Bacterial Communication and its Role as a Target for Nanoparticle-Based Antimicrobial Therapy

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BACTERIAL COMMUNICATION AND ITS ROLE AS A TARGET FOR NANOPARTICLE-BASED ANTIMICROBIAL THERAPY

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DEDICATION

This dissertation is lovingly dedicated to my parents, Nancy and Jeffery Publicover. Their love, support, and years of encouragement have been invaluable in the completion of this degree. I must also dedicate this work to my brother, Daniel Publicover, for showing me how to find joy in one’s profession and for his love, support, and humor. Lastly, this dissertation is dedicated to my husband, Philip Miller, who has patiently supported and encouraged me throughout this process.
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ABSTRACT

The goal of this dissertation is to establish the environmental and public health importance of alternative forms of antimicrobial therapy, specifically those that utilize nanotechnology to combat quorum sensing-controlled bacterial infections. Quorum sensing (i.e. chemical communication) is an inherent characteristic that is essential to bacterial pathogenesis and biofilm formation (where most infections occur). A thorough review of the literature has been conducted to establish an understanding of the state of nanotechnology research as it relates to combatting bacterial infections. This synthesis, provided in Chapter 1, demonstrates how the chemical and structural designs of nanoparticles can be manipulated to specifically target bacterial infections.

Next, an investigation into the development and novel use of nanoparticles engineered to shut down bacterial quorum sensing is given in Chapter 2. Inhibiting the quorum sensing process is significant because it does not kill the bacteria, and therefore does not exacerbate antibiotic resistance. Briefly, the model system demonstrates that β-cyclodextrin functionalized nanoparticles are able to persist in the bacterial cell environment and quench extracellular bacterial communication molecules, and effectively silence bacterial communication. The system neutralizes communication through chelation of common signaling molecules called acyl-homoserine lactones. The new technology described here provides a seminal step in developing anti-virulence therapies that will not contribute to antibiotic resistance, and do not rely on traditional
antimicrobials. Also, this technology utilizes non-toxic nanoparticles that can be functionalized with biologically-active compounds and tailored to meet specific needs. This study provides a scaffold and critical stepping stone that will promote more-tailored future developments in nanoparticle-based antimicrobial therapy.

Chapter 3 provides insight into the environmental importance of bacterial communication, and the steps taken by bacteria to protect the valuable signal molecules. Briefly, environmental biofilms consist of extracellular polymeric substances with a high concentration of nonreducing sugars, such as trehalose. Previous studies have shown that trehalose is commonly utilized by soil bacteria during periods of drought to maintain membrane stability and preserve the structure of proteins. The study presented in Chapter 3 demonstrates that trehalose plays a role in protecting quorum sensing signals during desiccation through the formation of an extracellular glass. Additionally, the study provides a survey of the complexity of microbial ecosystems and the role that biofilm components play in the natural environment. Together, the three chapters of this dissertation demonstrate the importance of quorum sensing to bacteria and as a target for nanoparticle-based antimicrobial therapy.
# Table of Contents

DEDICATION ........................................................................................................ iii

ACKNOWLEDGEMENTS......................................................................................... iv

ABSTRACT ................................................................................................................ v

LIST OF TABLES .......................................................................................................... ix

LIST OF FIGURES ...................................................................................................... x

CHAPTER 1: ENGINEERED NANOPARTICLES TO ATTACK BACTERIA ......................... 1

1.1 NANOPARTICLES AS DRUG CARRIERS: A BRIEF HISTORICAL PERSPECTIVE ........... 2

1.2 THE ANTIBIOTIC DILEMMA: THE MODE OF DELIVERY COUNTS ......................... 3

1.3 DIRECT AND INDIRECT ANTIMICROBIAL PROPERTIES OF INORGANIC NANOPARTICLES .............................................................................................................. 15

1.4 LINKING ANTIBIOTICS TO INORGANIC NANOPARTICLES: CHALLENGES IN SURFACE CHEMISTRY DESIGN ......................................................................................... 25

1.5 OVERCOMING THE BACTERIAL BARRIERS OF INFECTIONS: NANOPARTICLES AS ANTIMICROBIAL DELIVERY VEHICLES .......................................................... 45

1.6 CONCLUSION ....................................................................................................... 53

