Nanoengineered charge gradients as a high throughput tool to study antimicrobial drug tolerance

By

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LIST OF ABBREVIATIONS

Abbreviation	Full name
CFU	Colony forming unit
CLSM	Confocal laser scanning microscopy
GTAC	Glycidyl trimethylammonium chloride
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
OD	Optical density
ppAA	Plasma polymerised allylamine
QAC	Quaternary ammonium compound
XPS	X-ray Photoelectron Spectroscopy

ABSTRACT

Implantable medical devices are essential in healthcare systems to improve human health and life expectancy. However, the colonisation of pathogenic microorganisms on the surface of these devices is a significant concern as they should be removed or replaced frequently from the body of the patient to prevent complete colonisation. Currently, antibiotics have been the main method to prevent and control infections from pathogenic microorganisms; however, the overuse and misuse of antibiotics lead to the emergence of antibiotic tolerance, challenging in treatment with this conventional method. To combat this challenge, previous studies have modified the surface to change the surface properties by coating an antimicrobial compound on the material. Among these compounds, quaternary ammonium compounds (QACs) are demonstrated to show broad-spectrum antimicrobial activity and be less toxic to human cells. This compound is widely used to coat surfaces for the creation of contact-active killing bacterial material, killing harmful microorganisms upon direct contact. However, previous studies have not investigated deep into the potential of immobilised QACs in reducing antibiotic tolerance, especially in the context of electrostatic properties.

The surfaces with a strong positive charge are demonstrated to enhance the interaction with the negative charge cell membrane of bacteria. These interactions are critical as they facilitate the disruption of bacterial membranes, enhancing direct antimicrobial effects. In addition, these electrostatic properties can enhance the efficacy of antibiotics by facilitating their interaction with bacterial surfaces. Therefore, in this project, we investigated a biomaterial to address the antibiotic tolerance of creating an antimicrobial coating on surfaces with immobilised QACs gradient density. This modified surface was developed by the deposition of allylamine using plasma polymerisation to generate an amine-rich surface. Glycidyltrimethylammonium chloride (GTAC), a type of quaternary ammonium compound, was coated onto the plasma polymerised allylamine (ppAA) surface via the reaction between epoxy groups of QAC and amine groups of allylamine. The primary samples in this study were the surfaces with a concentration gradient of GTAC created by dip coating. The surface characterisation of the modified surface confirmed the presence of QAC and a gradient in QAC density on the surface through ellipsometry, water contact angle measurement and fluorescence analysis. In general, the addition of GTAC on ppAA surface changed the surface properties including thickness, wettability, fluorescence intensity due to the presence of NR_4^+ of QAC.

The antimicrobial surface tests demonstrated that increasing the concentration density of QAC significantly enhanced the antibacterial efficacy against *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 15692 compared to the control surface (ppAA only). Especially, after 3 hours of incubation with pathogenic microorganisms, the modified surface exhibited a

reduction in the viability of *S. aureus* (13.23% ± 1.55) and *P. aeruginosa* (23.78% ± 3.81) at the 8-10 mm position remained in the GTAC solution for the longest time (6 hours). In contrast, the lowest viability of fungi (*Candida albicans* ATCC 10231) was observed at 0-2 mm position, which corresponds to the region immersed in GTAC solution for the shortest duration. The data also revealed that QAC surface immobilisation density did not significantly impact the attachment of the microorganisms, indicating that enhanced antimicrobial effect was primarily due to reduced bacterial viability rather than decreased microorganism adhesion.

This study focused on the antibiotic tolerance of *S. aureus* on QAC surface concentration density gradient under vancomycin, daptomycin levofloxacin, and kanamycin treatment for 24 hours. Overall results revealed that the presence of QAC improved the antibacterial activity of antibiotics in reducing bacteria viability, especially at higher gradient positions (8-10 mm). Particularly, kanamycin reduced *S. aureus* viability by 55.21% ± 3.03, while vancomycin showed a similar reduction of 47.47% ± 1.21. Levofloxacin and daptomycin were the most effective, reducing viability by 31.08% ± 6.78 and 34.03% ± 2.14, respectively. In overall, the results demonstrated that the activity of four antibiotics was improved, highlighting the effect of QAC surface immobilisation density on antibiotic treatments in combating bacterial tolerance.

DECLARATION

I certify that this thesis:

1. does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university

2. and the research within will not be submitted for any other future degree or diploma without the permission of Flinders University;

3. to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

Signed: Gia Han Tran

Date: 31st October 2024

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CHAPTER 1. LITERATURE REVIEW

1.1. Introduction

Implantable medical devices are widely used in surgeries worldwide, with demand increasing due to aging populations, trauma and elective procedures. This rise also brings a risk of medical device-associated infections, which can lead to severe complications such as biofilm formation and chronic infections. The overuse of conventional antibiotics to prevent these infections may contribute to antibiotic tolerance. To address this, research has focused on developing coatings for the surface of implantable medical devices that offer both antimicrobial properties and biocompatibility.

In this study, a plasma polymerisation technique was employed to create an amine-rich layer on the surface. This surface was further coated with GTAC which is a QAC, known for its broad-spectrum antimicrobial activity. Dip coating, a high throughput coating tool, was used to create QAC immobilisation surface density. This method allows to generate QAC concentration density with antimicrobial properties on one surface by controlling the immersion time and the speed. These coatings would reveal the influence of electrostatic properties on antibiotic tolerance, providing a pathway for reducing biofilm formation and combating antibiotic tolerance.

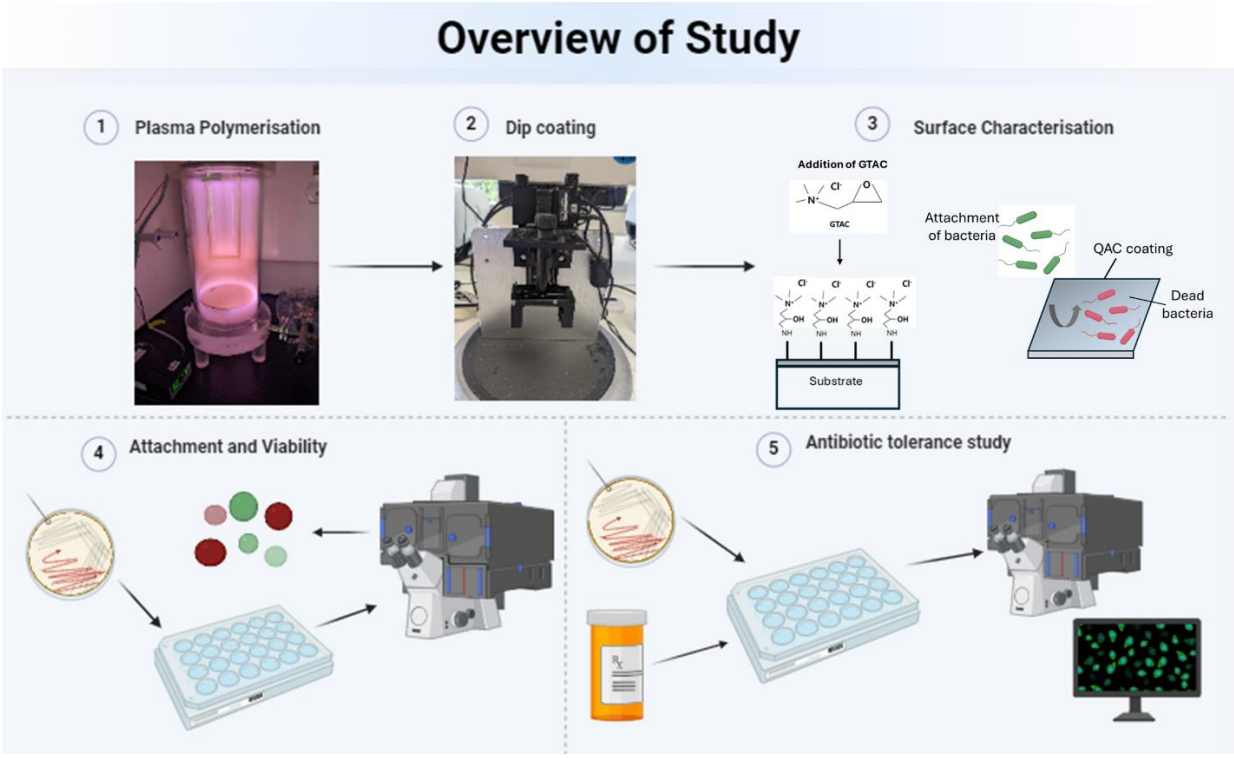

Figure 1. The schematic illustrating the main experiment in the study from surface modification using plasma polymerisation (1), followed by dip coating to achieve QAC immobilisation surface gradient

(2), surface characterisation to assess the presence of GTAC (3), the study of pathogen microorganism attachment and viability (4), and concluding with antibiotic tolerance study (5).

1.2. Implantable medical devices in healthcare

A medical device is an essential aspect of healthcare that contributes to life expectancy and improved efficacy of a wide range of treatment conditions. Medical devices include a variety of equipment, materials, and apparatus used for medical purposes (Khatoon et al., 2018). In recent years, the demand for medical devices has increased driven by some factors such as organ transplants, chronic diseases, and traumatic accidents (Khatoon et al., 2018). The market for medical devices in the US was reported to have a substantial growth of 90 billion USD (Caldara et al., 2022). With the wealth of functions, the industry is anticipated to reach a compound annual growth rate (CAGR) of 6.3% from 2019 to 2025 with approximately 140 billion USD of market value in 2025 (Caldara et al., 2022). The treatment of certain diseases was improved by the use of medical devices, which ultimately enhanced the quality of life for patients (Caldara et al., 2022). As a result, advancements in medical technology and their application continue to play a critical role in modern healthcare practices.

An essential part of medical devices is implantable medical devices which address patient morbidity and mortality. These devices, which are either fully or partially introduced into the human body through surgical or medical procedures, are designed to remain within the body post-implantation (Wilson et al., 2021). The advancements in microelectronics, biotechnology, and materials science over the past six decades have contributed to the improvement of the design, function, and reliability of these devices (Joung, 2013). Implantable medical devices have been applied across various medical specialties. For example, 8% to 10% of people in America, along with 5% to 6% of individuals in industrialised nations have used an implantable medical device (Joung, 2013). Also, approximately seven million Americans live with hip or knee replacements, enhancing mobility and reducing joint pain (Wilson et al., 2021). Additionally, over 500,000 procedures involving pacemakers or defibrillators are performed annually in the United States, addressing cardiac arrhythmias and preventing sudden cardiac events (Wilson et al., 2021). In coronary interventions, around 480,000 inpatient procedures, often involving cardiac stent placement, highlight the widespread use of these technologies to support vascular health (Wilson et al., 2021). These devices play an important role in restoring bodily functions, enhancing life quality, and extending life expectancy.

1.3. Infections associated with implantable medical devices

Most medical devices can be easily infected and contaminated by bacteria which poses challenges to current treatment. Contamination of these devices typically occurs during the surgical process, often from a small number of microorganisms originating from the patient's skin or mucous membranes (von Eiff et al., 2005). In some cases, pathogens can also be introduced by the hands of surgical or clinical staff. The severity of device-associated infections depends on patient characteristics, the type of microorganisms involved, and the specific device (von Eiff et al., 2005). Infections acquired from contaminated medical devices not only increase mortality rates but also lead to chronic infections and a higher risk of device failure which challenges conventional antibiotic treatment (Bera et al., 2023; Khatoon et al., 2018). In particular, indwelling medical devices were reported to be a primary source of nosocomial infections as microorganisms can easily attach, grow, and infect subsequently (Bera et al., 2023). The healthcare-associated infections have become a significant concern accounting for 2 million cases, among which infections from indwelling devices account for 50-70% (Bera et al., 2023). Once the medical devices are contaminated with bacteria, they must be promptly removed and replaced with new ones before complete bacterial colonization occurs (Bera et al., 2023; Cavallaro et al., 2016). However, this process is costly and inconvenient, with estimates suggesting that treatment involving the removal of infected prostheses and antibiotic therapy can exceed $50,000 (Khatoon et al., 2018). Also, the replacement of more complex permanent implants including hip or knee implants poses a significant challenge (Cavallaro et al., 2016). In general, the emergence of hospital-acquired infections, especially from medical devices might be life-threatening for patients and healthcare systems.

1.4. Biofilm formation on implantable medical devices

Biofilm formation is mainly responsible for implantable medical device-related infections. It is reported that approximately 40-80% of bacterial cells have the ability to develop a biofilm (Muhammad et al., 2020). The common pathogens associated with healthcare infections and biofilms on implantable medical devices are *Escherichia coli, Staphylococcus aureus, Klebsiella spp., Pseudomonas aeruginosa*, *Candida albicans*, among other microorganisms (Bera et al., 2023). Biofilm is a structural community of microorganisms living inside an extracellular polymeric matrix (EPS), fostering microbial adherence to surfaces (Khatoon et al., 2018; Muhammad et al., 2020). The extracellular polymeric substance consists of polysaccharides, proteins, and extracellular DNA, which significantly contribute to biofilm formation (Khatoon et al., 2018; Muhammad et al., 2020). In the matrix, nutrients are trapped for metabolic utilization by the bacteria and water is efficiently retained through H-bond interactions with hydrophilic polysaccharides (Kostakioti et al., 2013). Additionally, enzymes secreted in response to changes in nutrient availability allow them to tailor

biofilm architecture to the specific environment. As the structural components of the matrix are highly hydrated and tensile strength that keeps bacteria in close proximity, enabling intimate cell-to-cell interactions and DNA exchange, EPS serves as a protective barrier against external threats and captures exogenous substances surrounding the cell (Bera et al., 2023; Kostakioti et al., 2013; Muhammad et al., 2020). Hence, the ability to form the biofilm is considered an adaptability of microorganisms which helps them survive under unfavorable conditions (Muhammad et al., 2020). It can be seen that bacterial biofilm is a notable concern that healthcare is facing. Bacteria are capable of developing biofilm when attached to a surface to persist under stress conditions. This process occurs in a few main steps including initial attachment, microcolony formation, biofilm maturation and dispersal/detachment (Bera et al., 2023; Muhammad et al., 2020). **Figure 2** displays various stages as the microorganisms attach to the surface for biofilm formation.

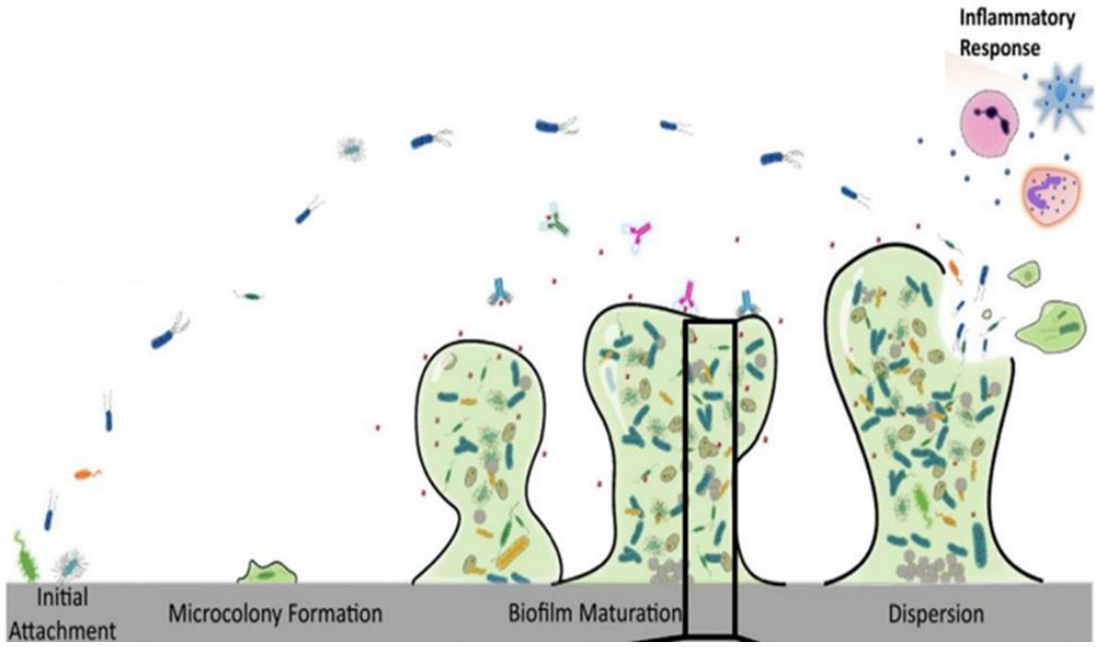

Figure 2. The main stages to form the biofilm of microorganisms on the surface (Bera et al., 2023)**.** Reproduced with permission.

1.4.1. Initial attachment

The initial step of biofilm formation is attachment, influenced by various environmental factors. The first stage of biofilm formation begins once the favorable interaction between surface and planktonic cells is generated based on repulsive and attractive forces (Muhammad et al., 2020). In particular, bacteria are transported to surfaces through mechanisms like Brownian motion, sedimentation, and convection, and this process is further affected by surrounding hydrodynamic forces (Kostakioti et al., 2013; Muhammad et al., 2020). Once bacteria are close to the surfaces, bacteria experience attractive or repulsive forces based on conditions such as nutrient levels, pH, ionic strength, and temperature (Kostakioti et al., 2013). The properties of the medium, along with the

composition of the bacterial cell surface, influence both the velocity and direction of movement toward or away from the contact surface (Kostakioti et al., 2013). Upon reaching the surface, bacteria attachment depends on the net effective forces, such as electrostatic interactions, hydrophobicity, and Van der Waals forces versus repulsive forces (Muhammad et al., 2020).

1.4.2. Microcolony formation

After weak adhesion forms on the surface, EPS is produced through the regulation of quorum sensing (QA) of bacterial cells to establish a stable microcolony (Bera et al., 2023). EPS is an important factor in surface adherence, biofilm formation, structural integrity, water retention, signal, protection, nutrient trapping, and genetic exchange (Muhammad et al., 2020). DNA in the EPS matrix, an integral part, acts as an intercellular connector and lipids can drive the attachment process (Muhammad et al., 2020). Additionally, the polysaccharides involved in the composition of EPS are essential for biofilm development, are typically neutral or polyanionic in Gram-negative bacteria, with anionic properties due to uronic acids or ketal-linked pyruvates that facilitate the binding of divalent cations like magnesium and calcium, leading to stronger biofilm cohesion (Muhammad et al., 2020). In contrast, the EPS of Gram-positive bacteria, such as *Staphylococci*, is primarily cationic (Muhammad et al., 2020).

1.4.3. Maturation

Biofilm maturation is considered a steady state that includes water channels facilitating nutrient distribution and signaling molecule exchange (Bera et al., 2023). The duration to form a mature biofilm typically takes from several hours to several weeks (Bera et al., 2023). The autoinducer (AI) signals drive the multiplication of bacteria within the protective EPS matrix, promoting the formation of microcolonies and the further maturation of the biofilm (Muhammad et al., 2020). As microcolonies form and EPS accumulates, gene expression changes occur, leading to the production of EPS components that serve as biological “glue” to bond bacterial cells within the matrix (Muhammad et al., 2020). The maturing biofilms develop water-filled channels, which function like a circulatory system to distribute nutrients and expel waste products, ensuring the survival bacterial community (Muhammad et al., 2020). Structurally, biofilm microcolonies often display pyramid or mushroom shaped multicellular arrangements (Muhammad et al., 2020).

1.4.4. Detachment/Dispersal

The final stage of biofilm formation is detachment, also known as dispersal which is a critical biofilm formation process as it facilitates the spread of bacteria from one site to another, leading to infection spreading (Khatoon et al., 2018; Muhammad et al., 2020). The detachment is considered an

adaptive strategy where bacterial cells detach from a biofilm and form new microcolonies on other substrates in response to environment or physiological signals (Kostakioti et al., 2013; Muhammad et al., 2020). Although detachment mechanisms differ among bacterial species, they generally follow three common stages including detachment of cells from the biofilm, movement to a new substrate, and reattachment to initiate new biofilm formation (Muhammad et al., 2020). The detachment process can occur actively like seeding, where cells initiate their own release due to environmental stress like nutrient starvation, antimicrobial presence, or enzyme activity (Muhammad et al., 2020). This form (seeding dispersal) involves the rapid release of planktonic cells or microcolonies from the biofilm's center, resulting in an empty cavity (Muhammad et al., 2020). Voids within the biofilm can lead to cell death which is considered an additional detachment mechanism, as observed in *P. aeruginosa* (Kostakioti et al., 2013). In the context of biofilm-associated medical device infections, detachment can lead to disseminated chronic infections, contributing to morbidity and mortality (Bera et al., 2023).

1.4.5. Clinical concerns

The incidence of infections related to implantable medical devices is a significant clinical problem, and challenging to treat. Within the host, the biofilm matrix protects bacteria from innate immune defences like opsonisation and phagocytosis, as well as antibiotic treatment, allowing bacteria to persist and grow (Kostakioti et al., 2013). As a result, biofilm-associated pathogens are responsible for chronic and difficult-to-treat infections, leading to severe complications and death in some cases (Kostakioti et al., 2013). In fact, critically ill patients in intensive care units (ICUs) are especially vulnerable to biofilm-associated nosocomial infections due to the frequent use of invasive devices such as ventilators, central lines, and catheters (Mishra et al., 2024). These infections often involve pathogens like *Staphylococcus aureus*, *Streptococcus* species, and *Enterococcus* species, leading to conditions such as infective endocarditis when bacteria adhere to heart valves (Mishra et al., 2024). Also, the clinical diagnosis and treatment of implantable device-associated infections still remain problematic due to the lack of clear diagnostic criteria. This issue is more challenging because of the difficulty in distinguishing between benign bacterial colonisation of the device and true infection (VanEpps & Younger, 2016). Current diagnostic methods can increase the detection of microorganisms but may also result in false positives (VanEpps & Younger, 2016). Hence, implantable medical device-associated infections not only complicate clinical management but also contribute to false assessments of patient health status.

1.4.6. Clinical treatment to prevent infection

The most effective treatment for biofilm infections associated with foreign bodies is the removal of implantable medical devices. However, this can challenge devices such as prostheses and pacemakers, complicating the management of infections in these cases (Dhole et al., 2023). Currently, antibiotics are the preferred treatment for bacterial infections in the context of reducing infection rates. The conventional antibiotics act through two main mechanisms such as inhibiting cell division (bacteriostatic) or killing bacteria (bactericidal) (Kostakioti et al., 2013). In fact, antibiotic prophylaxis is widely used to reduce bacterial populations at the surgical site. By minimizing the microbial load, these antibiotics create a less conducive environment for infections to establish, which is critical during the early stages of wound healing when the surgical site is most susceptible to bacterial invasion (Dhole et al., 2023). Additionally, antibiotics serve as a barrier against the local spread of bacteria, preventing migration to deeper tissues or body cavities where infections can become severe and more challenging to manage (Dhole et al., 2023).

