# Simulation and Synthesis of Functional, Self-Assembling Amyloid Complexes for the Development of Antimicrobial Coatings

by

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A thesis submitted in partial fulfillment of the requirements for the Degree of Master of Science in Biomedical Engineering at Brown University


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# 1 Signature Page

This thesis, by Quentin D. Altemose, is presented and accepted in its present form by the Center of Biomedical Engineering as satisfying the thesis requirement for the degree of Master of Science

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# 2 Vita

Quentin Altemose received his undergraduate degrees in Biology and Physics from Ursinus College, in Collegeville Pennsylvania, where he graduated Summa cum Laude with distinguished departmental honors. In his undergraduate years, Quentin participated in research in several laboratories, studying a wide assortment of topics ranging from quantum photonic entanglement to pollution's effect on local steam biodiversity. For his final two years of his undergraduate studies, Quentin studied in the lab of Dr. Casey Schwarz, where he studied the material sciences in the physics department. Specifically, he studied thin-film glass optics, working to develop thin-films of chalcogenide glass that could be applied to substrates and subsequently laser etched to induce crystallization, with the end goal of altering the optical properties of the materials. This work was part of a multi-institute collaboration between academic universities, private industry, and the American Department of Defense.

Following his undergraduate degrees, Quentin enrolled in the Brown University Scientific Masters Program in biomedical engineering. At Brown, he joined the lab of Dr. Anita Shukla, studying peptide synthesis and the fictionalization of peptides for medical applications. While at Brown, Quentin also began to engage in entrepreneurial ventures, winning several grants, fellowships, and awards for the development of new business concepts and ventures. Additionally, while at Brown, Quentin captained the Men's Equestrian Polo Team for the university.

# 3 Dedication

This thesis is dedicated to my grandmother, Carol "Jane" Altemose. A nurse who cared for others before herself, and worked tirelessly to improve the lives of those around her. Who served as an inspiration to me and my family. Her love is eternal and boundless, and this piece is written in her honor.

To my parents, Jennifer Altemose and Patrick Juliano, I would like to thank you for everything you have done to help get me to this point, and for putting up with my nonsense all these years without killing me. You two are the best parents in the world, and I love you more than I can say!

# Contents

# 4 List Figures

# List of Figures

# 5 Collaboration Notice and Distribution of Contributions

The data presented throughout this work is the result of a collaborative effort between the labs of Dr. Anita Shukla (Brown University), and Dr. Phanourios Tamamis (Texas AM). The Tamamis lab was responsible for the computational simulations and molecular dynamics calculations that went into the development of our peptide-based fibrils. The Shukla lab was instead responsible for all experimental studies. The results discussed in this thesis relate exclusively to the work done by the Shukla lab. Any mention of computational work or peptide sequence prediction was performed by the Tamamis lab.

# 6 Preface

I have always wanted to help people. It is one of the driving forces that has helped me get to where I am today! I love to learn, and I want to use the knowledge that I obtain to make the world a better place for everyone. That was why I was so excited to be able to join the Brown Biomedical Engineering Department to work on an exciting new project that eventually culminated into the thesis presented here. It was a challenging project, full of setbacks, self-doubt, confusion, and frustration. The thing that kept me going and drive my desire however was the idea that by the end of this series of experiments, we could develop a completely new therapeutic devices that could profoundly improve the lives of people across the world. I am proud to say that through dedication, and with great support from colleges, faculty, friends, and family, I was able to complete my research, and produce the thesis presented before you today.

This thesis is broken down into three parts. In the first part, I will begin by outlining the background information that will support your understanding of the field. This section will be written in a more general fashion, limiting unnecessary jargon and complexities that would make the material less obtainable to someone less familiar with the field. In the second part, I will take you through my actual project. This will be done in the form of a traditional journal article, with its own (more detailed) introduction, abstract, and discussion. In this section, I will report my findings and figures in a detailed fashion. Finally, in part three, I will discuss the findings of my paper in a more general manner. Here I will also discuss the implications of my findings, as well as the potential for future studies expanding on my findings.

The motivation behind this work is to develop short peptides that are capable of self-assemble into fibrils improving the treatment efficiency of bacterial infections, particularly in regard to medical devices. While this concept will be explored in more detail in subsequent sections, bacterial infections remain prevalent and dangerous throughout the medical

community. Through this work, it is my hope that we may be able to make the world a better place for everyone!

I would like to thank everyone who made this thesis possible, as well as you, the reader, for taking the time to review and consider my work! I wish you all health and happiness that I possibly can! Thank you! :)

# Part I

# Introduction and Background

# Design and Synthesis of Self-Assembling Amyloid Peptides for Specific Antibiotic Binding and Targeted Release for Improved Treatment of Microbial Infections


### Abstract

Infections borne from the implantation of medical devices has continued to be a global health concern. Bacteria are capable of binding to the surfaces of these implantable materials, which allows the formation of colonies that secrete a waxy, protective extracellular material known as biofilm, which limits the ability for healthcare providers to treat the infection after it is established. The presence of the biofilm prevents immune cells from phagocytosing the bacteria, while simultaneously blocking antigen receptors on the surface of bacteria, preventing attachment and function of antibiotic drugs. Current methods of preventing bacterial attachment on medical devices include the coating of said devices in antimicrobial films that either repel or kill bacteria on contact. Many of these films however are short-lived, degrading rapidly in aqueous environments. Additionally, many of these materials are limited in what bacterial targets they are capable of treating. In order to address these problems, we have developed a novel self-assembled material capable of binding and releasing targeted ligand drug molecules. Here, we present a novel peptide sequence capable of forming amyloid fibrils as well as binding to targeted, antimicrobial ligands. Our peptide was able to self-assemble and form amyloid fibrils *in vitro* as determined by Congo Red and Thioflavin T staining. Our self-assembled fibrils are shown to bind to the antibiotic agent gentamicin, preventing the adhesion of bacteria onto glass surfaces. Furthermore, upon exposure to a specific release molecule (e.g. curcumin), the self-assembled fibrils are shown to release gentamicin into the surrounding environment. This has significant implications for the future development of antimicrobial coatings on medical devices.

# 1 Introduction

Despite extensive pharmacological innovation over the last century, bacterial infection remains a prevalent threat in healthcare facilities worldwide [1] [2]. The inoculation of infectious organisms into patients during implantation of medical equipment is common throughout the medical field, particularly with catheters due to their functionality as trans-dermal devices [3] [4] [5]. Many times, these devices become contaminated with bacteria from the skin (staphylococci are a major contributor to medical-device-borne infection [6]) before or during implantation, resulting in infection. In addition to presenting a pathway for infectious agents to enter the body, certain materials commonly used in the construction of medical devices allows for easy bacterial attachment, and the subsequent formation of bacterial biofilms, which limit the ability to treat the infection after it has become established [4]. To combat this, recent efforts have been made to develop technologies to prevent bacterial adhesion to the surface of implantable medical devices, such as altering the surface composition, or coating the surface in an antimicrobial drug or molecule [7].

The aminoglycoside family of antibiotics are commonly utilized for general systemic as well as coating applications. Aminoglycosides are a family of antibiotics which possess a widespread antibacterial spectrum and highly potent antibacterial activity [8] [9] [10]. However, aminoglycosides suffer from suboptimal pharmacokinetics due to their rapid elimination rate (plasma half-life 2–3 hours) [11]. Additionally, owing to their hydrophilic nature, the efficacy of aminoglycosides is limited by their poor membrane permeability and slow cytosolic accumulation [12]. Due to these limitations, multiple daily high-dose injections are required for a prolonged period of time to achieve therapeutic efficacy, and this treatment regimen can have undesired side effects (e.g., nephrotoxicity and ototoxicity) and potentially induce drug resistance [13] [14]. Consequently, drug delivery systems that can enhance the cytosolic accumulation of aminoglycosides in the intracellular space might potentiate drug activity.

The development and production of functional synthetic oligopeptide sequences for use

in therapeutic drug delivery applications represents a unique and novel approach at treating infectious disease [15] [16]. The use of peptides in drug delivery vehicles (such as nanoparticles or liposomes) offers several unique advantages over general systemic delivery of drugs or use of drug delivery vehicles on their own, such as increased drug uptake by the target cells, improvement of drug stability, and the prevention of enzymatic degeneration of the drug/nanoparticle in the body [17]. This increased stability allows the therapeutic agent to survive in environments where it would otherwise be degraded, increasing the retention and bioavailability of the molecule to treat diseases and infections [18]. Beta-sheet forming oligopeptide sequences are particularly advantageous due in part to their propensity to self-assemble into amyloid fibril networks [19].

Amyloid peptide fibrils have been shown to be less soluble in aqueous solutions, while also increasing the structural integrity of their fibril networks [20] [21]. Furthermore, the self-assembly of these peptides allows for spontaneous and rapid formation of fibril networks. In some cases, amyloid peptides have even been shown to possess innate antimicrobial properties [22], which may improve treatment efficacy compared to alternative peptide delivery systems. Additionally, amyloid peptides have been shown in previous studies to be capable of self-assembling into hydrogel materials, which may be subsequently loaded with drugs for timed release [23]. Based on these findings, it is possible to create self-assembling amyloid networks that simultaneously provide support for the binding and release of antimicrobial drugs.

Developing a self-assembling, cross beta-sheet peptide networks that is capable of selectively binding to targeted molecules and subsequently releasing them at specified times is exceptionally challenging, as many naturally occurring amyloid sequences lack the functionalities we desire [15]. A more ideal solution would involve the use of computational proteomic simulations to identify short oligopeptide sequences capable of binding to and releasing target ligands while also forming beta-sheet secondary structures as well as larger amyloid fibril networks. Proteins and their structures are able to be computationally designed with relatively high precision [24] [25] [26], however to the knowledge of these authors,

a computational method for designing and simulating functional amyloid peptides has not yet been reported.

Here, we utilize the results of computational methods that are capable of developing amyloids that are functionally capable of binding to target ligands while retaining their ability to form fibril networks. Through a series of imaging experiments, binding experiments, and bacterial growth tests, we demonstrate that our novel peptide fibrils are effective in treating microbial colonization on the surface of medical devices. Furthermore, these molecules may be stimulated to release their cargo at specified time points when introduced to a specific release agent in order to better treat local infections. Through this work, we demonstrate an exciting and novel material for use in producing antimicrobial coatings, as well as verifying computational methods that allow for the production of custom peptide sequences.

## 2 A General Introduction to DANG Peptide

Computational evaluation of peptide sequences was performed by our collaborators in the group of Prof. Tamamis at Texas AM University. Using their proprietary simulation protocols, they simulated a wide assortment of potential peptide sequences that could self-assemble into amyloid fibrils, and had the ability to bind to targeted ligands. While many potential sequences were produced, the peptide DANGAIIGDIS (hereafter referred to as, "DANG") was shown to be the most interesting candidate for further experimental study. In molecular dynamic simulation and ligand docking predictions, this peptide's performance was superior to all other potential candidates. Additionally, DANG peptide showed high solubility in physiologically relevant buffer solutions, such as PBS, while other potential peptides only showed solubility in caustic and/or toxic buffers, such as DMSO or ammonia.

All simulated sequences underwent rigorous scrutiny during the computational analysis process in order to ensure that the experimental peptides would have our desired characteristics. DANG peptide is a peptide sequence eleven residues in length. It features an

acetylated N-terminus, and an amide C-terminus. The peptide is slightly negative in charge and slightly hydrophobic. Furthermore, computational studies showed that DANG peptide possess unique binding sites towards both terminal sides that allow for interactions either to other DANG peptides to create amyloid fibrils, or to the targeted ligand of choice.

# 3 A General Introduction to Infectious Diseases

Microorganisms, particularly bacterium, viruses, and fungi, co-inhabit our world alongside us. These microscopic organisms fulfill critical roles throughout the ecosystem, from recycling nutrients in soil to allow plants to grow, to producing breathable oxygen through photosynthesis, to assisting in the digestion of certain foods within our own digestive tracts [27]. Without these various organisms, life as we know it today would not be possible, and as such we depend on these small creatures just as much as they depend on us. Unfortunately, while many microorganisms have adopted a mutually beneficial symbiotic relationship with humans, some have instead opted for a more malicious survival strategy. In lieu of competing in a conventional ecological niche or depending on another organism, these creatures opt to take what they need by force, parasitizing another animals to obtain essential nutrients and reproductive space. These are the organisms that we know to cause various diseases, including but not limited to common colds, pneumonia, whooping cough, and Staph infections (including methicillin-resistant *Staphylococcus aureus*, or MRSA, infections) [28].