CHAPTER 2: FUNCTIONALIZED NANOPARTICLES SILENCE BACTERIAL COMMUNICATION .... 61

2.1 CHARACTERIZATION OF MONOLAYER NANOPARTICLES .................................... 63

2.2 CHARACTERIZATION OF POLYMER NANOPARTICLES ........................................ 64

2.3 NMR DETECTS BINDING OF HSLS AND β-CYCLODEXTRIN ................................ 64

2.4 β-CYCLODEXTRIN QUENCHES HSLS IN VITRO ................................................ 67

2.5 TRANSCRIPTION OF THE LUX OPERON DURING NANOPARTICLE EXPOSURE ........ 69
CHAPTER 3: EXTRACELLULAR GLASS WITHIN THE EPS MATRIX IS A PROTECTIVE STRATEGY FOR BIOFILMS AGAINST DESICCATION .......................................................... 92

3.1 RESULTS ......................................................................................... 97
3.2 DISCUSSION .................................................................................. 106
3.3 CONCLUSION ................................................................................. 108
3.4 MATERIALS AND METHODS .......................................................... 109

REFERENCES ......................................................................................... 124
LIST OF TABLES

Table 1.1 Description of major endocytosis pathways .................................................. 54

Table 2.1 Measured and corrected diffusion coefficients ($D$) for various mixtures of C8-HSL and cyclodextrin. .............................................................. 78

Table 2.2 Binding strength of β-CD and C6-HSL or C8-HSL as determined by NMR.... 78

Table 2.3 Calculated fold change of transcription by $V. fischeri$ after treatment exposures to 125 nM 3OC6- and 0.25 nM C8-HSLs................................................................. 79

Table 2.4 Primer sequences used in qPCR ........................................................................ 79

Table 3.1 Composition of sugar monomers within EPS extracted from natural mats.... 113

Table 3.2 C6-AHL activity in trehalose as determined by pigmentation response of $C. violaceum$ CV026................................................................. 114

Table 3.3 Acylase activity in sugar after heat treatment.................................................. 114
LIST OF FIGURES

Figure 1.1 Diagram of cell walls of Gram-positive and Gram-negative bacteria...........54

Figure 1.2 Schematic illustration showing the loading and release mechanism of an
anionic drug in TA modified MSN.................................................................55

Figure 1.3 (a) Schematic illustration showing the controlled drug release of a pNIPAAm-
grafted Au nanocage; (b) Atom-transfer radical polymerization of NIPAAm and acrylic
amide monomers.........................................................................................56

Figure 1.4 Major endocytosis pathways .......................................................57

Figure 1.5 Schematic illustration of multidrug release by MSN-polymer
nanocomposite .............................................................................................57

Figure 1.6 Schematic illustration of the synthesis of DOX-associated Fe$_3$O$_4$ nanoparticles
coated with a PEG modified porous silica shell ...........................................58

Figure 1.7 Schematic illustration of the synthesis of core-shell drug delivery vehicle .....59

Figure 1.8 Schematic illustration showing the one-pot self-assembly strategy for
synthesis of drugs@micelles@MSNs.............................................................59

Figure 1.9 Schematic of biofilm encased in extracellular polymeric secretions ..........60

Figure 2.1 N-acyl homoserine lactone .............................................................80

Figure 2.2 β-cyclodextrin chemical (A) and toroidal (B) structure ......................80

Figure 2.3 Schematic representation of monolayer β-CD coated fluorescent silicon
dioxide nanoparticle.....................................................................................80

Figure 2.4 $^1$H NMR spectra of the as-synthesized β-CD coated silica nanoparticles.....81

Figure 2.5 TGA of (a) dye-labeled monolayer carboxylic acid coated silica nanoparticles;
(b) dye-labeled monolayer β-CD coated silica nanoparticles................................82

Figure 2.6 Schematic representation of a polymer grafted silica nanoparticle with β-CD
side group........................................................................................................83
Figure 2.7 TGA of (a) dye-labeled poly(methacrylic acid) grafted silica nanoparticles; (b) dye-labeled poly(β-CD) grafted silica nanoparticles

Figure 2.8 Photograph of dye-labeled poly(β-CD) grafted silica nanoparticles in DMSO

Figure 2.9 N-Acyl homoserine lactone molecules synthesized and recognized by *V. fischeri*

Figure 2.10 *V. fischeri* JB10 cultures in marine broth. Bioluminescence induced with 3OC6-HSL and C8-HSL