1.5. The challenge to treat implantable medical device-associated infections

1.5.1. Emergence of antibiotic tolerance

Although antibiotics can be used to treat implantable medical device-associated infections, bacteria can be tolerant to antibiotics which leads to the failure of antibiotic treatment. Antibiotic tolerance refers to bacteria being unable to proliferate but can persist under antimicrobial treatment (Khatoon et al., 2018). It differs from antibiotic resistance which involves the ability of bacteria to withstand the effects of antibiotics and continue to proliferate (Khatoon et al., 2018). Bacteria within biofilm exhibit slower metabolism, resist host immune defences, and tolerance to high doses of antibiotics (Kostakioti et al., 2013). One of the main factors causing the recalcitrance to antibiotic treatment is the diffusion barriers posed by the biofilm's extracellular matrix, leading to antibiotic activation (Kostakioti et al., 2013). In fact, biofilm infections are difficult to eradicate with an increase in antimicrobial tolerance in biofilms to 100 to 1,000 times (Olsen, 2015). Biofilm formation, as well as antibiotic tolerance, is believed to be a natural state as the majority of bacteria (Olsen, 2015). Also, it is reported that the antimicrobial efficiency may be reduced due to the phenotypic characteristics of biofilms (Olsen, 2015). Tolerant cells have a selective advantage during transient or periodic antibiotic treatment as they exhibit a prolonged minimum duration of killing by antibiotics (Yan & Bassler, 2019). The presence of tolerant cells is believed to contribute to the challenges in eliminating biofilms during chronic infections (Yan & Bassler, 2019). Therefore, antibiotic therapy may no longer be sufficient to address biofilm infections.

1.5.2. Mechanisms of antibiotic tolerance in biofilm

Antibiotic tolerance occurs through various mechanisms, contributing to the failure of conventional antibiotic treatment. These protective mechanisms involve restricted penetration, slow growth rates, altered metabolism and the presence of persister cells (Olsen, 2015). Specifically, the biofilm matrix acts as a barrier that slows and restricts antimicrobial penetration, protecting microorganisms and delaying the drug's effect through biofilm (Olsen, 2015). Also, bacteria can find other retention sites where without the presence of antibiotics on medical devices and attach to them (Olsen, 2015). Differential physiological activity is another mechanism that causes antibiotic tolerance. In an environment with limited nutrients and oxygen penetration, bacteria with low metabolic activity can enhance their tolerance to antibiotics that only target actively growing cells (Ciofu et al., 2017). In addition to the above mechanisms, persister cells with low metabolic activity are unaffected under antibiotic treatment (Olsen, 2015). Persister cells are defined as bacterial cells that survive under antibiotic treatment due to their slow-growing, low-metabolic state. Hence, these persister cells can escape the antibiotic activity and then repopulate the infected location since most antibiotics target growing cells to kill them (Ciofu et al., 2017; Olsen, 2015). Overall, the mechanisms of antibiotic tolerance in biofilm such as low antibiotic penetration, differential physiological activity and the presence of persister cells present obstacles to effective antimicrobial therapy.

1.6. Role of material surface properties on biofilm formation

The process of bacterial adhesion to the surface is an initial step of biofilm formation; hence it is important to prevent bacterial adhesion. There are several factors influence bacterial adhesion to the surfaces including the exposure duration to the substrate, the bacterial population, and intrinsic bacterial characteristics such as cell wall composition, motility and nutritional environment (Zheng et al., 2021). Among these parameters, surface properties of the substrate such as charge density, wettability, roughness, stiffness, and topography significantly affecting initial bacterial adhesion have been reported to govern initial bacterial adhesion to surfaces as described in **Figure 3** (Zheng et al., 2021). In that context, numerous efforts have been made to design materials by modifying these surface properties to prevent biofilm formation at an early stage.

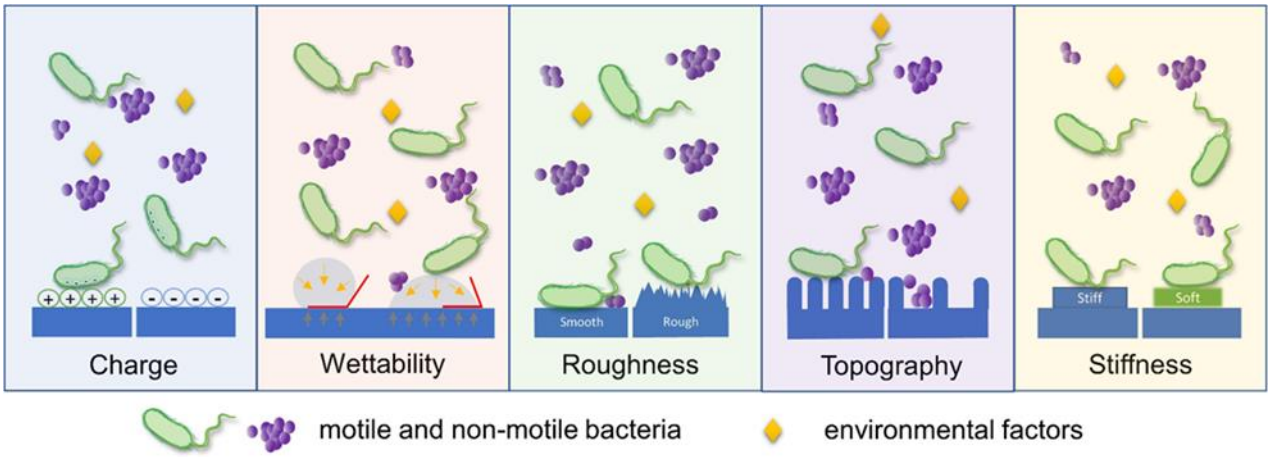

Figure 3. Schematic representation of the surface properties that affect bacterial adhesion. Bacterial adhesion is influenced by several surface parameters, such as surface charge density, wettability, roughness, topography, and stiffness (Zheng et al., 2021). Reproduced under the Creative Commons Attribution License.

Surface wettability, a key property, governs the behaviour of liquid-solid interactions within biological systems. The liquid phase wets a solid surface by spreading out to increase the contact area, which enhances the interaction between them (Zheng et al., 2021). Typically, low-energy surfaces and high-tension liquids decrease wettability, while high-energy surfaces and low-tension liquids increase it (Zheng et al., 2021). The energy of adhesion is critical in bacterial attachment which can be evaluated by combining Lifshitz-van der Waals interactions, electric double-layer forces, and acid-base interactions (Zheng et al., 2021). A negative free energy generally promotes bacterial adhesion, whereas positive free energy can inhibit it. It is indicated that bacteria with hydrophobic cell surfaces prefer hydrophobic material surfaces, while those with hydrophilic surfaces favour hydrophilic materials (Zheng et al., 2021). The relationship between wettability and bacterial adhesion is still complex. Previous studies show conflicting results for bacterial attachment on surfaces with moderate hydrophobic or hydrophilic properties (Zheng et al., 2021). However, the modified surfaces with superhydrophobic or super hydrophilic have shown promise in limiting bacterial adhesion. For instance, *S. aureus* adhesion was significantly reduced on superhydrophobic polyurethane sponges modified with zinc oxide and copper nanoparticles, and *E. coli* adhesion was suppressed on superhydrophilic TiO_2-coated stainless steel (Zheng et al., 2021).

Surface roughness is a key factor influencing bacterial adhesion, as it increases the available surface area and provides additional structural support for bacterial attachment. For instance, rough surfaces also protect bacteria from shear forces, making it more difficult for attached bacteria to detach under fluid flow (Zheng et al., 2021). In general, studies have shown that bacterial adhesion and biofilm formation increase as surface roughness increases. For instance, surfaces with larger surface areas or micro-textures promote higher bacterial attachment rates, as seen in species like

Staphylococcus epidermidis, *Pseudomonas aeruginosa*, and *Ralstonia pickettii (Zheng et al., 2021)*. However, the effect of surface roughness on bacterial adhesion is complex. In some cases, increased roughness has led to reduced bacterial adhesion, as seen with *P. aeruginosa* and *S. aureus* on unpolished stainless steel, which showed lower adhesion rates compared to smoother electropolished surfaces (Zheng et al., 2021).

Surface charge density plays a vital role in influencing bacterial adhesion to material surfaces. Van der Waals forces and electrostatic interactions are the main forces that help bacteria attach to the surfaces (Zheng et al., 2021). Studies have suggested that certain charge densities not only affect initial adhesion but also influence subsequent biofilm formation. For instance, surfaces with specific cationic groups, such as quaternary ammonium compounds and polyethylenimines, have antimicrobial properties that can prevent adherent bacteria (Zheng et al., 2021). Notably, the trends in initial bacterial attachment and biofilm growth may not always be consistent. For example, while *E. coli* adheres more to modified polyethylene sheets with the presence of positively charged functional groups, high charge density may reduce cell viability and slow biofilm growth over time (Zheng et al., 2021). This highlights the need for further research to understand how surface charge density affects both attachment and biofilm development across different bacterial species.

1.7. Contact-active surfaces

To reduce implantable medical device-associated infections, it is important to prevent contamination on high-contact areas of the surfaces and medical equipment. Researchers have developed surfaces that are either non-fouling or antibacterial offering a promising method to minimise cross-contamination. There are three forms of antibacterial surfaces including surfaces that prevent bacterial attachment (repelling surface), surfaces that release antibacterial agents (leachable surface) and surfaces that kill bacteria upon contact (contact-kill surface) (**Figure 4**) (Kaur & Liu, 2016). The first approach focuses on designing surfaces that resist bacterial adhesion through strategies like controlling hydrophobicity and surface roughness. Although this kind of surface effectively reduces biofouling, it cannot eliminate bacteria (Kaur & Liu, 2016). The second approach involves surfaces treated with leaching agents such as silver or triclosan, that kill bacteria as the agents are released. However, there are concerns about environmental impact and bacterial resistance limiting their sustainability (Kaur & Liu, 2016). The third and more eco-friendly method involves contact-killing surfaces where biocides are permanently fixed, ensuring that bacteria are killed on contact without environmental contamination or contributing to resistance.

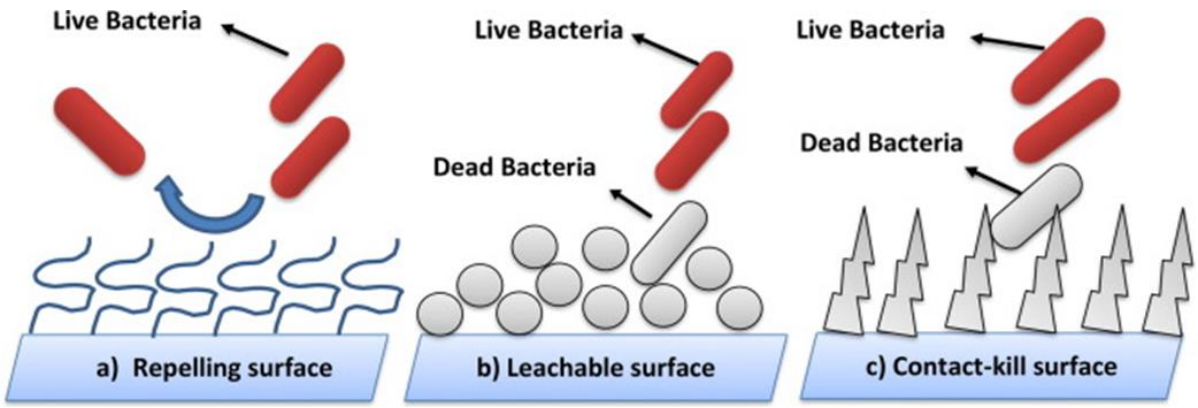

Figure 4. Three forms of antibacterial surface mechanism (a) Repelling surface, which prevents bacterial adhesion; (b) Leachable surface, which contains antimicrobial agents that leach out and (c) Contact-kill surface, where bacteria are killed upon direct contact with the surface (Kaur & Liu, 2016). Reproduced with permission.

1.7.1. Quaternary ammonium compounds-based surface coatings

1.7.1.1. Quaternary ammonium compounds

Researchers first attempt to modify the surface's physicochemical properties to prevent bacterial adhesion, and second, employ bactericidal properties to kill bacteria upon direct contact (VanEpps & Younger, 2016). Immobilised quaternary ammonium compounds (QACs), one kind of contact-kill surfaces, were demonstrated to provide long-lasting and contact-based antimicrobial action (Muhammad et al., 2020). Antimicrobial agents such as QACs exhibit antimicrobial activity and likely have low toxicity to human skin cells (Saverina et al., 2023). Structurally, this cationic compound consists of a quaternary ammonium nitrogen ion as the primary head, a lengthy hydrophobic alkyl chain tail, and a counterion (X^-) being primarily halogen negative ions or acids (Wang et al., 2023). The main structure of QAC is described in **Figure 5**. The positively charged region of QAC is believed to contribute significantly to its antimicrobial efficacy, particularly in its interaction with negatively charged bacterial cell surfaces (Wang et al., 2023). The antimicrobial action occurs when positively charged QAC interacts with negatively charged bacterial cell surfaces, and then replaces positively charged ions such as Ca^{2+} and Mg^{2+} on the bacterial surface to maintain charge neutrality (Asri et al., 2014; Wang et al., 2023). Based on these features, QAC has attracted particular attention because they exhibit antimicrobial action by interacting with bacterial cell surfaces and enhancing antibacterial activities.

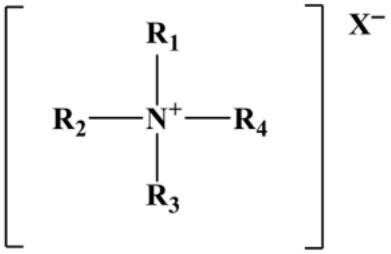

Figure 5. The generic structural formula of quaternary ammonium compounds (Wang et al., 2023)**.** Reproduced with permission.

The structure of QAC including cationic group, alkyl chain length and counterion may influence their antimicrobial activity. Specifically, the antimicrobial properties are associated with the interaction between the cationic head groups of QAC and the negatively charged head of acidic phospholipids in the cell membrane (Wang et al., 2023). Increasing the number of cationic groups has been shown to enhance bactericidal activity and absorption to bacteria, as demonstrated by research on synthesised gemini quaternary ammonium compounds with multiple cationic head groups (Wang et al., 2023). Additionally, the antimicrobial effectiveness of QAC against various microorganisms might be influenced by hydrophilic-hydrophobic balance with increasing alkyl chain length (Kaur & Liu, 2016). Studies on free QACs in solution have shown that those with 12-14 methylene units in their alkyl chains are most effective against Gram-positive bacteria, while chains with 14-16 methylene units exhibit greater activity against Gram-negative bacteria (Kaur & Liu, 2016). However, the bacterial killing of QAC mainly focuses on two primary mechanisms: one involves the penetration of bacterial membranes by QACs coated onto surfaces, and the other relies on the exchange of cationic ions between cell membranes and positively charged surfaces, so the impact of alkyl chain length in surface-bound QACs on their antibacterial properties is controversial (Kaur & Liu, 2016).

Among QACs, glycidyl trimethylammonium chloride (GTAC) containing an epoxide group on one end and a quaternary ammonium group on the other is commonly used to coat on the surface (**Figure 6**). The quaternary ammonium cation contains chloride ions that serve as Lewis's base sites, while the epoxide group enables GTAC to form strong covalent bonds, allowing tight attachment to support materials (Mukhayani et al., 2024). Covalent bonding is one of the most effective strategies for creating stable and durable contact-active antimicrobial materials (Elena & Miri, 2018). This strong chemical linkage ensures that the antimicrobial agent remains firmly attached to the surface, preventing its release into the environment (Elena & Miri, 2018). Hence, this unique structure provides both reactive sites and binding capacities, making GTAC suitable for applications requiring both stability and functional reactivity.

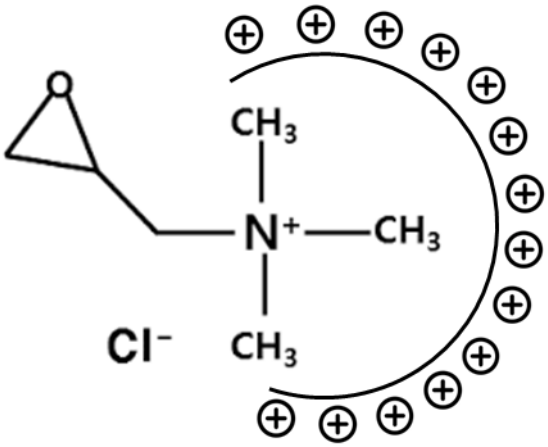

Figure 6. Structures and surface charge of glycidyl trimethylammonium chloride (GTAC) (Mukhayani et al., 2024). Reproduced under the Creative Commons Attribution-Non-Commercial 3.0 Unported Licence.

1.7.1.2. Electrostatic properties in antimicrobial efficacy

While a positive charge is generally associated with promoting adhesion, strong positive charges are also known for their antibacterial properties. For instance, research has shown that the amino cationic group of α-polylysine, a strong positive charge, can interact electrostatically with the anionic components of bacterial cell walls, leading to effective adhesion (Yan et al., 2021). This interaction can ultimately cause the rupture of bacteria and death by altering their membrane permeability (Yan et al., 2021). Overall, it was observed that surface charge density plays a significant role in antibiotic tolerance.

Quaternary ammonium compounds, common agents used to create contact-active surfaces, offer the potential for incorporation into antimicrobial materials and coatings, either to inhibit microbial adhesion or actively combat biofilm formation (Saverina et al., 2023). The efficacy of poly(vinyl-N-pyridinium bromide) when covalently attached to various surfaces of synthetic polymers achieves 99% bacterial kill with no inhibition zone and non-leaching antibacterial behaviour (Kaur & Liu, 2016). Similarly, a chitosan scaffold grafted with a quaternised compound, where the bacterial kill was observed only when direct contact occurred, improving contact-active mechanism (Kaur & Liu, 2016). The first killing mechanism of QACs immobilised on a surface involves damage to the cell membrane due to the penetration of long polymeric QAC grafted onto surfaces (Kaur & Liu, 2016). The other mechanism is related to the exchange of cationic ions between the cell membranes and positively charged surfaces occurs in short-chained QAC (Kaur & Liu, 2016). Another study also reveals that a particular threshold of charge density with cationic groups on the surface can kill the bacteria quickly. The charge density threshold varies depending on the bacterium type, its metabolic state, and the structure of the QAC coating (Kaur & Liu, 2016). Additionally,

researchers grafted glass surfaces with short-chain bromoalkane quaternised PVP chains to study the impact of charge density on cationic surfaces in bacterial death (Kaur & Liu, 2016). The result showed that the outer layer quaternary ammonium compound was relevant to induce bacterial death (Kaur & Liu, 2016). Additionally, a lower charge density allowed to kill *S. epidermidis* and *E. coli* effectively (Kaur & Liu, 2016). Similarly, another study investigated the correlation between surface positive charge density and antibacterial activity, revealing a pseudo-linear relationship between the density of surface positive charge and the number of killed *E. coli* upon contact (Kaur & Liu, 2016). These findings demonstrated a specific threshold of QAC coating surface is important to induce bacterial death.

1.8. Common techniques for producing QAC-based coatings

1.8.1. Solution casting

The solution casting technique, developed by Eastman Kodak in the 19th century, is one of the earliest methods used to create flexible polymer-based composite films (Saverina et al., 2023). This straightforward and adaptable approach was widely used to produce antimicrobial coating in laboratories (Saverina et al., 2023). In the solution casting process, a polymer solution containing antimicrobial additives is poured onto a flat surface and dried to remove the solvent, resulting in uniform polymer coatings with an evenly distributed antimicrobial component without the need for additional mechanical or thermal treatment. For instance, Kallitsis and colleagues used this method to create a dual-action QAC-based coating to prevent biofouling (Saverina et al., 2023). Despite its simplicity and cost-effectiveness, the solution casting method requires toxic solvents, which limits scalability. Another limitation of the solution casting technique is the use of volatile solvents, creating difficulties in completely removing residual solvents from the final film (Saverina et al., 2023).

1.8.2. Spray coating

Spray coating is another simple and widely used technique for coatings. In this method, an atomized polymer solution is sprayed onto a substrate, creating a film as it dries (Saverina et al., 2023). For example, Cornelius and colleagues modified reverse osmosis membrane surfaces with quaternary ammonium (QA)-containing polymers using alcohol-ionomer solutions (Saverina et al., 2023). While these coatings demonstrated strong antimicrobial activity against *E. coli*, they also led to a reduction in membrane flux due to the formation of excessively thick films (Saverina et al., 2023). A limitation of spray coating is its stochastic nature, leading to variability in the resulting film thickness and surface roughness, which can affect the consistency and performance of the coating (Saverina et al., 2023). These issues can also impact the durability and uniformity of antimicrobial properties.

1.8.3. Plasma polymerisation

Plasma technology:

Some surfaces lack the functional groups required for QAC attachment; hence, plasma technology can be used to introduce functional groups, resulting in covalent bonds with antimicrobial agents. Plasma is known as the fourth state of matter in the sequence of solid, liquid, and gas. Plasma is also referred to as ionised gas with no shape or volume similar to gas but has the unique characteristic of being shaped by magnetic fields due to its electrical conductivity (Benčina et al., 2021). The electrical conductivity of plasma results from the breakdown of bonds within gas molecules and atoms, which generates free electrons, charged ions, and other reactive species (Benčina et al., 2021). Plasma technology is widely used in medicine, particularly for enhancing biomaterial surfaces. The interaction of reactive plasma species with material surfaces allows for modification of the surface chemistry, topography, and other characteristics, which can enhance biocompatibility, improve cell attachment, and prevent bacterial adhesion (Benčina et al., 2021). A key advantage of plasma modification is that the bulk properties underneath is maintained while the top surface layer of a material is changed. This environmentally friendly process allows for precise adjustments to surface chemistry, nanoscale morphology, wettability, surface charge, and even crystallinity, all of which significantly affect biological responses (Benčina et al., 2021). Additionally, these surface parameters might influence bacterial adhesion and biofilm formation. The interaction of plasma technology and metal biomaterial surface is described in **Figure 7**. Plasma technology can be categorised into thermal and non-thermal types based on the Maxwell-Boltzmann thermodynamic equilibrium and how molecular bonds are broken (Benčina et al., 2021).

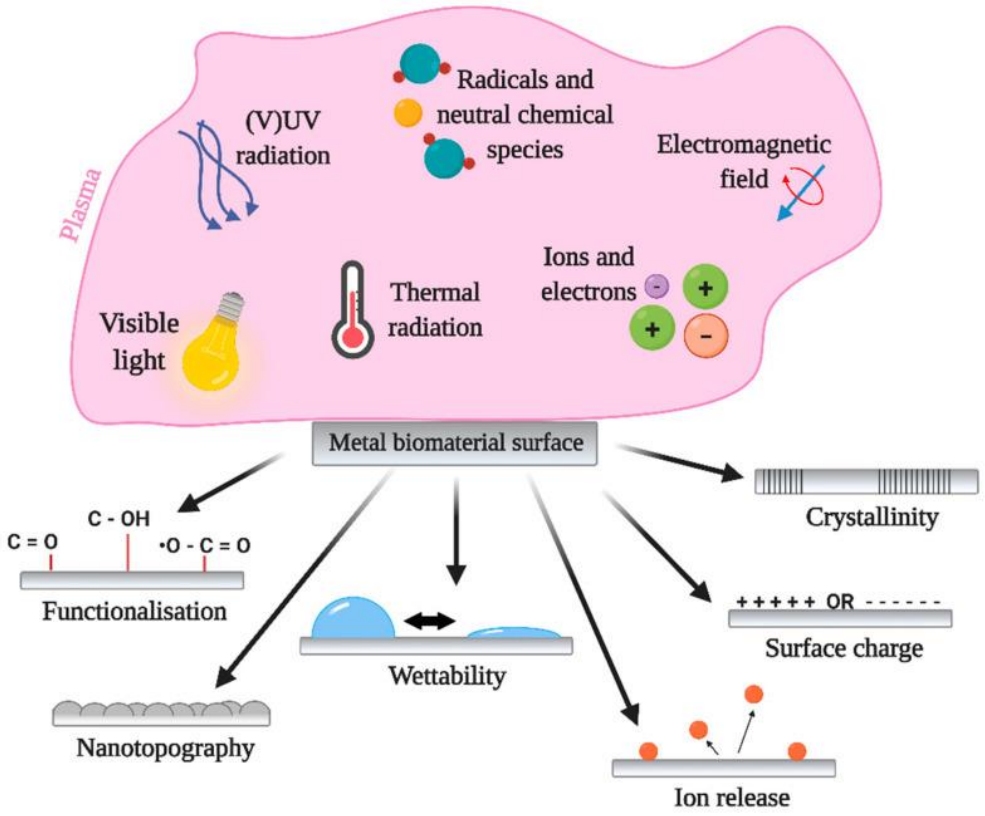

Figure 7. Effects of plasma-generated species on the surface parameters of metals for modification (Benčina et al., 2021). Reproduced under the Creative Commons Attribution license.

