detection, Differential pulse voltammetry (DPV) has proven to be a valuable tool for the electrochemical detection of bacteria. DPV is another widely used electroanalytical technique in biosensing applications, which consists in measuring the current response (I) in an electrochemical system as small amplitude pulses are applied on a linear ramp potential [75]. Figure 2.8 exhibited measurement of a target and obtained calibration curve using DPV.

Mathur et al. introduced a low-cost electrochemical paper-based analytical device (EPAD) (shown in Figure 2.9) for detection of *S. aureus* using graphene nano dots (GNDs) and zeolite (Zeo)

integrated with a specific DNA probe [76]. A linear current response with a limit of detection of 0.1 nM of target DNA was obtained using DPV technique in fruit juice samples.

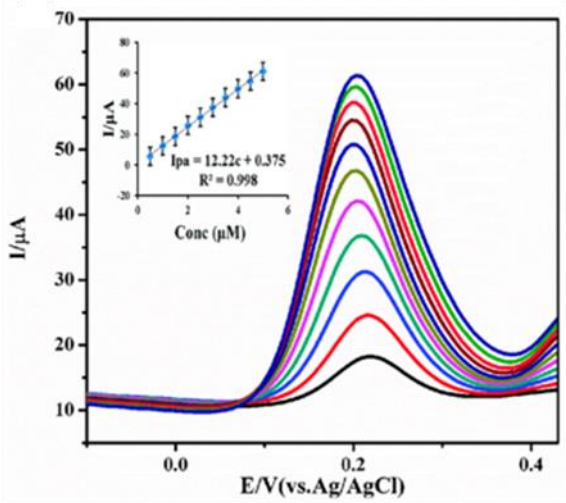

Figure 2.8 Differential pulse voltammogram responses to a different range of analyte concentrations. The inset is linear regression of DPV response vs analyte concentration [77].

Another example of DPV application in *S. aureus* detection is a simple method that uses a surface imprinted matrix, polyethyleneimine (PEI), as a molecular recognizer on a manganese dioxide thin film decorated with solver coating fluorine-doped tin oxide electrode [78]. The presence of bacteria leads to a change in DPV peak showing the dynamic range of detection of 10^3 to 10^7 CFU/mL.

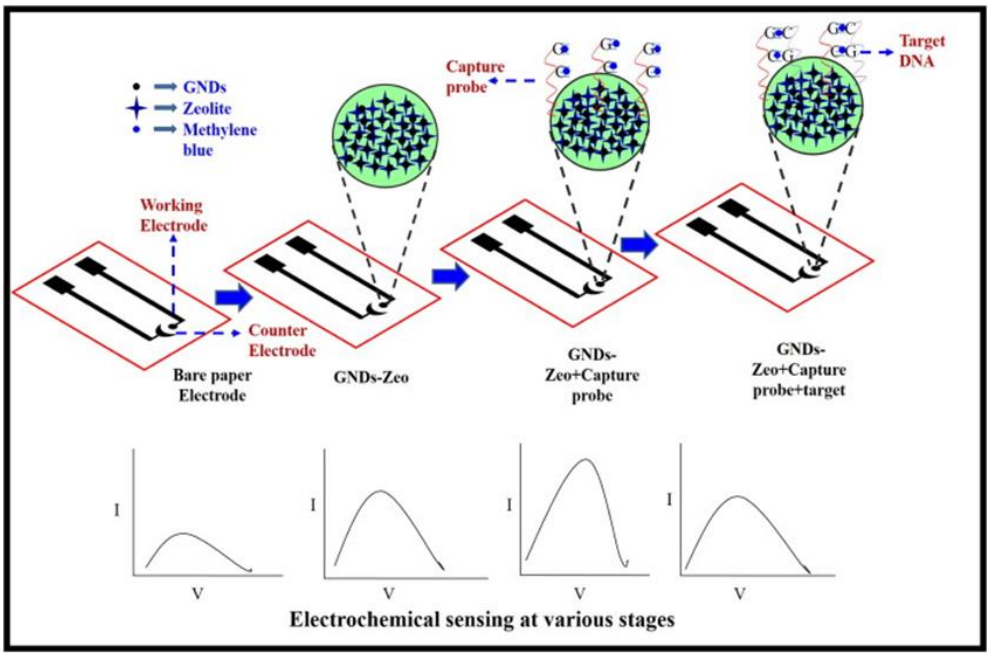

Figure 2.9 Development of the GNDs-Zeo /PAD, ssDNA/GNDs-Zeo/PAD and dsDNA/GNDs-Zeo/PAD and their corresponding DPV [76].

2.5.6 Electrochemical Impedance Spectroscopy

Electrochemical impedance spectroscopy (EIS) is a sensitive and powerful technique for the study of the interfacial properties including conductivity and resistivity. The technique is able to analyze mass and charge transfer and diffusion processes [79]. The small excitation, sinusoidal signal function, is applied to the electrode, and then the impedance (Z) response is calculated through voltage to current ratio. Due to this alteration a phase shift is acquired between I and E [80]. The obtained impedance is expressed by Nyquist Plot where X-axis is Z_{real} and Z_{imag} is plotted on Y-axis (Figure 2.10). The application of EIS in bacterial biosensing has been reported previously. In a study, an impedimetric immunosensor based on a self-assembly monolayer of 16-mercaptohexanoic acid was presented for rapid measurement and detection of stressed pathogenic *S. aureus* [81]. The recorded Nyquist Plots upon attachment of *S. aureus* to the antibody at different concentrations was curve fitted using Randle circuit resulting the LOD and linear range of 10 CFU/mL and 10^1 to 10^7 CFU/mL, respectively.

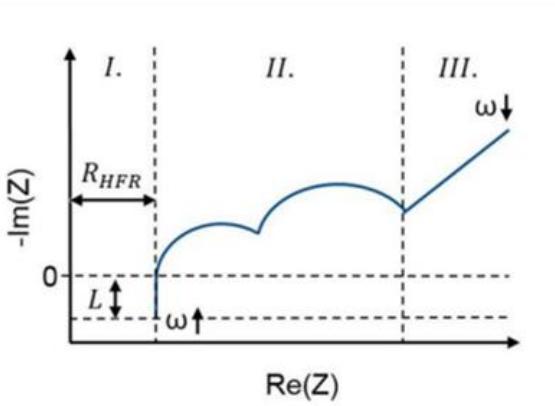

Figure 2.10 Nyquist plot representing impedance obtained at different frequencies [82].

2.6 Electrochemical detection of *S. aureus*

2.6.1 Electrochemical aptasensor for *S. aureus* detection

Aptamers are a class of oligonucleotide sequences that exhibit high specificity towards a diverse range of targets, including proteins, antibiotics, small molecules, peptides, nucleotides, and even whole cells [83]. This specificity is enabled by intermolecular interactions such as hydrogenic bonds, which facilitate selective recognition of the target. Aptamers are developed through a technique called systematic evolution of ligands by exponential enrichment (SELEX) [84], which involves the isolation of specific oligonucleotides from a random library of ssDNA or RNA molecules, typically comprising around 10^5 sequences, that are screened against the target of interest [85]. The SELEX process involves iterative rounds of binding, washing, and elution, with the goal of enriching the library for aptamers with high affinity and specificity for the target. This section provides a concise overview of recent advances in the aptamer-based electrochemical detection of *S. aureus*.

A dual-aptamer-based sandwich biosensing technique was developed by Abbaspour et al. to detect *S. aureus* in tap and river water. The technique involved the use of anti-*S. aureus* aptamer-conjugated magnetic beads to capture the bacteria, followed by the use of aptamer-conjugated AgNPs to provide an electrochemical signal [83]. The peak current of differential pulse voltammetry increased with the initial concentration of *S. aureus* in the samples. This sensor can measure samples containing 10 to 10^7 CFU/ml, with a LOD of 1 CFU/ml. Chen et al. designed an

electrochemical aptasensor for detecting *Staphylococcus aureus* in whole blood samples using a catechol-chitosan redox capacitor. The methodology involved the cathodic deposition of chitosan, succeeded by anode grafting of catechol with Fc-Ru^{3+} double mediator, resulting in a redox capacitor-based electrochemical system (Figure 2.11) [71].

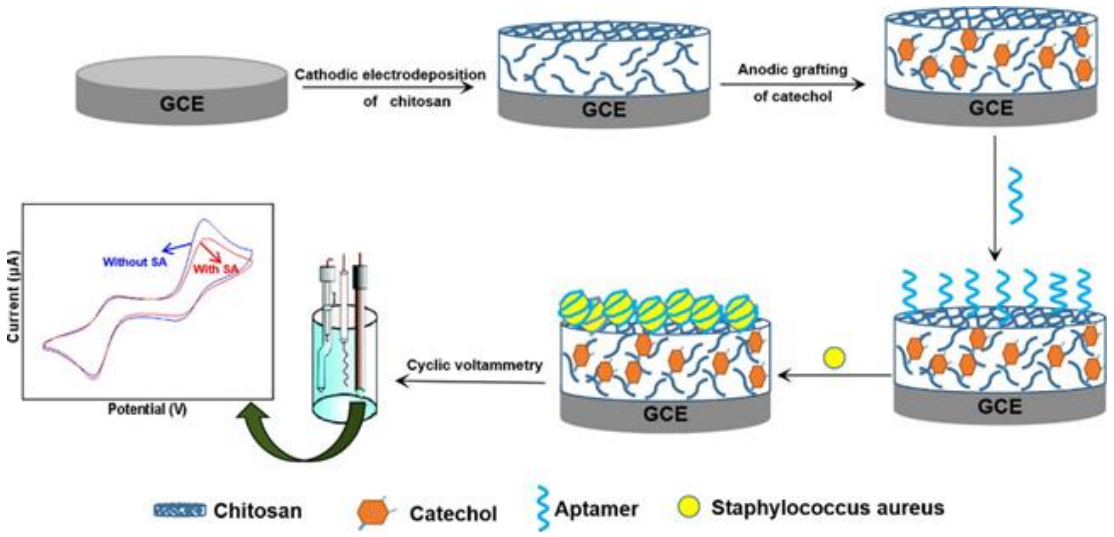

Figure 2.11 Schematic representation of aptamer immobilization on chitosan-catechol-glassy carbon electrode and electrochemical detection of *S. aureus* [71].