Figure 2.11 Maximum relative bioluminescence per OD (600 nm) of *V. fischeri* during exposure to β-CD and 2 μM 3OC6-HSL

Figure 2.12 Schematic of nanoparticle-based silencing of bacterial quorum sensing

Figure 2.13 Changes in bioluminescence by *V. fischeri* during exposures to 2 μM 3OC6-HSL, with either β-CD or β-CD functionalized Si-NPs

Figure 2.14 Maximum relative bioluminescence per OD (600 nm) of *V. fischeri* during exposure to 2 μM 3OC6-HSL and β-CD or β-CD functionalized 15 nm polymer nanoparticles

Figure 2.15 Mean relative bioluminescence per OD (600 nm) of *V. fischeri* during exposure to 125 nM 3OC6-HSL and 0.25 nM C8-HSL treated with 250 nM β-CD, bare 15 nm Si-NPs, 155 nM β-CD functionalized 15 nm Si-NPs, bare 50 nm Si-NPs, or 133 nM β-CD functionalized 50 nm Si-NPs

Figure 3.1 Natural microbial mats collected from Salt Pond on San Salvador Island, Bahamas showing wet- (A) and dry- (B, C) season sections of mat surface

Figure 3.2 SEM of dried surface layer of microbial mat

Figure 3.3 TEM of dry, natural microbial mats showing intact cells surrounded by a dense capsular layer of EPS

Figure 3.4 Vertical cross-section of microbial mat in Salt Pond showing distinct layering of microbial groups

Figure 3.5 Abundances of EPS isolated from surface layers of hypersaline mat in Salt Pond, San Salvador Island, Bahamas

Figure 3.6 DSC thermogram recorded upon heating of trehalose dihydrate at rate of 10°C/minute
Figure 3.7 DSC thermogram recorded upon heating of trehalose anhydrous at rate of 10°C/minute 

Figure 3.8 DSC thermogram recorded upon heating of Salt Pond EPS at rate of 10°C/minute 

Figure 3.9 TGA thermogram of trehalose dihydrate recorded upon heating at rate of 10°C/minute 

Figure 3.10 TGA thermogram of Salt Pond EPS recorded upon heating at rate of 10°C/minute 

Figure 3.11 Solid state NMR spectra of (a) untreated C6-AHL, (b) heat-treated C6-AHL with trehalose, (c) heat-treated trehalose, and (d) untreated trehalose 

xii
CHAPTER 1

ENGINEERED NANOPARTICLES TO ATTACK BACTERIA

The seminal realization of the nanotechnology, and its potential to be manipulated at molecular and atomic scales developed over a half century ago.\textsuperscript{1} However, largely due to a lack of technical capability, a time of obtuse contemplation followed. During this initial lull in nano-based research, the 1950s and 60s saw a surge of attention in the development of antibiotics – the wonder bullet cited to stop all bacterial disease. However, this phenomenon was quickly tempered by a rapid emergence of antibiotic resistance (AR) among pathogens, often in the form of multi-drug resistance (MDR). AR continues to grow today, and at present, the future utility of traditional antibiotics remains questionable.

The surge in nanoresearch, coupled with dramatic increases in technical capabilities, has allowed the disparate fields of nanochemistry and antibiotics to come together. Traditionally, nanoparticles and nanomaterials have been developed as carriers to deliver anticancer and other forms of drugs to eukaryote cells. Given this foundation, the delivery of antibiotics using engineered nanoparticles has become an emerging and realistic area of research. Overcoming the problem of antibiotic resistance, however, requires a more in-depth understanding of interactions between the biology of
microorganisms and the physical chemistry of nanoparticles. This understanding is necessary in order to ultimately target bacteria, and overcome their immense arsenal of defenses. This review centers on fundamental, RAFT-based and timed-release surface chemistry, which is currently being developed for nanoparticle delivery of antibiotics to bacteria. We also integrate approaches being developed to enhance the detection and quantification of bacteria within biological systems. Concurrent with this chemistry is a necessary overview of certain bacterial processes, as these directly and indirectly interact with the chemistry of nanoparticles.

1.1 NANOPARTICLES AS DRUG CARRIERS: A BRIEF HISTORICAL PERSPECTIVE

Credited for conceiving the idea of a “magic bullet” to selectively target toxic organisms in the body, Paul Ehrlich inspired many pioneers of the nanoparticle field. In the 1950s and 60s, Peter Speiser’s group worked on the development of polyacrylic beads, then microcapsules, and eventually the first nanocapsules. Their ultimate goal was to achieve sustained drug release from nanocapsules in the blood after intravenous injection.