Plasmas can be generated in various systems including adjusting pressure conditions from reduced to atmospheric levels, using different electromagnetic field sources like radio frequency (RF) and microwave, electrode configurations, and reactor designs (Carneiro de Oliveira et al., 2021). Plasma polymerisation involves creating high-molecular-weight thin films from low-molecular-weight precursors or monomers. This process allows for the polymerisation of both unsaturated and saturated organic compounds, resulting in plasma polymer thin films that have different chemical and physical properties compared to those deposited using conventional techniques (Carneiro de Oliveira et al., 2021). The deposition mechanism occurs when particles (monomer, oligomeric or fragmented molecules) are adsorbed onto the surface and then react with the substrate's surface, supporting the film-forming process. Initial adsorbates anchor to the surface, ensuring the growing film adheres to the substrate (Carneiro de Oliveira et al., 2021).

Plasma polymerisation in antimicrobial surfaces:

Currently, various polymerisation techniques are deeply investigated to develop antimicrobial surfaces incorporating quaternary ammonium compounds, especially to form the covalent bond when attaching quaternary ammonium compounds to different material surfaces (Saverina et al., 2023). Plasma polymerisation is an effective method for manipulating surface chemistry, aiming to induce

specific biological responses; hence, this technique is widely under intense investigation by many laboratories (Vasilev et al., 2011). **Figure 8** illustrates the plasma polymerisation setup in the lab. To achieve a longer antibacterial effect, covalently grafting antibacterial compounds onto biomaterial surfaces is considered a promising strategy. The excellent interlayers for covalently immobilising antimicrobial molecules can be generated by plasma polymer coatings (Vasilev et al., 2011). Additionally, on surface materials lacking necessary functional groups for QAC attachment, plasma technologies can be used for surface activation or introduction of functional groups. This enables the subsequent formation of a covalent bond with antibacterial agents (Saverina et al., 2023). In previous studies, researchers utilised the plasma polymerisation technique to coat glass coverslips with allylamine, creating an amine-rich surface. Subsequently, GTAC was applied onto this plasma-polymerised allylamine surface (Cavallaro et al., 2014). The study demonstrated that the modified surface not only showed potent antibacterial activity but also exhibited no observable cytotoxic effects on human dermal fibroblasts (Cavallaro et al., 2014). Another study also found that a specific threshold of immobilised quaternary ammonium compounds surface concentration was necessary to induce significant bacterial death (Cavallaro et al., 2016).

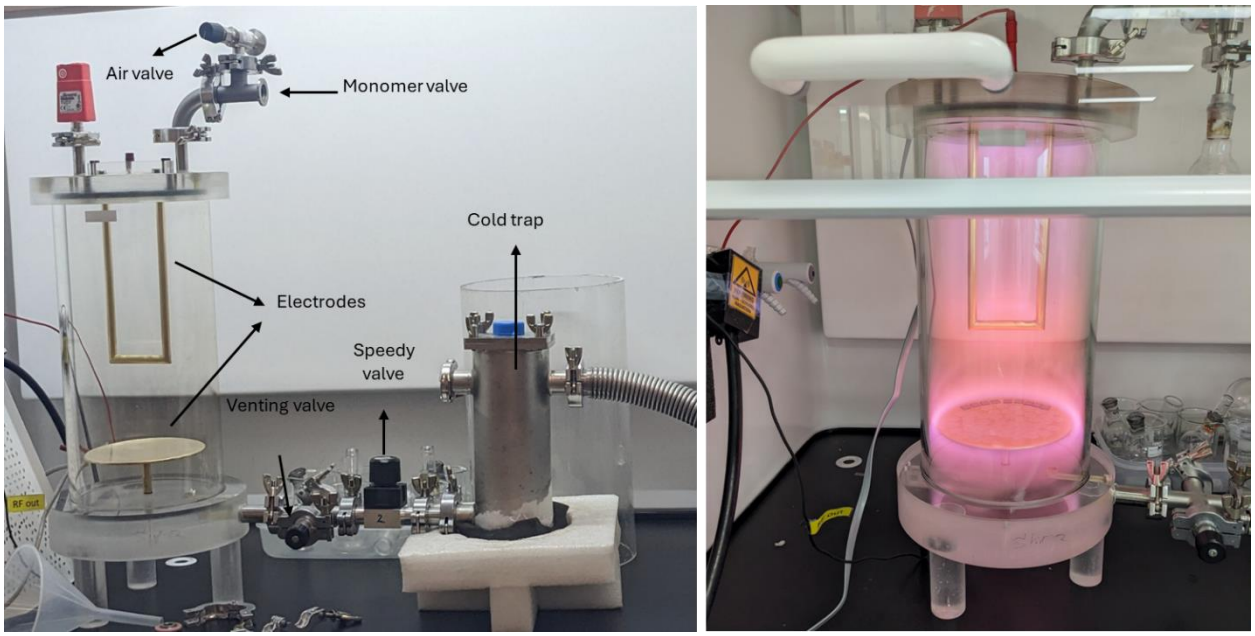

Figure 8. Photograph illustrating the main components of plasma polymerisation technique at Biomedical Nanoengineering Laboratory (Flinders University) and plasma process in the deposition of allylamine on the coverslips and silicon wafers

1.8.4. Dip coating

Dip coating is a cost-effective and straightforward method to generate a thin layer coating with a gradient of surface properties. This technique begins with immersing a substrate into a solution at a constant speed, maintaining it within the solution, and withdrawing the substrate at a constant speed,

followed by solvent evaporation and drying (Saverina et al., 2023). **Figure 9** depicts the main process of dip coating. Initially, the substrate is immersed at a controlled speed. During the start-up phase, the substrate remains in the solution for a set period before being lifted (Mohd Salleh et al., 2024). As it is withdrawn in the deposition stage, a thin film coating forms on the substrate surface, with the coating thickness inversely proportional to the withdrawal speed. At the final stage, any excess solution is removed from the substrates (Mohd Salleh et al., 2024). This allows for the creation of thin films with thicknesses ranging from 1 to 10 micrometers, making dip coating a popular choice for applications requiring precise and consistent coatings (Mohd Salleh et al., 2024). A study used dip coating to deposit copolymer coatings containing quaternary ammonium compounds and phosphorylcholine groups onto glass and polylactic acid substrates (Saverina et al., 2023). The coated surface with the combination of the bactericidal properties of quaternary ammonium compound and the antifouling and antithrombotic activity of phosphorylcholine groups resulted in the effective biofilm formation prevention without cytotoxic effects on endothelial cells of the human umbilical vein (Saverina et al., 2023). Also, one-step dip coating can be used for various textile materials. For example, a finding revealed that incorporating fluorinated and quaternary ammonium-functionalised mesoporous silica nanoparticles with polydimethylsiloxane as a binder can generate a “repel-and-kill” antibiofilm mechanism, contributing to hydrophobic, antibacterial, and antifouling properties of textiles (Saverina et al., 2023). Previously, QAC was typically mixed directly into the coating matrix after undergoing a specific process to generate antimicrobial properties (Wang et al., 2023). However, it has been observed that the antimicrobial efficacy of these coatings decreases during long-term periods (Wang et al., 2023). To address this issue, QAC has been grafted or compounded with other chemicals and incorporating them into coating substrates (Wang et al., 2023). This approach aims to create new coatings to protect material surfaces more effectively over extended periods.

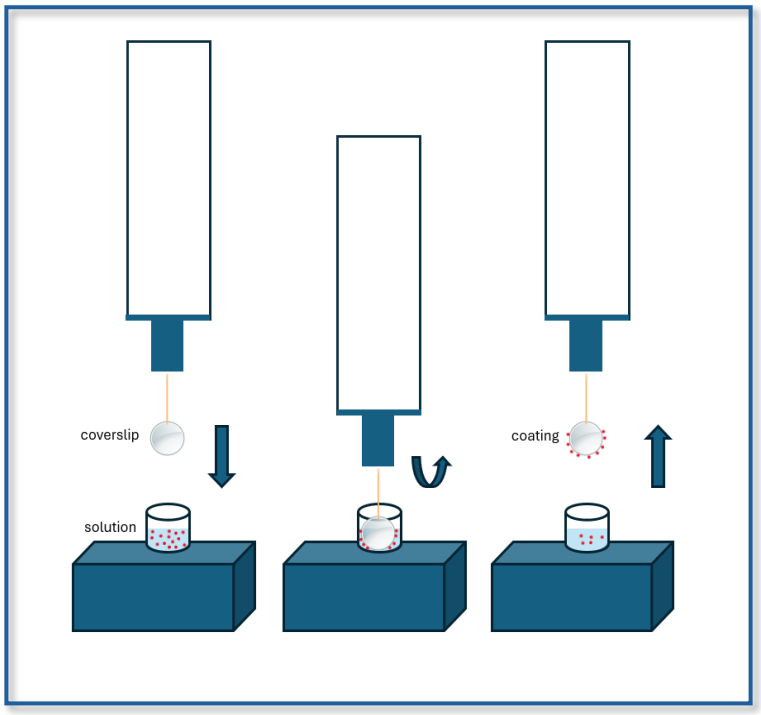

Figure 9. A schematic representing the rate-controlled exposure of coverslips and solution by dip coating.

1.9. Research gap

Previous studies have focused on the antimicrobial activity of immobilised QAC surfaces and found that there is required specific charge density to induce bacterial growth. However, there is limited understanding of how the electrostatic properties of these surface such as charge density gradients affect bacterial tolerance to antibiotics. This gap is particularly critical, as electrostatic interactions between the positively charged QACs and bacterial cell membranes or antibiotic molecules could play a significant role in enhancing antibiotic efficacy. Therefore, there is a need to investigate whether immobilised QAC can reduce bacterial tolerance to antibiotics by creating gradients to evaluate the influence of material electrostatic properties on tested microorganisms.

1.10. Hypothesis and aim

1.10.1. Hypothesis

• Hypothesis 1: By regulating the duration of exposure of an amine-rich substrate to a GTAC solution, GTAC density gradients can be effectively prepared.

• Hypothesis 2: Along the length of a GTAC density gradient, the rate of attachment and viability of pathogenic microorganisms is expected to show variation.

• Hypothesis 3: The GTAC density gradient serves as a viable platform to identify the optimal electrostatic properties aimed at reducing bacterial tolerance to antimicrobial drugs.

1.10.2. Aim

The main aim of this study is to fabricate and validate a tool capable of high-throughput analysis to investigate the influence of charge density on antimicrobial drug tolerance.

• Aim 1: Create GTAC density gradients on plasma polymerised allylamine samples by controlling the duration of exposure of an amine-rich substrate to a GTAC solution.

• Aim 2: Validate the charge gradient by using standard samples containing homogenous coatings of GTAC with known properties.

• Aim 3: Assess influence of GTAC density gradient on viability of pathogenic microorganisms

• Aim 4: Demonstrate the use of the platform by using it to characterise the outcome of various antibiotics in relation to the GTAC density

1.11. Significance to Medical Biotechnology

Medical devices play a significant role in the healthcare system with various functions including diagnostic, therapeutic, and rehabilitation. However, microorganisms can easily attach to the surface of these devices, necessitating frequent revisions and replacements. This can result in increased morbidity, mortality, and a substantial decline in patient well-being. Additionally, the biofilm phenotype gives rise to cells with high tolerance to drug treatments, making antibiotic therapy substantially less effective against infections associated with medical devise. Therefore, new strategies are required that can enhance the activity of antibiotics against device associated.

Recent advancements have focused on coating antimicrobial agents on the surfaces. Among antimicrobial agents, quaternary ammonium compounds, a positively charged molecule, are reported to exhibit antimicrobial activity. Moreover, a strategy such as grafting QAC with other chemicals to create covalent bonds through multiple steps of surface modification, including plasma polymerization and dip coating, has been developed to enhance their effectiveness. It has been observed that immobilised QAC poses no cytotoxicity to human skin cells, at concentrations that are therapeutically significant (Cavallaro et al., 2014). Another study showed that the charge density properties of the surface can affect antimicrobial efficiency (Kaur & Liu, 2016). Building on these findings and addressing the research gap, this investigation will create the charge density on the

immobilised QAC surface and discover how the properties of the material/pathogen interface influence antibiotic tolerance. This study could significantly contribute to the treatment of biofilm-associated infections and advance biomedical technologies.

CHAPTER 2. MATERIALS AND METHODS

The experiments in this study were mainly conducted at the Biomedical Nanoengineering Laboratory (BNL), Flinders University. Fluorescence AX70 Upright Microscope and the Zeiss LSM880 were accessed in the facilities housed by Flinders Microscopy and Microanalysis.

2.1. Materials

Round glass coverslips with a diameter of 13 mm were the main substrates to generate homogenous surfaces and gradient surfaces. A gradient surface also was created on a glass side with a length of 30 mm to measure the wettability. Silicon wafers served sample substrates for thickness measurement.

The following items obtained from Sigma Aldrich: allylamine (98%), glycidyltrimethylammonium chloride (GTAC; technical grade ≥ 90%), phosphate buffered saline tablet, fluorescein isothiocyanate (FITC).

The items purchased from Thermo Fisher Scientific: Tryptone Soya Broth (TSB), Tryptone Soy Agar (TSA), LIVE/DEAD™ Viability/Cytotoxicity Kit (Invitrogen).

Staphylococcus aureus ATCC 25923, *Pseudomonas aeruginosa* ATCC 15692 and *Candida albicans* ATCC 10231 were used as model organisms. Antibiotics used in antibiotic tolerance study were vancomycin, daptomycin, kanamycin, levofloxacin (Thermo Fisher Scientific, MA, USA).

2.2. Preparation of immobilised quaternary ammonium compounds surface

2.2.1. Plasma polymerisation

Principle of technique:

Plasma-based biotechnology has been widely applied to generate a coating layer for biomedical applications. This technique involves exposing a gas, typically a monomer or a mix of monomers, to low-pressure plasma, where the gas molecules undergo dissociation into reactive species like ions, radicals, and excited atoms or molecules (Carneiro de Oliveira et al., 2021). These reactive species interact with the surface of the substrates, leading to the formation of a polymer thin film (Carneiro de Oliveira et al., 2021). The properties of the resultant thin film, such as thickness, composition, and surface energy can be adjusted by process parameters like monomer type, plasma power, pressure and deposition time (Michelmore et al., 2013). This flexibility of plasma polymerisation contributes to the application of surface modification for biomedical coatings, drug delivery systems, and antimicrobial surfaces.

Additionally, the substrates are functionalised with various chemical groups such as amine, hydroxyl, and carboxyl groups to form the covalent attachment on the surface via plasma polymerisation to improve the biofunctionalization of surfaces, allowing for enhanced interactions with biological environments including cell attachment and inhibition of microbial growth. (Zhianmanesh et al., 2023). In this study, allylamine was used as a precursor for plasma polymerisation to generate functionalised surfaces. Plasma polymerised allylamine (ppAA) creates a thin coating rich in amine functional groups, which is favourable for further chemical modifications. These amine groups from covalent bonds of epoxy groups of quaternary ammonium compounds like glycidyltrimethylammonium chloride (GTAC), a type of quaternary ammonium compound, enhances surface properties and antimicrobial activity (Cavallaro et al., 2014).

Experiment detail:

GTAC was used as a representative quaternary ammonium salt in this study. Immobilised GTAC surface was generated following the method described by Cavallaro et al. (2016). All substrates were initially washed with acetone, ethanol and Milli-Q water for 4 minutes in each step with the support of an ultrasonic cleaner bath. Then, the nitrogen gas was used to dry all the substrates before plasma polymerization. The plasma polymerization was conducted in a plasma reactor with allylamine as a monomer to generate thin films rich in amine ($-NH_2$). Deposition of allylamine was carried out at a base pressure of 1.3×10^{-1} mbar, and plasma was applied at a power of 40 W for a duration of 2 minutes (**Figure 10**).

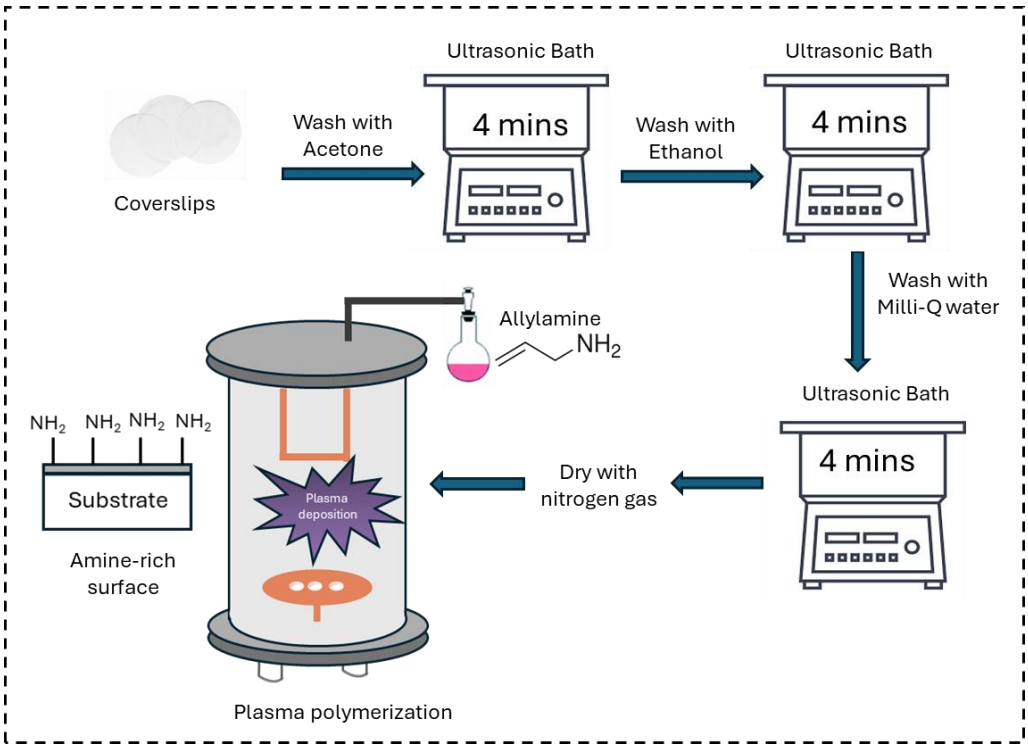

Figure 10. Schematic representation of the step-by-step process for creating an amine-rich surface. The process begins with cleaning substrates, followed by coating them with allylamine by plasma polymerization.

2.2.2. Dip coating for preparation of gradient surface

Principle of technique:

Dip coating is a widely used method for applying thin films onto substrates by immersing them in a coating solution, and then withdrawing them at a controlled rate (Faustini et al., 2014). In this process, a substrate is dipped into the solution, where the portion of the surface that stays immersed in the solution longer will accumulate more of the coating material, while the upper regions that are withdrawn earlier will have less. This difference in deposition creates a gradient of the coating material across the surface. This technique was applied previously to create immobilised QAC and nanoparticle density gradients (Cavallaro et al., 2016; Goreham et al., 2013).

Experiment detail:

GTAC was covalently bonded to the amine functional groups by immersing the amine-rich substrate into 10% (v/v) GTAC solution with pH 10 prepared in Milli-Q water, with the pH adjusted using KOH. Finally, Milli-Q water was used to remove any unattached GTAC and excess KOH in the washing step on the coated samples, followed by drying with nitrogen gas. The samples were divided into two groups including homogenous samples and gradient samples.

For the homogeneous surfaces, the samples were immersed directly in the GTAC solution for three different time periods: 1 hour, 3 hours, and 6 hours. These samples were used to characterise the surface in cases where the analysis technique was not compatible with gradient surfaces.

For the gradient samples, a concentration gradient of GTAC was created by controlling the movement of a dip coater (**Figure 11**). A linear motion drive (Zaber Tech T-LSM050A, Canada) and Zaber console 1.1.7 software was used to control the time and dipping speed of the ppAA substrates into the GTAC solution. Using a controlled rate of exposure, a GTAC density gradient was established with exposure durations spanning from 0 minutes to 6 hours.

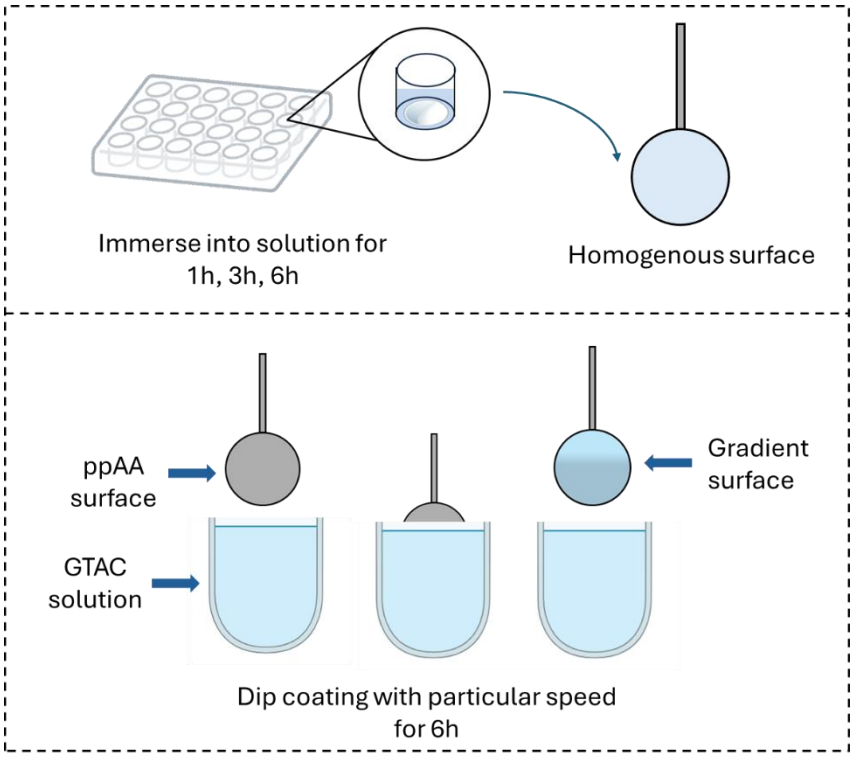

Figure 11. Schematic representation immobilised QAC surface from amine-rich surfaces. The substrates with ppAA film were then covalently bonded to GTAC compound through the reaction of epoxy group on GTAC with the amine groups on the ppAA surface. The ppAA film were soaked into GTAC solution with different time point to create homogenous surfaces and dip coated into GTAC solution using dip coating to prepare gradient surfaces.