Another detection technique based on dual-aptamer-based sandwich aptasensor developed by Gao et al. introduced the detection of *S. aureus* with EIS. An aptamer modified anti-fouling film, sulfonated polyaniline, was electro-plated on a glassy carbon electrode to specifically capture *S. aureus*. Then the detection signal was amplified by aptamer coated highly conductive gold nanoparticles. The developed biosensor exhibited the limit of detection of 2 CFU/mL [86]. In 2022, the work of Sohouli et al., continued the electrochemical biosensing of *Staphylococcus aureus* via a sensitive aptasensor comprised of nitrogen-doped carbon nano-onions (CNOs) and screen printed electrode coupled with gold nanoparticles [87]. CNO has received interest as a biosensing material due to its high conductivity, low toxicity, and high biocompatibility. However, they have low energy storage capacity [88] and low dispersibility in solvents [89]. This study targeted *S. aureus*, conducting electrochemical studies in human serum samples with a limit of detection of 3 CFU/mL. Ranjbar et al. reported the use of nanocomposite composing of cellulose nanofibers (CNFs), carbon

nanoparticles (CNPs) and gold nanoparticles (AuNPs) for an electrochemical impedance spectroscopy based aptasensor. The platform was able to detect live *S. aureus* cells in complex mediums such as human blood serum with a wide dynamic detection range of 1.2×10^1 to 1.2×10^8 CFU/ mL and limit of detection of 1 CFU/mL [90].

A recent study reported the development of an electrochemical signal-on biosensor based on sandwich of cognate aptamers pair for detection of *S. aureus*. The aptamers were conjugated onto a commercially screen printed electrode, enabling simultaneous binding to the target. The resulting chronoamperometry responses to the horseradish peroxidase (HRP) connected to the secondary aptamer leads to limits of detection of 39 and 414 CFU/ mL respectively, in buffer and tap water [91].

Tian and his co-workers proposed a new electrochemical aptasensing strategy for detecting *S. aureus* using copper-based metal-organic framework encapsulated with Cu_2O nanocrystals for food safety assessment. The ML-Cu_2O@Cu-MOF nanospheres showed strong anchoring ability toward aptamer and increased electrical conductivity, resulting in limit of detection of 2.0 and 1.6 CFU/mL using EIS and DPV, respectively [92]. A novel stepwise signal amplification was introduced by Zhou and his team for the development of a *S. aureus* electrochemical aptasensor. The amplification was carried out first by enzyme-mediated cyclic cleavage following by a cycle of catalytic hairpin assembly (CHA). The third-level signal amplification was done via fast scan cyclic voltammetry (FSCV) to achieve a linear range of $1 \sim 10^8$ CFU/mL and a detection limit of 0.3 CFU/mL [93].

Aptamers have emerged as a promising agent in biorecognition assays, due to favorable characteristics, including their small size, ease of modification, high shelf life and relative cost-effectiveness. In recent years a substantial collection of aptamers targeting diverse analyte has been developed by SELEX. However, despite this progress, there are still numerous analytes for which aptamers have not been synthesized, making the use of aptamer-based assays unfeasible for their detection.

2.6.2 Electrochemical Immunosensor for *S. aureus* detection

Antibodies are one of the most important biorecognition elements in bioanalytical systems [94]. Immunosensor rely on immobilized antibodies on electrode surfaces to achieve high selectivity by

forming high-affinity antibody-antigen complexes. The anti-*S. aureus* antibody is a biorecognition element that specifically targets *S. aureus* and has been extensively used for design of *S. aureus* electrochemical immunosensors.

Recently, a study reported utilizing graphene quantum dots (GQDs) in electrochemical assays for detecting *S. aureus*, owing to their high electrical conductivity [95]. This combines antibody coated GODs and screen-printed electrode with AuNPs for bacteria detection. Using cyclic voltammetry, a minimum of 1 CFU/mL of *S. aureus* in bacterial culture was detected within an hour. Kozitsina et al. utilized an electrochemical immunosensor incorporating Fe_3O_4–SiO_2–NH_2 magnetite nanoparticles for the detection of *Staphylococcus aureus* B-1266 in pure cultures. This immunosensor could detect concentrations as low as 8.7 CFU/mL of *S. aureus* [96]. In another study, Han et al. developed a novel label-free amperometric immunosensor for detection of *Staphylococcus aureus* [97]. The authors used a nanocomposite of functionalized multi-walled carbon nanotubes (MWCNs) with AuPdPt trimetallic nanoparticles and antibodies. The obtained limit of detection (LOD) was 39 with a linear range from 1.1×10^2 to 1.1×10^7 CFU/ mL. Wang's group developed an electrochemical immunosensor for detection of *S. aureus* using silver sulfide quantum dots (Ag_2S QDs) as well as dendritic mesoporous silica nanospheres (DMSNs) for signal amplification, showing the detection limit of 2 CFU/ mL [98]. Another electrochemical immunosensor was introduced by Braiek et al. for the detection of *S. aureus*. In this work, gold plate electrode was modified with self assembly monolayer (SAM) of 3-Mercaptopropionic acid to immobilize antibodies and then characterized by EIS and CV. The system has an LOD of 10 CFU/mL with a linear response between 10 CFU/mL to 10^6 CFU/ mL of *S. aureus* [81]. Bhardwaj et al. developed a paper-based electrochemical immunosensor for the label-free detection of *Staphylococcus aureus* in food samples. The immunosensor utilized functionalized single-walled carbon nanotubes (SWCNs) with anti-*S. aureus* antibodies on the working electrode to detect *S. aureus* by measuring the change in DPV peak current after formation of antigen-antibody complex, achieving a LOD of 13 CFU/mL in spiked milk samples [99].

2.6.3 Electrochemical DNA-sensor for *S. aureus* detection

The principle of DNA biosensors relies on the immobilizing single-stranded DNA (ssDNA) probes onto the electrode surface [100]. In terms of structure, aptamers and ssDNA are both nucleotide-

based, with this difference that aptamer is a synthetic short version of ssDNA or RNA, obtained through a selection process [101]. The complementary DNA, present in the sample, hybridizes with the immobilized probe, leading to a change in the electrochemical signal. This change can be detected and quantified, enabling the accurate and sensitive detection of the target bacteria. Figure 2.12 shows a general design of a DNA biosensor [102].

Use of DNA genomic biosensor with a signaling and a fixing probes for detection of *S. aureus* in fresh beef was investigated [103]. The signaling DNA was connected to lead sulfide nanoparticles, forming a sandwich type structure comprising of DNA–target DNA–capture DNA–PbS NPs. GCE functionalized with multiwalled carbon nanotubes–chitosan–bismuth was used resulting in a LOD of 3.17×10^{-14} M for the detection of *S. aureus.*

To decrease LOD of the nucleic acid systems, amplification techniques including strand displacement amplification (SDA) in combined with a real-time electrochemical detection of *S. aureus*. As an example, an immobilized triple-helix DNA structure on the gold electrode as a molecular switch was combined with SDA to detect *S. aureus* with a LOD of 8 CFU/mL. The interaction of amplified target ssDNA and molecular switch results in dissociation of the triple-helix DNA which triggers the release of G-quadruplex/hemin as a redox active agent [104].

Recently several studies introduced the remarkable application of CRSPR/cas in nucleic acid detection. Huang and his team established an effective, novel strategy by coupling saltatory rolling circle amplification (SRCA) and CRISPR/Cas12a system. The trans-cleavage activity of Cas12a is activated by the amplified target DNA, cleaving the immobilized ssDNA prob on electrode surface which leads to a signal change. The calculated LOD in pure culture were calculated 0.51 fg/μL for genomic DNA and 3 CFU/mL for *S. aureus* [105].

A summary of all the discussed techniques for *Staphylococcus aureus* detection and their performance is presented separately based on their biorecognition elements in Table 2.1, Table 2.2 and Table 2.3.

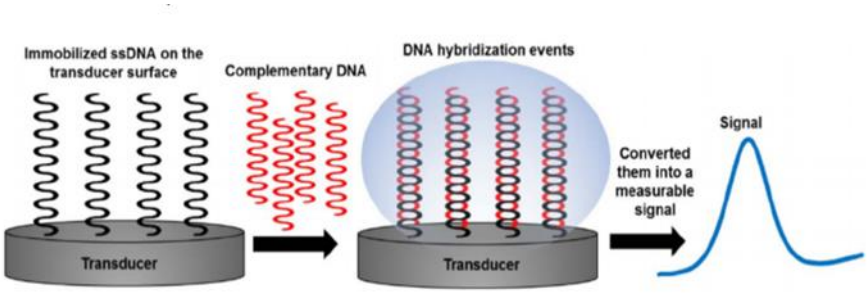

Figure 2.12 Schematic representation of DNA biosensors [102].

Table 2.1 Performance and technique of different aptamer based Electrochemical biosensor for *S. aureus* detection.