Since that time, nanoparticles have been used for pharmaceutical and medical applications, mainly for cancer treatment and enhancing the efficacy and targeting of cancer drugs. This has allowed for the use of lower concentrations of highly-toxic drugs in an effort to reduce side effects. More recently, and in conjunction with the important discovery that polyethylene glycol chains on nanoparticles prolong blood circulation and reduce liver uptake, studies have been conducted on the ability of
nanoparticles to cross the blood brain barrier, and target deep brain tumors or infections.\textsuperscript{11-14} The innovative research of the past 50 years has reinforced the roles of nanoparticles as drug delivery vehicles and has inspired the current diversity in nanoparticle research. Today, investigators focus their nanoparticle research on recognition, sensing, imaging, and delivery in biological systems with a broad range of core materials.

1.2 THE ANTIBIOTIC DILEMMA: THE MODE OF DELIVERY COUNTS

Antibiotics are molecules produced by microorganisms to inhibit other microorganisms. Several major classes of antibiotics, based on their chemical structure and mechanisms of actions, are currently known. Since bacteria produce antibiotics, inherently they also possess mechanisms to resist their inhibitory actions, which can be passed on to nearby cells. Bacteria have also demonstrated resistance towards the naturally antimicrobial compounds used to construct NPs, such as silver and copper. This section addresses the molecular structure of antibiotics, and provides a basis for understanding their key activity sites on molecules, and how these relate to their inhibitory action(s). It sets a stage for why and how NPs can be used to enhance delivery and activities of antibiotics.

Chemistry of antibiotics: structure and design

Selman Waksman coined the term ‘antibiotic’ to describe any small molecule made by a microbe intended to antagonize the growth of another microbe.\textsuperscript{15} Waksman, along with Fleming, Chain, Florey, and many others, was a pioneer of the ‘golden age’ of
antibiotics. Although we have been aware of the healing nature of moldy foods for thousands of years, it was not until the 19th and 20th centuries that scientists began to understand that the provenance of antibiotics was related to the antagonism between two microorganisms. In 1928, British scientist Alexander Fleming witnessed the fungus *Penicillium notatum* produce a compound, that he named penicillin, which prevented the growth of the bacterium *Staphylococcus aureus*. By 1939, Florey and Chain manipulated *P. notatum* to produce large quantities of a stable form of penicillin. 16 The cooperation of scientists from Great Britain and the United States resulted in clinical trials and the mass production of penicillin during the final, crucial years of World War II. Also during this period, Waksman and colleagues isolated several antibiotics from filamentous, soil-dwelling bacteria of the genus *Actinomycetes*.

Since the groundbreaking work of the 1940s, nearly 25,000 natural, synthetic, and semisynthetic antimicrobials have been discovered. 20,000 of these are naturally occurring drugs produced by soil-dwelling fungi and bacteria. 17 Although there is an excess of available bioactive and toxic compounds, less than 1% are clinically useful. There are two major classes of antibiotics: bactericidal and bacteriostatic. Bactericidal antibiotics, like the β-lactam compounds penicillin and cephalosporin, kill bacteria. Bacteriostatic antibiotics, like tetracycline and its many derivatives, inhibit the proliferation of bacteria. Compounds that act on both Gram-positive and Gram-negative bacteria are classified as broad-spectrum antibiotics. Compounds that act on only a specific group of bacteria are narrow-spectrum antibiotics. The most studied and relied upon antibiotics have been repeatedly proven to be nontoxic to humans and highly-toxic
to pathogens. For the purpose of this review, we will briefly discuss the pharmacokinetic and pharmacodynamic properties of the major classes of antibiotics.

**Major classes of antibiotics and mechanisms of action**

Pharmacokinetics describes the effects of the body on the actions of a drug. The pharmacokinetics of an antibiotic dictates its absorption, distribution, metabolism, and elimination by the body. Non-ionized molecules are more lipid soluble than ionized molecules and can readily diffuse across the cell membrane. Since ionized molecules are less able to penetrate the lipid membrane; their entry depends on the leakiness of the cell membrane, as related to electrical resistance. The transmembrane distribution of a weak electrolyte is influenced by its pKa and the pH gradient across the membrane, where pKa is the pH at which half of the compound is in its ionized form. Antibiotics rely on passive transport (paracellular transport or diffusion) or active transport (facilitated diffusion or drug transporters) to cross cellular barriers. Due to the presence of a carboxyl group (essentially a proton donor), β-lactams are considered weak acids and do not readily cross the cell membrane (of mammalian or bacterial cells).18

To understand the pharmacokinetic transport of an antibiotic to a bacterial cell, it is important to briefly consider the fundamental structural differences between Gram-negative and Gram-positive bacteria (Figure 1.1).