2.3. Surface characterisation

2.3.1. Thickness measurement using ellipsometry

Principle of technique:

Ellipsometry is an optical technique used to determine the thickness and optical properties of thin films by analysing how polarised light interacts with the surface of a material. The principle of

this method is directing a polarised light beam at the surface of the sample at a large oblique angle (Podraza and Jellison, 2017). Upon reflection, the polarisation state of the light changed, typically from linear to elliptical polarisation. An ellipsometer consists of primary components including a light source, a polarisation state generator, a sample, a polarisation state analyser and a detector. Ellipsometry collects data on the polarisation changes of reflected light to derive the thickness and optical properties of a thin film (Podraza and Jellison, 2017). The main components of ellipsometry technology are presented in **Figure 12**.

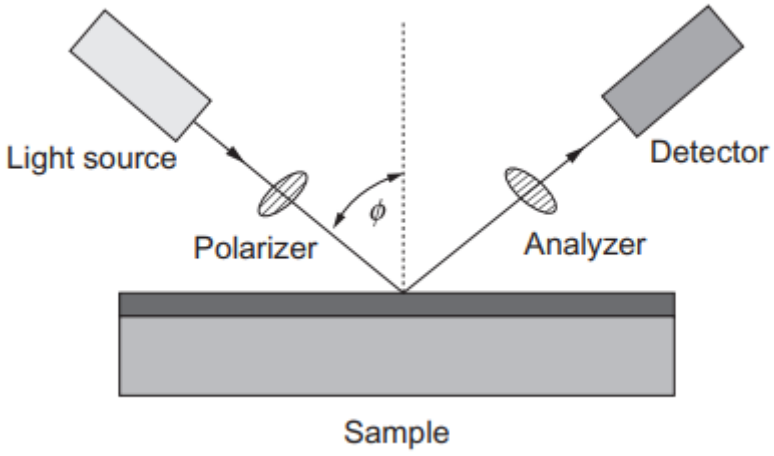

Figure 12. Schematic representation of an ellipsometer with main components (Airaksinen, 2015). Reproduced with permission.

Experimental detail:

The thickness of deposited plasma polymers was measured by an ellipsometer followed by the process of Cavallaro et al. (2014) with modification. For this purpose, silicon wafers were cut into pieces of 1cm x 1cm to serve as a substrate and coated with allylamine under conditions as described in plasma polymerisation section. The samples were ultrasonicated sequentially in acetone, ethanol and Milli-Q water for 4 minutes each, then stored in ethanol and dried with nitrogen gas before use. The cleaned samples were also coated with allylamine by plasma polymerisation and immersed directly into GTAC solution for 1 hour, 3 hours and 6 hours. After washing with Milli-Q water and drying in a stream of nitrogen, a spectroscopic ellipsometer (SENTECH SENresearch 4.0, Gmbh, Berlin, Germany) was used to determine the thickness with triplicate. The thickness (in nm) of thin film was recorded by SpectraRay/4 spectroscopic ellipsometry software of SENTECH.

2.3.2. Surface wettability

Principle of technique:

The sessile drop method is one of the most common approaches to measure the contact angle directly. It involves applying a droplet of distilled water or another test liquid from a syringe on the sample surface, forming a contact angle at the three-phase interface (solid-liquid-gas) (Ponomar et al., 2022). A camera is used to capture images of the droplet on the surface, and image analysis software determined the contact angles. The angle above 90° is hydrophobic material while the surface is hydrophilic if the angle is under 90° (Ponomar et al., 2022). For accurate measurements, the surface is typically required to be smooth, planar, and non-porous (Ponomar et al., 2022). The device to measure water contact angle is illustrated in **Figure 13**.

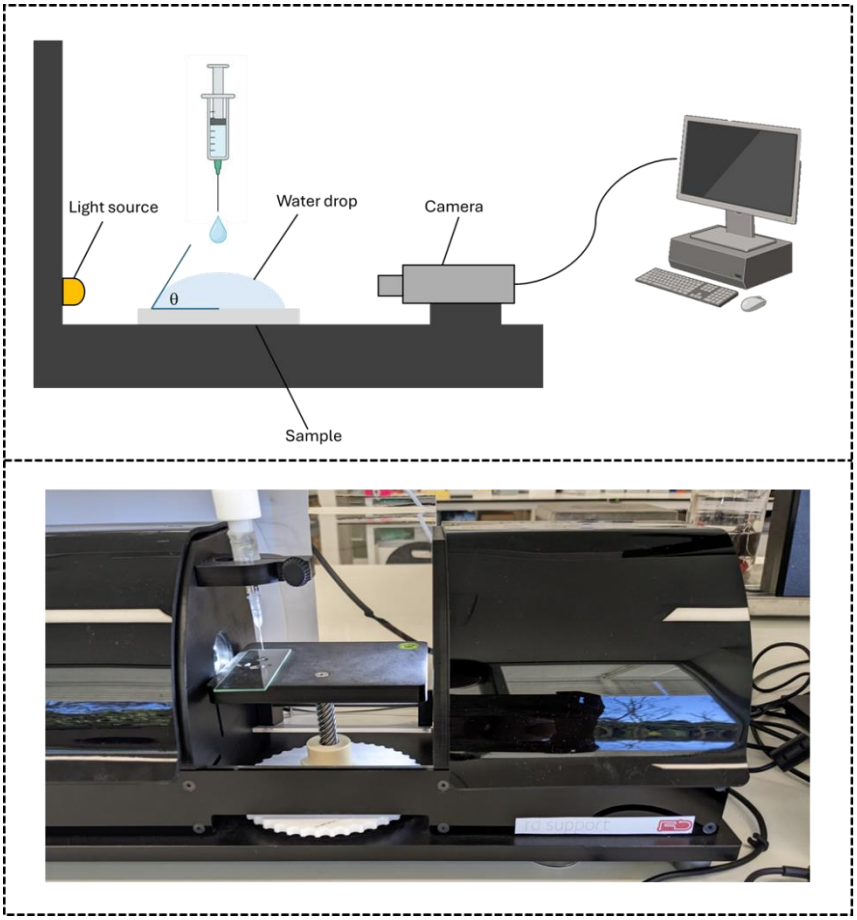

Figure 13. Schematic and photograph of the sessile drop method setup for measuring contact angle

Experimental detail:

Surface wettability was determined by water contact angle measurement (θ) using the sessile drop method (Cavallaro et al., 2014). Both coverslips with homogeneous surfaces and 30 mm glass slides with gradient surfaces were used to measure using contact angle measurement device (RD-SDM02, Denmark). A small droplet of liquid was injected from a syringe onto each surface, and

measurements were taken in triplicate. For homogeneous samples, a single measurement point was used, while for the 30 mm gradient samples, measurements were taken at 5 points in 6 mm increments. The image of water contact angles was captured directly and then analysed using the Drop Analysis-DropSnake plugin for ImageJ v1.53.

2.3.3. Fluorescent assay

Principle of technique:

The fluorescein dye used in this study to quantify the presence of QAC is fluorescein isothiocyanate (FITC). It is reported that FITC, a negatively charged dye, binds strongly to the positively charged molecules (Mahmudi-Azer et al., 1998). Previous studies indicated that once fluorescein dye was applied to the treated surfaces containing quaternary ammonium groups, it binds to the QAC assuming each fluorescein molecule binds to one quaternary ammonium unit (Huang et al., 2007). The amount of fluorescein dye bound to the surface produces a fluorescent signal, which can be measured using fluorescence microscopy or UV-visible spectroscopy (Elena & Miri, 2018). The fluorescence intensity directly correlates with the concentration of quaternary ammonium units present on the surface. By determining the amount of bound dye, the surface density of the QACs can be inferred.

Experimental detail:

The fluorescent analysis can be used to quantify the presence of GTAC due to the binding of fluorescent dye to the quaternary ammonium group of GTAC (Elena & Miri, 2018). The coverslips with homogenous surfaces were immersed in 400 µL of 0.05 mg/mL fluorescent dye (FITC) with a range pH from 7 to 9 for one hour at room temperature in the dark condition, followed by gently washing twice with PBS. The Fluorescence AX70 Upright Microscope (Olympus, Japan) connected to Zeiss Axiocam 506 monochrome camera with Zen Blue software was used to capture the stained samples. The sample is exposed to light of a specific wavelength (470 nm), which excites the fluorescent molecules in the sample. The photo was taken at three different areas with triplicate samples and analysed by using ImageJ v1.53 to quantify the fluorescence intensity. After identifying the pH at which the fluorescence intensity of the immobilised QAC surface was most effective, the same pH would be applied to the gradient samples with the same protocol. The coverslips were placed on the glass side with a 2 mm etching line as described in **Figure 14** to capture the photo for determination of fluorescence intensity at each 2 mm position along the gradient.

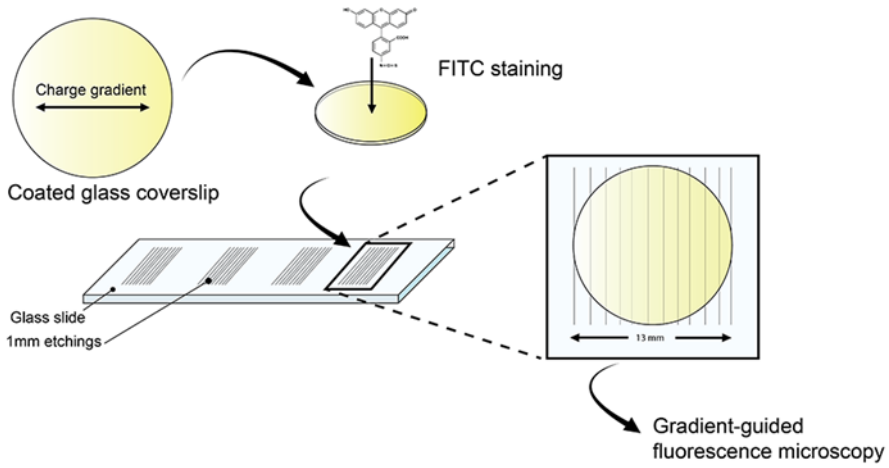

Figure 14. Schematic illustrating the determination of fluorescence intensity on gradient samples. The fluorescence intensity was measured at five points on the gradient samples. A 2 mm etching line was created on a glass slide, and round coverslips were placed on the glass slide, aligned with the etched line to form a gradient.

2.4. Rapid method for determination of attachment and viability

2.4.1. Cultures and conditions

This experiment focused on testing immobilised QAC gradient surfaces with three types of pathogens, namely *S. aureus*, *P. aeruginosa* and *C. albicans*, which were chosen to represent Gram-positive, Gram-negative bacteria and fungi, respectively. Before the experiment, *S. aureus*, *P. aeruginosa*, *C. albicans* from -80°C stock were streaked onto TSA and incubated at 37°C overnight. The following day, isolated individual colony of *S. aureus* and *P. aeruginosa* was obtained and transferred to 5 mL of TSB while single colony of *C. albicans* was aseptically transferred to 50 mL of TSB. After the incubation of overnight at 37°C, the cell concentration of all pathogenic microorganisms was determined by optical density at 600 nm (OD_{600}) using a cuvette by spectrophotometer (Nanodrop 2000, Thermo Scientific, MA, USA).

2.4.2. LIVE/DEAD assay

Principle of technique:

In this research, LIVE/DEAD assay was applied to evaluate bacterial viability. This approach used Bacterial Viability Kit involving two fluorescent dyes: SYTO9 and propidium iodide (PI). SYTO9 can pass through both live and dead cells, binding to nucleic acids and emitting green fluorescence with excitation/emission maxima of 480/500 nm (Robertson et al., 2019). In contrast, PI can only enter cells with disrupted membranes, displacing SYTO 9 and binding to nucleic acids to

emit red fluorescence (excitation/emission maxima: 490/635 nm) (Robertson et al., 2019). The ratio of green (live cells) to red (dead cells) fluorescence allows differentiation between viable and non-viable bacterial populations. **Figure 15** shows that the bacterial viability can be determined by using mixed solution containing SYTO 9 and PI.

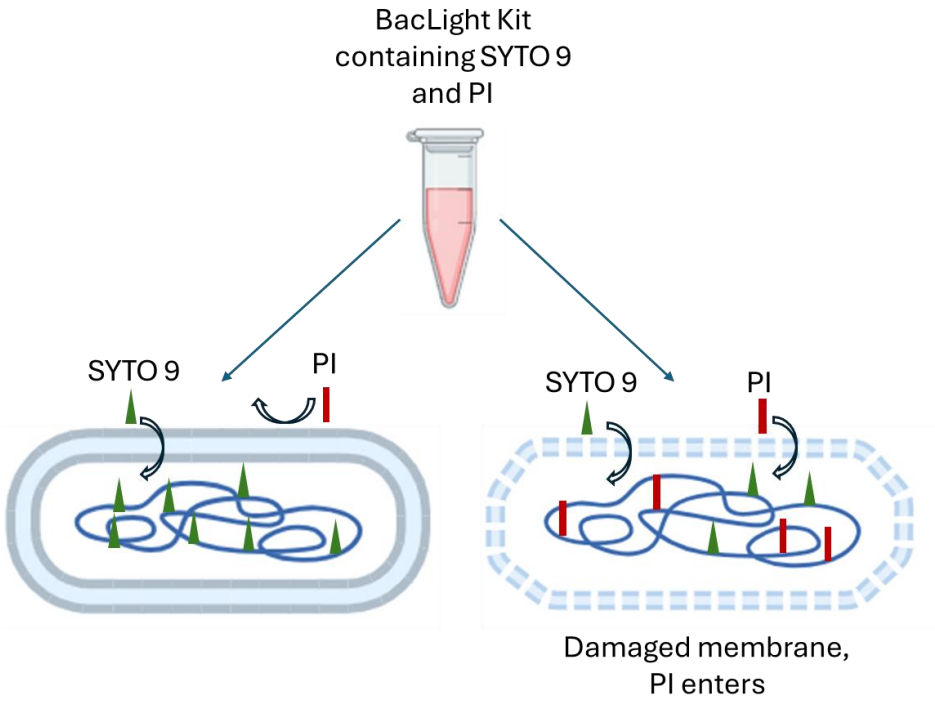

Figure 15. Schematic illustrating the LIVE/DEAD assay involves the use of two staining, STYO 9 (green triangle) and PI (red rectangle). The figure depicts intact live cells stained green (left) by SYTO 9 and dead cells with permeabilised membranes showing red fluorescence (right) by PI.

Experimental detail:

Bacterial and fungal pre-culture were diluted to approximately 10^8 CFU/mL (OD_{600} = 0.1 for bacteria and OD_{600} = 1 for fungi) in TSB and then added to each well of 24-well plate containing coverslips. The plate was incubated for 3 hours at room temperature. BacLight Live/Dead stain was prepared by mixing SYTO9 and Propidium Iodide (PI) from LIVE/DEAD kit at a concentration of 1.5 µL/mL in PBS. After 3 hours of incubation, each coverslip was immersed in 400 µL of staining solution and incubated for 15 minutes in the dark at room temperature. Subsequently, samples were imaged by confocal laser scanning microscope (Zeiss LSM 880, Germany) at 40X magnification. The image of each sample was taken at each 2 mm distance along the length of the density gradient, with at least three distinct areas per sample. To record measurements at each 2 mm position, the "X-position" on the Touch Pad Microscope Control was used (**Figure 16**). The excitation and emission wavelengths were set to 480/500 nm for SYTO9 and 490/600 nm for PI, respectively.

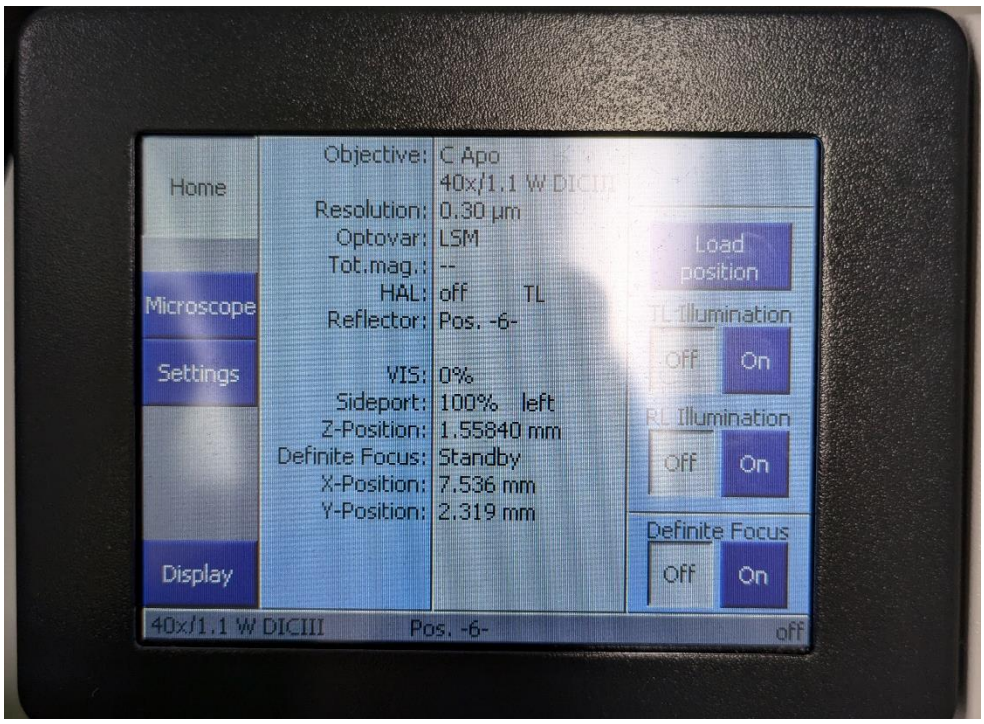

Figure 16. Touch-Pad Microscope Controls screen showing settings of microscope system

2.4.3. Cell count and viability

To determine the number of live cells and dead cells, image of pathogen microorganisms after LIVE/DEAD assay was processed in ImageJ v1.53 using the splitting channel function. In the image, green cells represent alive cells while dead cells are shown in red colour. The number of green and red cells were counted by "Find Maxima" tool. The percentage of cell viability was calculated using an equation:

$$Cell\ viability\ (\%) = \frac{Number\ of\ live\ cells}{Total\ cells} \times 100$$

2.5. Antimicrobial susceptibility testing

Prior to determining the required concentration of antibiotics for the antibiotic tolerance study, minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) tests were used to assess the effectiveness of antibiotics against bacteria. In this study, the MIC of antibiotics against *S. aureus* was followed the protocol of the guidelines outlined by the Clinical and Laboratory Standards (CLSI) with modification of media. MIC is defined as the lowest concentration of a drug that inhibits the visible growth of a microorganism (CLSI, 1999). In contrast, MBC refers to the lowest concentration of an antibacterial agent required to kill 99.9% of bacterial population (CLSI, 1999).

Bacterial suspension and antibiotics were prepared in TSB instead of cation-adjusted Mueller-Hinton Broth. Briefly, *S. aureus* was treated with two-fold serial dilutions of each antibiotic in 96-well plate, with concentrations beginning at 32 µg/mL for vancomycin and daptomycin and 62 µg/mL for kanamycin and levofloxacin. The plates containing antibiotic dilutions and bacterial suspension were incubated at 37°C for 24 hours. After incubation, the final cell density was assessed using a Synergy HTX Multi-Mode microplate reader (BioTek Instruments, Winooski, Vermont, USA) at 600 nm (OD_{600}). The lowest concentration of antibiotic that visibly inhibits bacterial growth was determined to be the MIC. The MBC result was determined by transferring aliquots from the MIC plate to the sterile agar plate, starting from MIC concentration and including several concentrations higher than MIC. The lowest concentrations at which no colonies were observed on the agar plate was determined to be the MBC.

2.6. Evaluation of antibiotic tolerance in *S. aureus*

To determine the tolerance of adherent *S. aureus* to antibiotics, bacterial culture was prepared according to the inoculation procedure as described in the attachment and viability assessment. Following the addition of cultures on the coverslips and incubation of 3 hours, samples were rinsed with PBS for 10 seconds twice. The concentration of vancomycin, daptomycin, levofloxacin, and kanamycin was based on the MIC/MBC result obtained from **Part 2.5**. The coverslips were immersed in TSB supplemented antibiotics with a concentration 4X the minimum bactericidal concentration for 24 hours. Post-treatment viability was assessed using LIVE/DEAD assay described previously. For each sample, images were also captured at 2 mm intervals along the length of the density gradient with at least three different areas. The percentage of cell viability was determined based on the number of live cells (green colour) and dead cells (red colour) processed on ImageJ v1.53 software. The summary of the experiment is described in **Figure 17**.

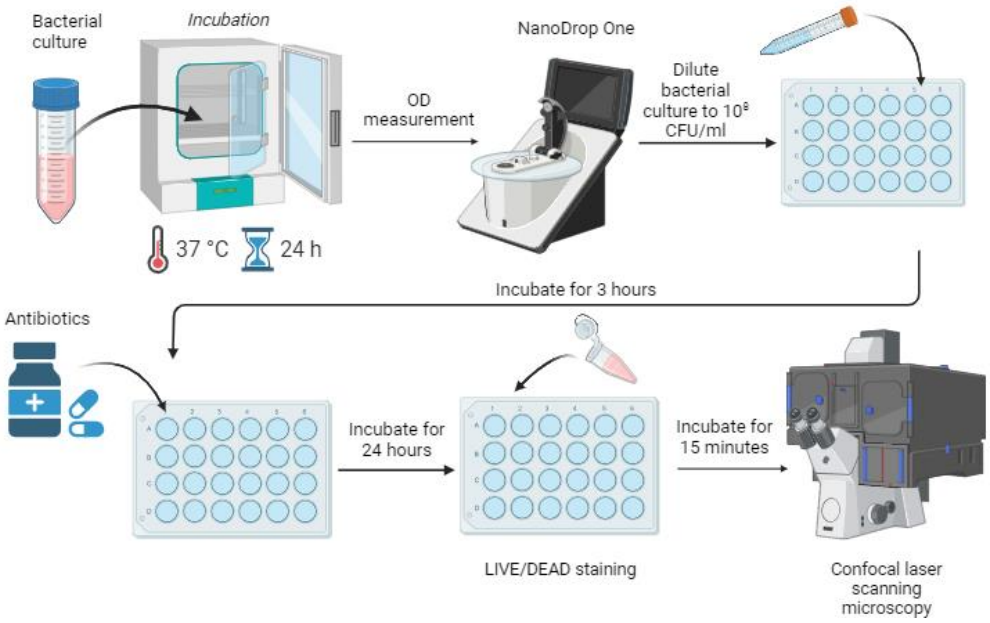

Figure 17. Experimental workflow for antibiotic tolerance study using LIVE/DEAD assay and confocal laser scanning microscopy

2.7. Statistical analysis

The data was expressed as mean ± standard deviation (SD). Statistical differences between groups were evaluated using One-way analysis of variance (ANOVA), followed by Fisher's LSD to determine pairwise comparisons between groups. The software Origin 2024b was used for statistical analysis and plotting the graph.