Infectious diseases are frequently defined as any organism (bacterial, viral, fungi, parasite, etc.) that is capable of pathogenesis within another organism [29]. Infectious diseases have consistently been, and remain to be, one of the major sources of illness and mortality across the world. At the start of the $20^{th}$ century alone, roughly 30% of all cases of mortality in the United States were due to one of three diseases: tuberculosis, pneumonia, and diarrhoeal diseases [30]. Throughout the following century, improvements in medical understanding, clinical tools, and diagnostic technology allowed researchers to better understand the sources

of many of these conditions. Various bacterial and viral species were identified to cause disease when allowed to infect the body, and by limiting exposure to these organisms, doctors were able to significantly reduce the overall morbidity and mortality of these diseases that once plagued humanity [31]. Despite the overall reduction in mortality however, infectious diseases remain a prevalent threat to the medical community.

There are many mechanisms by which infectious diseases are able to spread, and increased globalization throughout the $20^{th}$ century has facilitated more rapid spread of diseases around the globe [32] [33]. Infectious agents can spread through liquid droplets in the air (known as aerosols), through vectors (that is, animals that carry the organism but do not necessarily show symptoms, such as mosquitoes for example), contaminated drinking water, and physical contact with contaminated surfaces or people (sexual contact, child birth, and even shaking hands could facilitate the spread of infectious diseases) [34]. The ease of transmission for these organisms makes them dangerous, as patients in close proximity, particularly in hospitals, and very susceptible to transmission and spread of the diseases, causing illness.

Interestingly, these infectious diseases are not exclusive in their ability to infect and colonize a patient. In some cases, when an individual contracts a disease, their immune system becomes somewhat weaker initially as the body begins to mount a response to the specific pathogen [35]. It is also possible for some infectious diseases (such as human immunodeficiency virus (HIV) for example) to deliberately attack and weaken the immune system [36]. During this period, the patient may contract another infection from a disease-causing organism, which could make their overall condition deteriorate [37]. As such, it is essential that medical professionals work to prevent infection in the first place. However, in the event of infection, it is pivotal that the patient be protected from secondary infection, while simultaneously receiving rapid and effective treatment for their initial disease state.

Infectious diseases consistently have a broad impact on human society, from humanitarian, economic, and sociological perspectives. As stated above, infectious diseases have historically been one of the leading causes of mortality throughout the centuries [38]. While

medical advancements have significantly reduced the overall mortality of many diseases, they remain one of the leading causes of mortality and hospitalization across the world, particularly in more susceptible groups such as the elderly, very young, and immunocompromised [39]. Apart from the direct human impact of infectious diseases, there is also a significant economic burden associated with these organisms. Every year, billions of dollars are lost due to missed work from illness, and trillions are spent on healthcare systems to treat these diseases [40]. Costs for these diseases include hospitalization fees, drug costs, necessary surgeries, and in some cases, the cost of new medical devices [41]. Overall, the economic burden associated with these diseases is substantial. Finally, these diseases influence our society and how we interact with each other, as well as the world around us. When a disease is spreading, special societal measures, such as vaccination programs or social distancing, are frequently employed to limit its spread throughout the population [42] [43]. While these measures can be effective, it may possibly result in social and psychological effects on the global populations, which in turn may lead to continued economic and humanitarian hardships following the treatment of the disease [44].

When taken as a whole, it becomes clear that infectious diseases remain a prevalent and notable threat in the world. These parasitic organisms do more than simply cause a cold. They are capable of disrupting society, causing wide-scale panic, and causing suffering across the world. That said however, researchers have fought back throughout the centuries, developing novel treatments and cures to these diseases. Two major breakthroughs in the fight against infectious diseases has been the development of vaccines and antibiotics [45] [46]. These two tools have dramatically limited the spread and infection potential of many viral, fungal, and bacterial pathogens. In recent years however, antibiotic resistance has become a more prominent threat in medicine [47]. Antibiotic resistance refers to the phenomenon by which bacteria are able to resist the effects of antibiotics, and continue to grow despite their presence. There are many mechanisms by which this may occur, which are beyond the scope of this introduction, however it is important to understand the impact of this trend.

A lack of antibiotic treatment options may eventually lead to situations where infectious organisms are not treatable, much like they were before the discovery of antibiotics [48]. This could result in increased mortality and transmission of these infectious diseases. In order to combat this, researchers have begun to develop novel treatments, using alternative molecular compounds or strategies, that more effectively treat infections, and ideally, prevent bacterial colonization in the first place [49]. As such, the continued advancement of treatments and treatment options is critical to the medical community, as it protects global health, economic securities, and society as a whole.

In summary, infectious diseases represent a major source of human, societal, and economic hardships across the world. Continued efforts to develop alternative treatments to prevent infection, or treat infection, remain the best method of combating these pathogens. The research presented within this paper continues this effort, in that I present a novel treatment to prevent infection from various strains of gram-positive and gram-negative bacteria. Specifically, I show data illustrating the efficacy of my materials at preventing the growth of the bacterium *Staphylococcus aureus* (discussed in more detail in subsequent sections). It is important to note however that gentamicin is a broad acting drug, and bacterial growth inhibition is not limited to *Staphylococcus aureus* exclusively. Through this work, I aim to reduce the incidence of infection from this organism, resulting in fewer economic losses, a reduction in societal fears stemming from this infectious disease, and a decrease in human suffering resulting from this disease.

# 4 A Brief Review of Antimicrobial Coatings for Implantable Medical Devices and Biofilms

The concept of coating the surface of implantable medical devices with a secondary compound in order to garner some additional effect has been prevalent in medicine for centuries,

however recent trends within the last 100 years have focused primarily on producing surface coatings that improve the functionality of the devices [50]. One of the major benefits of surface coatings for devices such as catheters is that it renders potentially porous or reactive surfaces more inert [51]. This helps prevent surface fouling and the accumulation of biomolecules in the surrounding tissues, which in turn helps protect the device while also limiting clotting and inflammatory effects [52]. Apart from surface fouling/clotting benefits, surface coatings also have the potential to improve the innate physical characteristics of the implantable device. For example, surface coatings may reinforce the material to prevent leakage, or could improve overall device shear strength, limiting breakage and wear of the implant itself [53]. By preventing wear on the physical implant, patients would see a prolonged implant lifespan, improved general functionality of the device, and more limited leeching of material surface particles into the body.

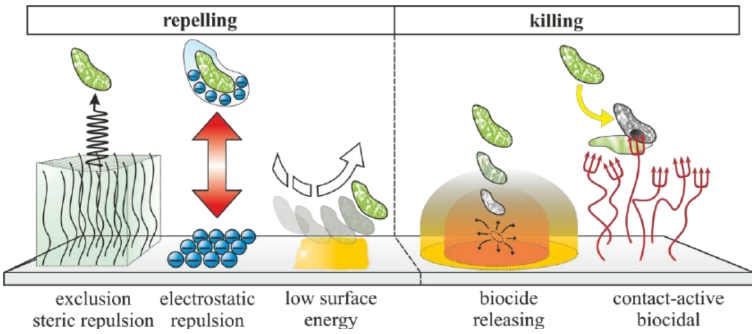


**Figure 1:** Current antimicrobial coatings function by either repelling or killing bacteria upon contact with the surface of the material. Repulsion may occur via electronic or steric interactions. Killing of bacteria may result contact with biocides, such as antibiotics, on the coating. Reproduced without edits from Siedenbiedel *et al*, 2012, creative commons attribution license, https://creativecommons.org/licenses/by/3.0/legalcode.

While improvements to physical factors such as those listed above are necessary and beneficial, one of the most cited reasons for coating the surfaces of medical devices is to prevent the adhesion, growth, and subsequent biofilm formation resulting from infectious microorganisms colonizing the surface of the implantable device [54]. By their very nature,

implantable devices are highly susceptible to colonization from infectious diseases, with millions of infections occurring yearly as a result of implant-borne infection [55]. While any implantable device is theoretically susceptible to colonization from infectious agents, some devices, such as trans-dermal catheters, tend to be higher risk devices due to the location and design of the implantable device [56] [57] [58].

There are several reasons why implantable medical devices are more susceptible to bacterial colonization and result in subsequent infection compared to alternative routes of microorganism entry. Perhaps the simplest explanation, implantable medical devices provide a simple and effective means for the infectious agent to enter the body [56] [59]. By placing an external object into the patient, bacteria are able to effectively traverse the skin of the patient without a need for an entry point, giving them the ability to colonize successfully. In addition, because implantable medical devices are foreign to the cell of the body, an immune response is stimulated immediately following implantation. While it may seem counter-intuitive, this immune response actually ultimately limits the body's natural ability to fend off bacterial infection. Because the implanted device is so large relative to the cells of the body, it is not phagocytosable (that is, it cannot undergo lysis by immune cells like a virions or bacteria can). The failure of the immune cells to destroy the implanted device results in a down-regulation of immune responses to the local implant site, as well as the death of local immune cells. This helps create an environment where invading bacteria are able to thrive, as the local immune cells are not longer functioning at maximum capacity and can no longer inhibit their growth and spread [60]. As the bacteria begin to colonize the local materials and tissues, many begin to secrete exopolysaccharides and other biomolecules that ultimately form biofilms [61] [62] [63].

Biofilms are a common form of bacterial colonization in clinical settings, and refers to the aggregation of bacteria on a surface, where they are able to grow in close proximity to each other [64]. While some planktonic bacteria may float freely in solution in nature, and some infections may feature freely floating bacteria, biofilms grow when bacteria are

colonizing near each other and spread outward from a single central location. The other major characteristic associated with biofilm formation is the development of extracellular polymeric substances on the surfaces of the bacteria [65]. These substances protect the bacteria from immune cells, and limit the functionality of chemicals that the bacteria come into contact with, such as antibiotics [66]. This is particularly hazardous, as increases in bacterial resistance prevents effective and efficient treatment of the infection, and can lead to exacerbation of the patient's condition. Furthermore, because treatment is significantly more challenging, the implantable device frequently has to be removed, resulting in the loss of the device, and the need for additional expenses and surgeries for the patient [67].

Because of the increased risk of bacterial colonization associated with medical devices, many researchers have focused their efforts on producing device coatings that are able to counteract bacterial adhesion and biofilm formation in these materials [68]. While each coating functions thorough its own unique mechanism (Figure 1), almost every antimicrobial coating for medical devices can be organized into one of two categories of coating: 1) that prevent bacterial adhesion, 2) that kill bacteria upon adhesion [54]. Coatings that prevent bacterial adhesion function primarily by interacting with the surface of the microorganisms, perhaps through static interactions or electrochemical interactions, repelling it from the surface. By comparison, coatings that kill microorganisms function by disrupting or lysing the cell when it binds to the surface, by releasing a biocide for example. It is also possible that these biocide materials disrupt the polymeric network of the bacteria, leading to the eradication of infection. Each method has its own benefits and drawbacks that must be considered when producing a novel device. For example, materials that prevent bacterial adhesion via repulsion may have longer-lasting effects than biocide coatings, due to the fact that bacteria are not directly utilizing the coatings on the surface of the implant as they would if they interacted with a coating that released a biocide molecule for treatment. The major downside to this however is that because the bacteria is not killed, it is free to potentially bind to an alternative material or tissue in the body, and infection may still occur.

By comparison, one of the major problems facing biocide coatings is the breakdown and loss of biocide molecules in the body over time. This may be due to the use of the molecules in preventing infection (that is, bacteria taking up the materials that inhibit their growth), but could also be due to natural degradation resulting from the physiological conditions in which the implant resides (body temperature denaturing the material, renal system filtration, erosion of material due to aqueous flow in the bloodstream, etc.) [69]. As such, one of the predominate goals of researchers producing these device coatings is to create a material that is able to effectively prevent bacterial infection in general (that is, either on the device or in the local environment), while also is able to retain its efficacy over a longer period of time, limiting degradation due to environmental conditions.

Here, we seek to develop a novel antimicrobial coating for medical devices that is functionally superior to coatings currently available on the market. We seek to address the shortcomings of lifespan and efficacy that some current biocide coatings have. Simultaneously, we seek to produce highly effective coatings that prevent infection at both the implant site, as well as nearby tissues that may be subsequently infected. Finally, we seek to produce coatings that are customizable, allowing many different antimicrobial compounds to be coated to the surface of implantable devices. By doing this, we seek to prevent infection from a wider assortment of pathogenic organisms.

# 5 A Brief Introduction to *Staphylococcus aureus*

A member of the *Micrococcaceae* family, *Staphylococcus aureus* (frequently abbreviated as *S. aureus*) is a gram-positive bacteria specie that is commonly found on the surface of the skin in animals in a commensal relationship [70]. While there are many species of *Staphylococcus*, one of the most common species that is frequently the cause of infection is *S. aureus*, which is linked to over half a million infections in the US alone each year [71]. As such, obtaining a better understanding of this particular specie is essential for understanding the driving

motivations behind the studies presented in this study. In this section, we will briefly review this bacterium, including its general mechanisms of infection, basic appearance and cellular structure, and current methods of treatment.