Pharmacodynamics refers to the study of the biochemical and physiological effects of drugs and their mechanism of action. In the case of antibiotics, the barriers inflicted by human anatomy, the chemical properties of the antibiotic, and the presence of multidrug transporters among the many types of cells along this pathway influence the
efficacy of antimicrobial therapy. The penetration of physical barriers by antibiotics is directly related to the octanol-water partition coefficient ($K_{ow}$) of the antibiotic. Generally, hydrophobic drugs (with high $K_{ow}$s) become concentrated in the lipid membrane of cells, while hydrophilic drugs typically concentrate in aqueous areas such as the blood or cytosol. Additionally, the larger and more negatively charged an antibiotic is, the more difficult it is for that drug to penetrate cell membranes and physical barriers.\(^{20}\)

The extracellular concentration of an antibiotic directly affects the intracellular concentration in a concentration- and time-dependent fashion,\(^{21-24}\) depending on the drug and microorganism. An antibiotic intended to target an intracellular infection must cross the cell membrane and retain activity in the cytosol.\(^{25}\) The equilibrium of free and bound (to protein, stabilizing agent, serum, etc) antibiotics directly alters active transport, pH partitioning, and accumulation in the host, and therefore influences its ability to cross the cell membrane and target an intracellular infection.\(^{26}\) Additionally, the intracellular activity of antibiotics is largely influenced by the pH of the vacuoles and lysosomes in which it becomes localized, and the level of bacterial (\textit{i.e.} growth rate, presence of persister cells, and small colony variants\(^{27-29}\)) and cellular activity.\(^{25}\) Alternatively, the antibiotic oritavancin (\textit{i.e.} a semi-synthetic glycopeptide) is unaffected by pH, but shows decreased intracellular activity, likely due to binding with intra-lysosomal constituents.\(^{23}\)

\textbf{$\beta$-lactams}

To date, $\beta$-lactam compounds are the most important broad-spectrum antibiotics and the most commonly prescribed. The most well-known examples of the fungal-
derived drugs include penicillin and cephalosporin. Simply put, β-lactams bind irreversibly to enzymes intended to catalyze the transpeptidation of the peptidoglycan layer of the bacterial cell wall. β-lactams prevent proper reconstruction of a cell wall, eventually weakening and lysing the bacterial cell.\textsuperscript{30-31}

More specifically, β-lactams are a large class of drugs that primarily target the penicillin-binding proteins (PBPs) required for bacterial cell wall synthesis. This class is characterized by a highly reactive, four-membered carbonyl lactam ring containing azetidinone\textsuperscript{32} and includes penicillins, narrow- and extended-spectrum cephalosporins, monobactams and carbapenems.\textsuperscript{33} β-lactams target peptidoglycan biosynthesis by blocking the insertion of glycan units into the cell wall to inhibit cell wall development and by blocking transpeptidation linking and maturation.\textsuperscript{34} Peptidoglycan, found in both Gram-negative and Gram-positive bacteria, is an integral part of the cell wall that is required by bacteria to maintain the structural integrity of the cell and to survive changes in environmental osmotic pressure.\textsuperscript{35} β-lactams form a covalent complex with enzymes (the PBPs) that generate the mature peptidoglycan molecule. The β-lactam antibiotic, containing a cyclic amide ring, mimics the D-alanyl-D-alanine region of the PBP, forms a covalent acyl-enzyme complex with the activated serine of the PBP that inhibits the peptide bond formation reaction catalyzed by PBPs, and is mistakenly incorporated into the cell wall.\textsuperscript{30-31,36} This complex inhibits transpeptidation activity and weakens the integrity of the cell wall, eventually resulting in cell lysis.\textsuperscript{36} Specifically, the PBP inhibition occurs by penicilloylation of the active site, which blocks the hydrolysis of the bond created with the now open-ringed drug and disables the enzyme.\textsuperscript{32,37-38} A review by Kohanski and colleagues additionally explores alternative targets of β-lactam activity.
such as cell division, autolysin activity, the SOS response, the TCA cycle, Fe-S cluster synthesis, ROS formation, and envelope and redox-responsive two-component systems.\textsuperscript{32}