CHAPTER 3. RESULTS

3.1. Preparation of QAC immobilisation surface density

The 13 mm round coverslips with immobilised QAC surfaces were the primary model samples in this research (ppAA+GTAC samples). These surface with various gradients allows to examine a wide concentration density range of surface bound GTAC on a single substratum. To achieve such gradients, a precise dip coating was applied to generate the epoxide-amine reaction based on the time-dependent nature. The Zaber device controller, connected to a computer, operates automatically based on software commands. The software allows for the precise setting of constant speed and duration, enabling substrates to move slowly from top to bottom, dipping into the solution for a specific period of time. This controlled movement creates a gradient surface, as the exposure time of the substrates to the coating solution varies along the length of the sample. The original system, equipped with six holders, could only process six samples per round, limiting the capacity for the experiment requiring a large number of samples. Additionally, the immersion process required 6 hours to complete, further extending the preparation time. To address these limitations, the lid of a 24-well plate was modified and attached to the system, with toothpicks and blue tack used to secure the coverslips (**Figure 18**). This modification allows for the simultaneous processing of 24 samples, significantly increasing the experiment throughput. The coverslips were immersed in GTAC solution along the z-axis, resulting in a gradient coating. The side of the substratum that remained in the solution for the longest time (6 hours) had the maximum concentration of quaternary ammonium functionalities. Each 2 mm distance of the surface was examined, with the 10 mm position being the region that had been exposed to GTAC solution for the full 6-hour duration. At this position, it was expected that the positively charged NR_4^+ would increase due to the presence of the large amount of GTAC. To confirm the success of GTAC surface grafting density, the method for surface characterization was applied.

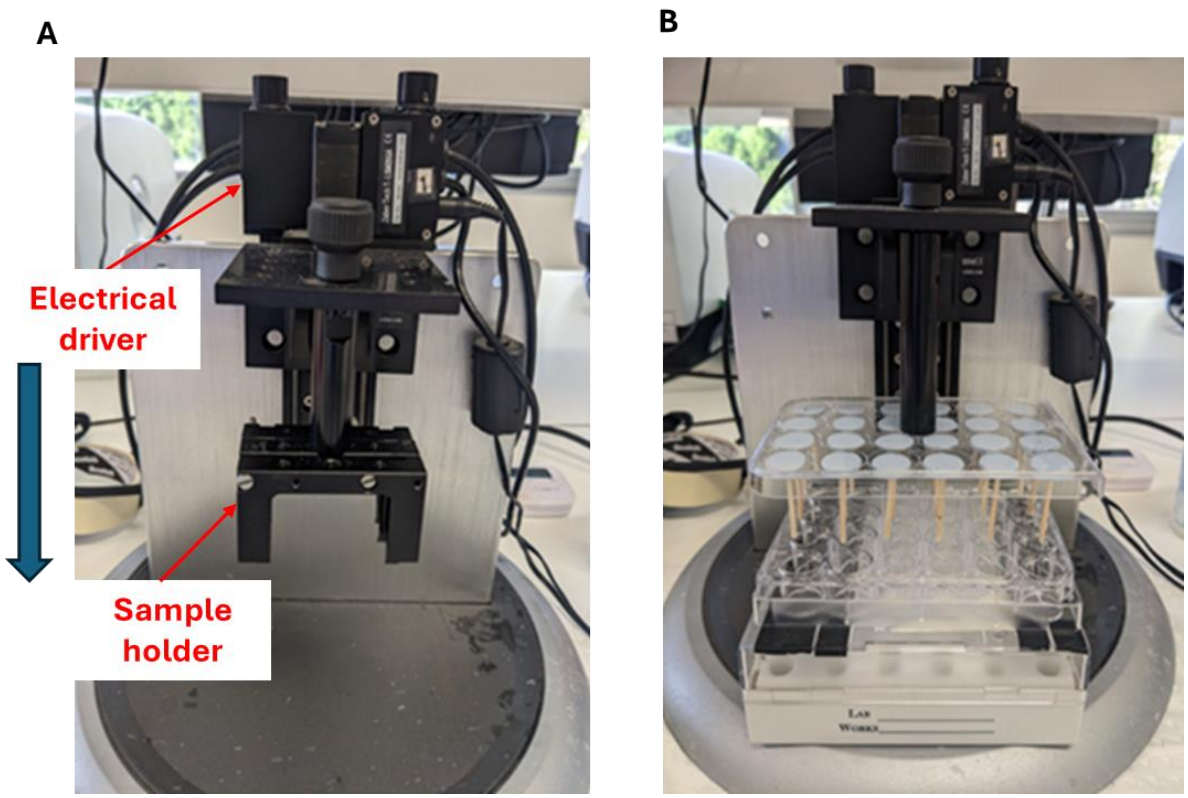

Figure 18. The modified system was designed to enhance the number of coverslips that can be processed simultaneously. A) The original system with the holder, which only allowed for six samples to be processed in one round, was modified to accommodate more samples. The blue arrow represents the direction of holder movement. B) To achieve this, a 24-well plate was used to increase throughput and efficiency in processing multiple samples at once.

3.2. Thickness measurement

To quantify the presence of GTAC after coating, thickness measurement using ellipsometry was performed. This is because GTAC containing epoxy groups forms covalent bonds with the amine groups of allylamine on the surface, which leads to an increase in the overall thickness (**Figure 19A**). In this experiment, silicon wafers were used as the substrates and homogenous layers due to their favourable properties for thickness measurement using ellipsometry. This method is highly sensitive to slight changes in thickness and requires uniform surfaces to ensure accurate and reliable results (Podraza and Jellison, 2017). By comparing the measurements taken before and after GTAC coating, the successful formation of a covalent bond between the epoxy and amine groups can be inferred from the increase in layer thickness. **Figure 19B** shows the changes in thickness due to the presence of GTAC at different time points, indicating the surface was being covered with a layer of GTAC. In general, an increasing trend in thickness was observed after coating with GTAC. Specifically, a significant increase in thickness was observed on the samples that were immersed in the solution at 3 hours and 6 hours compared to 1 hour and samples with ppAA only (control). It is clearly shown

that by increasing the time of immersion, a greater number of GTAC molecules are bound to the available amine groups on the surface, resulting in an increase in thickness. In general, this result confirms the final surface was covered by covalent bonds between allylamine and GTAC molecules and shows the dynamic interaction between GTAC and the amine groups over immersion time.

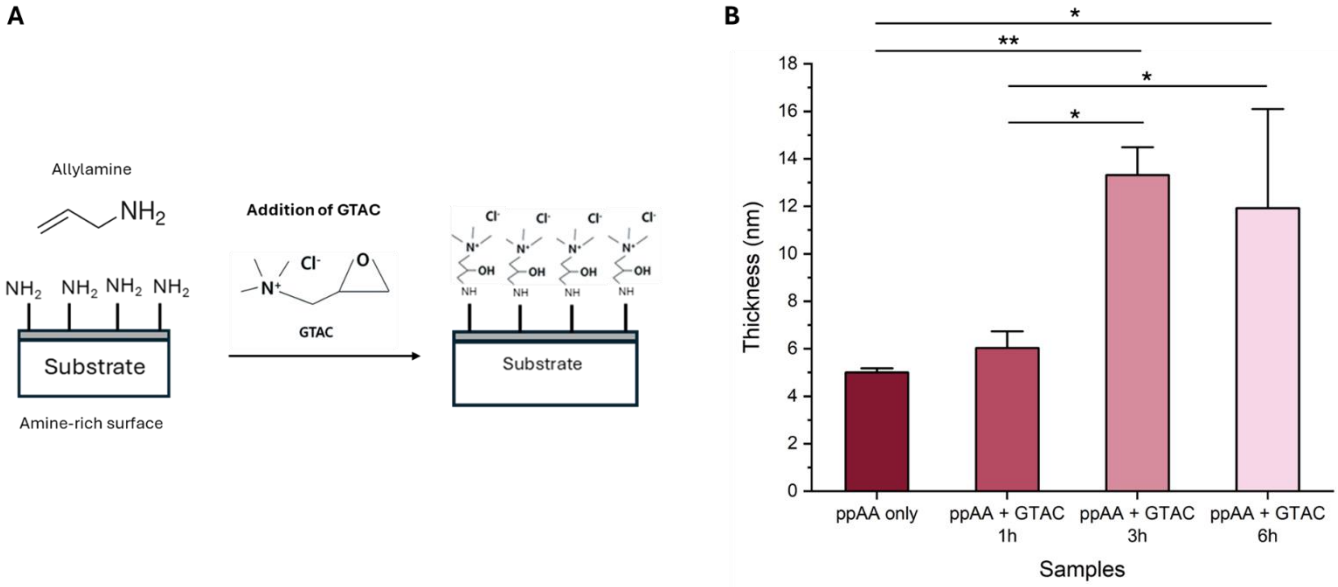

Figure 19. Thickness measurement of silicon wafers coated with GTAC for different periods of time. (A) Covalent attachment between epoxy group of GTAC and amine group of amine-rich surface. (B) Thickness changes of immobilised GTAC surface with different coating time. Thickness was determined using ellipsometry at each time point (1 hour, 3 hours and 6 hours) to assess the covalent attachment of GTAC to the amine-rich surface. Measurements were conducted on the samples with polymerised allylamine (ppAA only), and those subsequently immersed in the GTAC solution for different durations (ppAA+GTAC). The bars represent the average thickness of the samples at each time point. Statistical tests were performed with One-way ANOVA using Fisher's LSD. Error bars represent the standard deviation of three independent measurements (n = 3). * $p < 0.05$, ** $p < 0.01$

3.3. Surface wettability

The wettability of the modified surfaces was determined by water contact angle measurement using both the homogenous surfaces of coverslips and the gradient surface of the 30 mm-glass side. We measured both types of samples to validate that the gradient is actually functioning as a gradient that can be verified by using the homogenous surface with known exposure duration. The surface is considered hydrophilic if the water drop is spread over a large area of the surface and the contact angle is smaller than 90°. The decreasing trend in the contact angle from 1 hour to 6 hours of immersion time indicated that the surface becomes more hydrophilic as more GTAC molecules coat the surface. The static water contact of samples coated only with allylamine (control) exhibited the highest contact angle (37.61° ± 3.15), reflecting the hydrophilic nature of allylamine. Once ppAA

samples were immersed in GTAC solution, the water contact angle decreased due to the hydrophilic groups present in GTAC. To be specific, the angles of the samples in 1 hour, 3 hours and 6 hours were 22.09° ± 1.75, 16.38° ± 2.07, and 13.24° ± 1.29, respectively (**Figure 20**). In general, the reduction in contact angle can be observed on the samples in 1 hour, 3 hours, and 6 hours, reflecting the presence of positively charged GTAC molecules (NR_4^+).

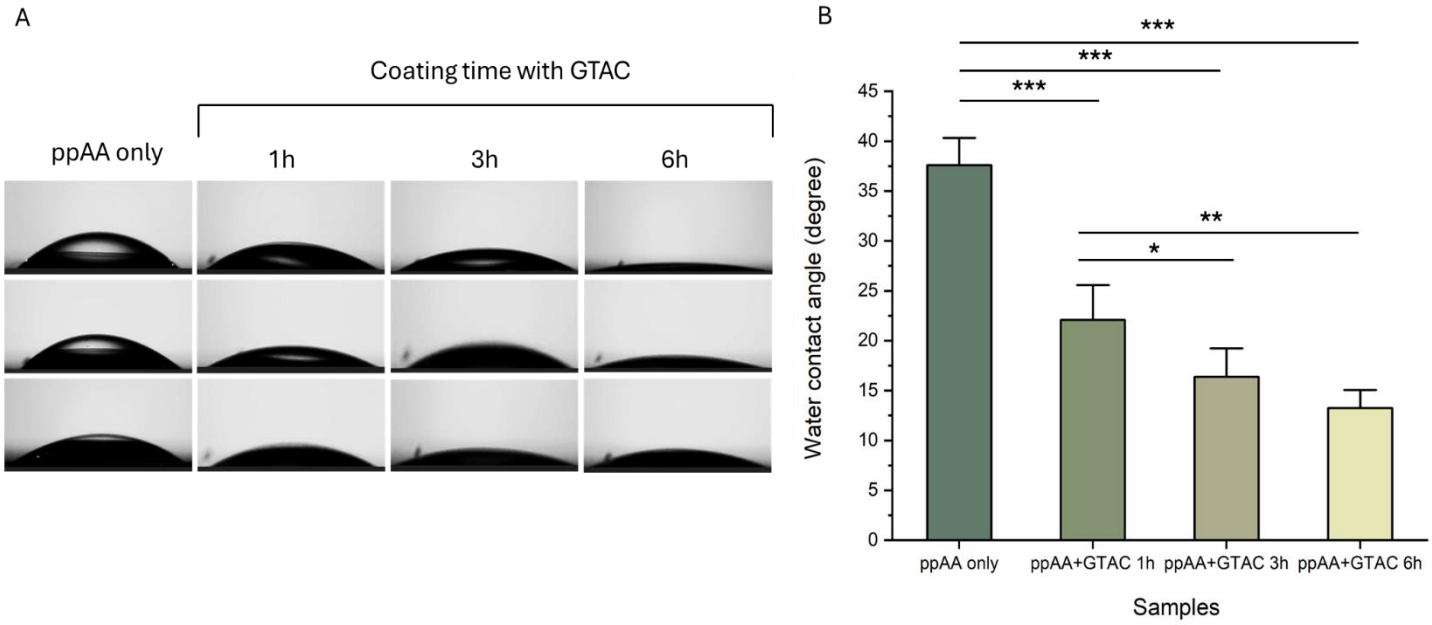

Figure 20. Water contact angle measurement with various coating GTAC duration on ppAA surface. (A) Image illustrating water droplets on coverslips at each time point (1 hour, 3 hours and 6 hours). (B) The contact angles quantification using a goniometer for different coating period. Measurement was conducted on the round glass coverslips. The samples coated with allylamine only represent a control (ppAA only) while ppAA+GTAC refers to ppAA samples immersed in GTAC solution. Data were expressed as means ± SD (n = 3 for each variable) and statical significance was analysed using one way ANOVA with Fisher's LSD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Water contact angle measurements were also performed on a 30-mm glass side with gradient surfaces since 13 mm round coverslips did not provide enough space for water droplets along the gradient. The measurements were recorded at each 6 mm distance. **Figure 21** illustrates a decreasing trend along the gradient, with contact angles ranging from 22.35° ± 2.79 at 6 mm to 14.28° ± 0.71 at 30 mm positions. This change in contact angle reflected the varying concentration of GTAC along the surface. It was obvious that the position at 30 mm (longest exposure to GTAC solution) was more hydrophilic than the position at 6 mm (shortest incubation time).

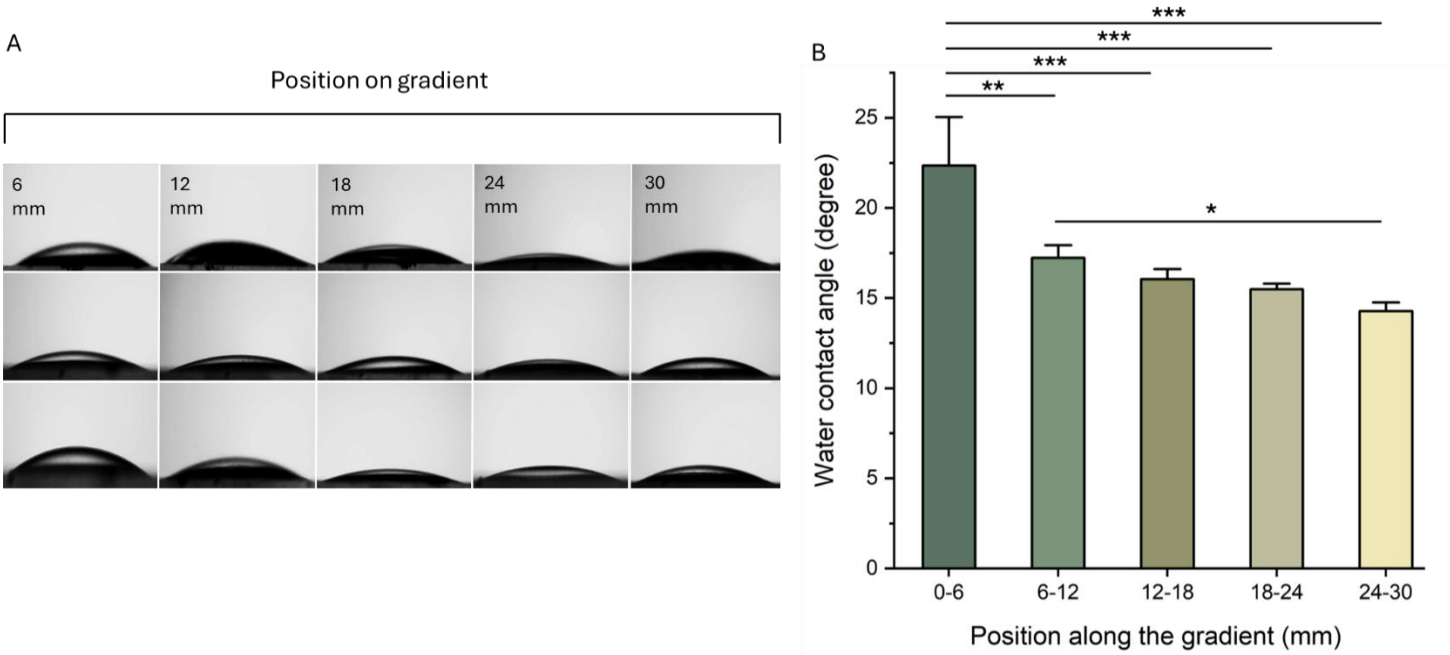

Figure 21. Water contact angle measurements at different positions along the gradient on 30-mm glass side. (A) Image displaying the water droplets at different positions along the gradients (6 mm, 12 mm, 18 mm, 24 mm, 30 mm). (B) Quantitative water contact angle measurements at representative locations of the gradient surfaces. The angle was measured on the 30-mm glass side with gradient surface. Data was analysed by one way ANOVA with Fisher's LSD and reported as means ± SD from triplicates for each position (n = 3 for each variable). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The relationship between the homogenous and gradient surfaces can be seen in the hydrophilicity trends. Both surfaces exhibited increasing hydrophilicity over time and position along the gradient due to GTAC coating. For the homogenous samples, the highest hydrophilicity was observed after 6 hours of immersion. Similarly, the gradient samples showed the highest hydrophilicity at the 30 mm position, which corresponds to the longest exposure time in the GTAC solution (6 hours). These results confirm that the wettability of materials can be affected by changing the surface chemical compositions. It is concluded that the surface immersed in GTAC for longer periods which means more positively charged GTAC bound to the surface, enhancing the hydrophilicity of the surface. This also demonstrates that the method of creating a gradient effectively mimics the time-dependent increase in hydrophilicity observed on both surfaces.

3.4. Fluorescence Assay

The fluorescence intensity along the gradient reflects the presence of GTAC on the surface, as the negatively charged ionic fluorochrome of fluorescent dye (FITC) used in this study electrostatically binds to positively charged moieties (NR_4^+ of GTAC) (Elena & Miri, 2018; Mahmudi-Azer et al., 1998). To determine the optimal pH for fluorescence binding, homogeneous

samples coated with GTAC for 6 hours were tested with a pH range from 7 to 9. The data showed a significant increase in fluorescence intensity at pH 9 (approximately 4.2 x 10^6), compared to the values at pH 7 and pH 8 (**Figure 22A**). This suggests that GTAC molecules might more effectively interact with the negatively charged FITC at this pH level. After establishing pH 9 as optimal, homogenous samples immersed in GTAC for different times (1 hour, 3 hours and 6 hours) were tested at this pH. The fluorescence intensity increased significantly with longer immersion times, particularly, the value at 6 hours was approximately twice as high as the value observed at 1 hour (**Figure 22B**). From the trend, it is assumed that regions with higher GTAC concentration density on gradient samples would show correspondingly higher fluorescence intensity, reflecting a high amount of quaternary ammonium groups.

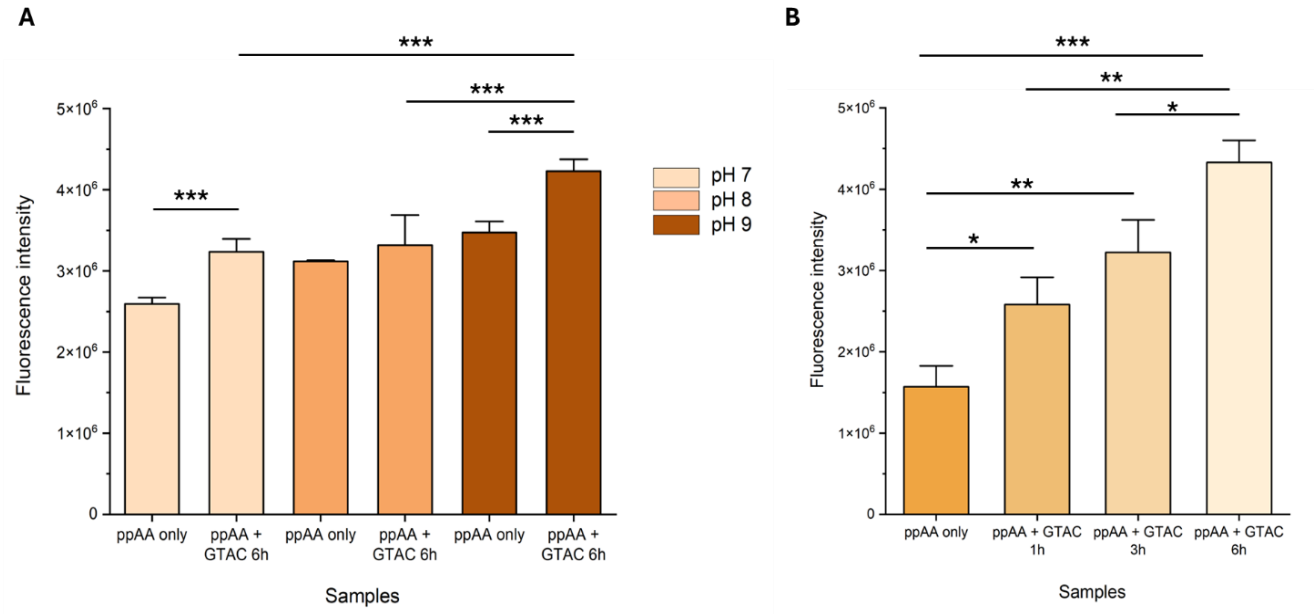

Figure 22. Indirect quantification of GTAC on the coated surface using FITC dye. (A) Fluorescence intensity of homogeneous samples coated with GTAC for 6 hours tested at pH of 7, 8 and 9. The result shows that pH 9 was the most favourable condition for optimal fluorescence assay to indirectly quantify the presence of GTAC. (B) Fluorescence intensity of homogeneous samples coated with GTAC for 1 hour, 3 hours and 6 hours at pH 9. The result shows a significant increase in fluorescence intensity with longer GTAC immersion times. The ppAA samples served as controls for comparison with those coated with GTAC. Data was analysed using One-way ANOVA followed by Fisher's LSD. Error bars represent the standard deviation ± SD (n = 3 for each variable). Statistically significant difference is indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The fluorescence intensity was also measured on coverslips with gradient surface at each 2 mm distance. As shown in **Figure 23**, the control samples, which were not exposed to GTAC, showed the

lowest fluorescent intensity. This low signal suggests minimal binding of the fluorescent dye, indicating the absence of GTAC. Differing from the control group, the fluorescence intensity of gradient samples steadily increased from 0-2 mm position, where the low intensity was observed, to the 10 mm position, where the highest intensity was recorded. This suggests that more GTAC molecules were coated along the gradient and bound to fluorescent dye. Theoretically, the ppAA samples immersed in GTAC solution for the longest time (6 hours), particularly at 10 mm position, formed more electrostatic interactions between NR_4^+ of GTAC and fluorescent dye, resulting in an increase in fluorescence intensity.