Probe	**Biorecognition element**	**Electrochemical technique**	**Dynamic range (CFU/mL)**	**Limit of detection (CFU/mL)**	**Sample size (µL)**	**Reference**
Magnetic beads and AgNPs	Anti-*S. aureus* aptamer	DPV	10 to 10^7	1	100	[106]
catechol-chitosan film	*S. aureus* aptamer	CV	10 ~ 10^8	2	-	[71]
GCE- SPANI- AuNPs	*S. aureus* aptamer	EIS	10 to 10^5	2	20	[86]
SPCE- NCNOs-AuNPs	*S. aureus* aptamer	EIS	10 to 10^8	3	-	[87]
AuNPs/CNFs/GCE	*S. aureus* aptamer	EIS	1.2×10 to 1.2×10^8	1	100	[90]
SPGE	Cognate *S. aureus* aptamers	Chronoamperometry	-	39 and 414	5	[91]
ML-Cu2O@Cu-MOF	*S. aureus*-targeted aptamer	EIS and DPV	10 to 1×10^8	2.0 and 1.6	10	[107]

MGCE	*S. aureus* aptamer	fast scan cyclic voltammetry	1 to 10^8	0.3	200	[93]

Table 2.2 Performance and technique of different electrochemical immunosensor for *S. aureus* detection.

Probe	**Biorecognition element**	**Electrochemical technique**	**Dynamic range (CFU/mL)**	**Limit of detection (CFU/mL)**	**Sample size (µL)**	**Reference**
Graphene Quantum Dots	Anti-*S. aureus* antibody	CV	1 to 10	1	100	[64]
Fe3O4–SiO2–NH2 nanoparticles	Anti-*S. aureus* antibody	CV	10 to 10^5	8.7	200	[96]
GCE-MWCNTs-AuPdPt	Anti-*S. aureus* antibodies	Amperometry	1.1×10^2 to 1.1×10^7	39	10	[97]
Ag2S/DMSNs	Anti-*S. aureus* antibody	DPV	10 to 10^8	2	1000	[108]

AuE/MPA	Anti-*S. aureus* antibody	EIS	10 to 10^6	10	-	[81]
SWCNTs/ CPE	anti-*Staphylococcus aureus* antibody	DPV	10 to 10^7	13	-	[109]

Table 2.3 Performance and technique of different electrochemical DNA based biosensor for *S. aureus* detection.

Probe	**Biorecognition element**	**Amplification method**	**Electrochemical technique**	**Dynamic range (CFU/mL)**	**Limit of detection (CFU/mL)**	**Sample size (µL)**	**Reference**
GCE-MWCNTs/PbS NPs	*S. aureus*-targeted cDNA	PCR*	DPV	10^1 to 10^5	2.44×10^{-14} M	100	[103]

Gold electrode/ magnetic bead	*S. aureus*-targeted cDNA	SDA*	DPV	30 to 3×10^8	8	100	[110]
GCE-AuNPs	CRISPR/Cas12a	SRCA*	SWV	6.2×10^2 to 6.2×10^5	3	-	[105]

Saltatory rolling circle amplification (SRCA)

Strand displacement amplification (SDA)

Polymerase chain reaction (PCR)

CHAPTER 3 PROBLEM IDENTIFICATION, RESEARCH OBJECTIVES AND GENERAL METHODOLOGY

3.1 Problem identification

Upon reviewing the literature on electrochemical detection of *S. aureus*, it was found that certain methods exhibited high sensitivity for detecting *S. aureus*. However, in order to achieve low limits of detection, complex fabrication techniques were employed. For instance, biological amplifications such as PCR and rolling circle amplification (RCA) were combined with DNA electrochemical biosensors [111, 112], rendering the preparation steps more intricate and time-consuming. Furthermore, most of the literature focuses on detecting *S. aureus* as a foodborne pathogen in food products, and there is a lack of research on detecting *S. aureus* for medical applications, particularly PJI. Moreover, the majority of aptasensors and immunosensors rely on whole-cell detection, which presents a major challenge in diagnosis of biofilm infections including PJI as the responsible bacteria mostly grow on the surface of the film and detecting the presence of the bacteria in body fluid such as blood and saliva is challenging [7]. Additionally, due to the relatively large size of bacteria (0.5-1.5 µm in diameter) [113] compared to single proteins and molecules, capturing the bacteria poses a greater challenge.

In conclusion, despite the progress made in developing electrochemical detection methods for *S. aureus*, there is still a gap in the availability of methods that are suitable for real-time and point-of-care applications using a small size of body fluid sample. Thus, there is a continuous need for a simple electrochemical detection technique that can serve as an alternative to conventional methods for detecting bacterial infections.

3.2 Objective of the thesis

The aim of this thesis is to develop a highly sensitive but low-cost and simple immunosensor for detection of aureolysin, as a biomarker for *S. aureus* infection, using screen printed gold electrode (SPGE). The research objectives of this study are three-fold as follows:

- **S.O.1** Firstly, designing and developing an aureolysin electrochemical immunosensor and investigating the effect of gold nanospikes on the electrochemical properties of the SPGE.

- **S.O.2** Second, studying the performance and sensitivity of the immunosensor for Aur detection.
- **S.O.3** Finally, measuring sensitivity of the immunosensor for *S. aureus*, exploring its selectivity by challenging it with other interfering bacteria, and studying its stability.

3.3 Thesis organization

This thesis is comprised of the following 6 chapters:

Chapter 1 offers an insightful introduction to Prosthetic Joint Infection (PJI), examining its significant impact on both the healthcare system and individuals. The chapter highlights the existing challenges associated with diagnosing PJI, including identifying the responsible microorganisms that contribute to its development. Moreover, it emphasizes the crucial importance of early detection of the causative bacteria associated with PJI.

Chapter 2 provides a comprehensive literature review of both conventional and emerging methods for detecting bacteria, with a particular focus on biosensors. The chapter explores the principles of various biosensor types, providing a detailed analysis of commonly used electrochemical techniques in biosensing. Additionally, the chapter digs into the electrochemical detection of *S. aureus*, covering the use of aptamers, antibodies, and DNA in this process.

Chapter 3 covers the limitation and disadvantages of the existing literature, the objective of the thesis and general methodology.

Chapter 4 is the manuscript of the submitted article: Early warning diagnostics for *Staphylococcus aureus* infection: Aureolysin point-of-care biosensing devices. In this chapter the experimental methods and procedures for the development of the Aureolysin immunosensor and the characterization and electrochemical measurement results are presented.

Chapter 5: Summary and contribution of the work, limitation and recommendation are discussed in this chapter.

3.4 General methodology

3.4.1 Choice of target: Aureolysin

Aureolysin is a soluble extracellular zinc metalloprotease belonging to the family of thermolysin that is secreted specifically by *S. aureus* [114]. This enzyme is believed to play a pivotal role in the pathogenesis of *S. aureus* by inhibiting the complement response in the host and cleaving surface-associated proteins to facilitate bacterial spread [27]. With a molecular weight of 40 kDa, aureolysin is a single-chain enzyme that is present in the body fluids of patients with PJIs [115]. Due to its extracellular nature, aureolysin can be used as a biomarker to detect the presence of *S. aureus*.

Considering the larger size of bacterial cell compared to aureolysin, a higher number of antibodies to capture *S. aureus* bacterial cell on the electrode surface is required compared to the number of antibodies required to capture aureolysin. This means *S. aureus* antibody based immunosensors will reach saturation at a lower concentration. To compensate for this, a larger electrode surface area with a higher number of immobilized antibodies is required. However, this leads to more complex and costly electrode fabrication.

Therefore, using aureolysin as a biomarker to detect *S. aureus* in PJI offers several advantages. Its smaller size allows for more effective immobilization and its presence in body fluids enables its detection in patients.

3.4.2 Choice of nanoparticles: Gold nanospikes

The use of Au nanostructures has been extensively studied in electrochemical biosensing platforms [116-123]. Gold nanostructures enhance the electrochemical activity and electron transfer rate, while providing a larger surface area for efficient immobilization of antibodies, thus resulting in higher sensitivity [124]. Electrochemical deposition is a facile and versatile technique that enables control over the growth and geometry of a wide range of nanostructures [125]. The high aspect ratio morphologies such as rod, nanocornal and nanospikes have been successfully synthesized by electrodeposition [125-127]. In this thesis, we will employ an electrochemically deposited hierarchical gold nanostructure, namely gold nanospikes, to modify screen printed gold electrode surface due to several advantages associated with this specific geometry. First, the high electrocatalytic activity of the gold nanospikes is attributed to the properties of its dominant crystal

orientation, namely (111) [128]. Additionally, high density of atomic steps and kinks indicated by high index facets such as (311) on Au nanospikes which increases active sites [129].

3.4.3 General experimental steps

The development of proposed immunosensor is composed of two main parts, improving the transducer which is a commercially available screen-printed gold electrode, and the conjugation of bioreceptor to the electrode. Here the Aur-antibody will be used as a specific bioreceptor for aureolysin.

The immunosensor will be fabricated through these steps:

- Frist, the screen-printed gold electrode (SPGE) will be modified with gold nanospikes. To do so an electrochemical deposition technique, with a control potential and time, will be applied according to a previously reported protocol [127].
- Then, a self-assembly monolayer (SAM) layer for easy immobilization of antibody will be adhered on the surface of the modified electrode via sulfur-gold chemistry and activated by EDC/ NHS coupling [130].
- Subsequently, antibody will be conjugated to the nanospikes on the surface of the screen printed gold electrode via the link to the SAM.
- Finally, a blocking agent to block nonspecific interaction will be adsorbed to the area of the electrode where there is no antibody attached.