As stated above, *S. aureus* is a gram-positive bacterial species. This means that these bacteria have a Cytoplasmic lipid membrane, a significantly reduced volume of periplasm compared to gram-negative bacteria (that is, the gel-like fluid between the inner and outer membranes is dramatically lower), and a thick peptidoglycan layer (which is a tough outer membrane layer of the cell consisting of polymer polysaccharides and amino acids) [72]. Because gram-positive bacteria possess a thicker outer membrane, they are better able to take-up extracellular compounds, such as antibiotics. As a result, they are generally much easier to treat using conventional antibiotics compared to their gram-negative relatives. Furthermore, because they are generally easier to treat via traditional antibiotic protocols, these species of bacteria tend survive at lower frequencies, limiting selective pressures operating on them that could lead to the development of resistance, and thus limiting the development of resistance to antibiotics compared to gram-negative species. It should be noted however that resistances will develop in these species as well over time if left unchecked [73]. One of the most common examples of a hazardous resistant strain of *S. aureus* is methicillin-resistant *Staphylococcus aureus* (also commonly known as MRSA) [74].

All members of the *cocci* classification of bacteria (including Streptococci, Staphylococci, and Diplococci) are defined by spherical or oval shapes that are reminiscent of grapes or berries (the greek translation of the name cóccos is "berry") [75]. These bacteria generally grow in chains or clusters, giving the appearance of a group of grapes on the vine. The *S. aureus* genome is circular, spanning approximately 2800 bp in length [76]. Humans are a natural reservoir for the bacteria, with about 50% of adults being colonized at any given time, and 10%-20% [77] [78]. Transmission frequently occurs through contact with a contaminated individual or surface, and infections may occur when the bacteria are allowed to enter the body, either through inhalation, through a surface wound, or via an implantable device

[79]. Skin infections caused by *S. aureus* generally result in abscesses, impetigo, cellulitis, and/or folliculitis however the predominate threat associated with these microorganisms is bacteremia (or the spread of bacteria into the bloodstream). When bacteria enter the bloodstream, they are able to infect virtually any tissue throughout the body, such as the bone (osteomyelitis) or the lung (resulting in pneumonia) [80] [81] [82].

Conventional treatment for *S. aureus* infections involves the prescription and consumption of antibiotic drugs [76]. Penicillin remains the most effective and common medical standard for treating these infections, however other frequently prescribed drugs include vancomycin, linezolid, tedizolid, quinupristin plus dalfopristin, ceftaroline, telavancin, or daptomycin [83]. It is important to note however that in the event of infection from resistant strains of *S. aureus*, such as MRSA, alternative antibiotic therapies such as trimethoprim/sulfamethoxazole, clindamycin, minocycline, or doxycycline, combined with ointment containing bacitracin, neomycin, and polymyxin B, or mupirocin [76] [83]. When infection enters the bone, or colonizes on an implantable medical device, surgery may be required to remove the infected tissues/materials, while the antibiotic rifampin may be administered to help treat the infection and prevent/treat any cases of bacteremia [83].

Due to the various health hazards that *S. aureus* presents, and the ease of transmission of the bacteria from patient to patient, continual efforts are needed to develop effective therapeutics to prevent or treat infections [84]. High treatment efficacy ensures a speedy and thorough recovery in the patient, while also limiting the development of resistant strains of bacteria [85]. Furthermore, treatments that prevent or limit bacterial adhesion to medical devices can prevent biofilm formation (see preface Section 4 for more detail on biofilms) and subsequent loss of the implantable medical device. This would ultimately save the patient from a potentially hazardous infection, as well as limit the economic strain placed upon the patient from the loss of the device, its removal, and its replacement. As such, the development of effective therapeutics for medical devices targeting the bacteria *S. aureus* represents a critically important field of study that merits a more comprehensive analysis.

Part II

# Thesis: Experimental Procedures and Results

# 1 Materials

DANGAIIGDIS peptide was purchased from Genscript (Piscataway, NJ), Gentamicin, curcumin, Dulbecco's phosphate-buffered saline, dimethyl sulfoxide (DMSO), Gelzan, Thioflavin T were purchased from Sigma-Aldrich (St. Louis, MO), Milli-Q purification system obtained from Millipore (Burlington, MA), scintillation vials ordered from DWK Life Sciences (Millville, NJ), filter syringe and Congo Red powder ordered from Fisher Scientific (Waltham, MA), TS100 light microscope from Nikon (Melville, NY), PowerShot s110 digital camera ordered from Canon (Huntington, NY), 24-well plate and 5mL reagent reservoirs ordered from Genesee Scientific (San Diego, CA), class II, type A2, biological safety cabinet ordered from Nuaire (Plymouth, MN), BBL Mueller Hinton II broth (CMHB) ordered from BD (Franklin Lakes, NJ), SPECTRONIC 200 Spectrophotometer ordered from ThermoFisher (Waltham, MA), Cytation3 Plate Reader ordered from BioTek (Winooski, VT), 12mm circular glass coverslides ordered from Electron Microscopy Sciences (Hatfield, PA).

# 2 Methods

## 2.1 Peptide Synthesis

The amino acid sequence of DANG was DANGAIIGDIS, with an acetylation at the N-terminus and an amidation of the C-terminus, as spec. The purity of the peptides was determined to be >95%.

## 2.2 Fibril Formation and Stock Solutions

A standard 1× PBS (phosphate-buffered saline) solution was prepared from a 10× stock solution of Dulbecco's phosphate-buffered saline diluted using deionized water. 2 mM concentrations of DANG fibrils and 2 mM concentrations of gentamicin were prepared by massing appropriate quantities of each respective substance into individual scintillation vials,

and mixing with 1× PBS buffer solution. Stock solutions were vortexed to ensure complete homogeneity. When not in use, stock solutions were kept in a -20°C freezer to ensure the stability of the molecules. For samples containing curcumin, the concentration of curcumin was 10000 $\mu$g/mL in 5% v/v dimethyl sulfoxide (DMSO) in 95% 1× PBS buffer.

Imaging was performed on a Nikon TS100 light microscope, and imaging were acquired via Canon PowerShot s110 digital camera. Images of solutions were acquired, as well as images of solutions being mixed and imaged simultaneously.

## 2.3 Congo Red Staining and Imaging

Our protocol was based on previous reported work [86]. A staining solution was prepared consisting of 80% v/v ethanol in deionized water. A saturating amount of NaCl was added, and stirred for several minutes. After, the remaining undissolved NaCl was filtered out using a 0.22 $\mu$m filter syringe. Following this, a saturating amount of Congo Red powder was added to the solution, and again was filtered using the previously mentioned methodology. The resulting staining solution was kept at 4°C until use.

A 2% w/v gellan in warm MilliQ water was mixed and allowed to solidify into a hydrogel. Following solidification, small rectangles of hydrogel segments were cut and transferred to a glass microscope slide. Once on the slide, 20 $\mu$L of our experimental solution of interest was transferred onto the surface of the hydrogel. The solution was left to soak into the hydrogel for 5 minutes, allowing the fibrils to remain on the hydrogel surface. The molar ratio of gentamicin to DANG peptide was always mixed 1:1 (2 mM gentamicin to 2 mM DANG peptide). Following this, an equal volume of Congo Red stain (20 $\mu$L) was added to the surface of the hydrogel, and was allowed to soak as well over 5 minutes before further investigations.

Imaging was performed on a Nikon TS100 light microscope, and imaging were acquired via Canon PowerShot s110 digital camera. Premixed Dang and gentamicin solution were

used as controls at 1 mM concentrations. Note that all experimental solutions were mixed at 4 mM concentrations.

While imaging, three individual images were taken at different locations across the samples. When imaging samples that are being mixed during the actual imaging process (compared to those that are premixed and then subsequently imaged), hydrogels were prepared on glass microscope slides using the methods described above. The DANG was added and allowed to sit, followed by the addition of Congo Red. After allowing the solutions to soak for 5 minutes, 20 $\mu$L of gentamicin was added directly to the surface of the hydrogel via micropipette. Images were taken before and after mixing.

## 2.4 NMR Experimentation

DANG peptide, gentamicin, and DANG-gentamicin mixtures were all mixed at 2mM concentrations. Samples were loaded into 3 mm NMR tubes and taken to a Bruker Avance-III 300 NMR. Samples were loaded into the NMR, and the standard proton spectra protocol built into the Bruker NMR control software was utilized. Following the acquisition of the one-dimensional proton NMR spectra, the DANG-gentmaicin sample was loaded and the standard ROESY two-dimensional NMR was performed according to the protocols outlined by the Bruker software.

## 2.5 Thioflavin T Staining and Fluorescent Analysis

Our staining protocol was based on the methods and information previously reported in literature [87]. A stock solution of Thioflavin T was prepared at a 20 $\mu$M concentration in 1$\times$ PBS. The mixture was then filtered through a 0.22 $\mu$m filter syringe and stored in the dark. Experimental solutions of peptides and gentamicin were mixed at 2 mM concentrations. 1 mL of each mixture was transferred to a 24-well plate and 1 mL of stock Thioflavin T was then added to each well. Each sample was made in triplicate. In the dark, the plate was

lightly shaken to allow for mixing.

Imaging was performed on a Nikon TiE inverted fluorescence microscope using a 470/40 excitation and 525/50 emission (FITC) filter. While imaging, three individual images were taken at different locations across each sample.

## 2.6 Minimum Inhibitory Concentration Assay (MIC)

All bacterial work was performed in a class II, type A2, biological safety cabinet, and all equipment and fluids used in bacterial work was autoclaved prior to use. 1× and 2× stock solutions of BBL Mueller Hinton II broth (CMHB) were prepared.

The bacteria strain utilized throughout this work was *Staphylococcus aureus* 25923. When not in use, the bacteria were kept in a -80°C freezer. The bacteria were removed from the freezer and were brought into the bacteria hood. A small toothpick was used to scrape away a small quantity of bacteria, which were then transferred to a culture tube containing 5mL of 1× CMHB. The tube was partially capped to allow air to enter the tube, and the sample was placed onto a shaker set to 120RPM within an incubating oven set to 37°C. The bacteria were then allowed to grow uninterrupted for 16-18 hours overnight.

The next morning, 1:500 and 1:1000 bacteria dilutions were prepared from the initial bacteria stock solution. The methods utilized in this process were similar to those employed above, excepting that bacteria inoculation was performed by transferring the bacteria at known volumes to the new culture tubes to result in the desired volumetric dilutions specified above. These diluted samples were again placed on the shaker within the incubator for approximately 4 hours.

Four 25mL reagent reservoirs were prepared and filled with 1× CMHB, 2× CMHB, and 1× PBS, while the final reservoir was left empty. Experimental solutions were filtered using sterile 0.22 $\mu$m syringes. A 96-well plate was prepared by pipetting 300$\mu$L of experimental sample into the first row, 300 $\mu$L of 1× PBS into the first rows of the positive and negative

controls, 150 $\mu$L of 2$\times$ CMHB into the second rows, and 150 $\mu$L of 1$\times$ CMHB into the remaining rows. Finally, 150 $\mu$L was taken from the first row and was subsequently serially diluted down the plate. Note that all volumes were held consistent, so the final row had 150 $\mu$L removed and discarded instead. All experiments were run in triplicate. Here, we tested the antimicrobial efficacy of DANG peptides, as well as gentamicin. Furthermore, this experiment provided insight into whether binding was occurring between the DANG peptides and gentamicin molecules.

Following the 4 hour growth period, the optical density of the diluted bacterial solutions were measured compared to a 1$\times$ CMHB control. Measurements were taken using a SPECTRONIC 200 Spectrophotometer with a 600nm wavelength. Bacteria were utilized during their logarithmic growth period. The bacteria solution was diluted to result in a concentration of $8.0 * 10^6$ CFU/mL. Following this, 10 $\mu$L of this bacterial solution was transferred into each experimental well. 1$\times$ PBS was used as a negative control. The final concentration of bacteria in each well was approximately $5.0 * 10^5$ CFU/mL

The plate was placed on the shaker in the 37°C incubator for 16-18 hours. The next day, the optical density was measured in each well using a Cytation3 Plate Reader set to a 600nm wavelength.

## 2.7 Bacterial Attachment and Drug Retention Assays

A scheme of these experimental procedures is depicted in Figure 2. Thin films of DANG peptide were deposited on 12 mm circular glass coverslides. Slides were first washed in 100% ethanol, ensuring that the slides were completely submerged in liquid and allowed to move freely when shaken. Slides were placed onto a shaker operating at 100RPM, inside of an incubator set to 37°C. The slides were allowed to remain in the incubator until all the ethanol was evaporated.

Slides were then transferred to a 6-well plate using sterile tweezers. Experimental solu-

tions of 2 mM DANG-gentamicin complexes, curcumin, or gentamicin were then added to the wells until the slides were completely submerged. It was ensured that slides were allowed to move freely when the plate was shaken. The plate shaken within the incubator at 37°C, where the slides remained until the experimental solutions were completely evaporated. Note that negative control samples (that is, samples without a coating of experimental solution) were prepared using 1× PBS.