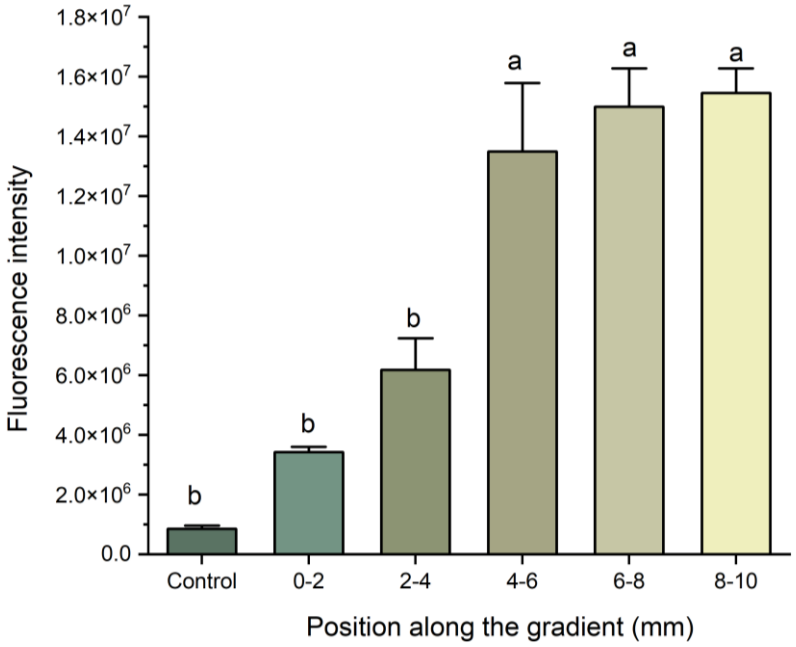

Figure 23. Indirect quantification of GTAC using fluorescent dye binding along the gradient. The fluorescence was measured in 2 mm positions along the 10 mm gradient surface, with the control representing the coverslips without GTAC coating. The bars represent the mean fluorescence intensity of control samples and ppAA+GTAC samples at each position along the gradient. The letters above the bars (a, b) denote statistically significant differences between groups ($p < 0.01$). Error bars represent the standard deviation ± SD ($n = 3$ for each variable). Statistical analysis was conducted using One-way ANOVA followed by Fisher's LSD test.

3.5. The influence of immobilised QAC on attachment and viability of common pathogens

After successfully coating ppAA samples with GTAC using a dip coater, the antimicrobial activity of these surfaces against Gram-negative bacteria (*P. aeruginosa*), Gram-positive bacteria (*S. aureus*) and fungi (*C. albicans*) was investigated by using LIVE/DEAD assay. They are known to form biofilm on implantable medical devices and tolerant to antibiotics (Hayles *et al.*, 2024; Obłąk

et al., 2014). In this investigation, live cells were stained with SYTO9, which fluoresces green colour, and propidium iodide (PI) stains dead cells with damaged membranes, which fluoresces red (Robertson *et al.*, 2019). Confocal laser scanning microscopy images were taken to visualise the live and dead cells on the surface at various positions along the gradient, allowing for the quantification of both cell attachment and viability.

S. aureus, Gram-positive bacteria, is one of the most common pathogens forming the biofilm on medical devices and responsible for 20-40% of orthopaedic implant-related infections (Hayles et al., 2024). Quantitative analysis of cell attachment revealed that *S. aureus* attachment was significantly higher at the 0-2 mm position compared to the control ($p < 0.05$) (**Figure 24B**). However, the number of attached cells was not stable along the gradient with a sudden increase and decrease. It can be explained by the bacterial distribution might vary and the area to capture the image was random, resulting in this inconsistent trend. In general, the density of QAC had no effect on rate of attachment. The viability results demonstrated a significant reduction in bacterial viability with increasing QAC concentration along the gradient. While the viability of 99.64% ± 0.06 was observed in the control surface (ppAA only), the viability began to drop at the 0-2 mm position (45.63% ± 1.52). There was no statistically significant difference in viability at the 0-2 mm, 2-4mm, and 4-8 mm position. However, at the 6-8 mm and 8-10 mm positions that remained in the GTAC solution for a longer time, *S. aureus* was killed with a survival rate of 17.96% ± 4.13 and 13.23% ± 1.55, respectively ($p < 0.001$) (**Figure 24C**). It showed that the immobilised QAC effectively reduced *S. aureus* viability at higher gradient positions with a high concentration of QAC density.

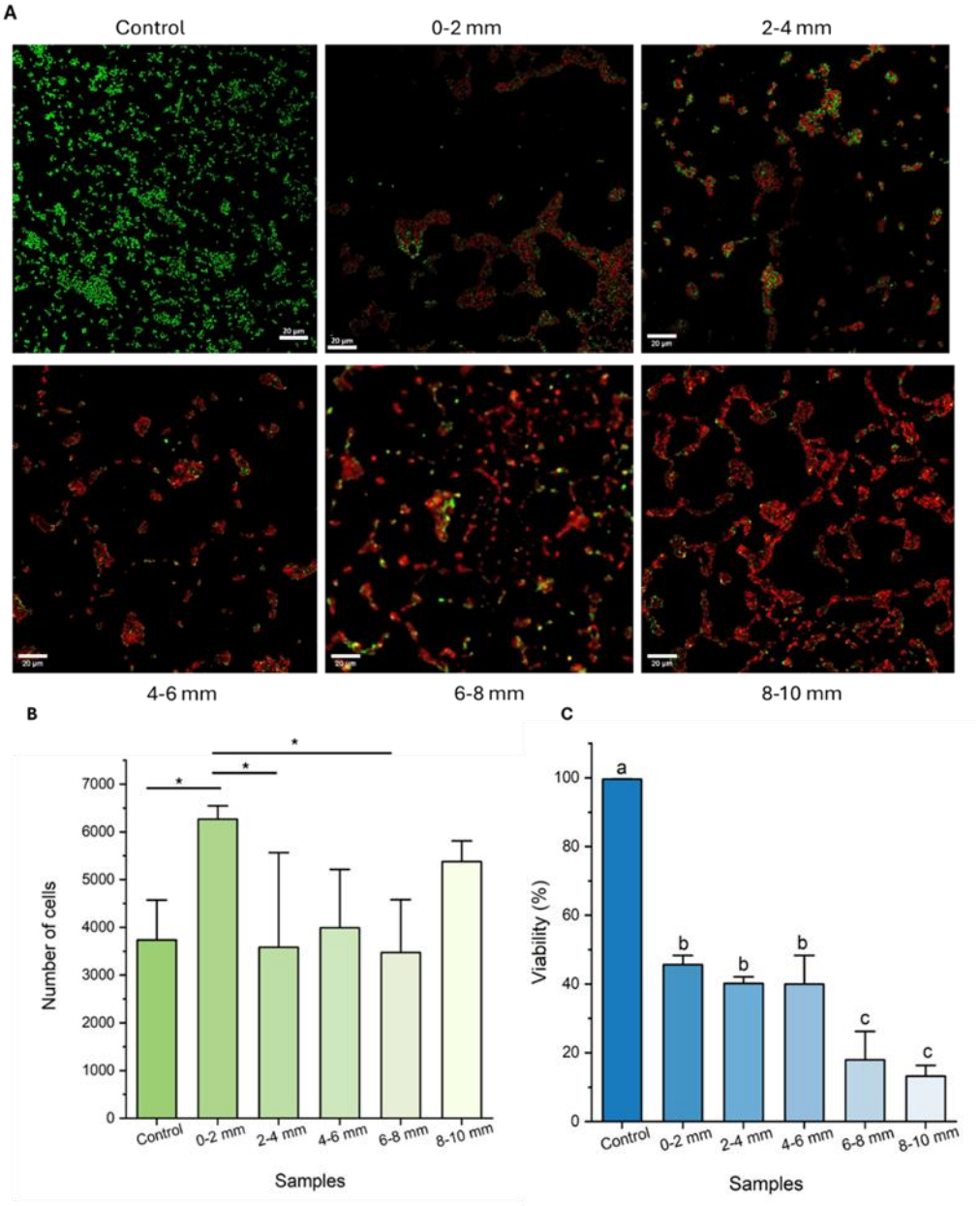

Figure 24. Evaluation of *S. aureus* attachment and viability using LIVE/DEAD assay. (A) Confocal laser scanning microscopy images showing live (green) and dead (red) *S. aureus* cells attached to a surface coated with allylamine (control) and gradient surface with different positions (scale bar = 20 μm). Images were taken at 0-2 mm, 2-4 mm, 4-6 mm, 6-8 mm, and 8-10 mm positions along the gradient of ppAA+GTAC samples under 40X magnification. (B) Quantification of *S. aureus* cell attachment at various positions on the QAC gradient. Statistical significance is denoted by a single asterisk (* $p < 0.05$). (C) The percentage of viability of *S. aureus* quantified from fluorescence images. Different letters represent statistically significant differences between groups ($p < 0.001$). Statistical significance was assessed using one-way ANOVA with Fisher's LSD. Error bars represent ± SD (n = 3 for each variable).

P. aeruginosa is a Gram-negative bacteria associated with nosocomial infection by forming biofilms (Obłąk et al., 2014). The structural features such as fimbriae and capsules enhance its adhesion to surfaces, contributing to the challenges in clinical settings (Obłąk et al., 2014). The study revealed the number of attached cells including live and dead cells using LIVE/DEAD assay. **Figure 25B** indicated that the GTAC-coated surfaces likely did not affect to attached *P. aeruginosa* cells. Specifically, a high number of attached cells was observed on ppAA+GTAC samples at the 0-2 mm region compared to the control (ppAA only) and other regions ($p < 0.05$). A reduction in attached cells at 4-6 mm and 8-10 mm position compared to position at 0-2 mm was recorded; however, this trend was not consistent along the gradient. Interestingly, the attached cells at 2-4 mm, 4-6 mm, 6-8 mm, and 8-10 mm position were not statistically different compared to the control ($p < 0.05$). Regarding *P. aeruginosa* viability, a decreasing trend across the GTAC gradient was observed. On the control surface, viability was 100%, indicating a large population of live cells. As the surface was coated GTAC for a longer duration from 0-2 mm region to the 8-10 region, viability dropped from 45.11% ± 1.20 to 23.78% ± 3.81 (**Figure 25C**). In general, the data suggested that the immobilised GTAC surface did not prevent bacterial attachment while can enhance the antibacterial effects on the surface to kill the Gram-negative *P. aeruginosa*.

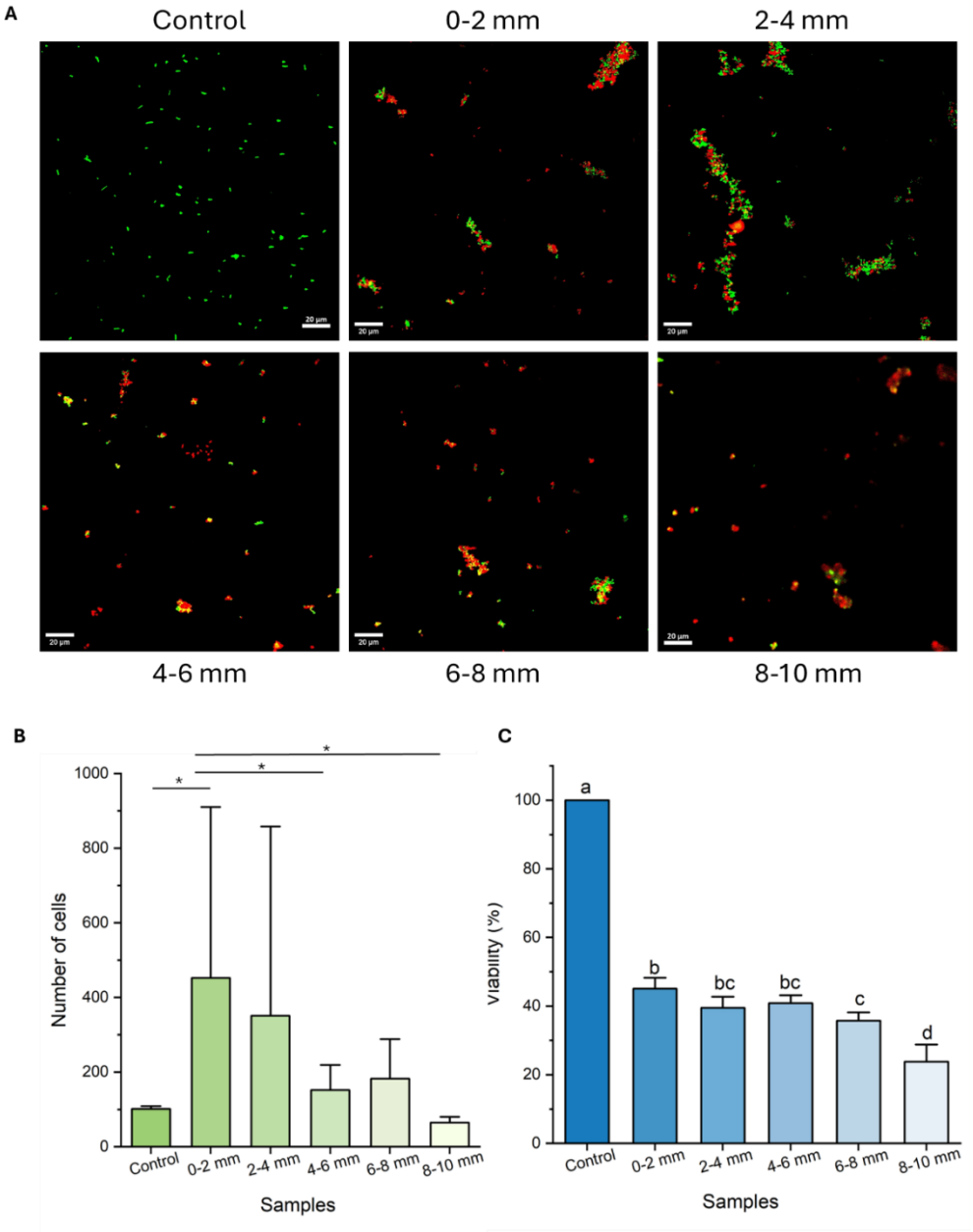

Figure 25. Evaluation of *P. aeruginosa* attachment and viability using LIVE/DEAD assay. (A) Confocal microscopy images showing the live cells and dead cells of *P. aeruginosa* on the GTAC-coated surfaces along the gradient. Green fluorescence indicates live cells, and red fluorescence indicates dead cells. Scale bar: 20 µm. Magnification: 40X. (B) Quantification of *P. aeruginosa* attachment across the surface GTAC gradient. Statistical significance is indicated as * $p < 0.05$. (C) Quantification of *P. aeruginosa* viability across the surface GTAC gradient. Control represents the ppAA samples without GTAC coating. Different letters (a-d) on the top of the bars represent statistically significant differences between groups ($p < 0.01$). Statistical analysis was performed using One-way ANOVA followed by Fisher's LSD. Error bars represent ± SD ($n = 4$ for each variable).

C. albicans, one of the most common fungal pathogens of humans, produces highly structured biofilms (Chakraborty et al., 2021). To investigate the effect of immobilised GTAC surface on fungal attachment and viability, LIVE/DEAD also was used in this experiment. In the region from 0-6 mm, where the GTAC concentration density was low, there was a significant reduction in the number of attached *C. albicans* compared to the control with ppAA only (**Figure 26B**). As the surface progressed to regions with higher GTAC amount (6-8 mm and 8-10 mm), the attached cells increased gradually compared to the 0-2 mm position, particularly in the 6-8 mm region where a marked rise in cell number was observed ($p < 0.01$). Regarding the ability to kill *C. albicans* on immobilised GTAC surface, **Figure 26C** reveals that the cell viability at 0-2 mm position was significantly reduced to 3.7 times compared to the control ($p < 0.01$). However, more alive *C. albicans* cells were observed at 2-4 mm and 4-6 mm position where the GTAC concentration density increased but did not return to the high levels seen in the control group. The number slightly rose at 6-8 mm and 8-10 mm position with the viability of $87.20\% \pm 0.52$ and $88.26\% \pm 1.57$, respectively, which were not significantly different compared to the control ($p < 0.01$). This indicated that immobilised GTAC surface affects the viability of *C. albicans*, with low concentration density of GTAC being more effective across the gradient while did not impact the attached cell that much.

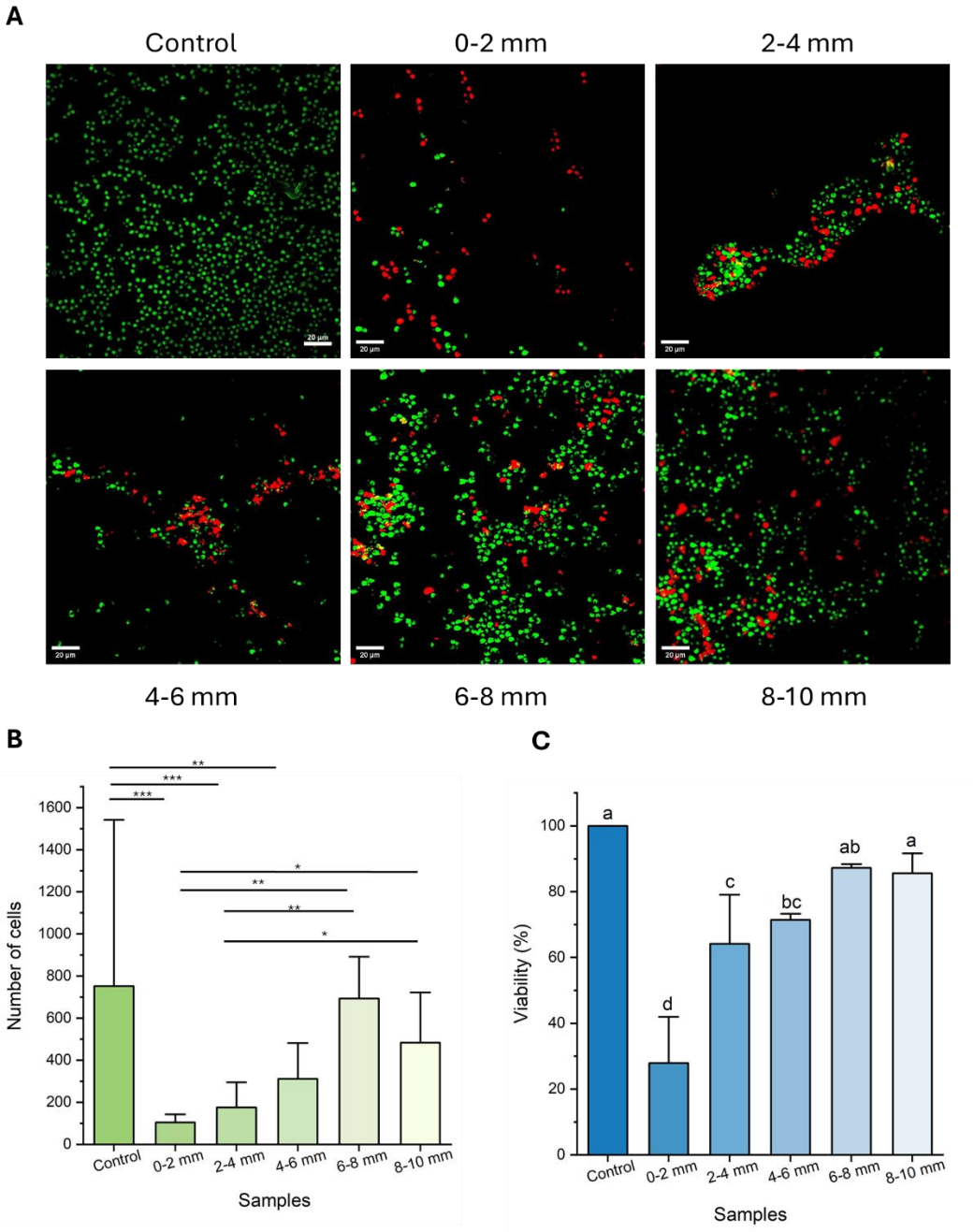

Figure 26. Evaluation of *C. albicans* attachment and viability using LIVE/DEAD assay. (A) Representative confocal microscopy images of *C. albicans* on the GTAC-coated surface along the gradient. (B) Quantification of *C. albicans* cell attachment on the surface with GTAC coating. Statistical significance was indicated by p values (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (C) Cell viability assessment on the GTAC-coated surface across the gradient. Control represents the ppAA samples without GTAC coating. Different letters indicate significance differences between groups ($p < 0.01$). Statistical analysis was performed using One-way ANOVA with Fisher's LSD. Error bars represent the standard deviation (n = 5, independent samples).

It is concluded that the attachment and viability of *S. aureus*, *P. aeruginosa* and *C. albicans* vary significantly in response to QAC coatings. Gram-positive bacteria such as *S. aureus* and Gram-negative bacteria including *P. aeruginosa*, the results demonstrated a decrease in viability along the gradient as GTAC concentration density increased. This highlights that the coated surface effectively inhibits the survival rate of bacteria across the gradient. However, GTAC modified surface did not influence on the attachment of both Gram-negative and Gram-positive bacteria in this study. Unlike the bacteria, immobilised QAC surface displayed a different trend on both attachment and viability of *C. albicans* across the GTAC gradient. This suggests that the electrostatic properties of modified surfaces did not contribute to the fugal inhibition such as *C. albicans*, likely due to their differences in their cell wall structure and adhesion mechanisms.

3.6. Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of antibiotics against *S. aureus*

The antimicrobial susceptibility of *S. aureus* to vancomycin, daptomycin, kanamycin, and levofloxacin was determined by evaluating the MIC and MBC values. MIC represents the minimum concentration of antibiotic required to inhibit visible bacterial growth (Hayles *et al.*, 2024). As shown in **Figure 27A**, vancomycin displayed an MIC of 1 μg/mL, indicating effective inhibition at this concentration. Regarding daptomycin, this antibiotic exhibited a higher MIC which was 2 μg/mL, revealing its effectiveness at this concentration (**Figure 27B**). Kanamycin showed the lowest MIC of 0.125 μg/mL (**Figure 27C**) and levofloxacin exhibited an MIC of 1 μg/mL similar to vancomycin (**Figure 27D**).