The successful fabrication and impact of gold nanospikes will be confirmed after each step by scanning electron spectroscopy, CV and DPV. The performance of the resulting biosensor will be tested to measure aureolysin in samples containing different concentrations of aureolysin and phosphate buffer saline (PBS). To do so, 20 µL of sample will be applied to the modified SPGE and allowed to stay. After 1h the signal will be recorded. These measurements will be carried out by deferential pulse voltammetry in a redox active solution of $[Fe(CN)_6]^{3-/4-}$. Furthermore, the device sensitivity will be studied for *S. aureus* culture samples by recording DPV at different concentrations of *S. aureus*. The regression equation for change in signal vs target concentration will be calculated. The limit of detection and linear range will be obtained accordingly [131]. Finally, stability and cross-reactivity of the developed immunosensor will be explored using two commonly encountered bacteria to prove specificity of the biosensor platform. During all the

experiment the SPGE will be connected to a potentiostat which is an analytical instrument with several internal circuits that applies controlled potential to the working electrode and measure the current consequently. The detailed methodology will be discussed in the next chapter within submitted manuscript.

CHAPTER 4 ARTICLE 1: EARLY WARNING DIAGNOSTICS FOR STAPHYLOCOCCUS AUREUS INFECTION: AUREOLYSIN POINT-OF-CARE BIOSENSING DEVICES.

This section contains the final manuscript of the paper submitted to the Journal of Biosensors and Bioelectronics. The article presents the development of an aureolysin biosensing device for the early-stage diagnosis of *Staphylococcus aureus* infections. A brief review of the current state of the art, including recent advances in *S. aureus* electrochemical immunosensors, is provided. The experimental section includes a list of materials and equipment, stepwise fabrication methodology, and characterization techniques. Proof of successful fabrication was provided using scanning electron spectroscopy, cyclic voltammetry, and differential pulse voltammetry. The results and discussion section presents the differential pulse voltammetry measurements of aureolysin and *S. aureus* samples. The references for the article are grouped with those of the thesis presented at the end of the document.

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Keywords: Electrochemical immunosensor, Screen printed gold electrode, *S. aureus* electrochemical detection, Aureolysin electrochemical biosensor, Gold nanospikes

4.1 Abstract

One of the most significant challenges in the field of microbiology is the detection of pathogenic bacteria, such as *Staphylococcus aureus* (*S. aureus*), which is a major cause of infections in humans. Aureolysin (Aur) is an extracellular metalloprotease of *S. aureus*, which plays an important role in the pathogenesis of *S. aureus* infections. In this work, we have developed a rapid and low-cost biosensing device based on an electrochemical immunosensor for label-free detection of aureolysin, using antibody (Ab)-Au bioconjugates. Anti-aureolysin antibodies were covalently attached to the Au nanospikes, via the N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS) coupling chemistry. These Ab-Au bioconjugates were immobilized on screen printing electrodes, and the presence of aureolysin and

S. aureus was detected by analyzing the change in peak current following antigen-antibody complex formation. Differential pulse voltammetry was performed with an increased bacterial concentration. A selectivity test using *Escherichia coli* and Pseudomonas Aerugonisa to identify if any cross-reactivity confirmed that the aureolysin immunosensor was specific to *S. aureus* and showing a good linear relationship (R^2 = 0.974) between the increase in peak current and logarithmic *S. aureus* concentration, with a rapid detection time (1h) and a limit of detection of 5 pg mL^{-1} in buffer and 28.5 CFU mL^{-1} in complex bacterial culture samples. This low-cost immunosensor can be used for rapid detection of pathogens in body fluid samples with high sensitivity and specificity. Overall, our immunosensor offers an early warning point-of-care diagnostic tool to provide rapid and precise results for *S. aureus* infections in clinical settings.

4.2 Introduction

Staphylococcus aureus (*S. aureus*), a gram-positive bacterium, is the leading cause of severe, invasive infections with high morbidity and mortality rates, including prosthetic joint infection (PJI), pneumonia, nosocomial bacteremia and foodborne infection [33, 132, 133]. Rapid and accurate detection of *S. aureus* is crucial for appropriate clinical management and infection control measures. Currently available methods for detecting *S. aureus* include culture-based methods, molecular methods such as PCR [134], matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) [135] and enzyme-linked immuno-sorbent assays (ELISAs) [41].The gold standard culture-based test is time-consuming (3 days to weeks), laborious, and requires technical skills [12]. PCR has a high sensitivity but remains costly and lacks the ability to differentiate between live and dead bacteria [99, 134]. ELISAs have a short detection time (less than 6h) compared to the culture-based method but suffer from high potential for cross-contamination and poor stability, which restrict their application for real-time *S. aureus* detection [10].

An alternative to these traditional methods is based on electrochemical detection, which have been established to be more rapid, highly sensitive, reliable, and easy to miniaturize, thus making them an attractive solution for real-time pathogen detections [97, 111, 136-140]. There are several electrochemical sensors used to detect bacterial infections, each with their own advantages and limitations. Several biorecognition elements including antibody, enzyme, or nucleic acid choices have been used in electrochemical sensors for bacterial detection depending on the specific

application and the bacterial target. Among them, antibody-based electrochemical sensors have been applied for *S. aureus* detection [97, 141, 142]. A two dimensional MOF nanozyme-amplified electrochemical biosensor using dual sensing method, containing vancomycin and anti-*S. aureus* antibody, was reported for the sensitive and selective detection of *S. aureus* with a detection limit of 6 CFU mL^{-1} [143]. In another example, modifying a silanized ITO electrodes with AuNPs on the surface, an electrochemical impedimetric immunosensor was used to detect *Staphylococcus aureus* with a limit of detection of 3×10^3 CFU mL^{-1} [144]. In a recent study, an immunosensor was developed using a layer-by-layer film of chitosan and carboxylated MWCNTs, which successfully detected *Staphylococcus aureus* in milk samples with a limit of detection of 2.6 CFU mL^{-1} [145]. Other works on nucleic acids or aptamers have gained special attention for signal amplification. For example, Cai and co-workers developed a DNA walker and DNA nanoflowers based electrochemical biosensor to determine *S. aureus*; they used rolling circle amplification (RCA) to amplify signal achieving a limit of detection of 9 CFU mL^{-1} [111]. Wu et al. reported the application of dual recognition units, a DNA walker and pb^{2+}-specific DNAzyme for detecting *S. aureus*. Using this approach, the linear range for monitoring *S. aureus* was $10–10^7$ CFU mL^{-1}, with the LOD at 1 CFU mL^{-1} [140]. Consequently, the detection signal and sensitivity could be expected to be significantly enhanced with low limit of detection (LOD) but fabrication is complex, use of DNA involves complex biological preparation steps and immunosensor focused on whole bacteria limiting the sensitivity of biosensor (*S. aureus*, ~ 0.5-1.5 μm in diameter). Most studies have focused on electrochemical detection of *S. aureus* in food products, and there is a lack of research for medical applications, particularly to detect microbial biofilms in PJI, where the causative bacteria tend to concentrate on the prosthesis surface [7].

Au surface has been extensively used as biosensing surface due to its high electrical conductivity, biocompatibility, and ease of functionalization with biomolecules [118, 119, 123, 146]. Electrochemical deposition of gold nanostructures has been successfully used to produce high aspect ratio morphologies, such as rods, pyramids, and spikes [122, 125, 127]. Among them, Au nanospikes can act as a stable substrate for antibody immobilization and can enhance electrochemical performance due to their dominant crystal orientation of (111), which increases

electrocatalytic activity [128], and high index facets (311), indicating a high density of kinks and atomic steps on the surface that create more active sites [117, 129].

There are other strategies to improve the detection sensitivity while keeping the biosensing device architecture simple and low-cost for widespread implementation.

Here, we report an ultra-sensitive label-free immunosensor to detect *S. aureus* through the measurement of aureolysin for the first time. Aureolysin is an extracellular, single-chain, metalloprotease with the size of 40 kDa [114], specifically secreted by *S. aureus*. Aureolysin has been extensively studied for its role in the virulence of *S. aureus* and has been found to inhibit the complement response in the host immune system [27] .To the best of our knowledge, this is the first time that aureolysin is used as an early marker of bacterial infection. First the surface of a screen-printed gold electrode (SPGE) was modified with gold nanospikes through electrochemical deposition to act as a signal amplifier by increasing the electrochemically active surface area. Then a self-assembled layer of 11-Mercaptoundecanoic acid (MUA) was attached to the Au surface using thiol chemistry followed by the grafting of anti-Aur antibody. The effectiveness of our approach is demonstrated by detecting aureolysin in phosphate-buffered saline (PBS) and *S. aureus* in bacterial culture. The electrochemical signals were significantly enhanced and were proportional to the concentration of *S. aureus* and the selectivity against other bacteria was confirmed.

4.3 Experimental section

4.3.1 Materials and reagents

11-Mercaptoundecanoic acid (MUA), Phosphate buffer saline (PBS: 0.01 M, pH: 7.4), Tetrachloroauric(III) acid trihydrate ($HAuCl_4 \cdot 3H_2O$), Lead(II) acetate trihydrate ($(CH_3COO)_2Pb. 3\ H_2O$), Bovine serum albumin (BSA), 1-Ethyl-3- (3-(dimethylamino)propyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), sulfuric acid (98%), 4-Morpholineethanesulfonic acid (MES), potassium ferrocyanide ($K_4[Fe(CN)_6]$, 99%), potassium ferricyanide ($K_3[Fe(CN)_6]$, 99%) were purchased from Sigma-Aldrich. The Recombinant *Staphylococcus aureus* Zinc metalloproteinase aureolysin (Aur) was achieved from MyBioSource and the Zinc metalloproteinase aureolysin antibody was purchased from CUSABIO. The antibody and enzyme solutions were prepared with 0.01 M PBS. *E. Coli*, *S. aureus* and *Pseudomonas aeruginosa* were

provided by the chemical engineering school of Polytechnique Montreal, and LB broth was purchased from VWR chemicals. For all the steps the ultrapure water obtained from a Milli-Q system (18.2 MΩ cm^{-1}) was used. All other chemicals were analytical grade.