Although β-lactam antibiotics are highly effective against susceptible bacteria, they are most useful against extracellular infections within the body. Intracellular pathogens, such as \textit{Listeria monocytogenes} and \textit{Salmonella} species, are protected from the immune system by phagocytes and are less accessible to these antibiotics. Reports show poor accumulation of β-lactams in phagocytic and nonphagocytic cells, possibly due to the acidic conditions of the cytosol and the presence of multidrug efflux pumps in both human and bacterial cells.\textsuperscript{39,40} The free carboxylic group on β-lactams is essential to activity, and makes them a weak acid. Due to the acidic nature of the cytosol of phagocytic and nonphagocytic cells, β-lactams cross the membrane very slowly, if at all. The efficacy of β-lactams is dependent upon time of exposure above MIC, so slow penetration renders them less effective at targeting intracellular pathogens.\textsuperscript{41}

Resistance to β-lactams arises through mutations in PBPs,\textsuperscript{42} acquisition of new PBPs with lower affinity for the drug,\textsuperscript{43} production of β-lactamases that inactivate the drug by hydrolyzing the β-lactam ring,\textsuperscript{33,42,44-45} changes in cell wall porins that limit entry of the drug to the target site, and active efflux of the drug out of the cell by energy-dependent pumps.\textsuperscript{34} Bradford\textsuperscript{46} provides an in-depth review of the characterization and epidemiology of plasmid and transposon mediated β-lactamases. Information on β-lactamase inhibitors, compounds used concurrently with β-lactam antibiotics to fight resistant bacteria, can be found in papers by Drawz & Bonomo and Therrien & Levesque.\textsuperscript{33,47} A detailed overview of bacterial resistance to β-lactam antibiotics was published by Fisher and colleagues.\textsuperscript{48}
**Tetracyclines**

Tetracyclines are another group of medically-relevant antibiotics. They are bacteriostatic against a broad spectrum of Gram-positive and Gram-negative bacteria, and several protozoans. Tetracyclines act through interrupting protein synthesis by inhibiting the aminoacyl-tRNA from binding to the 30S ribosomal subunit of bacterial RNA. The antibacterial activity of tetracyclines is derived from the linear fused tetracyclic nucleus of six-membered carbocyclic rings, and their strong chelating ability.\(^\text{49}\)

The basic structure of the tetracycline group of antibiotics consists of a linear fused tetracyclic nucleus with a variety of constituents. These structural features confer antibacterial activity to the tetracyclines.\(^\text{49-52}\) Several structure-activity studies have demonstrated that the rings of tetracycline must contain six members and be purely carbocyclic to retain antibiotic activity.\(^\text{49}\) The review by Chopra\(^\text{49}\) has detailed the mode of action of tetracycline. Briefly, in Gram-negative bacteria, which have both an outer- and inner-membrane, tetracycline diffuses across the outer membrane through porin channels as a positively-charged metal complex. The metal ion-antibiotic complex accumulates in the periplasm where it dissociates, and the uncharged tetracycline diffuses through the lipid bilayer of the cytoplasmic (i.e. inner) membrane. At this point, tetracyclines bind to the small ribosomal subunit and prevent the association of aminoacyl-tRNA with the ribosome and inhibit protein synthesis. Tetracyclines are strong chelators and are likely to become chelated within the cytoplasm. Therefore, the complex that binds to the 16S rRNA likely contains magnesium. Association of tetracycline and the ribosome is reversible and may explain the bacteriostatic properties of the drug.\(^\text{49, 53-54}\)
Aminoglycosides

Aminoglycosides (AGAs) were one of the first discovered and clinically used antibiotics. Hence, they are also one of the most resisted by human pathogens. The structure of AGAs varies. In general, AGAs consist of an inositol derivative linked to an amino-sugar and a variety of free hydroxyl and amino groups, potentially containing further substituents. These free groups interact with the 30S subunit of ribosomal RNA and interfere with protein translation. More specifically, AGA binds three adenines present in the A-site of the 30S ribosomal subunit. Binding stabilizes the 30S subunit, which subsequently allows the noncognate tRNA to bind and initiate a misreading of the mRNA, followed by the synthesis of faulty proteins. It is unknown how the loss of translational fidelity leads to cell death, but it has been suggested that the use of faulty proteins in the inner membrane destabilizes the cell. Resistance against AGAs by bacteria is inferred by aminoglycosidic-modifying enzymes (acyltransferases, phosphotransferases, nucleotidyltransferases, methyltransferases), target modification by mutation, change of uptake and efflux, and membrane proteases. A recent review by Becker and Cooper goes into specific detail.