Following sample evaporation, the slides were transferred to a sterile petri dish containing deionized water, where they were allowed to soak for 10 minutes. It was ensured that each slide was completely submerged and was free to move. The slides were then transferred either to a new 6-well plate, or a 24-well plate depending on whether we were performing direct bacterial adhesion testing or determining antibiotic retention, respectively. Note that if curcumin was added to a sample, 50 $\mu$L of stock was added to each slides surface, and was allowed to evaporate before inoculation. That is, the initial coating was evaporated onto the surface, followed by the curcumin being added and evaporated, and finally the inoculation of bacteria. Each sample was made in triplicate.

For bacterial adhesion, samples were taken into the bacterial hood, where they were individually inoculated with 36 $\mu$L of bacteria at a concentration of $1.0 * 10^6$ CFU/mL. This volume was chosen to ensure adequate coverage of the experimental side of the slide with bacterial solution, without inoculating the non-coated side of the slide. The plate was transferred to an incubator at 37°C, where it remained for approximately 2 hours until the bacteria solution was completely evaporated. The slides were then briefly washed with 1× PBS to remove any unbound bacteria. Washing occurred by gently pipetting PBS across the slide. Slides were then placed on a 1× CMHB agar plate experimental side down and were incubated at 37°C for 16-18 hours. Colony growth was determined visually the following day and was recorded by taking a digital photograph of each well.

When determining the antibiotic retention capacity of the films, each slide was placed into an individual well. These wells were then filled with 2.5 mL of bacteria and 1× CMHB,

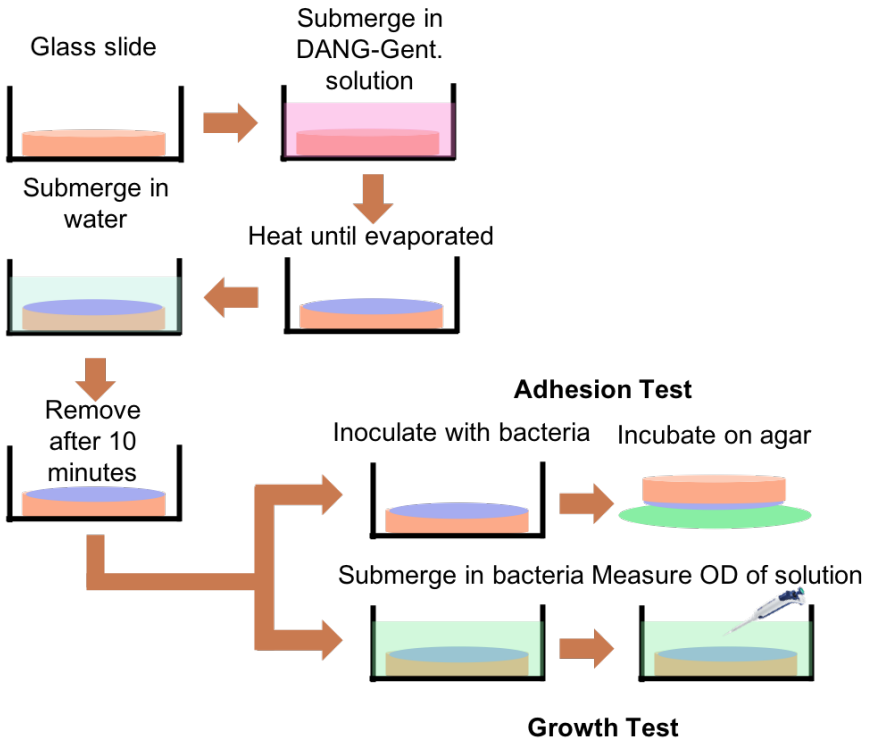


**Figure 2:** Scheme of the methods utilized in the bacterial attachment and bacterial growth tests. Glass slides were prepared by evaporating experimental films onto the surface of the slides. Slides were then washed in DI water for 10 minutes to remove any unbound materials and molecules on the surface of the slides. If bacterial attachment was being tested, slides were inoculated with bacteria, and placed experimental-side down on agar, where colony growth was subsequently quantified. If bacterial growth was being tested, sides were submerged in bacterial growth solutions containing bacteria. The samples were allowed to grow for 24 hours, after which the optical density of the resulting solution was measured to determine the amount of bacterial growth inhibition observed in the system.

resulting in a final bacterial concentration of $1.0 * 10^4$ CFU/mL. It was ensured that each slide was completely covered and the plate sealed with parafilm and placed on a shaker at 120 RPM inside of an incubator at 37°C for 16-18 hours. Following incubation, 1mL of liquid from each well was transferred to a new 24-well plate, and the optical density was measured at 600 nm using a Cytation3 Plate Reader. The slides were then removed using sterile

tweezers, and were placed in a new 24-well plate, and the procedure was repeated using the methods described above, incubating the slides again with a fresh solution of bacteria for further 24 hours and then measuring again the optical density of each well.

# 3 Results

## 3.1 Amyloid Fibril Formation

In order to determine whether DANG peptides were capable of forming the anti-parallel $\beta$-sheet structures that are characteristic of amyloids, Congo Red staining of a DANG solution was utilized (Figure 3). Through staining, we aimed to visually confirm the presence of amyloid fibrils, as predicted by computational studies.

When observed without Congo Red stain, DANG fibrils could not be observed visually (Figure 3B). Similarly, Congo Red stain observed without the presence of DANG peptide

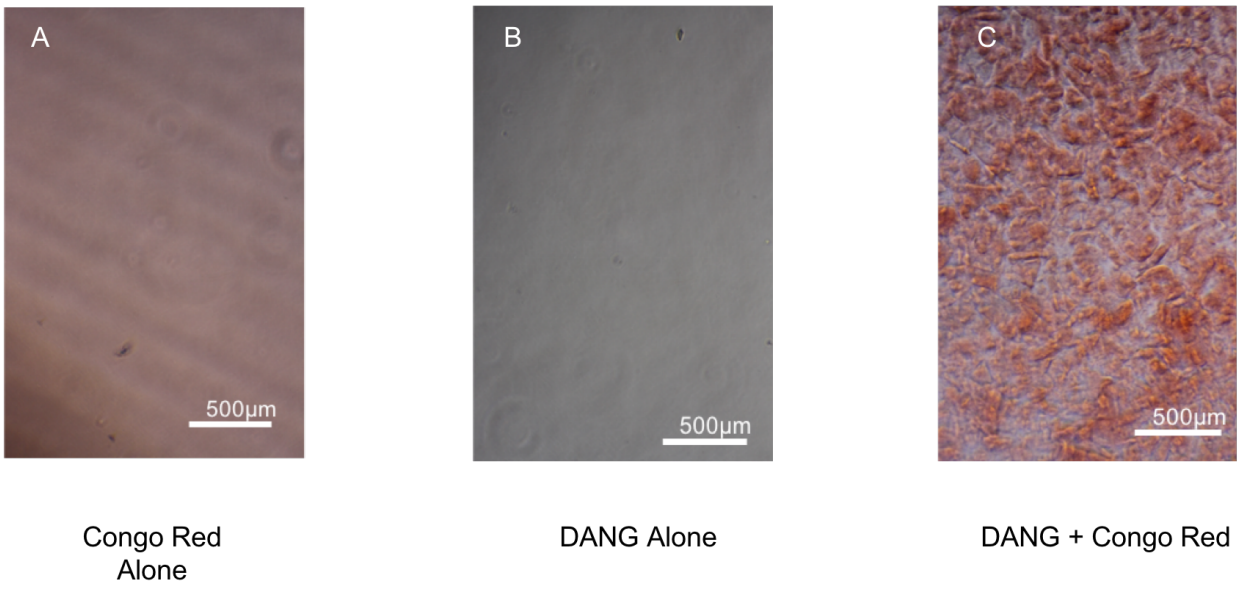


**Figure 3:** Identification of DANG fibril formation using Congo Red staining. Optical images of 100% saturated Congo Red (A), 4 mM DANG peptide (B) and 4 mM DANG peptide previously mixed with 100% saturated Congo Red (C), deposited on a gellan hydrogel.

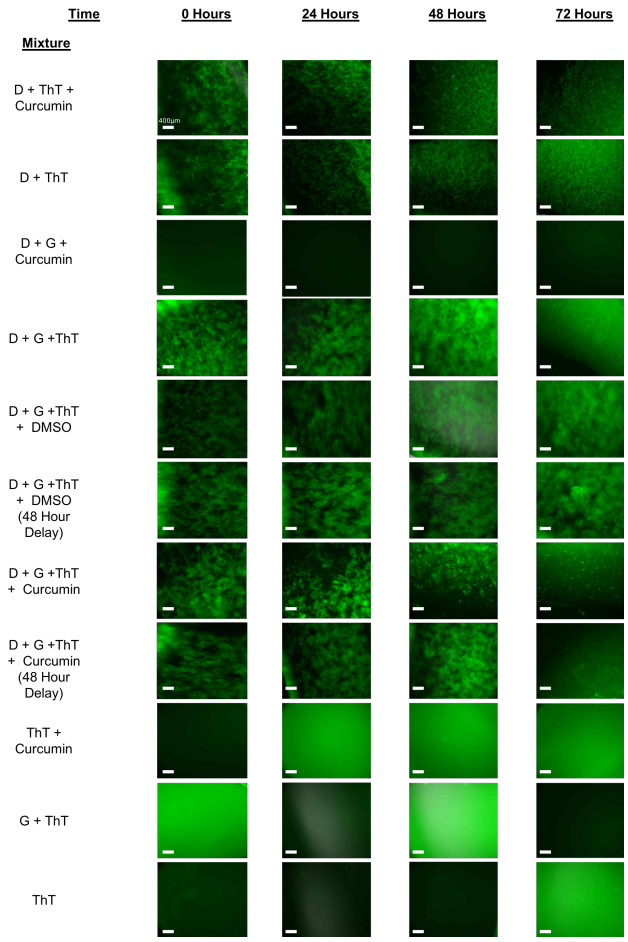


**Figure 4:** Curcumin induced effects on DANG-gentamicin fibril conformations over time. DANG peptides (2 mM, labeled as "D") were mixed with gentamicin (2 mM, labeled as "G") and/or curcumin (10000 $\mu$g/mL) and/or DMSO which acted as a control group. Samples labeled "48 Hour Delay" indicate that curcumin or DMSO were not added until just before the 48 hour images were taken. All results were obtained and observed using thioflavin T fluorescent dye (20 $\mu$M, labeled as "ThT") on a Nikon TiE inverted fluorescence microscope using FITC filter. DANG peptide alone shows fibril formation with limited clumping and aggregation into larger fibril networks, whereas DANG-Gentamicin complexes show more aggregation.

yielded no visibly identifiable amyloid complexes (Figure 3A). When Congo Red was mixed and observed with DANG peptide (4 mM), individual fibril complexes were observed (Figure 3). DANG concentrations lower than 4 mM were not capable of producing visually

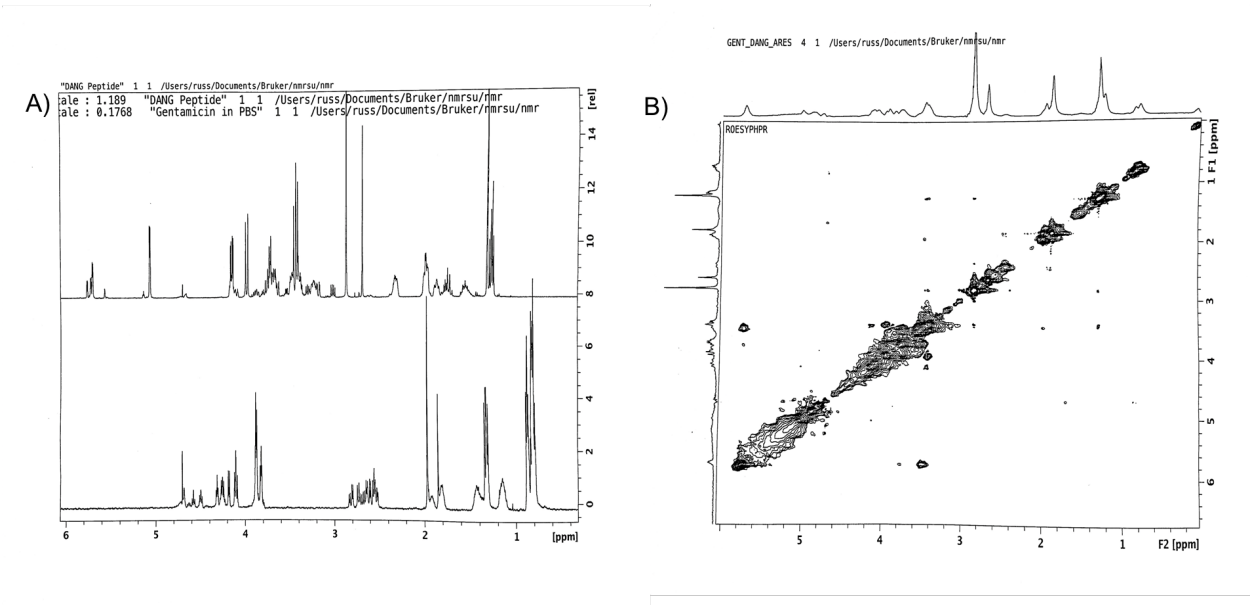


**Figure 5:** Two dimensional ROESY NMR shows the presence of gentamicin and DANG peptides following mixing. ROESY NMR was performed for 8 hours at 300 MHz. A) $^{1}$H-NMR shows two spectra featuring unique peaks, signifying the presence of gentamicin and DANG peptide. B) 2D analysis shows several proton interactions, suggesting potential interaction between DANG and gentamicin, however spectral overlap prevents making any definitive statement from this data alone.

identifiable fibrils, regardless of the presence of Congo Red.