4.3.2 Preparation of aureolysin immunosensor

The fabrication of the sensor can be seen in Figure 4.1. Au nanospikes were deposited on the SPGE using electrochemical deposition, as described in the method reported by Plowman et al. [127]. The SPGE was immersed in a solution of 0.5 mM Pb $(CH_3COO)_2$ and 6.9 mM $HAuCl_4$, and a potential of 50 mV was applied for 10 minutes.

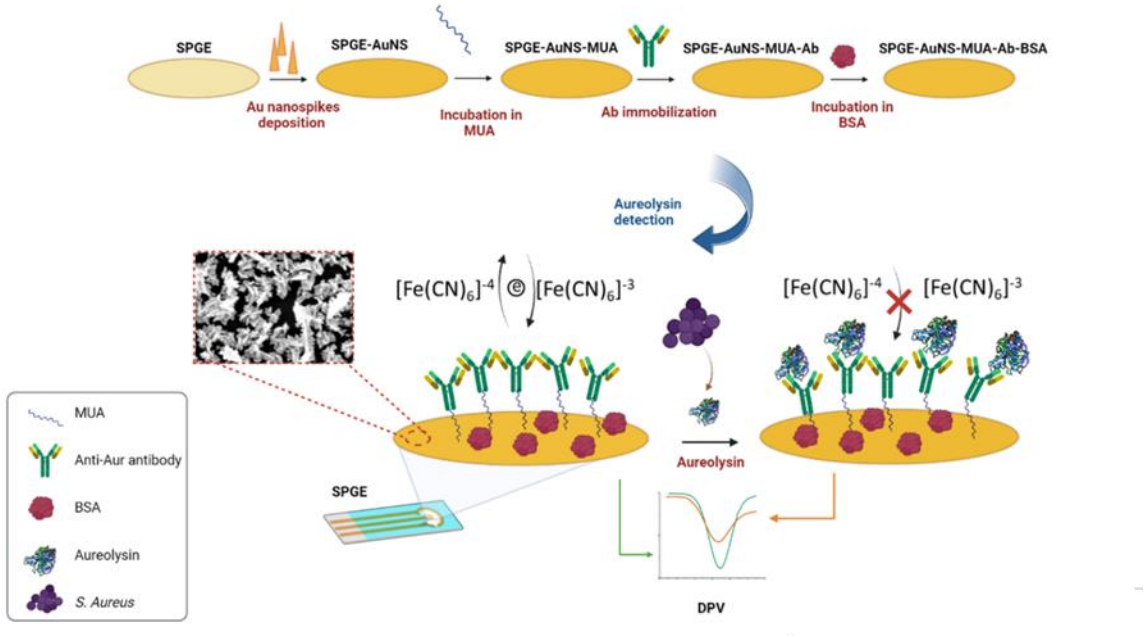


Figure 4.1 Schematic representation of the steps in fabrication of Aur immunosensor utilizing SPGE

The resulting nanospiked SPGE was then scanned in a 0.1 M H_2SO_4 solution using cyclic voltammetry with a potential range from 0.5 V to 1.4 V with a scan rate of 0.5 V s^{-1}. After scanning, the electrode was thoroughly rinsed with ultra-pure water and dried for the next step. The immunosensor was fabricated using the clean Au nanospiked SPGE. The Au Nanospikes-SPGE was immersed in a 10 µM solution of MUA in ethanol for 2h to obtain a self-assembled monolayer on the electrode surface. Then, it was thoroughly rinsed with ethanol and ultra-pure water and dipped in EDC/NHS (400 mM/100 mM in MES, pH 6) for 1h to activate the carboxyl groups of

MUA. After activation, 20 µL of anti-Aur antibody (100 µg mL^{-1}) was drop-casted on the SPGE surface and kept for 2h for antibody binding, afterwards it was rinsed by PBS. A solution of BSA (1%) subsequently was used for 30 minutes to block non-specific interaction. All the steps were done at room temperature. After fabrication BSA-Antibody-MUA-Aunanospike-SPGE immunosensor was stored at 4°C for future use.

4.3.3 Characterization

The surface morphology of the modified screen-printed electrode was investigated using scanning electron microscopy (SEM, Inspect F50, FEI Company, Hillsboro, OR, USA). All the electrochemical measurements were carried out by a potentiostat (VersaSTAT 4, Princeton Applied Research, Oak Ridge, TN, USA) and Screen-printed gold electrodes (SPGE, ϕ = 4 mm) with Ag as reference electrode and carbon as counter electrode (Metrohm Canada). Cyclic voltammetry (CV) was carried out in the range of -0.4 to 0.6 V and at the scan rate of 50 mV s^{-1}. Differential pulse voltammetry (DPV) was performed with a potential range of -0.4V to 0.4 V, with a step of 5 mV, a modulation amplitude of 50 mV, a modulation time of 0.05 s, and an interval time of 0.2 s in a 5 mM/5 mM $[K_3Fe(CN)_6]/[K_4Fe(CN)_6]$ solution with 0.1 M PBS (pH 7.4).

The PBS solution (pH 7.4) of aureolysin at different concentrations of 1pg mL^{-1}, 10 pg mL^{-1}, 40 pg mL^{-1}, 50 pg mL^{-1}, 70 pg mL^{-1}, and 100 pg mL^{-1} were prepared. 20 µL of each solution was drop casted on the surface of BSA-Antibody-MUA-Aunanospike-SPGE biosensor and allowed to stay for 1h at room temperature. Following incubation, the electrode was rinsed with ultra-filtered water and PBS solution to remove unbound Aur and dried. Then, differential pulse voltammetry measurements were recorded in a solution of 5 mM/5 mM $[K_3Fe(CN)_6]/[K_4Fe(CN)_6]$, as the redox couple, in 10 mM PBS (pH 7.4).

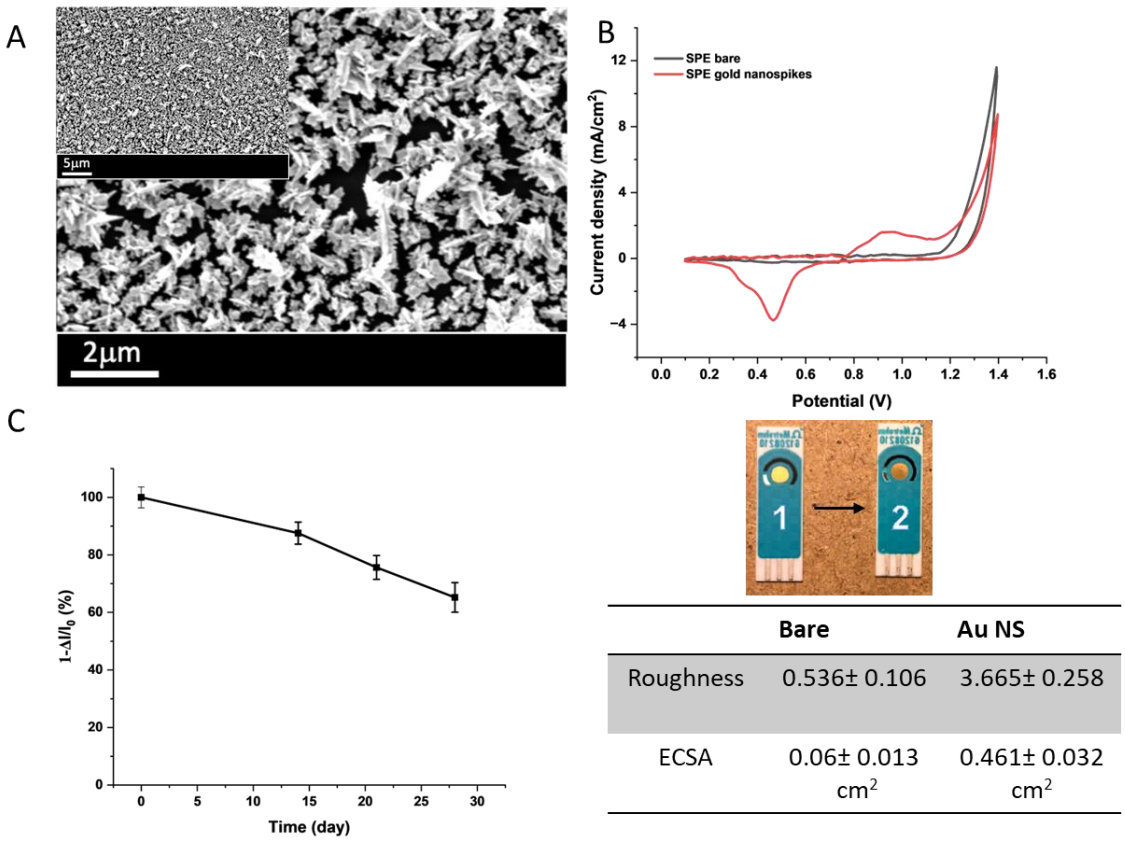
E. Coli, *S. aureus* and *P. aeruginosa* were cultured in LB broth medium at 36°C for 24 hours. The cells were suspended and serially diluted to desired concentrations (10^2-10^8 CFU for *Staphylococcus aureus* and 10^5 CFU for *Escherichia coli* and *Pseudomonas aeruginosa*) in a suspension medium (1/500 NB). The number of cells present in the diluted samples was calculated by utilizing a bright line hemacytometer cell counting chamber (Sigma Chemical Co., St. Louis, USA). For *S. aureus* measurement, 20 µl of diluted samples ranging from 10^2 to 10^8 CFU *S. aureus* were drop casted on the surface of the prepared immunosensor at room temperature. After 1h the

immunosensors were gently rinsed with ultra-filtered water and the DPVs were conducted in a solution of 5 mM/5 mM $[K_3Fe(CN)_6]/[K_4Fe(CN)6]$, as the redox couple, in 10 mM PBS (pH 7.4).