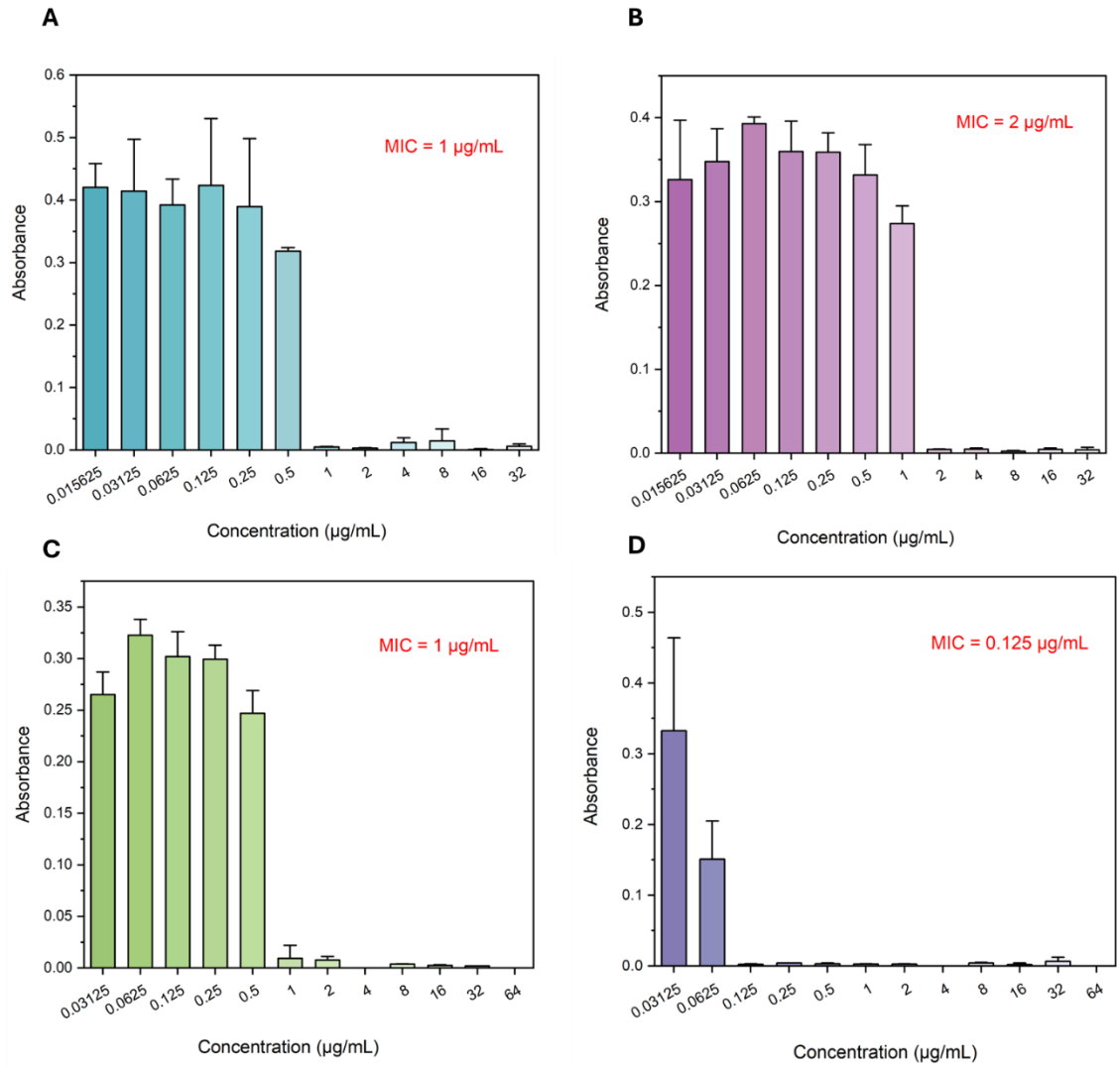

Figure 27. Minimum Inhibitory Concentration (MIC) values for *S. aureus* exposed to various antibiotics. (A) MIC of vancomycin against *S. aureus* tested at various concentrations from 0.016 µg/mL to 32 µg/mL. (B) MIC of daptomycin against *S. aureus* tested at various concentrations from 0.016 µg/mL to 32 µg/mL. (C) MIC of kanamycin against *S. aureus* tested at various concentrations from 0.031 µg/mL to 64 µg/mL. (D) MIC of levofloxacin against *S. aureus* tested at various concentrations from 0.031 µg/mL to 64 µg/mL. The absorbance was measured at wavelength 600 nm, mean ± SD and n = 3.

Differ from MIC, minimum bactericidal concentration (MBC) represents the concentration at which bacterial viability is completely eliminated. As shown in **Table 1**, vancomycin and kanamycin exhibited MBC of 2 µg/mL. Daptomycin required 4 µg/mL to achieve bactericidal effect, whereas levofloxacin showed the lowest MBC at 0.25 µg/mL.

Table 1. The MBC of antibiotics against *S. aureus* tested for 24 hours

Antibiotics	MBC (µg/mL)
Vancomycin	2
Daptomycin	4
Kanamycin	2
Levofloxacin	0.25

3.7. The influence of immobilised QAC on the antibiotic tolerance of *S. aureus*

Antibiotic tolerance is a significant concern in healthcare systems, particularly concerning implantable medical devices. To examine the effect of immobilised QAC surface on the antibiotic tolerance of *S. aureus*, four antibiotics including daptomycin, vancomycin, levofloxacin and kanamycin with different mechanisms targeting biofilm formation were tested. The antibiotic sensitivity of adherent cells was evaluated using LIVE/DEAD assay, where the proportions of green and red cells were visualised via confocal laser scanning microscopy for all four antibiotics. This method enables quantification of bactericidal activity of the antibiotics on the modified surfaces, establishing the relation between charge gradient and antibiotic tolerance. The tested concentration of antibiotics in this experiment was based on the MBC of antibiotics against *S. aureus*, with the dosage set at 4X MBC (**Table 2**). Once the concentration for each antibiotic was established, the control surface (ppAA only) and surface with immobilised QAC after 3 hours of incubation with *S. aureus* were treated with the antibiotics for 24 hours.

Table 2. The concentration of antibiotics used to test the antibiotic tolerance in *S. aureus*

Antibiotics	Concentration (µg/mL)
Vancomycin	8
Daptomycin	16
Kanamycin	8
Levofloxacin	1

After administration of daptomycin into *S. aureus* cultured immobilised GTAC surface, the control group of *S. aureus* mostly remained alive, as indicated by the lack of red colour (**Figure 28B**). However, the addition of GTAC on the surfaces resulted in a decrease in the number of live cells, as evidenced by the enhanced red fluorescence signal, suggesting that the immobilised QAC had decreased the survival of bacterial cells when daptomycin was administered compared to the control. The viable cell population at 8-10 mm position decreased to 34.03% ± 2.14, which was approximately half the value observed at the 0-2 mm position and nearly 3 times lower than that of the control ($p < 0.01$). The results suggested that *S. aureus* becomes more susceptible to daptomycin at higher GTAC concentration density. In other words, the surface coated with GTAC significantly increased the effectiveness of daptomycin against *S. aureus*.

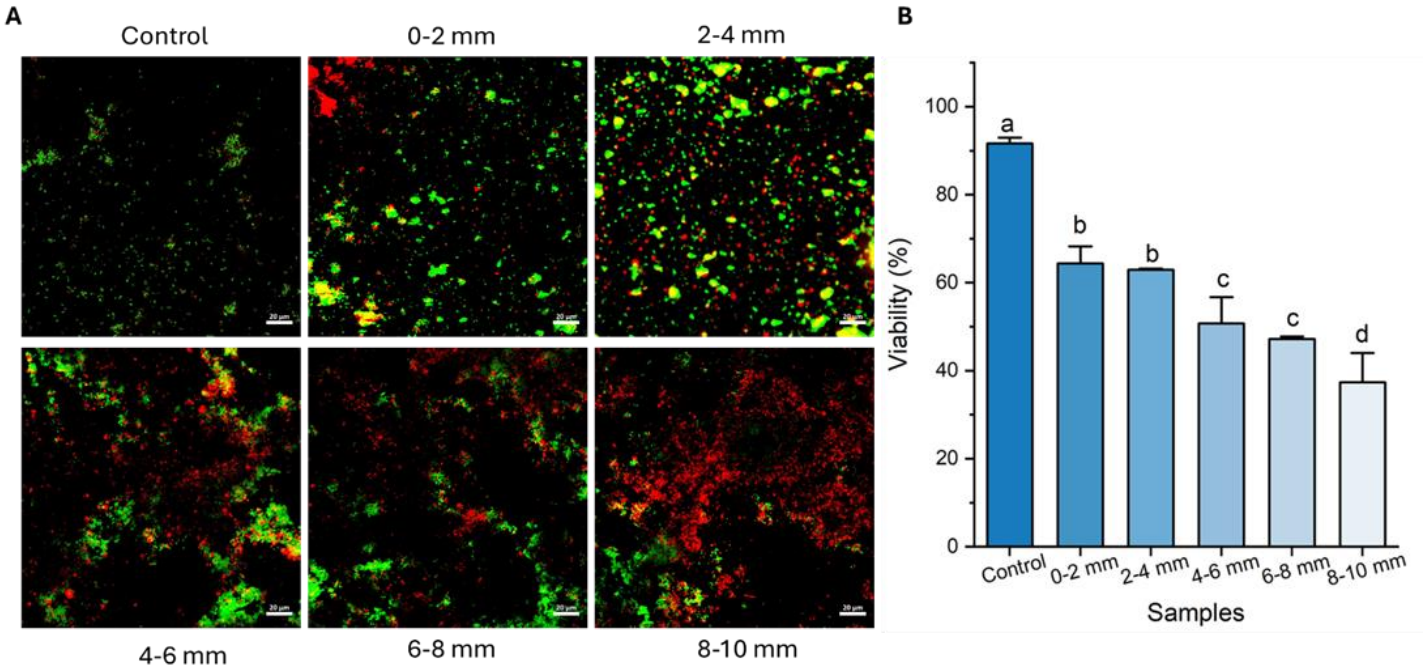

Figure 28. *S. aureus* viability on GTAC-coated surfaces after daptomycin treatment using LIVE/DEAD assay. (A) Confocal laser scanning microscopy images of *S. aureus* treated with daptomycin (16 μg/mL) for 24 hours on various positions along the gradient (0-10 mm). The images display live cells and dead cells in green and red colour, respectively. Scale bars represent 20 μm and magnification is 40X. (B) Quantification of bacterial viability along the gradient. The position at 8-10 mm inhibits *S. aureus* growth under daptomycin treatment compared to the control (ppAA only) and other positions. Different letters indicate statistically differences between groups ($p < 0.01$, One-way ANOVA with Fisher's LSD). Error bars indicate the standard deviation ($n = 5$, independent samples).

Similarly, the vancomycin tolerance of *S. aureus* was evaluated along the GTAC gradient using LIVE/DEAD assay. Vancomycin, a glycopeptide antibiotic, disrupts cell wall synthesis in susceptible bacteria by binding to the terminal D-alanyl-D-alanine of peptidoglycan precursors (Cong et al., 2020). In **Figure 29B**, *S. aureus* viability decreased from 0 mm to 10 mm on the coverslips under the treatment with the lowest survival rates recorded at the 8-10 mm position (47.47% ± 1.21). In this region, the viable cell population dropped significantly compared to the control (ppAA only) and other regions, demonstrating that the higher concentration density of GTAC on the surface enhanced the bactericidal activity of vancomycin. In contrast, *S. aureus* showed greater tolerance to vancomycin with a higher percentage of live cells after treatment in the control samples without GTAC (99.14% ± 0.83).

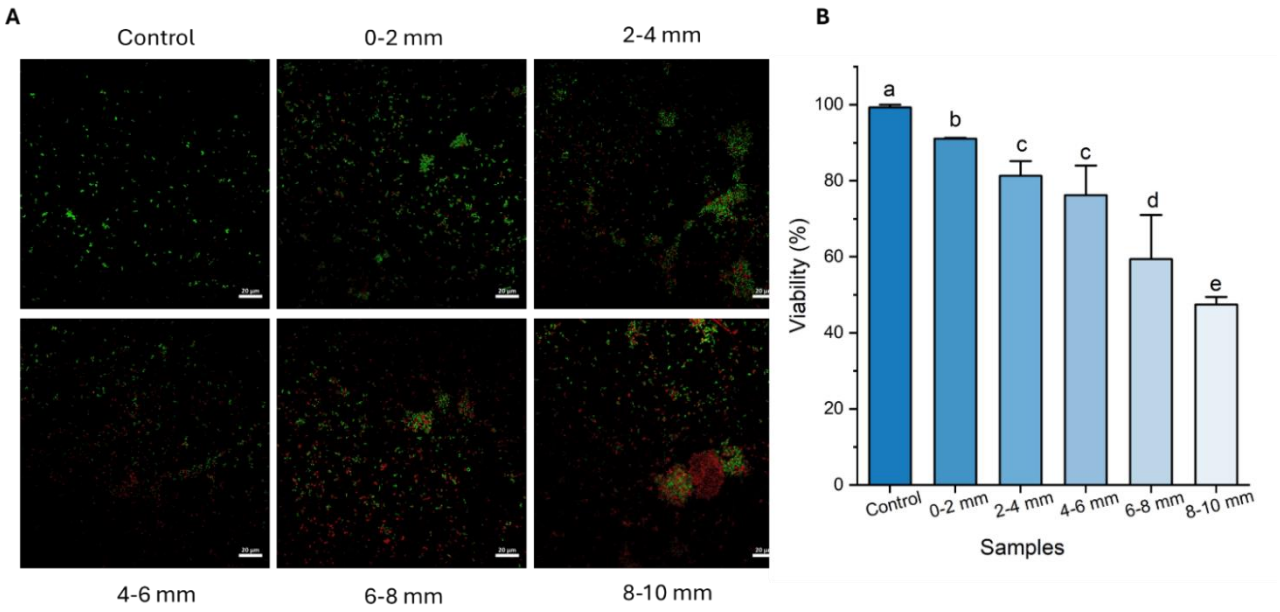

Figure 29. *S. aureus* viability on GTAC-coated surfaces after vancomycin treatment using LIVE/DEAD assay. (A) Confocal laser scanning microscopy images of *S. aureus* treated with vancomycin (8 µg/mL) for 24 hours across different positions along the GTAC gradient (0-10 mm). Live cells are stained green while dead cells are shown in red colour. The bacterial growth under vancomycin treatment reduces at 8-10 mm position compared to the control (ppAA only) and other positions. Scale bars represent 20 µm, and images were captured at 40X magnification. (B) The viability percentage of *S. aureus* along the gradient. Statistically significant difference is indicated by different letters ($p < 0.01$, One-way ANOVA with Fisher's LSD). Error bars indicate the mean ± SD ($n = 5$, independent samples).

Kanamycin, an aminoglycoside antibiotic, was tested on GTAC-coated surfaces to assess its effect on *S. aureus* tolerance. As shown in **Figure 30**, the result reveals a reduction in *S. aureus* viability as the GTAC concentration increased along the gradient surface. In the control group (ppAA only), *S. aureus* displayed high tolerance to kanamycin, with 100% viability. However, as the GTAC compound was coated on the surface with increasing exposure time along the gradient, a decrease in viability was observed. The percentage of alive bacteria obtained at 0-2 mm and 2-4 mm position was 85.08% ± 2.74 and 81.96% ± 2.75, respectively. The lowest survival rate was recorded at 6-8 mm and 8-10 mm position (55.43% ± 0.66 and 55.21% ± 3.03), where the GTAC concentration density was higher than other regions.

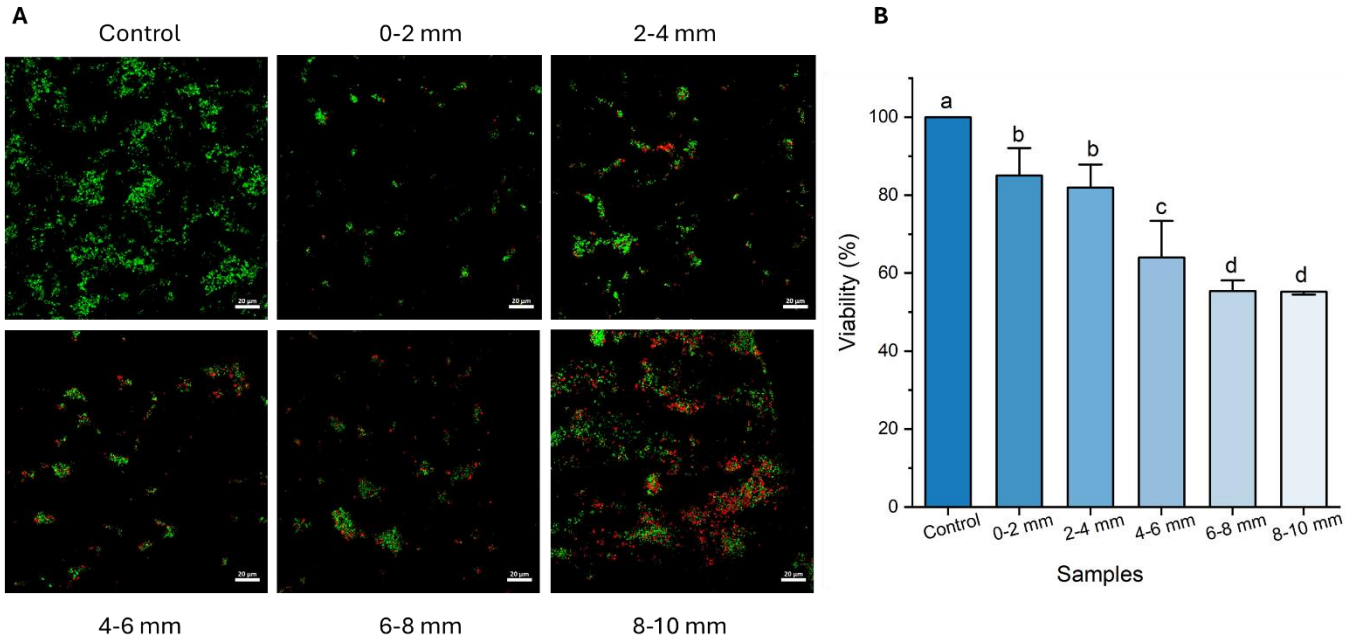

Figure 30. *S. aureus* viability on GTAC-coated surfaces following treatment with kanamycin using LIVE/DEAD assay. (A) Confocal laser microscopy images of S. aureus exposed to kanamycin (2 µg/mL) for 24 hours along different regions of the GTAC gradient (0-10 mm). Live bacteria appear green colour, while dead bacteria are stained red colour. The image displays a reduction in cell viability as the GTAC concentration increases compared to the control (ppAA only). Magnification: 40X, scale bar: 20 µm. (B) Quantitative analysis of *S. aureus* viability across the GTAC gradient. Different letters above the bars indicate statistically significant differences ($p < 0.01$, One-way ANOVA with Fisher's LSD test). Error bars represent the standard deviation ($n = 5$, independent samples).

Another antibiotic used to evaluate the antibiotic tolerance of *S. aureus* was levofloxacin, a fluoroquinolone antibiotic. As illustrated in **Figure 31**, the fluorescence intensity of the PI stanning was not significantly different from 0-8 mm position ($p < 0.01$). However, a reduction in *S. aureus* viability was observed at the beginning of the GTAC gradient (0-2 mm) with the viability of 59.79% ± 3.85, nearly 2 times lower than that of the control (99.71% ± 1.27). It was obvious that there was a significant decrease in the number of live cells at 8-10 mm region, as indicated by the increase in red fluorescence, reaching 31.08% ± 6.78.

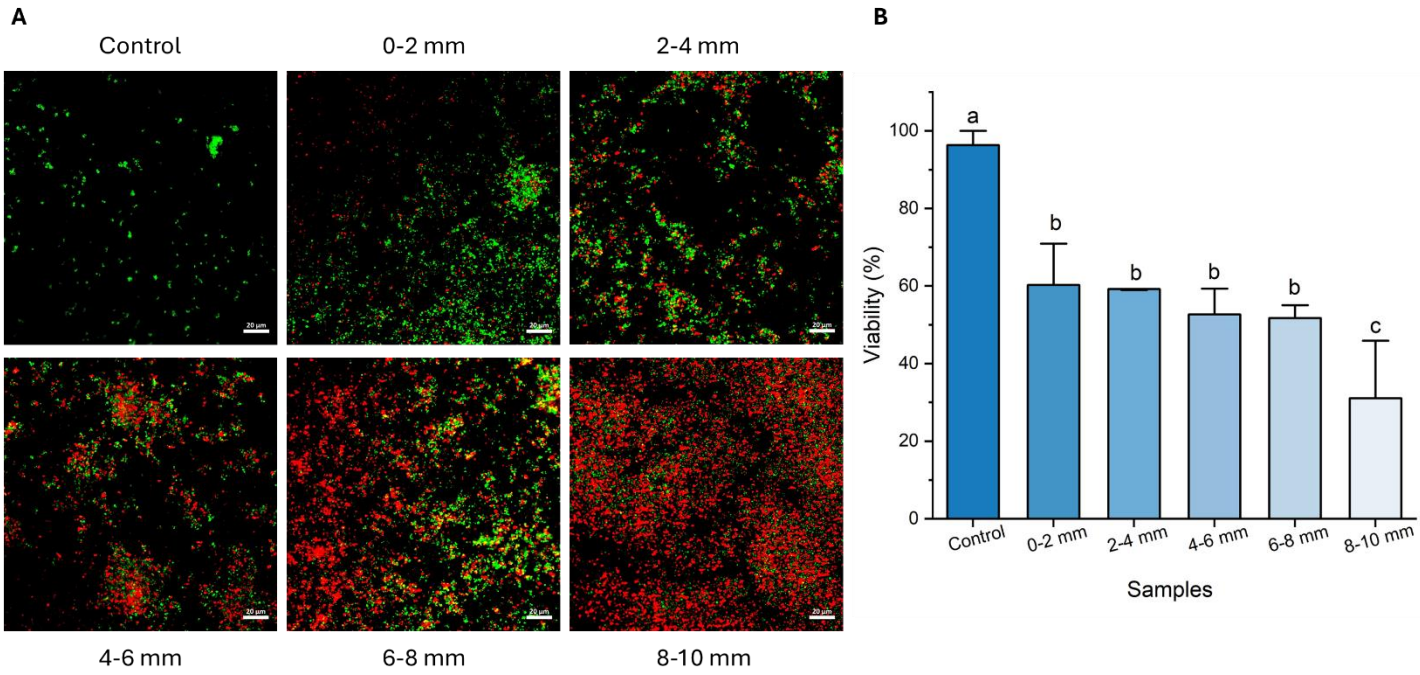

Figure 31. *S. aureus* viability on GTAC-coated surfaces following levofloxacin treatment as assayed by LIVE/DEAD cell staining. (A) Confocal laser scanning microscopy images of *S. aureus* treated with levofloxacin (0.25 μg/mL) across regions along the GTAC gradient (0-10 mm). Green colour indicates live cells, and red colour indicates dead cells. As GTAC concentration increases along the gradient, a reduction in bacterial viability is observed compared to the control (ppAA only). The scale bar is 20 μm and magnification is 40X. (B) *S. aureus* viability quantification along the GTAC gradient on coverslips. One-way ANOVA, followed by Fisher's LSD test, was performed and shows a statistically significant difference by different letters above the bars ($p < 0.001$). Data was shown as mean ± SD of n = 5 (field of views for each distance).

Overall, the antibiotics including vancomycin, daptomycin, kanamycin, and levofloxacin used in this study demonstrated that the presence of GTAC on the surface enhances their antimicrobial activity against *S. aureus*. The result highlights the highest charge density regions (8-10 mm, corresponding to 4.8 – 6 h GTAC exposure) improved the outcomes of antibiotic treatment more

effectively than lower GTAC densities. Table 3 lists a summary of the optimal antibiotic outcomes of each antibiotic investigated.