To better observe DANG fibrils and study the interaction with gentamacin and curcumin, we used Thioflavin T stain. Using this method, DANG fibrils were strongly visible by fluorescent microscopy at 20 $\mu$M concentrations. When DANG peptides were allowed to form amyloid structures without the presence of gentamicin, small fibrils were observed freely floating homogeneously throughout the solution. On the contrary, when fibrils were formed in the presence of gentamicin allowing the formation of peptide-drug complexes, larger structures were observed (Figure 4).

The contrast between the homogeneous distribution of smaller DANG amyloid fibrils and the aggregated DANG-gentamicin fibril complexes suggests that DANG peptide is capable of independently self-assembling into anti-parallel $\beta$-sheet amyloid structures. The presence

of gentamicin appears to facilitate interactions between fibrils, leading to increased binding affinity and subsequently, the highly aggregated complexes observed here. This is likely due to the gentamicin effectively increasing the total number of binding sites accessable to DANG fibrils. Based on computational expectations, each peptide possesses a single binding site for amyoid aggregation, as well as a binding site for gentamicin. As such, when gentamicin is present, secondary fibrils now have an additional place of binding, increasing the binding affinity of the system. This would likely result in the aggregation effect that we are observing here.

## 3.2 DANG-Gentamicin Complexes

The capability of DANG fibrils to bind to the targeted ligand gentamicin was determined using NMR analysis in combination with visual fluorescent microscopy techniques. Through these processes, we aimed to show that DANG fibrils are capable of interacting with and binding to the antibiotic gentamicin. Additionally, in the event that fibrils were binding to gentamicin, we sought to determine if fibril-drug complexes were capable of surviving long-term without noticeable degradation in an aqueous solution.

Gentamicin and DANG peptides were first analyzed using one-dimensional proton ($^1$H) NMR (Figure 5A-B). Due to the complexity of their spectra, we did not enter in the in-depth determination of each proton of the two molecules. We investigated instead the interactions of gentamicin and DANG in a mixture using a two-dimensional ROESY NMR and we compared it with the $^1$H-NMR spectra of gentamicin and DANG alone to determine if any interaction between the two molecules was present to confirm the complexation. All off-diagonal proton-proton interactions observed correspond to sections of spectral overlap on the one-dimensional NMR spectra for both DANG peptide and gentamicin. As such, we are able to conclude that there are potential interactions occurring between the two molecules, however because there is such overlap, we cannot completely rule out the possibility that the

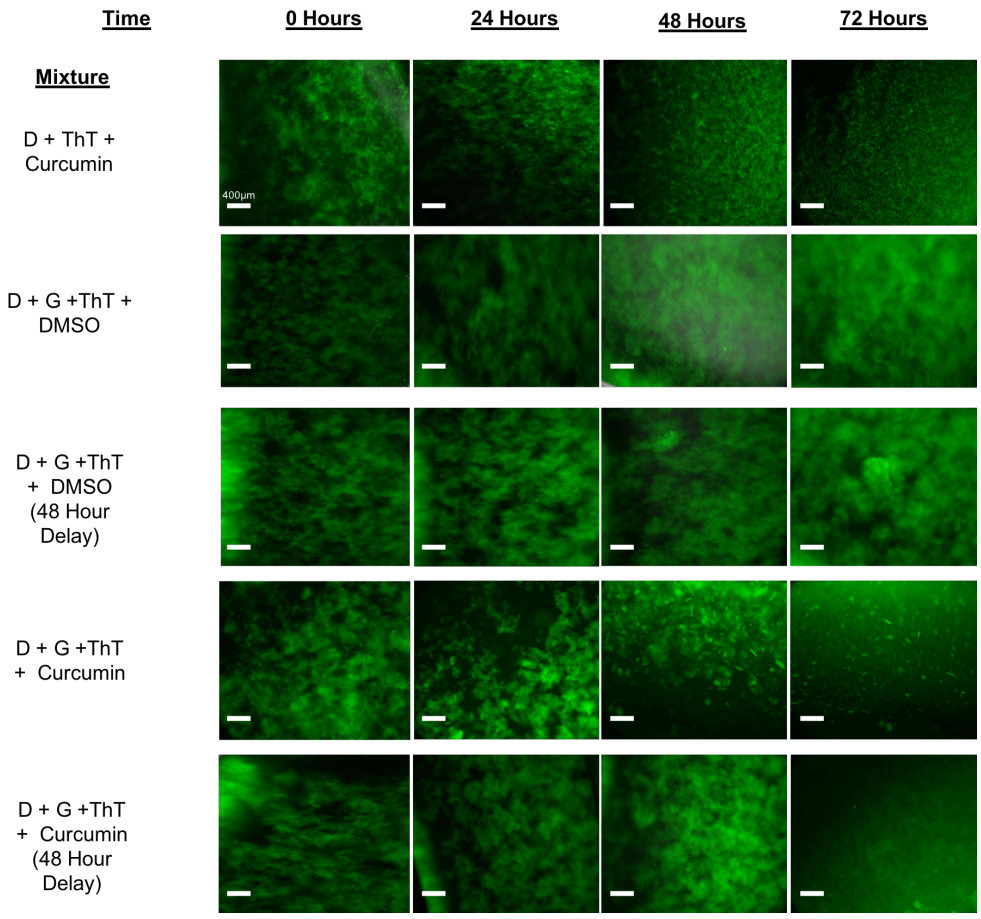


**Figure 6:** Thioflavin T (ThT) fluorescent imaging showing DANG (D) fibrils and DANG-Gentamicin (D+G) complex breakdown due to the introduction of curcumin. Upon introduction, curcumin appears to result in disassociation between large fibril networks when compared to DMSO control groups. Furthermore, samples appear to remain stable over time, showing little to no degradation without a disruptive agent (in this case, curcumin.

protons are interacting with themselves in the solution. We are able to confirm that protons are interacting in the DANG-gentamicin mixed solution, however further experimentation would be needed to determine whether the interactions were between the peptides and themselves, gentamicin and itself, or the peptides and gentamicin with each other.

Thioflavin T staining showed the presence of DANG amyloid fibrils both with and without the presence of gentamicin (Figure 4). Interestingly, when DANG fibrils were allowed to self

assemble without the presence of gentamicin, the resulting fibrils were more homogeneously dispersed in solution. By comparison, DANG fibrils that had been mixed with gentamicin showed significant aggregation. Solutions containing both molecules appeared cloudy (data not shown), and larger networks of fibrils could be observed, while smaller individual fibrils

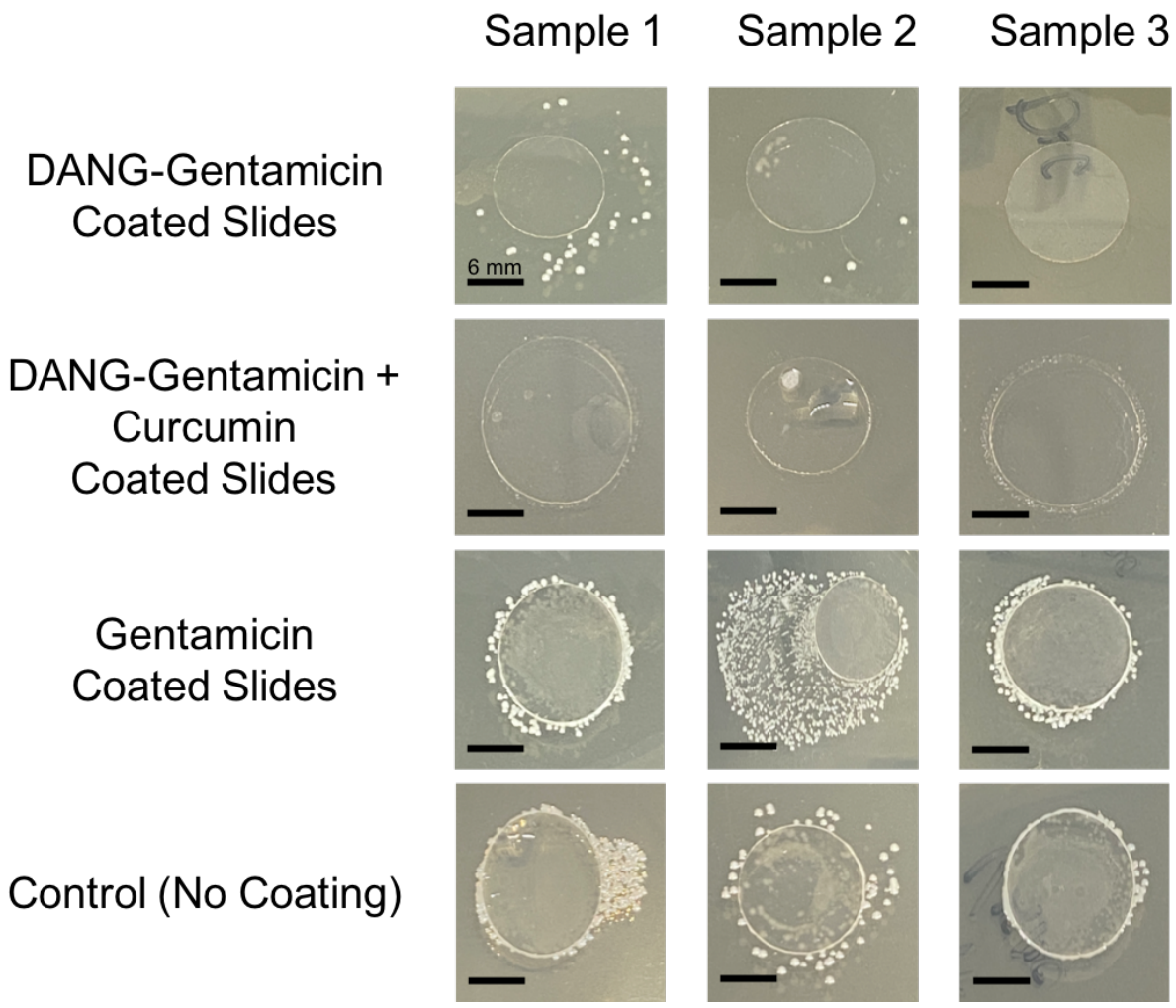


**Figure 7:** Bacterial attachment to glass coverslide is reduced in slides coated with DANG-gentamicin complexes compared to gentamicin coating. Coating were prepared by evaporating DANG-Gentamicin solution on the surface of the substrates. Following a 10 minute wash in DI water, slides were inoculated with bacteria and allowed to grow on agar overnight. This suggests that DANG is capable of retaining antibiotic on surfaces in aqueous environments.