4.4 Results and discussion

4.4.1 Electrochemical and physicochemical characterization of Au nanospike modified SPGE

The nanostructured surfaces were prepared electrochemically using fixed precursor concentrations of 6.8 mM $HAuCl_4$ and 1 mM $Pb(CH_3COO)_2$ [127]. To confirm successful growth of multi-directional Au nanospikes on the electrode, the surface morphology of Au nanospike- electrode was observed with SEM (Figure 4.2A). The electrochemically treated surface is rough and uniformly covered with three-dimensional nanospikes with prismatic tapering ends and the top view of Au nanospikes indicates a length of approximately 500nm and a base thickness dimension of about ~300 nm. In Figure 4.2, the photograph of bare SPGE (1) and Au-nanospike-SPGE (2) showed that the deposition of Au nanospikes causes a color change from yellow to brown [147]. Figure 4.2B shows the cyclic voltammetry of SPGE before and after electrochemical treatment. Larger redox peaks for nanospike represent that the corresponding Au nanospike nanostructures have a greater surface area in comparison to the bare Au electrode. To determine the electrochemical active surface areas (ECSA), both the bare Au control and Au nanospike nanostructures were subjected to cyclic voltammetry in a 0.1 M H_2SO_4 solution.



	Bare	Au NS
Roughness	0.536± 0.106	3.665± 0.258
ECSA	0.06± 0.013 cm^2	0.461± 0.032 cm^2

Figure 4.2 Photographs of SPGE and Aunanospike-SPGE A) Scanning electron micrographs of Aunanospike-SPGE at low and higher magnification. B) CVs obtained at 0.5 V s^{-1} in 0.1 M H_2SO_4 with (red) and without (black) Au nanospikes and associated ECSA and roughness. C) Stability of the Aunanospike-SPGE electrochemical signal for 28 day period.

Given that the cathodic peak's area under the curve is directly proportional to the real surface area (ECSA = Q/390 µC.cm^{-2}) and is a measure of surface roughness, ECSAs for both electrodes were calculated from the cathodic peak [148]. This peak is attributed to the oxide monolayer removal formed on the surfaces of the electrode during the anodic CV scan. As expected, the ECSA of the Au nanospike nanostructures (0.461 ± 0.032 cm^2) is significantly higher compared to that of bare Au electrode (0.060 ± 0.013 cm^2), as shown in Figure 4.2. Given the geometrical area of the bare Au electrode ca. 0.126 cm^2, a roughness factor (Rf, the ratio of ECSA to geometrical area) of 3.665 ± 0.258 and 0.536 ± 0.106 were calculated for the Au nanospike nanostructures and bare Au electrode, respectively, confirming the effective and successful deposition of gold nanostructures on the electrodes [122]. Stability of the electrode surface was investigated via DPV in

$[K_3Fe(CN)_6]/[K_4Fe(CN)_6]$ acting as electron transfer, for 28 days. The stability was represented as $(1-\Delta I/I_0) \times 100$ where $\Delta I/I_0$ is $(I_{day(0)}-I_{day(n)})/I_{day(0)}$ .The results depict a slow decay of the current stability over time with only 24% of initial electrochemical activity after 3 weeks, reflecting the high stability of Au nanospikes as a substrate structure for the electrochemical immunosensor (Figure 4.2C).

4.4.2 Characterization of BSA-Antibody-MUA-Aunanospike-SPG electrode

The fabrication of the immunosensor was characterized at each step using CV and DPV in $[K_3Fe(CN)_6]/[K_4Fe(CN)_6]$ as an electron transfer mediator. Figure 4.3A depicts the DPV curves for SPGE (blue), Aunanospike-SPGE (black), MUA-Aunanospike-SPGE (red), Antibody-MUA-Aunanospike-SPGE (green), BSA-Antibody-MUA-Aunanospike-SPGE (purple) and BSA-Antibody-MUA-Aunanospike-SPGE at 10 pg mL^{-1} of aureolysin (yellow). As shown by the DPV curves, the peak current density increased from 1.79 mA. cm^{-2} (SPGE) to 3.59 mA. cm^{-2} following the deposition of Au nanospike structures onto the SPGE, indicating an enhanced electron transfer between the solution and the electrode. Then, following the self-assembly monolayer of 11-Mercaptoundecanoic acid (MUA) on the surface of the Au nanospike electrode the peak current density significantly decreases to 1.14 mA. cm^{-2} due to the insulating behavior of the alkane chains. Subsequent to the grafting of the antibody (Ab) and adsorption of BSA, the peak current density lowers down further to 915 µA. cm^{-2} (Ab) and 629 µA. cm^{-2} (BSA) probably caused by the insulation of the electrode by Ab and BSA that retard or block the electron transfer from the solution to the Au electrode. In Figure 4.3B, a pair of well-defined redox peaks attributed to the $[Fe(CN)_6]^{3-/4-}$, with the anodic and cathodic peak potential of 0.19 V and 0.05 V, respectively, and a peak potential difference of 140 mV were observed in CV (blue curves in Figure 4.3B). The results from the CV measurements were consistent with those from the DPV measurements. A decrease in the peak potential difference from 140 mV to 93 mV was calculated, indicating increased electron transfer which can be attributed to the high electrical conductivity and surface area of Au nanospikes [149]. The changes in peak current density observed in the CV and DPV measurements for each step are depicted in the insets of Figure 4.3.

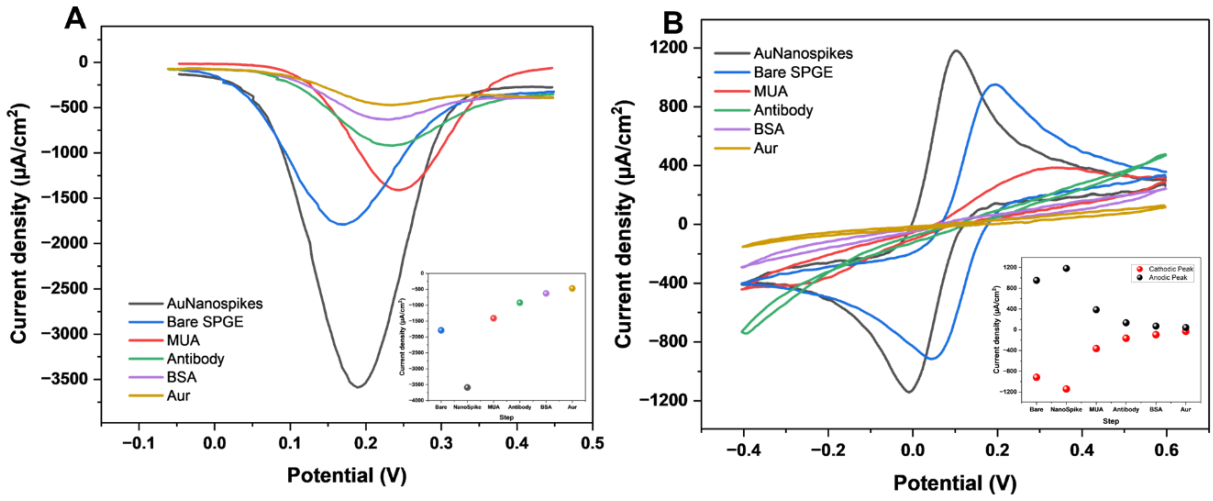


Figure 4.3 A) DPV- and B) CV- curves in 5 mM/5 mM $[K_3Fe(CN)_6]/[K_4Fe(CN)_6]$ as the redox medium for characterization of SPGE (blue), Aunanospike-SPGE (black), MUA-Aunanospike-SPGE (red), Antibody-MUA-Aunanospike-SPGE (green), BSA-Antibody-MUA-Aunanospike-SPGE (purple) and the immunosensor after incubating in 10 pg mL^{-1} of aureolysin (yellow). The inset A) shows the reduction peak current density and B) shows reduction and oxidation peak current density change in each step.

4.4.3 Differential pulse voltammograms for aureolysin and *S. aureus* detection

For analyzing the biosensor performance considering LOD and dynamic range, DPV experiments were conducted in PBS solution with increasing concentration of aureolysin ranging from 1 pg mL^{-1} to 100 pg mL^{-1}. The current density change, ΔJ, was calculated as the difference between the peak current density after incubation in Aur solution, J_{Aur}, and the baseline peak current density, J_{BSA}. The peak current density decreases with increasing concentration of Aur, resulting from the attachment of Aur to the Ab immobilized onto the electrode surface causing steric hindrance and reduction of electron transfer from solution to electrode. Figure 4.4B demonstrates a linear relation between DPV current density (ΔJ) and C_{Aur}. Three independent electrodes were used to acquire the calibration line. The obtained regression equation was $Y = 2.791\ C + 91.610$ with $R^2 = 0.995$. A low limit of detection, using the IUPAC method (LOD= 3SD/S), of 5 pg ml^{-1} (S/N=3) was achieved due to the high electrochemical activity and large surface area of the Au nanospikes [150]. The high surface area of the nanospikes provides a larger binding interface for the antibodies, while their high conductivity facilitates the efficient transfer of electrons during the binding process

[151]. This improves the sensitivity of the assay, enabling the detection of lower concentrations of the Aur.