Macrolides

The macrolides are a class of natural and semisynthetic bacteriostatic antibiotics that are often used to treat respiratory, skin, and soft tissue infections. Macrolides contain a large lactone ring (12-16 atoms) attached to sugars with glycosidic bonds and substituted by hydroxyl or alkyl groups. Macrolides are subdivided into four classes based on the number of atoms in the lactone ring (12, 14, 15, and 16-membered rings).
Although the precise mechanism is unknown, macrolides bind to the 23s rRNA portion of the 50S ribosomal subunit to block protein synthesis.\textsuperscript{61} Kannan & Mankin\textsuperscript{62} provide a review of the binding and action of macrolide antibiotics in various microorganisms. They explain that macrolides bind in the nascent polypeptide exit tunnel of the large ribosomal subunit close to the peptidyl transferase center (PTC). In this position, macrolides inhibit the movement of proteins out of the ribosome and prevent translation elongation.\textsuperscript{61, 63-64} The macrolide establishes hydrophobic interactions with the rRNA residues belonging to domains II and V of the 23S rRNA. Crystallographic evidence has demonstrated that the macrolactone is positioned flat against the wall of the ribosome with the side chains protruding up towards the PTC active site and down the tunnel towards the exit. Although highly conserved, the slight variation in rRNA sequences among bacteria contributes to the spectrum of activity of macrolide antibiotics and indicates that the binding sites and modes of action may be species-specific.\textsuperscript{65} A brief review by Gamerdinger & Deuerling\textsuperscript{66} summarizes that inhibition of protein translation in the nascent peptide exit tunnel may be regulated by small metabolites and may play an important global role in cellular physiology. The inhibitory action of macrolides on the bacterial ribosomal tunnel is selective. Kannan \textit{et al.} found that macrolides allow selective synthesis of full-sized and truncated proteins in bacteria,\textsuperscript{67} an action that influences the cellular proteome and may enhance toxicity.

\textbf{Sulfonamides}

Sulfonamides are a structurally-related group of broad spectrum, bacteriostatic, synthetic antibiotics used for systemic application. Originally known as sulfa drugs,
compounds of this class of drugs contain a 4-aminobenzene sulfonamide backbone that inhibits the growth and reproduction of bacteria by competing with \textit{p}-aminobenzoic acid during folic acid synthesis.\textsuperscript{68} Sulfonamides are unique polar molecules with amphoteric properties. At pH 2-3, the amino nitrogen is protonated, while at pH 4.5-11, the amide nitrogen is deprotonated.\textsuperscript{69} Application of these drugs began in the early 1940s, but was quickly phased out due to frequent bacterial resistance. Because of widespread resistance, sulfonamides are rarely used alone. Currently, combination drugs containing both sulfonamides and diaminopyrimidines are used to treat both Gram-positive and Gram-negative infections.

A brief review of the mechanisms of action and resistance of sulfonamides are presented by Skold\textsuperscript{70}. Sulfonamides interrupt the essential folic acid synthesis pathway in bacteria by mimicking \textit{p}-aminobenzoic acid (\textit{p}ABA) and inhibiting dihydropteroate synthase. Often used in combination with sulfonamides, diaminopyrimidine next targets dihydrofolate synthase (DHPS), an essential enzyme of the folic acid synthesis pathway. DHPS catalyzes the condensation of \textit{p}-aminobenzoic acid and 7,8-dihydro-6-hydroxymethylpterin-pyrophosphate (DHPPP) to form dihydropteroic acid (the second to last step in the folic acid synthesis pathway). At this point, the sulfonamide is bound to DHPS. Crystallography shows that the sulfonamide is sandwiched between the main chain of Arg 200 and the side chain of Lys 221 on one side and the side chain of Arg 63 on the other of DHPS. The sulfonamide NH\textsubscript{2} donates a hydrogen bond to the carbonyl of Ser 219 and one sulfonamide oxygen accepts a hydrogen bond from the guanidinium of Arg 63 of DHPS.\textsuperscript{71} The competitive inhibition of DHPS by sulfonamides halts the folic
acid synthesis pathway and inhibits the growth of bacteria by eventually starving the cell of folic acid.