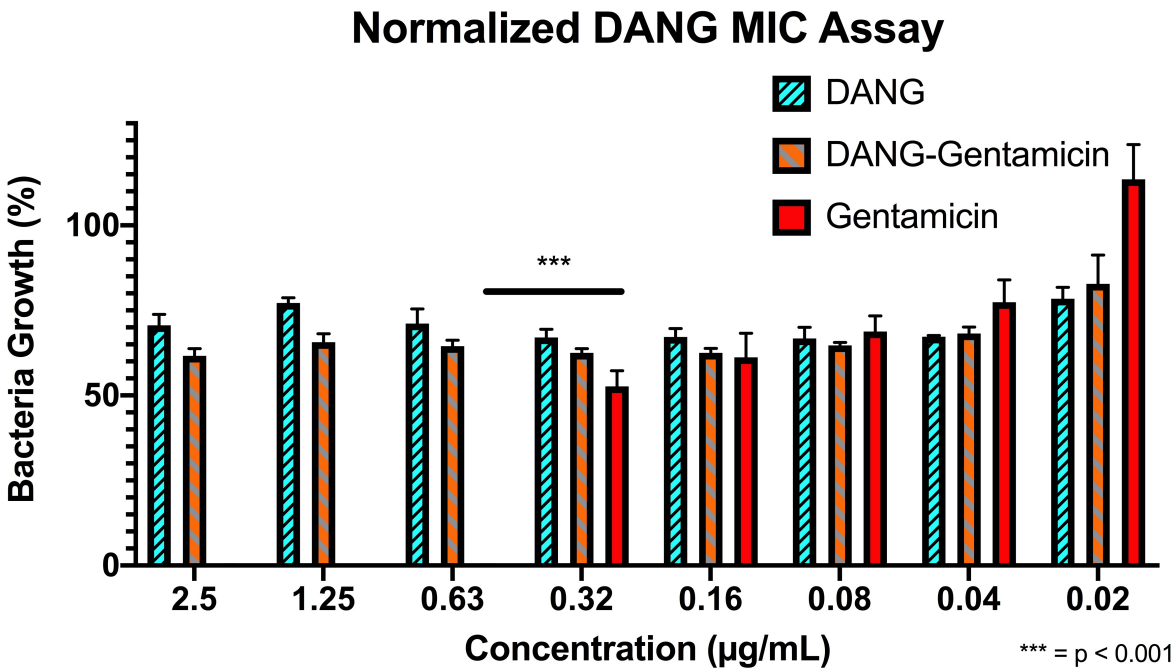


**Figure 8:** Minimum inhibitory concentration assay shows inhibition of *S. aureus* 25923 by gentamicin, yet no inhibition of growth when complexed to DANG fibrils. Bacterial concentrations were diluted to $5 * 10^5$ CFU/mL. DANG peptides show no antimicrobial effect, whereas gentamicin shows inhibition up to 0.32 $\mu$g/mL. When gentamicin is complexed to DANG fibrils, there is no evident antimicrobial effects at the same concentration as gentamicin alone.

were less distinguishable. This suggests that the presence of gentamicin in solution facilitates the aggregation of fibril complexes. Furthermore, these results suggest that DANG peptides are interacting with gentamicin to some extent, resulting in the larger aggregates observed here.

The exact mechanism of interaction between DANG fibrils and gentamicin is not fully understood, and requires further future investigation. Based on the computational simulations made by the group of Prof. Tamamis, we hypothesize that gentamicin is capable of binding to DANG fibrils in a unique location that is exclusive from the binding zones where individual peptides interact. Because this binding site is more unique from other binding regions, it may allow additional peptide molecules to bind to the peptide complex, even if

the peptide-peptide binding site is already occupied. Furthermore, the gentamicin molecule may provide additional physical space between the peptide molecules, lowering electrostatic or steric interactions that may limit binding, while increasing overall binding affinity, potentially resulting in the formation of the larger fibril networks observed here.

## 3.3 DANG-Gentamicin Complex Stability and Ligand Release

The long-term stability of antimicrobial compounds is essential for implantable device coatings, particularly for devices that frequently operate in aqueous environments, such as catheters. The stability of DANG-gentamicin complexes was observed via Thioflavin T staining using fluorescent microscopy (Figure 4).

Thioflavin T staining indicated the presence of amyloid fibrils, both with and without gentamicin (Figure 4). These fibrils are observed throughout the full 72 hour duration of the experiment. This indicates that DANG fibrils are capable of retaining their binding affinity and structure while suspended in solution over significant periods of time. This result is favorable, as it suggests that these compounds will be able to retain their form in harsher physiological environments, such as those encountered by implantable medical devices.

DANG fibrils formed in solution without the presence of gentamicin showed the formation of small fibrils, which were homogeneously distributed throughout the experimental solution (Figure 6). In contrast, DANG peptides that were allowed to self-assemble in the presence of gentamicin showed significantly more aggregation of fibrils, resulting in visibly larger networks of fibrils clumped together, with few to no individual fibers freely floating in solution outside of the larger network of peptides (Figure 6). This data suggests that DANG peptides are binding to the targeted ligand gentamicin, and the presence of gentamicin increases the aggregation occurring between DANG peptides. We believe this observed phenomenon of increased aggregation is due to the binding of gentamicin to DANG fibrils. When the gentamicin binds, the total number of potential binding sites on the complex increases,

allowing creating another active zone where other fibrils can bind, which would lead to a reduction in the presence of smaller, free-floating fibrils.

Computationally, curcumin is believed to interact with amyloid fibrils, creating competitive binding between the fibrils and their ligands, based on prior examples in the literature [88]. This is believed to stimulate the release of the ligand from the complex. Interestingly, when curcumin is added to the experimental solutions, we observe a conformational change over time, where larger aggregated networks of fibrils break down into smaller, more distributed fibrils (Figure 6). That is, the networks deteriorate into conformations that more closely resemble fibrils that were formed without the presence of gentamicin. Overall, this suggests that curcumin is acting as a release agent by interfering with the bonds between gentamicin and the DANG fibrils, causing the disassociation of the ligand, and thus a breakdown of the larger peptide-drug complexes. Similarly, when samples were allowed to sit without curcumin for 48 hours, there was no change in complex aggregation size or the distribution of fibrils in solution. When curcumin was added to these samples however, the same effects could be observed, with the larger aggregates degrading into smaller fibrils that were more similar to fibrils formed without the presence of gentamicin. This suggests that curcumin stimulated the release of gentamicin from these complexes, resulting in the deterioration of the amyloid complexes into smaller, more distributed fibrils.

## 3.4 Prevention of *Staphylococcus aureus* Adhesion and Growth

Treating and/or preventing microbial infections on the surfaces of biomedical devices is essential for improving the overall lifespan of the device, as well as ensuring the overall health and well-being of the patient. The primary method of preventing or treating infections related to implantable devices is to apply a thin-film (or coating) of antibacterial agents to the device to prevent the adhesion and growth of bacteria on the material. These coatings may consist of various compounds and molecules, and may be deposited through different

techniques, such as thermal evaporation, spin coating, or dip-coating. To determine the ability of DANG and DANG/gentamicin complex to inhibit bacteria growth we performed MIC assay on DANG and gentamicin alone or complexed.

The MIC of gentamicin was determined to be approximately 0.32 $\mu$g/mL (Figure 8), which is in accordance with literary standard values for that antibiotic [89]. DANG peptide however did not show inhibition of growth at any physiologically relevant concentration. Interestingly, when DANG peptide was mixed with gentamicin, no inhibition of growth was observed, suggesting that the gentamicin was bound and encapsulated in the DANG fibrils, preventing its function in preventing the growth of bacteria (Figure 8). This data further supports our claims that DANG fibrils are binding to gentamicin. Additionally, this data shows that any observed antimicrobial effects that are observed are the result of gentamicin, as DANG fibrils have no detrimental effect on the bacteria.

In order to determine whether the DANG-gentamicin fibrils could be applied to biomedical devices, thin-films of the complexes were deposited on the surfaces of glass slides. In bacterial adhesion tests, bacterial growth was inhibited in all samples containing DANG peptides and gentamicin, while gentamicin coatings alone failed prevent bacterial adhesion (Figure 7). This suggests that the gentamicin-only coatings were completely solubilized during the washing phase of the experiment due to the soluility of gentamicin in water, removing all traces of the antibacterial compound and preventing subsequent inhibition of growth. By comparison, coatings containing DANG peptides retained gentamicin on the surface of the slide, where it was free to inhibit the growth of bacteria. This data further confirms that DANG fibrils are binding to gentamicin, and demonstrates that DANG fibrils are capable of retaining its bound cargo in aqueous environments.

In order to ensure that the peptide complex coatings were capable of inhibiting the growth of bacteria that were not initially adhered to the surface, additional bacterial growth inhibition assays were performed. On the first day of testing, all coatings containing DANG peptides showed inhibition of bacterial growth, compared to groups without the peptides.

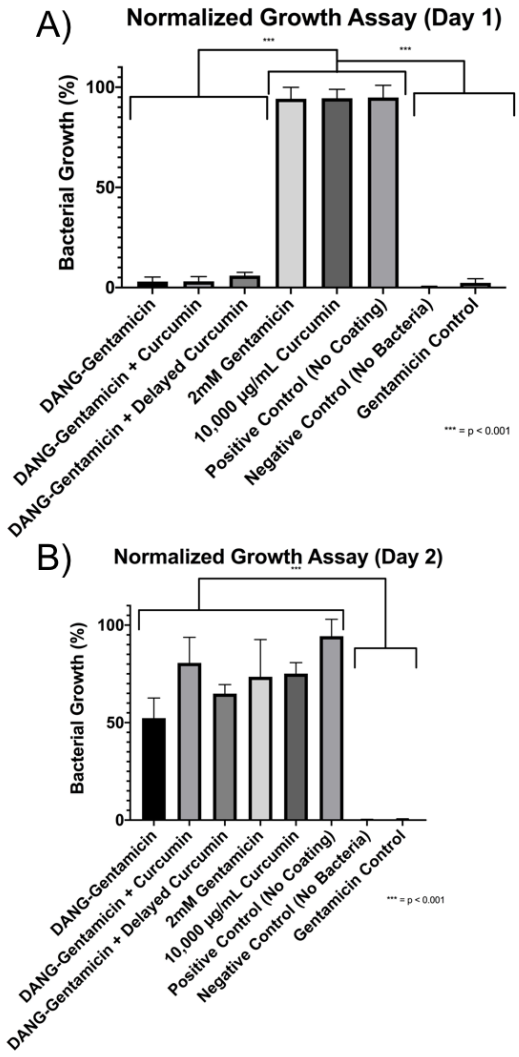


**Figure 9:** Bacterial growth is inhibited in solutions exposed to substrates coated with DANG-gentamicin complexes. A) Upon first exposure, glass slides coated with DANG-Gentamicin complexes showed significantly reduced bacterial growth after being submerged in growth media containing $1 * 10^4$ CFU/mL of *S. aureus* compared to gentamicin alone. Curcumin was added to the "DANG-gentamicin + Curcumin" 10 minutes prior to inoculation. B) When re-exposed to the same concentration of bacteria for a second time, no significant bacterial inhibition compared to controls was observed. Curcumin was added to the "DANG-gentamicin + Delayed Curcumin" 10 minutes prior to secondary inoculation.

Additionally, the degree of bacterial growth inhibition was found to be equivalent to samples inoculated with gentamicin directly (Figure 9-A). When these samples were transferred to a new plate and reinoculated however, no coating showed significant inhibition of bacteria compared to control groups (Figure 9-B). Additionally, these results were found to be consistent regardless of whether the release agent curcumin was present or not. This suggests that while curcumin may act as a competitive binding agent that stimulates the release of gentamicin from DANG peptides, other compounds may exist that perform similar functions. Additionally, it is also possible that some gentamicin remained complexed to the DANG fibrils after the first day, and were subsequently released on the second day. The total amount released however may not have reached the necessary MIC value for gentamicin, resulting in the lack of growth inhibition observed in this study. Further studies will be required to better understand what physical characteristics are necessary to facilitate gentamicin release in this system.

Overall, these results demonstrate that DANG fibrils are capable of binding and retaining gentamicin on the surface of substrates. Furthermore, these complexes are capable of preventing bacterial adhesion, and are capable of preventing bacterial growth in solution as well. MIC analysis shows however that the prevention of growth is due to the release of gentamicin, rather than the peptides themselves. This suggests that our DANG complexes are capable of releasing gentamicin when in the presence of secondary compounds that disrupt the bonds between the fibrils and the antibiotic. While these compounds are effective however, once the gentamicin is utilized, the coatings are no longer capable of preventing bacterial growth. In order to address this problem, future studies will be required to better understand the release of gentamicin from the system. By understanding how gentamicin is released, it may be possible to control the rate of release, thereby limiting the amount of antibiotic lost during treatment, and increasing the overall useful lifespan of the device coating.

Part III

# A General Review and Discussion of the Data

# 1 A Brief Review of the Major Findings and Results

Throughout this report, I have presented several novel and unique findings pertaining to the topic of antimicrobial coatings for medical devices. Throughout this section, I will briefly review, in general terms, the major findings presented in this paper.

## 1.1 DANG Peptide is Capable of Self-Assembling into Amyloid Fibrils

Amyloid peptides are cross beta-sheet structures that are capable of self-assembling into fibril networks when placed in solution [90]. Amyloid fibrils are generally insoluble and posses immense strength between their chemical bonds, making them very robust peptide complexes. Frequently, amyloids are associated with the development of disease, as their presence within cells and the extracellular matrix can inhibit proper cellular function [91]. In some instances however, amyloid peptides have been shown to have beneficial (that is, functional) impacts on the organism in which they form, acting as support structures for other cells and tissues for example [92]. In this work, we demonstrated that our peptide sequence, referred to as the DANG peptide, was capable of producing functional amyloid complexes.