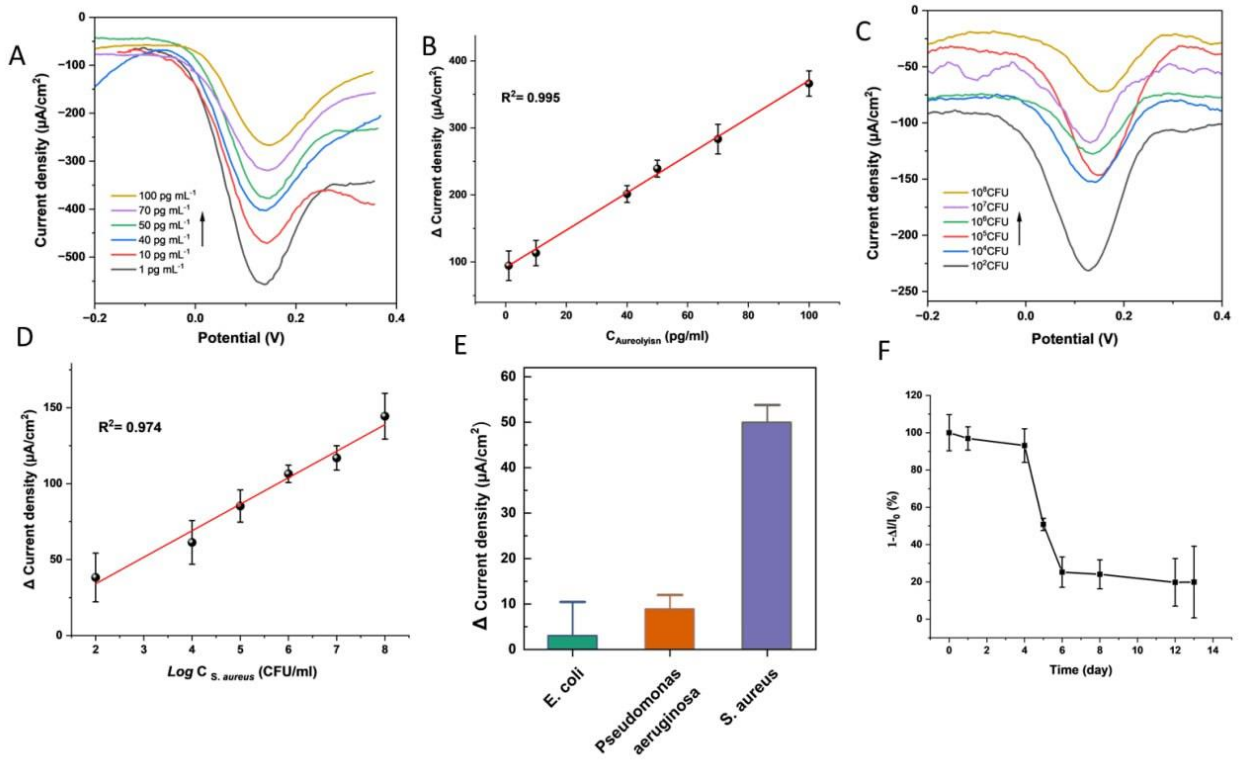


Figure 4.4 A) Differential pulse voltammograms of the SPGE immunosensor after exposing to Aur solutions with different concentrations of 1 pg mL^{-1}, 10 pg mL^{-1}, 40 pg mL^{-1}, 50 pg mL^{-1}, 70 pg mL^{-1}, and 100 pg mL^{-1} (measured in 5 mM/5 mM $[K_3Fe(CN)_6]/[K_4Fe(CN)_6]$ solution in 10 mM PBS (pH 7.4). B) Linear relationship between ΔJ and C_{Aur}. C) Differential pulse voltammograms of the SPGE immunosensor after exposing to *S. aureus* solutions with different concentrations of 10^2 CFU mL^{-1}, 10^4 CFU mL^{-1}, 10^5 CFU mL^{-1}, 10^6 CFU mL^{-1}, 10^7 CFU mL^{-1}, and 10^8 CFU mL^{-1} (measured in 5 mM/5 mM $[K_3Fe(CN)_6]/[K_4Fe(CN)_6]$ solution in 10 mM PBS (pH 7.4). D) Linear relationship between ΔJ and log ($C_{S.\ aureus}$). E) Selectivity of the immunosensor to *S. aureus* compared to *E. Coli* and *Pseudomonas aeruginosa*. F) Stability of the biosensor after 13 days.

Subsequent to Aur detection, DPV electrochemical characterization was conducted for *S. aureus* detection in bacterial culture onto BSA-Antibody-MUA-Aunanospike modified SPGE (with bacterial concentrations from 10^2 to 10^8 CFU mL^{-1}). The blank response was measured after immersion and incubation in PBS without *S. aureus*. After 1h of incubation, the voltammograms were recorded and displayed in Figure 4.4C. The peak current decreased gradually as the bacterial

concentration increased (Figure 4.4C). This change in peak current is the result of the formation of the Aur–Ab complex immobilized at the electrode surface, that inhibits the electron transfer due to conformational change of electrode surface and hindrance effect of electron transfer of ferrocyanide. These findings confirm the applicability of the biosensor in detecting Aur secreted by *S. aureus* in complex environment. A linear regression curve was generated in Figure 4.4D, showing a good linear relationship in this range between the Aur secreted *S. aureus* concentration and redox probe value. The LOD of fabricated biosensor was estimated to be 28.5 CFU mL^{-1} in bacterial culture. The regression equation was Y= 17.467 log(C) -0.876, with R^2= 0.974.

4.4.4 Selectivity and stability assay

A key performance factor for biosensors is their specificity to the target of interest. Next, the selectivity test of the fabricated biosensor was carried out by testing other commonly encountered bacteria, i.e. *E. Coli* and P. Aerugonisa. Cultured samples of these bacteria were diluted to a concentration of 10^5 CFU mL^{-1} and incubated for 1 hour with the biosensor. The resulting changes in current density were measured using DPV under the same conditions as for the *S. aureus* samples. As shown in Figure 4.4E, there was little electrochemical signal change observed for other bacteria compared to the distinct current density change observed for the *S. aureus* sample. Sensor stability was assessed by analyzing the DPV response of 3 prepared BSA-Antibody-MUA-Aunanospike-SPGE for 13 days. During this time the electrodes were stored in PBS at 4 °C. Figure 4.4 F shows that after 1h incubation, the biosensor retained 93% of its initial electrochemical signal after 4 days to finally dropped to 50% after 6 days. The sensor performance comparison between present fabricated biosensor and other electrochemical biosensors for detection of *S. aureus* reported is summarized in Table 4.1. As shown in Table 4.1, this aureolysin biosensor can detect *S. aureus* at a low concentration compared to that of other screen printing electrode system– based biosensors published to detect whole bacteria [91].

Table 4.1 Comparison of different Electrochemical biosensor for *S. aureus* detection

Working Electrode	Probe	Technique	Detection range	LOD	Interference	Ref
SP* gold electrode	Pair of aptamers, sandwich-type	Signal-on chronoamperometry	1.9×10^2-2.5×10^4 CFU mL^{-1}	39 and 414 CFU mL^{-1}	*S. saprophyticus*, *S. typhimurium*, *L. monocytogenes*, *and E. Coli*	[91]
MWCNT@CS/GCE*	Aptamer	DPV	$10–10^7$ CFU mL^{-1}	1 CFU mL^{-1}	*E. Coli*, Sal. Typhimurium, B. subtilis, C. sporogenes, S. pneumoniae	[140]
Gold electrode	dsDNA	DPV	60 to 6×10^7 CFU mL^{-1}	9 CFU mL^{-1}	E. faecium, L. monocytogenes, *P. aeruginosa*, *E. Coli*, E. faecalis, S. enteritidis	[111]
ITO/PDDA/PSS/Au*	Anti-*S. aureus* antibody	EIS	3×10^3–3×10^7CFU mL^{-1}	10^3CFU mL^{-1}	*E. Coli*, S. typhimurium, B. pumilus, K. pneumoniaeand B. subtilis	[144]

GCE/PDDA/PSS/GNR	Anti-*S. aure us* antibody	EIS	1.8×10^3 - 1.8×10^7 CFU mL^{-1}	10^2 CFU mL^{-1}	*E. Coli, S. typhimurium, B. pumilus, B. subtilis*	[107]
GCE/AuNPs/MOF	anti-*S. aureus*rosenbach tropina antibody	DPV	$10-7.5\times10^7$ CFU mL^{-1}	6 CFU mL^{-1}	*E. Coli,Bacillus cereus, and Pseudomonas aeruginosa*	[143]
AuNP-modified SP electrode	Mouse monoclonal anti-MRSA antibody	DPV and EIS	$10–10^6$ CFU mL^{-1}	13 CFU mL^{-1}	-	[141]
Aunanospike-SPGE	**Aur Antibody**	**DPV**	**10^2-10^8 CFU mL^{-1}**	**28.5 CFU mL^{-1}**	***E. Coli, P. aeruginosa***	**This work**

SP electrode: screen printed electrode

MWCNT: multiwalled carbon nanotubes

GCE: glassy carbon electrode

PDDA: Polydiallyldimethylammonium chloride

PSS: polystyrene sodium sulfonat

Comparing to another study, an electrochemical immunosensor based on self-assembled gold nanorods with LOD of 2.4×10^2 CFU mL^{-1}, our immunosensor exhibits a significantly lower LOD [107]. Cai et al. reported the use of the strand displacement amplification (SDA) reaction in an electrochemical biosensor based on triple-helix molecular switch to achieve lower LOD of 8 CFU mL^{-1} *S. aureus* [110]. Our proposed immunosensor can be used with only 20 µl of sample without any preparation or amplification step. Given its excellent analytical performance and ease of preparation, the Au nanospike-based immunosensor shows a great potential as a reliable and rapid tool for detecting *S. aureus* in bacterial culture samples, suggesting its suitability for clinical applications.