Table 3. Summary of enhanced antibiotic outcomes of four investigated antibiotics

Antibiotic	Concentration (μg/mL)	Mechanism of action	Lowest cell viability (%)	Gradient position (GTAC adsorption time)
Vancomycin	8	Inhibition of peptidoglycan synthesis	47.47% ± 1.21	8-10 mm (4.8 – 6 h)
Kanamycin	8	Membrane disruption + inhibition of protein synthesis	55.21% ± 3.03	8-10 mm (4.8 – 6 h)
Daptomycin	16	Membrane disruption	34.03% ± 2.14	8-10 mm (4.8 – 6 h)
Levofloxacin	1	Inhibition of DNA synthesis	31.08% ± 6.78	8-10 mm (4.8 – 6 h)

CHAPTER 4. DISCUSSION

4.1. Characterisation of immobilised QAC surface

To create GTAC immobilisation surface density, allylamine was grafted on the surface via plasma polymerisation, resulting in an amine-rich layer onto the substrates. The purpose of using allylamine as a precursor in plasma polymerisation is to provide an amine functionalised layer, enhancing the biocompatibility of biomedical materials (Mangindaan et al., 2013). Additionally, plasma polymerised allylamine is often used as an underlayer for enhancing surface functionality before further modifications such as nanoparticle or biomolecule coatings (Mangindaan et al., 2013). Hence, allylamine was used as an underlayer for subsequent QAC coating, further enhancing the antimicrobial properties and modifying surface properties. In this project, ppAA samples were immersed in a GTAC solution (10% v/v in Milli-Q water) with a pH of 10 based on the data of previous studies. The study indicated that pH 10 was the optimal condition for maximising the attachment of GTAC to the surface (Cavallaro et al., 2014). In the previous report, XPS analysis revealed that 7.5% ± 1.7 of the total nitrogen content on ppAA+GTAC samples was in the form of NR_4^+ (Cavallaro et al., 2014). The final QAC modified surface was created by the covalent attachment between amine group of allylamine and the epoxy group of GTAC molecules. Although we used a higher concentration of GTAC, the confirmed presence NR_4^+ of GTAC (1% v/v in Milli-Q water) in Cavallaro et al. (2014) is strong support for the expected presence of NR_4^+ in our study.

The thickness measurement using ellipsometry on homogenous surfaces (without a gradient) also confirmed the presence of this covalent attachment, showing an increase in thickness after the addition of GTAC. The necessity of using homogenous surfaces rather than gradient surfaces arises from the issues related to light scattering and polarisation. Inhomogeneous surfaces can cause light scattering, which leads to partial polarisation of reflected light (Fujiwara, 2007). When the structural inhomogeneity exceeds about 30% of the measurement wavelength, the reflected light becomes partially polarised, increasing the measurement error (Fujiwara, 2007). The thickness increases only during the first 3 hours of exposure; however, the thickness did not increase any further at 6 hours. It can be explained that GTAC can only attach to amine groups, which are present as a monolayer on the surface. Thus, during the initial 3 hours, GTAC molecule gradually occupy the available amine function groups, resulting in an increase in thickness to a certain point. However, after 3 hours, any additional GTAC that binds to the surface does not contribute an increase in coating thickness because it just fills in the remaining amines available on the ppAA layer.

Surface wettability of QAC immobilisation surface density in this study was determined using water contact angle measurement. Particularly, the wettability analysis of both homogenous and

gradient samples showed a consistent relationship between immersion time and QAC concentration density. Also, these consistent results helped to validate that the gradient samples were behaving as expected with properties that are dependent on the exposure duration. In the homogenous samples, the contact angle of samples immersed for the shortest time (1 hour) was 22.09° ± 1.75, which was comparable to the gradient surface at the 0-6 mm region of a 30 mm gradient (22.35° ± 2.79), which corresponds to approximately 1.25 h of GTAC adsorption. As the immersion time increased, the contact angle decreased to 13.24° ± 1.29 at 6 hours on the homogenous samples and 14.28° ± 0.71 at the 30 mm point along the gradient samples, validating the notion that greater exposure duration leads to an increased adsorption of GTAC. This trend aligns with previous studies, particularly, a contact angle of 53.09° ± 0.29 of ppAA thin films decreased to 45.99° ± 0.67 on the ppAA+GTAC surfaces (Cavallaro et al., 2014). The reduction in contact angle of modified surface is attributed to the hydrophilicity of NR_4^+ groups. Surface wettability plays a crucial role in bacterial adhesion and biofilm formation, which can be explained using thermodynamic models based on surface free energy and interaction energy (Zheng et al., 2021). Hydrophilic surfaces, like those with higher NR_4^+ density, generally exhibit lower bacterial adhesion due to positive free energy, which resists bacterial attachment (Yang et al., 2022; Zheng et al., 2021). Conversely, hydrophobic surfaces often promote bacterial adhesion because of stronger Van der Waals and Coulomb interactions (Yang et al., 2022; Zheng et al., 2021). The higher bacterial adhesion on hydrophobic surfaces is primally due to stronger interaction between the bacteria and the surface. Hydrophobicity can reduce the velocity of bacteria upon surface collisions, thus promoting bacterial adhesion (Yang et al., 2022). This is evident in studies where *E. coli* showed reduced adhesion to superhydrophilic surfaces on the stainless-steel plates coated with TiO_2 (Zheng et al., 2021). Hence, the hydrophilicity of QAC immobilisation surface density might contribute to reducing bacterial adhesions and decreasing biofilm formation.

To further validate the presence of GTAC at varying densities, we developed a fluorescence detection assay using the fluorescent probe FITC, which has a negative charge and therefore strongly interacts with the positively charged NR_4^+ of GTAC. Based on this electrostatic interaction, an increased fluorescence intensity corresponds to an increased presence of GTAC. The principle of this experimental design has been validated in previous research, where FITC has been used as a fluorescent label in immunofluorescence assays to detect positively charged proteins, particularly in eosinophils (Mahmudi-Azer et al., 1998). The control sample (ppAA only) exhibited the lowest fluorescence intensity. However, the 4-10 mm position on a 10 mm gradient (corresponding to 2.5 - 6 h GTAC adsorption) showed increasing fluorescence intensity, indicating an increasing density of GTAC. These results are further support for the expected relationship between GTAC adsorption time and immobilised GTAC density.

This study did not conduct X-ray Photoelectron Spectroscopy (XPS) to quantify the presence of GTAC; however, the XPS data from prior research provides evidence to confirm the successful immobilisation of QAC like GTAC on the surface. In previous research, the presence of NR_4^+ of GTAC was identified by the deconvolution of the N1s spectra (Cavallaro et al., 2014). The control surface (ppAA only) typically showed a single N1s peak at approximately 339 eV, which corresponds to N-C bonds from amines group of allylamine via plasma polymerisation (Cavallaro et al., 2014; Cavallaro et al., 2016). However, when GTAC was added to ppAA surfaces, a new peak appeared at 402 eV, which is attributed to the quaternary ammonium group (NR_4^+) (Cavallaro et al., 2014; Cavallaro et al., 2016). This peak confirms the presence of immobilised GTAC, showing that the QAC had been successfully incorporated onto the surface. These studies also revealed that the oxygen content on the surface increased after GTAC coating, likely due to the presence of oxygen from the epoxide ring in GTAC (Cavallaro et al., 2014). Therefore, as our study followed the same surface fabrication protocol, it is expected that NR_4^+ is indeed present on the surface at increasing concentrations along the gradient. This expectation is further supported by the indirect measurements (fluorescence analysis, water contact angle and coating thickness), which all presented data that would be expected from the presence of NR_4^+.

4.2. The effect of immobilised QAC on bacteria and fungi

Previous research has demonstrated the potential antimicrobial properties of quaternary ammonium compounds. QACs have been shown to be effective against both Gram-negative, Gram-positive bacteria as well as fungal pathogens. In this study, LIVE/DEAD assay was performed to assess the relationship between antimicrobial efficacy and the density of immobilised GTAC. Based on the confocal scanning microscopy images and the data analysed from ImageJ software, the immobilised GTAC showed good inhibition against both Gram-positive bacteria and Gram-negative bacteria, particularly, *S. aureus* and *P. aeruginosa*. The percentage of cell viability decreased with a longer immersion time of GTAC on the surface. At the 8-10 mm position (corresponding to approx. 5 – 6 h GTAC adsorption) the *S. aureus* viability dropped to approximately 13% while ppAA thin films showed about 99% viability. Similar results were observed for Gram-negative bacteria (*P. aeruginosa*) in which the lowest viability of approximately 24% was observed at the 8-10 mm (5 – 6 h GTAC adsorption). In general, increasing surface density of immobilised GTAC was associated with an increased bactericidal activity. Consistent with this result, previous study reported that once QAC content increased, charge density also increased, leading to the enhanced bactericidal activity of coatings (Liu et al., 2015). In particular, the coatings with synthesised photo curable QAC with ≥ 8 wt% of high content (charge density of ≥ 8.6 mol cm^{-2}) exhibited 5-log reduction and almost 100% killing efficiency against both *E. coli* and *S. aureus* (Liu et al., 2015). The antibacterial activity of

immobilised QACs is supported by the strong positive charge of the NR_4^+. When QACs with their positively charged quaternary ammonium moieties make contact with bacterial membranes, they may interfere with the electrostatic properties of the cell envelope, disrupting the membrane potential or displacing essential cations required for cellular function.

Additionally, this study revealed that immobilised QAC coating was more effective against Gram-positive bacteria (*S. aureus*) rather than Gram-negative bacteria (*P. aeruginosa*). Similarly, Peyneau et al. (2020) reported that QAC showed a weaker activity on Gram-negative than on Gram-positive. The differences in cell envelope structures between Gram-positive and Gram-negative bacteria significantly influence their susceptibility to QACs. The cell wall of Gram-positive bacteria such as *S. aureus* consists of a peptidoglycan layer containing charged teichoic acids (Peyneau et al., 2022). This negative charge makes Gram-positive bacteria more susceptible to cationic antimicrobial agents like QACs (Peyneau et al., 2022). In contrast, Gram-negative bacteria like *P. aeruginosa* have an outer membrane covered in lipopolysaccharides (LPS), which may provide a shielding effect against immobilised QACs (Peyneau et al., 2022).

In fact, QAC does not show the same effect among microorganisms, particularly when comparing bacteria and fungi such as *C. albicans*. In this study, GTAC concentration density did not share the same effect on *C. albicans* cells compared to bacterial cells. Fungal viability increased from about 28% at low QAC concentration density (0-2 mm, corresponding to 0 – 1.2 h GTAC adsorption) to nearly 88% at high QAC concentration density (8-10 mm, corresponding to 4.8 – 6 h GTAC adsorption). This suggests that the optimal bactericidal activity of immobilised GTAC against *C. albicans* is achieved at low GTAC densities. This indicates that fungal pathogens and bacterial pathogens have a fundamentally different interaction with immobilised GTAC, which may be due to differences in the cell envelope structure. *C. albicans* has a multilayered cell wall structure that is composed mainly of polysaccharides like chitin, β- glucans, and mannoproteins. Under this is a lipid bilayer membrane, similar to bacterial membranes but without peptidoglycan (Chakraborty et al., 2021). This thick wall provides mechanical protection and limits the access of many antimicrobial agents. Another possibility is the difference size of *S. aureus*, *P. aeruginosa* and *C. albicans*. *C. albicans* cells are approximately 3-8 μm in diameter (Wiles & Mackenzie, 1987) while the size of bacteria is typically 0.5-1.5 μm (*S. aureus*) and approximately 1-5 μm in length and 0.5-1 μm in width (*P. aeruginosa*) (Akanbi et al., 2017; Diggle & Whiteley, 2020). For the larger size of fungal cells, the damaging effect of the NR_4^+ in GTAC may need to be more localised. When GTAC density is increased, the envelope-damaging effect of NR_4^+ may be analogous to the 'bed of nails effect', where the individual points of damage are reduced in impact by being distributed across a broader area, instead of being localised to individual points on the cell. By distributing the harmful effect across

the cell, it may be less likely that the damage to any one location passes the critical threshold required to kill the cell. Overall, it seems likely that the susceptibility of different pathogens to immobilised GTAC is influenced by a combination of factors, including cell envelope structure and cell size.

Furthermore, we observed no trend in surface attachment as a function of GTAC density, indicating that the variation in electrostatic charge and hydrophilicity had no effect on the propensity of pathogens to attach to the surface, at least under the conditions we investigated.

4.3. The effect of immobilised QAC on antibiotic tolerance

In this study, we explored the impact of immobilised quaternary ammonium compounds on the antibiotic tolerance of *S. aureus* when exposed to four different antibiotics operating on different bactericidal mechanisms, including vancomycin, daptomycin, kanamycin, and levofloxacin. It is known that antibiotic tolerance causes the failure of conventional antibiotic treatment and a risk of relapsing infections. Tolerance refers to the capacity of a bacterial population to survive transient exposure to bactericidal antibiotics, even when these drugs are administered at concentration above MIC (Brauner et al., 2016). Unlike resistance, tolerance is a phenotypic adaptation that applies only to bactericidal antibiotics and is not relevant to bacteriostatic antibiotics (Brauner et al., 2016). Our results indicate that the presence of immobilised QAC on surfaces elicit different effects on bacterial tolerance depending on the mode of action of the antibiotic used.

Vancomycin, a glycopeptide antibiotic that inhibits cell wall biosynthesis by binding to the D-Ala-D-Al terminal of peptidoglycan (Wang et al., 2018). The outermost peptidoglycan of bacteria is a key structural target for vancomycin, and the influence of surface-bound GTAC may enhance this effect via electrostatic interactions with the newly forming peptidoglycan layers. Our results showed that when vancomycin was administered to cells attached to GTAC-bearing surfaces, the outcome of treatment was enhanced compared to surfaces devoid of GTAC. The highest antibacterial effect (about 48% viability) was observed at the 8-10 mm position (corresponding to 4.8 – 6 h GTAC adsorption) suggesting that the enhanced bactericidal activity of vancomycin is facilitated by the increased GTAC concentration density. In contrast, the viability of nearly 99% on ppAA thin films indicates that the absence of GTAC allows for greater survival of *S. aureus*, thereby emphasizing the potential of GTAC to reduce antibiotic tolerance.

Daptomycin is a cyclic lipopeptide antibiotic that plays a vital role in the treatment of Gram-positive infections (Heidary et al., 2018). Its mechanism of action involves disrupting the cell membrane which is underneath the peptidoglycan layer. This action leads to rapid membrane depolarisation, which inhibits essential processes such as synthesis of protein, DNA and RNA (Heidary et al., 2018). This is particularly significant in the context of biofilm-associated infections,

where extracellular matrices protect bacteria like *S. aureus* from host defences and antibiotic treatment, allowing them to accumulate at high densities (Heidary et al., 2018). In the experiment assessing the efficacy of daptomycin against *S. aureus* on immobilised QAC, results indicated a significant reduction in bacterial viability, with the viability dropping to 35% at high GTAC concentration density (8-10 mm, corresponding to 4.8 – 6 h GTAC adsorption) compared to control samples with viability of approximately 91% following daptomycin treatment. The significant reduction highlights that the presence of GTAC enhances the antimicrobial efficacy of daptomycin. In other words, increasing GTAC concentration density on the surface could enhance the ability of daptomycin to penetrate biofilms and disrupt bacterial cell membrane integrity.

Kanamycin, a polycationic aminoglycoside antibiotic, exhibits its bactericidal effect by multiple mechanisms, including disruption of the bacterial cell membrane and inhibition of protein synthesis via interactions with the ribosome (John et al., 2017). For the disruption of cell membrane, the hydroxyl groups and amino groups of kanamycin bind to the membrane head groups and establish strong electrostatic interactions. In this binding process, the hydroxyl groups form hydrogen bonds with the phosphate and ester carbonyl groups of lipids, which disorder the bacterial membrane (John et al., 2017). However, this effect is only supplementary to the principal antibacterial effect involving inhibition of protein synthesis, which relies on the antibiotic entering into the intracellular space. (John et al., 2017). In our study, when kanamycin was administered to *S. aureus* attached to the QAC gradient surface, the greatest antibacterial effect was observed at 8-10 mm (4.8 – 6h GTAC adsorption). This brought the cell viability down to approximately 55%, compared to approximately 100% when kanamycin was administered to *S. aureus* on the ppAA surface devoid of GTAC.

Levofloxacin, a newer generation of fluoroquinolone antibiotic, inhibits both Gram-positive and Gram-negative bacteria by targeting two essential bacterial enzymes including DNA gyrase and topoisomerase IV (Kherroubi et al., 2024). The fluoroquinolone antibiotics enact their bactericidal effect by binding to the complex formed between cleaved bacterial DNA and type II topoisomerase (DNA gyrase or topoisomerase IV) (Kherroubi et al., 2024). This interaction prevents the ligation of the cleaved DNA, resulting in the accumulation of DNA breaks which inhibits bacterial replication and leads to cell death (Kherroubi et al., 2024). Similar to the results observed for other antibiotics, the greatest enhancement of bactericidal activity was identified at the 8-10 mm position on the gradient (corresponding to 4.8 – 6h GTAC adsorption), which brought the viability down to 31%, compared to approximately 95% when levofloxacin was administered to *S. aureus* on the ppAA surface. The survival rate dropped to about 3.2 times compared to ppAA thin film. The result reflected the effects of QAC's electrostatic interactions on levofloxacin's activity, supporting the hypothesis

that the positive charge of GTAC enhance the antibiotics' penetration and interaction with bacteria cells.

In general, the increased antibiotic sensitivity of *S. aureus* on our GTAC-bearing surfaces may be due to alterations in cell envelope integrity and permeability, which influence how each antibiotic interacts with the bacteria. The two most effective antibiotics were levofloxacin and daptomycin. The mechanism of action of levofloxacin involves events that exclusively occur within the bacterial cell. The increase in cell permeability, as indicated by high intake of propidium iodide, likely triggers a greater influx of levofloxacin into the cell and thereby improves the overall activity of the treatment. For daptomycin, the mechanism of action involves disruption to the cell membrane. Again, the high rates of intake of propidium iodide indicate greater disruption to the cell membrane, which could plausibly act synergistically with the membrane disruption associated with daptomycin. Although vancomycin and kanamycin activity were both improved by the presence of GTAC, they were comparatively less effective than daptomycin and levofloxacin. For vancomycin, this may be because its mechanism of action relies on the inhibition of peptidoglycan synthesis in growing and dividing cells, as opposed to internal cellular activities. Although immobilised GTAC appears to improve membrane permeabilization, this is unlikely to promote the binding of vancomycin to D-Ala-D-Ala residues within the growing peptidoglycan layer. The small enhancement of vancomycin activity may instead be attributable to non-specific detrimental effects such as increases in the generation of reactive oxygen species (ROS) and subsequent oxidative stress. Similarly, immobilised GTAC only moderately improved the activity of kanamycin. Kanamycin itself is known to disrupt the cell membrane, so the ability for the antibiotic to enter the cell may not be a limitation to its activity. If this is the case, although the enhanced membrane disruption of GTAC may promote antibiotic influx, this may not translate to a more potent bactericidal effect. Similar to vancomycin, the moderate increase in bactericidal activity of kanamycin following exposure to immobilised GTAC may simply be due to general oxidative stress.

To sum up, the results from testing four different antibiotics including vancomycin, daptomycin, kanamycin and levofloxacin on *S. aureus* adhered to QAC immobilisation surface density demonstrated that surface charge has the potential to improve the outcomes of antibiotic treatment. A common trend observed on four antibiotics was higher GTAC concentration along the gradient led to a higher rate of cell death. This is expected to be due to the presence of high amounts of positively charge NR_4^+ on GTAC. The result aligned with previous research where surface modification aimed at generating a positive charge enhance the interaction between antibiotics and bacterial membranes (Da Costa et al., 2021). In that study, the positively charged rifampicin-loaded PLA nanoparticles showed a stronger retention and interaction within *S. aureus* biofilms, significantly

improving antibiotic delivery and reducing bacterial viability (Da Costa et al., 2021). Similarly, the positive electrostatic interactions generated by the GTAC surface coating in this study could enhance the permeation and efficacy of the antibiotics, particularly in regions with higher GTAC concentration density. These effects appear to be largely influenced by the properties and mechanism of action of the antibiotic being used in combination with GTAC. These findings highlight the potential of surface charge modulation to combat antibiotic tolerance and improve treatment outcomes.

CONCLUSION, LIMITATIONS AND FUTURE VISION

Implantable medical devices are widely used to improve the quality of patient health, but infection is a common complication associated with their use. To treat infections, broad-spectrum antibiotics are frequently used, causing the emergence of antibiotic tolerance. In this context, this study proposes a process to address this issue by developing a surface coating using quaternary ammonium compounds (QACs) via dip coating. The use of dip coating is beneficial to observe the differentiation on one substratum. This coating is a promising material containing antimicrobial properties, biocompatibility, and adaptability to various surfaces. The immobilised QAC coatings showed a notable decrease in the viability of bacteria including *S. aureus* and *P. aeruginosa* at the highest QAC concentration density. This suggests that the electrostatic interactions facilitated by the positively charged QACs help in disrupting bacterial defence mechanisms. However, the influence of electrostatic properties on fungi like *C. albicans* is still questioned. In particular, although GTAC coating reduced viability, the lowest survival rate was observed at the position with the lowest QAC concentration density. The results also indicated that the effect of immobilised QAC surface on attached cells including *S. aureus*, *P. aeruginosa* and *C. albicans* was irregular. Antibiotic tolerance, the ability of bacteria to survive transient exposure to high antibiotic concentrations is still a challenge in treatment. The study underscores the importance of electrostatic interactions in this phenomenon, particularly in Gram-positive bacteria like *S. aureus*.

By creating different charge densities on one surface, we can explore which position or at which condition is favourable to reduce antibiotic tolerance, allowing for systematic investigation of how different charge densities influence microorganism viability, microorganism attachment, and antibiotic tolerance. This approach enables the identification of the optimal surface charge conditions to enhance antimicrobial and antibiotic effects. Furthermore, it provides a platform to study complex interactions between surface properties and microorganisms under varied electrostatic conditions, offering a deeper understanding of how to tailor surface modifications for specific applications.

Despite the progress made in this study, several important research questions should be further investigated. For instance, it would be important to examine how the density of immobilised GTAC affects antibiotic tolerance in Gram-negative bacteria and fungi, both of which have fundamentally different cell envelope structures. Another important consideration is the effect of immobilised QAC on mammalian cells. Additionally, to fully understand the physicochemical properties of the surface, future studies should include zeta potential measurements to quantify surface charge, and X-ray photoelectron spectroscopy (XPS) and Fourier Transform Infrared Spectroscopy (FTIR) to quantify the surface chemical composition in more detail. Characterisation of the surface chemical composition is particularly important, as the presented data rely on the GTAC adsorption duration

and indirect measurements of NR_4^+. To comprehensively evaluate the performance of immobilised GTAC on the outcomes of antibiotic treatment, it is imperative to directly and quantitatively characterise the density of GTAC as a function of its adsorption duration. Furthermore, in order to fully understand how GTAC improves antibiotic activity, further microbiological mechanistic analyses are required. These will include quantification of membrane polarization and intracellular generation of reactive oxygen species. This will facilitate a comprehensive understanding of how the effects of GTAC can be combined with antibiotics possessing different mechanisms of action. Lastly, the limits of performance of GTAC-bearing surfaces can be further evaluated using time-kill assays that will measure bacterial survival across a range of antibiotic concentrations and adsorption times. These questions open important directions for future research, which we intend to explore further in order to refine the current findings.

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