Generally, the observation of natural amyloids is done from histological samples harvested from animal models, however some techniques do exist for monitoring the formation of amyloids *in vitro* [93]. Through the utilization of standard microscopy staining techniques, we were able to demonstrate the formation of amyloid complexes in experimental solutions containing DANG sequences. Dry peptide, when placed into aqueous solutions, spontaneously formed visible complexes. When observed using dyes that preferentially bind to amyloid structures (that is, beta-sheet structures), large fibril networks could be observed. Congo Red stain, in addition to Thioflavin T staining, is considered a gold standard technique for determining the presence of amyloids in a system [86] [94]. By observing both of these dyes in

our peptide mixtures, it is extremely likely that the DANG peptides are forming cross beta-sheet structures associated with amyloids. Additionally, visible size of the amyloid complexes appears to be dependent on the initial concentration of DANG peptide placed in solution. Smaller molar concentrations of DANG resulted in smaller, harder to observe/image complexes, while larger concentrations produced larger, more visible complexes. This further supports the claim that these complexes are resulting from supramolecular complexation of DANG peptide into fibrils.

Overall, we demonstrate that the DANG peptide sequence is capable of self-assembling into amyloid fibril networks. This is supported by visual confirmation using various forms of microscopy and amyloid-specific dyes. Additionally, we show that the initial concentration of DANG influences the size of the resulting amyloid complexes, supporting the claim that these complexes are arising from DANG sequences. Finally, these results are expected from the computational simulations, which predicted the self-assembly and amyloid nature of the DANG peptide sequences.

## 1.2 DANG Peptides are Capable of Binding and Retaining the Targeted Ligand, Gentamicin

The propensity for certain peptide and protein sequences to bind to external molecules, known as ligands, is well studied [95] [96] [97]. Within the body, peptide-ligand interactions drive cellular processes, allow immune cell function, and carry out many other essential bodily functions [98] [99]. In laboratory settings, these interactions are frequently exploited, for example to purify samples or identify targeted molecules in solutions [100]. This is accomplished by allowing the peptides to bind to the target molecules, and then separating the complexes from the solution by some separation technique, such as centrifugation. Similarly, we demonstrate that DANG peptide is capable of binding to the antibiotic gentamicin, in accordance with the computational designs. Furthermore, DANG peptide is able to retain

the antibiotic in physiologically relevant environments, such as aqueous environments.

When DANG peptide is mixed in solution, we are able to observe the formation of fibrils using amyloid-specific dyes and microscopy techniques (as described in the previous sections). Interestingly however, when DANG peptides were mixed with gentamicin at 1:1 molar ratio, we were able to visually confirm the presence of amyloid-drug complexes with the naked eye (data not shown), suggesting that more aggregation was occurring between the two molecules, resulting in larger fibril networks. These results were supported by computational simulations, which suggested that gentamicin interacted with DANG peptides at an alternative binding site from where the peptides interacted to form the cross beta-sheet structure. As such, the presence of gentamicin effectively increased the number of binding sites on the peptides where binding interactions could occur, resulting in more aggregation. When DANG-gentamicin complexes were observed using the microscopy techniques described previously, we observed more aggregation of fibrils and reduced homogeneity in solution. This indicated that more fibrils were binding with each other to form larger structures, and as a result there were fewer free-floating fibrils in solution. Finally, two-dimensional ROESY NMR analysis suggested that some proton-proton interaction was occurring in the DANG-gentamicin system. The complexity of the system however prevented us from making definitive claims from these results as to whether interactions were occurring between DANG-DANG peptides or between DANG and gentamicin ligands. Taking together the results obtained microscopy and NMR studies we can conclude that the fibrils observed are the results from both peptide-peptide interactions (i.e. the formation of the cross beta-sheet fibril structure that is characteristic of amyloids) and the interactions between DANG peptides are the target molecule gentamicin.

We are also able to identify the binding of gentamicin to DANG fibrils through our antimicrobial testing and drug-retention experimentation (that is, the coating experiments). Using a simple evaporation-application technique, we applied thin films of our DANG peptides, gentamicin, and DANG-gentamicin complexes to the surface of glass substrates [101].

The use of thin films for biomedical applications is broadly discussed in literature [102], and is a useful technique for studying the potential applications of our materials. Our coated glass substrates were placed in a water bath, removed and dried, and then inoculated with our experimental bacteria. Our results demonstrated that gentamicin coatings dissolved when placed in solution, and subsequently allowed the colonization and growth of bacteria on the surface of the substrate. On the contrary, when DANG peptides were mixed with the gentamicin, we observed no bacterial colonization or growth, suggesting that gentamicin remained bound to the surface of the substrate thanks to the interaction with dang and were subsequently able to interact with the bacteria to inhibit their binding and growth.

Finally, our MIC assay supports our hypothesis that binding is occurring between DANG peptides and gentamicin. When DANG peptide is mixed with our target bacteria at physiologically relevant concentrations, we observe no inhibition of growth. This indicates that DANG peptides are not antimicrobial, and should have no effect on the growth of the bacteria. In contrast, when gentamicin is mixed with bacteria, we see the inhibition of growth at similar concentrations as literary standards [103]. When DANG peptides were mixed with gentamicin however, we observed no inhibition of growth in the bacteria. This suggests that binding is occurring between the the two molecules in such as a way that prevents the activation of gentamicin.

Overall, we were able to successfully demonstrate that our designed DANG peptide is capable of binding to the targeted compound gentamicin. Furthermore, we show that the presence of DANG peptide increases the retention of gentamicin on substrates placed in aqueous environments.

## 1.3 Curcumin Appears to Facilitate the Release of Gentamicin

The use of secondary compounds to stimulate the breaking of bonds between peptides and ligands is common throughout the medical sciences [104]. Secondary compounds are useful

tools for stimulating release as they can be selectively added at different time points to control release more effectively, and can be chosen to limit unintended secondary effects [105]. The computational simulations performed by Prof. Tamamis suggested that the molecule, curcumin, may be a molecule that can inhibit the bonds between gentamicin and our DANG peptides, without impacting the bonds between the DANG fibrils that make up the cross beta-sheet structure.

Experimentation using the microscopy techniques described in previous sections demonstrated that DANG-gentamicin complexes formed large aggregates in solution, with few observable free-floating fibrils. By comparison, DANG fibrils that were formed without gentamicin produced noticeably smaller fibrils that were more homogeneously distributed throughout the experimental solution. When curcumin was added to the DANG-gentamicin solution, we observed a change in aggregate distribution and conformation. The larger aggregates broke down and spread throughout the solution, appearing similar to DANG fibrils that were formed without the gentamicin present. This suggests that curcumin was stimulating the release of gentamicin, limiting the number of accessible binding sites on the fibrils for interactions, and thus preventing large aggregates from forming. This would explain why the resulting aggregates appeared more similar to fibrils that never were exposed to gentamicin. In addition, DANG-gentamicin complexes that were allowed to sit for multiple days without curcumin showed little to no change in conformation or distribution, suggesting that these compounds are stable for long periods of time in solution. Additionally, this suggests that the conformational changes observed were not due to natural degradation over time, but were rather caused by the presence of a secondary release compound.

Overall, we demonstrate that curcumin appears to be capable of disrupting the bonds between DANG peptides and gentamicin, causing the release of gentamicin. The reduction in fibril aggregation and increased distribution suggests that while amyloid fibrils are maintained, there are fewer binding sites where interactions can occur, thus limiting aggregation. The resulting distributions appear more similar to DANG fibrils that are formed without the

presence of gentamicin. Thus, this data suggests that curcumin may function as a targeted release agent specific to the bonds between gentamicin and the DANG peptides.

## 1.4 DANG-Gentamicin Complexes Retain Antimicrobial Functionality and Demonstrate Increased Surface Binding and Drug Retention

The development of antimicrobial surfaces has allowed researchers to manufacture devices and therapeutics, particularly implantable devices, that are able to carry out their intended function while simultaneously being capable of resisting colonization from infectious microbial organisms [106]. With our end application being the development of medical device coatings that prevent the adhesion of bacteria, it is essential that we demonstrate antimicrobial efficacy in our DANG-gentamicin complexes.

As described in previous sections, MIC assays indicate that gentamicin has innate antimicrobial effects when mixed in solution on its own. When gentamicin is mixed with DANG peptides however, there is no observed inhibition of binding, due to the binding of the two molecules. Additionally, thin films created from DANG-gentamicin complexes show significant inhibition of growth compared to gentamicin-only thin films. This demonstrates that the presence of DANG fibrils increases the retention of the water-soluble gentamicin on the surface of substrates. Similarly, when coated substrates were completely submerged in solutions inoculated with bacteria, DANG-gentamicin solutions completely inhibited growth of bacteria on day one, compared to gentamicin only samples. Interestingly however, an analysis of the same slides on the subsequent day showed the DANG-gentmaicin surfaces no longer inhibited the growth of bacteria. This suggests that all (or a majority of) the gentamicin was phagocytosed by bacteria on day one. Additionally, the presence of the release agent, curcumin, did not appear to have an impact on the inhibition of bacteria growth on either day one or day two.

Overall, this data demonstrates that DANG peptides improve the binding and retention of gentmaicin to the surface of substrates. Furthermore, DANG-gentmaicin complexes are able to release gentamicin to effectively inhibit the growth of targeted bacterial organisms. It is important to note however that while the data demonstrates a release of gentamicin, the exact mechanism of release is not fully understood. While our previous results suggest that curcumin is capable of disrupting the bonds between DANG peptides and gentamicin, causing the release of gentamicin, it is unclear if curcumin is unique in being able to do so. That is, it is possible that other compounds are similarly able to spur the release of gentamicin without disrupting the fibril forming bonds between the DANG peptides. Other release agents, either in the bacterial growth medium or molecules secreted by the bacteria themselves could explain the lack of effect curcumin appears to have in these experiments. Further research is necessary to better understand these phenomenon.

# 2 Significance and Impact of the Results

Throughout this report, I have presented an extensive background outlining the incidence and hazards of infectious diseases, particularly when associated with medical devices. Additionally, I have discussed the importance of antimicrobial coatings for medical devices as effective solutions to prevent and limit the spread of infection through this transmission pathway. Here, I present a novel peptide sequence that is capable of binding to both self-peptide sequences in a cross beta-sheet orientation (that is, an amyloid peptide) as well as targeted antimicrobial molecules. Furthermore, these peptides are capable of adhering to substrates in aqueous environments while retaining their cargo (that is, retaining the bound antibiotic). This is directly relevant and significant to the development of antimicrobial coatings for medical devices, which I will briefly discuss in more detail throughout this section. Apart from the direct benefit to implantable devices however, these findings represent significant developments in computational proteomics as well as peptide engineering. Throughout

this section, I will briefly outline the significance of the findings presented within this paper, as well as their lasting implications on the future of the medicine and experimental biology/engineering.

Coatings for medical devices are an effective means of preventing the colonization and spread of infectious organisms [107]. Currently, there are many compositions and types of antimicrobial coatings, some of which target specific pathogens, and other that function using unique chemical/physical mechanisms [108]. One of the central factors that unites these films however is the need for efficacy over time in a physiological environment. In order for antimicrobial coatings to be effective at preventing colonization on a medical device, they must be localized on the surface of the device, and must remain there long term, as a breakdown of the coating on the surface of the device would compromise the functionality of the film [109]. The DANG peptide presented here demonstrates successful binding to glass substrates, as well as retention of the bound antimicrobial molecules long term while in solution or while bound to the surface of the substrate. As such, DANG peptides appear to have significant use as a novel antimicrobial coating for implantable devices.

When directly applied, our results show that these novel coatings are useful for preventing colonization and infection resulting from pathogenic bacteria that are treatable with the antibiotic gentamicin. Since gentamicin is a broad spectrum antibiotic, there are many potential infections that could be limited by our DANG-gentamicin coatings, such as infections caused by Pseudomonas, Acinetobacter, Enterobacter, and Mycobacteria species [110]. The increased retention of drug on the surface of the implantable devices would allow the device to remain in an "antimicrobial state" longer than direct drug-coating techniques [63]. Additionally, coating the surfaces of implantable devices offers several other unique benefits to the device that may be improved using our novel DANG peptide solution. For example, natural deterioration of the medical device occurs over time, as the surface of the material oxidizes and erodes, which can result in the failure of the device, and has the potential to cause disease in the patient [111]. Device coatings have been shown however to limit the

breakdown of devices resulting from chemical or physical erosion [112]. Since our coatings are comprised of amyloid peptides, which are insoluble in aqueous environments and have strong bonds (and thus a strong structural integrity), it is possible that our coatings could prevent degradation of the device and increase its overall lifetime for the patient. Determining if this is true however requires additional testing, and may be considered in future experiments.