4.5 Conclusion

In this work, a highly sensitive label-free immunosensor was designed to detect *S. aureus* using anti-Aur antibody. The electrochemical deposition of Au nanospikes on SPGE successfully provides a stable substrate with a larger active surface area, allowing a higher Ab immobilization and thus an increased sensitivity. Anti-Aur antibodies as biorecognition elements were covalently bound to self-assembly monolayer of MUA. The biosensing platform demonstrated not only an excellent sensitivity, and great specificity towards *S. aureus* but also a fairly good stability. Our results demonstrate a linear range and LOD of 1-100 pg mL^{-1} and 5 pg mL^{-1} for Aur in PBS buffer, and 10^2-10^8 CFU mL^{-1} and 28.5 CFU mL^{-1} for *S. aureus*. The combination of a low limit of detection (LOD) and a wide detection range makes this immunosensor a highly promising tool for rapid and reliable detection of *S. aureus* and holds great potential for clinical applications, particularly in improving early and point-of-care diagnosis in PJI.

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CHAPTER 5 GENERAL DISCUSSION

The aim of the thesis was to develop an electrochemical biosensing device to detect a causative bacterium of PJI, *S. aureus.* To this end, a gold-antibody conjugate based sensor was designed to measure a biomarker for *S. aureus*, aureolysin and the successful fabrication of the immunosensor was demonstrated.

One of the main challenges in developing the electrochemical biosensor was improving the conductivity to increase sensitivity. To address this challenge a screen-printed electrode as a working electrode was modified with electrodeposited gold nanospikes. The following results after Au nanospikes electrodeposition were observed: a decrease in the difference between cathodic and anodic peaks potential (ΔE_p), an increase in cathodic (I_{cp}) and anodic peaks (I_{ap}) for CV, and an increase in peak current (I_{peak}) in DPV. The results were as expected since Au nanospikes led to an increase in electrochemical activity and surface area of the electrode. A self-assembly monolayer of MAU was adsorbed on the gold surface, followed by Aur-antibody conjugation on the electrode through linking to MUA molecules. Then the surface of the electrode was covered by BSA as an anti-fouling agent. Before and after each step, differential pulse voltammetry and cyclic voltammetry were performed to confirm the successful fabrication of each layer. The steps, from MUA formation to BSA adsorption, caused a decrease in I_{cp} and I_{ap} in CV and a decrease in I_{peak} in DPV due to the electron transfer hindrance. The CV and DPV results suggest that the immunosensor was successfully fabricated.

Another interest of the project was to study the impact of gold nanospikes on the electrochemical properties of the SPGE. The studied electrochemical properties were: electrocatalytic activity, electrochemical surface area (ECSA), and roughness of the electrode (Rf). The catalytic activity, after and before electrodeposition of gold nanospikes, was measured by cyclic voltammetry in a 0.1 M solution of H_2SO_4. The reduction peak current density of modified SPGE was approximately 15 times higher than that of bare SPGE (from -0.25 mA cm^{-2} to -3.74 mA cm^{-2}). Then the area under the cathodic peak was calculated to obtain charge transfer that is corelated to the ECSA (ECSA = Q/390 µC cm^{-2}). ECSA increased by 7.7 times from 0.060 ± 0.013 cm^2 to 0.461 ± 0.032 cm^2. Next, roughness was determined by calculating the ratio of ECSA to the geometrical surface area, changing from 0.536 ± 0.106 to 3.665 ± 0.258. Therefore, the electrochemical deposition of

gold nanospikes had an impact on increasing the active surface area, roughness, and electrochemical activity of the electrode.

To evaluate the performance, the limit of detection and dynamic range of detection of immunosensor for Aur were found. The DPV measurements in different Aur concentrations in PBS buffer were carried out. A linear regression was performed to analyze the change in current density versus Aur concentration, resulting in Y = 2.791 C+ 91.610 with an R^2 value of 0.995. Using IUPAC, the LOD was estimated 5pg mL^{-1} with the linear range of 1-100 pg mL^{-1}. Therefore, the sensor has a good sensitivity to Aur and the second subobjective was also successfully achieved.

The sensitive and selective detection of *S. aureus* in culture samples was investigated. The results showed a linear range of 10^2-10^8 CFU mL^{-1}, limit of detection of 28.5 CFU mL^{-1} and linear regression of Y= 17.467 log(C) -0.876 (R^2= 0.974). The results confirmed sensitivity of the developed sensor toward *S. aureus* and low limit of detection in a wide range of concentration.

Subsequently, two culture samples of *E. Coli* and *P. aeruginosa* with a concentration of 10^5 CFU mL^{-1} were applied to the immunosensor and compared to a sample of *S. aureus* with the same concentration. The results demonstrated that the change in current density (ΔJ) response was negligible for *E. Coli* and *P. aeruginosa* in comparison to *S. aureus*. Finally, a stability test was performed, showing the sensor is storable up to 4 days after fabrication with 93% initial response.

CHAPTER 6 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

In conclusion, a sensitive anti-Aur antibody based immunosensor to detect *S. aureus* was developed using Au nanospikes on SPGE.

A selectivity test using *E. Coli* and *P. Aerugonisa* to identify if any cross-reactivity confirmed that the immunosensor was specific to *S. aureus* showing a good linear relationship ($R^2 = 0.974$) between the increase in peak current and logarithmic *S. aureus* concentration, with a rapid detection time (1h) and a limit of detection of 5 pg mL^{-1} in buffer and 28.5 CFU mL^{-1} in complex bacterial culture samples. This low-cost immunosensor can be used for rapid detection of pathogens in body fluid samples with high sensitivity and specificity. Our immunosensor offers an early warning point-of-care diagnostic tool to provide rapid and precise results for S. aureus infections even in the presence of biofilm.

The immunosensor developed in this thesis shows promise for the development of highly sensitive biosensors capable of detecting various target analytes. The straightforward and facile fabrication process of the immunosensor offers a foundation for future studies aimed at advancing electrochemical biosensors.

A notable contribution of this thesis is the development of the first electrochemical immunosensor for the detection of aureolysin, which exhibits remarkable detection capability for the target *S. aureus*. The immunosensor demonstrates a broad linear range and a relatively low LOD compared to previous studies. With its outstanding performance, the developed immunosensor has the potential to detect low-concentration target analytes such as gram-positive and gram-negative bacteria, making it highly suitable for real-world applications.

The developed immunosensor holds the potential for a point-of-care test for bacterial infections which could benefit patients by providing a rapid and accurate diagnosis. It could potentially enable the monitoring of treatment progress, allowing clinicians to adjust the course of therapy as needed, particularly in cases where antibiotics are used to prevent the development of antibiotic-resistant strains of bacteria. It is less expensive than laboratory-based testing and reduces the need for expensive hospital stays and follow-up visits.

This achievement represents a significant contribution to the field of electrochemical biosensors and offers new avenues for the detection of various target analytes with high sensitivity.

6.2 Limitations of the work

- One of the main challenges was that the roughness and surface pattern of the commercially available screen printed gold electrodes were variable from one electrode to another, therefore the baseline signal for each immunosensor was different, leading to error in measurements.
- The proposed Aur immunosensor was tested in only buffer solution and bacteria culture. We did not take into account the complex environment of body fluids with presence of many other nonspecific proteins.
- All measurements were conducted using a benchtop potentiostat which has higher sensitivity than a portable potentiostat, limiting the application of the device as a point of care test.
- The developed immunosensor was designed to detect one single bacteria. The PJIs are polymicrobial infections with multiple causative bacteria rather than only one. Decisions regarding appropriate antibiotic treatment are made based on all the main responsible microorganisms [152]. Plus, PJIs cases involving *S. aureus* infection account for only 19-29% [30].

6.3 Recommendations and potential future work

- For real clinical applications, the research must be continued to calculate the accuracy of the device for detection of *S. aureus,* performing sensing measurements in hospitalized PJI patients' blood samples. Also the precision of the immunosensing platform can be calculated by repeatability test under constant condition and reproductivity test under different condition such as different operators and labs.
- For real time detection, the device can be designed as a wireless 3D printed handheld surgical pen projected to be used by surgeons or a health care specialist in the operating room. This would enable surgeons to monitor potential bacterial infection during the arthroplasty operation.

- One solution for detecting polymicrobial infections is upgrading the device to a microarray of electrodes, where each electrode was functionalized for different targets for multiplex detection of more than one bacterium simultaneously.
- It would be interesting to combine the antibody with ssDNA probs as a dual detection method and using CRISPR-Cas 12 with trans cleavage activity to improve the sensitivity and accuracy of the device.
- To address the lack of portability, the cellphone based potentiostat or portable mini potentiostat can be used as a closer platform to point of care diagnosis.

6.4 Closing remark

I would like to end by expressing my sincere gratitude to my advisor, Prof. Geraldine Merle, colleagues, and loved ones who have supported me throughout this journey. This work would not have been possible without their guidance and encouragement. It is hoped that this work, conducted at Polytechnique Montréal, will inspire future research in this field and contribute to the development of innovative devices for medical diagnosis.

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