The benefits of this work go beyond direct medical applications as well. For example, the ability to design customized and functional peptide sequences has the potential to dramatically impact research laboratories across the world. Functional proteins are used on nearly a daily basis on the lab to perform a wide variety of tasks, ranging from isolating and purifying solutions via protein binding, to tagging cells and molecules in microscopy, to culturing organisms (such as bacteria and viruses) for analysis [113]. Giving researchers the ability to design custom peptide sequences to carry out a specific function in their lab could accelerate research progress and allow for new experimental methods to be developed. New microscopy techniques or tagging tools may be developed as a result, which has untold potential to the scientific community.

Collectively, this work represents preliminary steps in the development of exciting new therapeutic tools. Peptide therapeutics, such as DANG, offer several unique benefits over alternative treatments such as synthetic or artificial coatings, such as increased lifespan and broad acting efficacy. These peptides also allow the use of drugs that otherwise would be impossible to use in aqueous environments due to their high solubility. Overall, we are excited by the application prospects resulting from this study. These methods have highly significant and high impact potential in the biological research and medical communities.

# 3 Future Work and Experimental Directions

The DANG peptides studied throughout this project represent a unique candidate in the field of implant-borne infections. The results presented here show that DANG peptides are not only capable of retaining their fibril structure in aqueous environments, but are also able to bind and remain on the surfaces of substrates. Furthermore, DANG peptides are able to successfully bind to the antibiotic gentamicin, as well as retain the bound drug until it is forced to release via a secondary release compound. There are several aspects of this system that require further study however before additional application-based experimentation can occur. First, it is essential that the release of gentamicin be better quantified utilizing quantitative experimental methods. While it does appear as though curcumin functions as a release agent, disrupting the bonds between gentamicin and the DANG peptides, and subsequently causing their release, it is not yet known how this is occurring, or if other molecules are capable of stimulating the release of gentamicin as well. Since the bacterial growth tests indicated release of the antibiotic regardless of the presence of curcumin, it would be logical to test the compounds present in the bacterial growth solutions as potential release agents. Additionally, it may be possible that the bacteria themselves are secreting some compound that is causing the disassociation between DANG and gentamicin. This potential cause should be considered and analyzed as well.

The secondary structure of the DANG peptides is predicted to be cross-beta sheet, however experimental attempts to determine if the secondary structure changes as a result of gentamicin binding have proven unsuccessful. Understanding this would be extremely to the project, as it would provide important insight into how substrate binding and ligand release may be occurring. Understanding how the secondary structure changes as a result of ligand binding also may provide some insight into how functionality of the peptide could be affected by molecular interactions, which then could be factored back into the computational simulations to produce a better approximation of ideal peptide sequences to carry out a particular

task. A better understanding of structural changes could also provide additional insight into why our MIC results show a lack of bacterial growth inhibition at low concentrations, but successful inhibition of growth during adhesion testing.

The experiments performed in this report with DANG peptides were extensive, however limited in their depth. This was due in part to several logistical challenges with experimental methods and equipment that were unique to this system. Because of this, more work is required to understand how these peptides behave in alternative systems and environments. For example, experimenting with alternative concentrations, different buffer conditions, and fluctuating ambient temperatures may result in different peptide behaviors. Another limitation that should be analyzed in greater depth is the breadth of infectious organisms used in the growth inhibition studies. Because this study was only preliminary, the bacterium *S. aureus* 25923 was the only organism studied with our experimental complexes. Gentamicin is a broad spectrum antibiotic and can inhibit the growth of many gram-positive and gram-negative, which they should also be considered and studied so that the potential limitations and benefits of our complexes can be better understood. Studying polymicrobial infections may also be useful here, as they also pose a significant risk to patients, and may result from implantable device instillation [114].

Following the more fundamental aspects of the DANG study that are described above, further experimentation on applications of this technology can be performed. Alternative substrates should be considered and studied, especially if the end goal is to apply these materials as coatings for implantable devices. Examples of materials that may be interesting to test include metals such as titanium and aluminum, alternative glass compositions, and polymer materials such as polyethylenes, polyetheretherketones (PEEK), bioabsorbables—polylactic acid (PLA), polyglycolic acid (PGA), and the various copolymers associated with these materials [115]. It may also be interesting to consider alternative applications of the DANG complex technology, such as its use as a drug delivery vehicle, or perhaps using the complexes in the production of other materials to increase their structural integrity (maybe surgical

sutures for example). If the end goal of the project remains the production of medical-grade coatings and devices, animal models will need to be studied in depth, followed by human trials assuming the animal models show promise. The cytotoxicity of the DANG fibrils will also need to be analysed in greater detail.

Overall, there are many potential uses of DANG fibrils in medical applications. By increasing antibiotic retention and allowing medical researchers to use alternative drugs for antimicrobial coatings, DANG peptides show remarkable potential in both research and clinical settings. While further experimentation is necessary, and much additional work is needed, these results have the ability to make a significant impact on the world for the better, preventing the spread of infectious organisms, and improving the lives of everyone across the world.

# Part IV

# Appendices

# 1 A General Introduction to Experimental Techniques and Methods Utilized in this Paper

Throughout this section, I will provide a brief introduction to some of the various experimental techniques that I used throughout the data acquisition phase of my project. Note that this section is meant to be a general overview of the machines and theory behind my data acquisition, and is in no way a complete overview of this information. Furthermore, for a more detailed analysis of the specific variables used in my experiments, please see the materials and methods section of part two.

## 1.1 Microscopic Imaging and Staining

Since its invention around the late $16^{th}$ century, the microscope has remained an essential tool for understanding the microscopic world around us [116]. While many variations of microscopy technology and techniques exist, the fundamental theory behind it has remained relatively consistent throughout the years. The central goal of microscopy is to use some technique, whether it is electrostatic, optical, or photonic absorbance, to visually magnify a small object that otherwise would be difficult to observe with the naked eye [117] [118]. Ideally, this process should allow the experimenter to scale up an image while simultaneously maintaining resolution, so that smaller details can be observed in the image (this is particularly true to electron microscopy techniques) [119].

Throughout this paper, the microscopy techniques employed include optical light microscopy, as well as fluorescent microscopy. Optical light microscopy is perhaps the most common form of microscopy, and is the technique that most people are familiar with when it comes to microscopy. Optical light microscopy works by using a light source to illuminate a specimen on the surface of the microscope. The light passes through the specimen and travels through the microscope's objective lens. The objective lens is curved in such as way

to produce magnification. The light then travels through the microscope to the eye piece, where it may be magnified again by another series of lenses, after which it will be viewed by the observer [120] [121].

While light microscopy can be useful on its own to view microscopic objects, there are certain limitations of the technique that limit its effectiveness in research. One such limitation is its limit on resolution. While light microscopes are great an magnifying an image, many are incapable of obtaining finer details in the image, making understanding what you are looking at more difficult. Furthermore, many objects appear similar in color, or are completely transparent when viewed under a microscope, making it difficult to differentiate what you are looking at, or making it impossible to locate a specific object/molecule in a sample. To help solve this problem, stains and fluorescent microscopy is frequently utilized [122].

Stains can be thought of like paints on a paint-by-numbers picture. They only go to specific places, and once they are there, they provide color to that region [123]. For example, if you are looking for the presence of a specific protein in a cell, you can stain the cell with a targeted stain that only will bind to the protein. If the protein is present in your sample, you will observe the presence of that color in the microscopic image. If the protein is not present, you will not see any color. This is a useful tool for visually identifying specific molecules in a sample. Similarly, fluorescent microscopy functions by binding molecules that emit light when they are excited by light of a specific wavelength (these molecules are called fluorophores) to the targeted molecule [124]. This is beneficial over light microscopy because it generally offers higher resolution and more control over appearance of the resulting image (specific colors to represent different molecules for example) [125].

When used in tandem, stains and fluorescent microscopy allow researchers to improve the resolution of their microscopic images, and makes the identification of specific molecules much easier. Collectively, microscopy offers researchers a unique glimpse into a microscopic world so that they can better understand what is occurring in these systems.

## 1.2 Nuclear Magnetic Resonance (NMR)

NMR is an extremely powerful tool for understanding the chemical composition of molecules, as well as their structure and organization [126]. NMR technology was invented in the mid-$20^{th}$ century, and functions on the general principals of quantum mechanics. Many atomic nuclei have some form of spin as well as an electronic charge [127]. During experimentation, an external magnetic field is applied to the system, which effectively increases the local energy in the molecules. This "elevates them" to a higher spin state, essentially meaning that they are more energetic than they would be normally. The atoms want to reach a more stable energy level however, and decay occurs where the energy the atoms were storing is released in the form of a radio wave [128]. That released energy can then be measured by the machine, and frequently is unique to specific atoms in specific orientations. As such, by studying the amount of energy released, and the intensity of the released energy, researchers are effectively able to determine the composition of the molecules in a sample, and are able to get a general understanding of the structure of the molecules [129].

There are many parameters that can be altered to obtain different results during NMR experimentation. Additionally, there are a vast number of alternative tests, which can be layered over each other (frequently called multidimensional NMR) [130] [131]. Understanding these techniques is beyond the scope of this paper, however one NMR experimental technique that is relevant to the context of this paper is two-dimensional rotating frame nuclear Overhauser effect spectroscopy (ROESY NMR). ROESY NMR is used frequently for determining the secondary structure of bio-molecules, such as proteins for example. It functions by determining the dipolar interaction of spins between protons. The correlation between two spin states is dependant on their distance from each other, so by measuring their correlation, this technique allows researchers to determine how far apart certain atoms are from each other [132]. ROESY is also particularly useful for measuring molecules with a lower overall molecular mass (note that NOESY, which functions very similarly to ROESY,

is used for larger molecular mass molecules) [133] [134].

Overall, NMR allows researchers to better understand the composition of the molecules they are working with. In addition, this technique provides researchers with an understanding of the structure and layout of bio-molecules, such as proteins, and can provide insights into how multiple molecules are interacting in solution.

## 1.3 Minimum Inhibitory Concentration (MIC) Assay

A minimum inhibitory concentration (MIC) assay is a medical/pharmacological technique used to determine the smallest amount of a bio-molecule needed to inhibit the growth of a microorganism [135]. This an extremely important for understanding if a particular molecule has any inhibitory effect on bacteria or other pathogens that have the potential for causing disease, and if so, how much of that molecule is needed to treat an infection [136]. MIC assays can be performed in a variety of ways, however the main technique used in this paper is a broth microdilution assay. As such, this section will predominately focus on outlining this technique.

MIC assays are generally performed in a welled-plate, which are essentially just plates with a large number of isolated cells where liquids can be kept for experimental analysis [137]. It is important to note that almost any pathogenic organism can be studied using an MIC assay, however the specific components that are used in each experiment (growth media, experimental molecules, etc.) will vary depending on what organism is being used. Each experimental well is loaded with growth media for the organism being studied, as well as increasingly lower concentrations of the inhibition compound being studied. The infectious organism (in the case of this study, bacteria) is then loaded at known concentrations into each well, as well as control wells. Finally, the bacteria are allowed to grow overnight, and the resulting growth is quantified using a technique such as optical density measurements (OD600) [138].

If the drug is functionally able to inhibit the growth of the pathogenic organism, you will see almost no change in the broth color or opacity. In contrast, if the concentration of the drug is too low or the drug is non-functional, you will see an increase in opacity as well as the presence of greyish, white contaminants in the broth [139]. This is an exceptionally powerful tool for determining the efficacy of drugs at treating infections from specific organisms. Furthermore, it allows researchers to better understand how much of a specific molecule is needed to treat infection, which prevents over or under dosing of medications.

## 1.4 Bacterial Growth Assay

A bacterial growth assay is a general term to refer to experiments that measure the growth potential of bacteria in various conditions [140] [141] [142]. In the context of this report, bacterial growth assays refer to experiments that measure the growth of bacteria in liquid solutions as well as on glass substrates. For liquid growth, procedures would be extremely similar to those used for MIC assays, with growth inhibition being measured via optical density. For substrate growth, inhibition can be visually determined by looking for the presence of bacterial colony growth on the surface of the substrate [143]. These experiments allow researchers to better understand what conditions are necessary for inhibiting or promoting the growth of infectious organisms.

Apart from determining the growth potential of infectious organisms in different environmental conditions, these experiments are also useful for measuring the adhesion potential of infectious organisms to treated surfaces [144]. As reviewed in the previous sections, infectious organisms have the potential to bind to surfaces, where they are subsequently able to form biofilms and cause infections. Preventing the adhesion of infectious agents to surfaces limits their ability to cause infection. Through experiments such as bacterial growth assays, researchers are able to determine if surfaces that have been treated with experimental compounds are capable of being colonized by the infectious agents [145].

Overall, these experiments represent various techniques used to measure the growth and adhesion potential of infectious agents on surfaces in different environments. By better understanding these factors, researchers are better able to determine how their experimental drugs functions, benefits of their drugs, and potential applications of their drugs and solutions.

# Part V

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