Bacteria exhibit resistance to sulfonamides through point mutations that affect binding of the inhibitor or through the acquisition of drug-insensitive enzymes that bypass the antibiotic-sensitive step of folic acid synthesis.\(^{34}\)

**Lipopeptides**

Lipopeptides are a class of drugs including both natural and semi-synthetic antibiotics that target bacterial cell wall synthesis in Gram-negative and Gram-positive species. Lipopeptides are used in a range of applications as antimicrobial, antitumor, immunosuppressant, and surfactant agents. The lipopeptides are a structurally diverse class of antibiotics that contain a short linear peptide or an oligopeptide of either net negative or positive charge. The peptide portion of the lipopeptide varies in length and amino acid composition, and is covalently attached to a fatty acid at the N-terminus of the lipid moiety. In general, lipopeptides target the bacterial cell membrane and are highly active against multi-drug resistant bacteria. Specifically, lipopeptides form pores in the bacterial cell membrane, which leads to an imbalance in transmembrane ion flux of K\(^+\), H\(^+\), and Ca\(^{2+}\), and cell death.\(^{72-73}\)

Lipopeptides, such as the anionic amphomycin and daptomycin, bind directly to the bacterial cell membrane where they interact with the lipid bilayer via electrostatic and hydrophobic interactions to cause rapid depolarization of the bacterial membrane potential.\(^{74-77}\) Upon interaction with the membrane, the peptide adopts a three dimensional structure and becomes amphiphilic, with the positively-charged side
interacting directly with the lipid head groups. The formation of a secondary structure is essential for the interaction of amphiphilic molecules with the bacterial cell membrane.\textsuperscript{78}

The anionic compound daptomycin is active against Gram-positive bacteria, and requires calcium to lock into an active conformation. The addition of calcium ions leads to oligomerization of daptomycin and induces the formation of micelle-like structures.\textsuperscript{78-79} The lipid tails point inward and the calcium ions help hold the charged side chains of daptomycin from different monomers together. Calcium is weakly bound (i.e., the system requires 1000 times more calcium than daptomycin) so the micelle can readily dissociate at the cell membrane surface.\textsuperscript{80} The initial binding event between the daptomycin aggregate and the bacterial membrane may be with the membrane lipid phosphatidyl glycerol.\textsuperscript{81} Daptomycin binds strongly to phosphatidyl glycerol head groups in the bacterial cell membrane.\textsuperscript{82} The large aggregates alter the membrane shape, leading to membrane leakage and eventual cell death. The daptomycin aggregates may also interfere with membrane-associated processes such as cell wall synthesis, cell division, and energetics.\textsuperscript{83} A detailed review of daptomycin in clinical microbiology is given by Humphries and colleagues.\textsuperscript{83}

**Glycopeptides and glycolipopeptides**

The glycopeptides and glycolipopeptides are a class of natural and semi-synthetic drugs derived from actinobacteria that target peptidoglycan synthesis in Gram-positive bacterial cell walls. The glycopeptides and glycolipopeptides act by binding to peptidoglycan units at the D-alanyl-D-alanine dipeptide terminus to block transglycosylase and PBP activity.\textsuperscript{84-85} These compounds act as steric inhibitors of
peptidoglycan maturation and reduce the mechanical strength of the cell. Some semi-synthetic glycopeptides have been shown to interact directly with transglycosylase.\textsuperscript{86} Kahne and colleagues have extensively reviewed the structure and function of glycopeptides and glycolipopeptides.\textsuperscript{84}

1.3 DIRECT AND INDIRECT ANTIMICROBIAL PROPERTIES OF INORGANIC NANOPARTICLES

Unique physical-chemical properties of inorganic nanoparticles have been utilized for hundreds of years. More recently, certain types of inorganic NPs have been found to exhibit strong antimicrobial properties. However, their applications as antimicrobial agents are limited by their apparent toxicity to other biological systems (e.g. human cells). This section examines major types of inorganic NPs, and discusses the chemistry of their toxic- and/or non-toxic properties and antimicrobial mechanisms of action. This section also provides an overview of the methods used to determine efficacy of inorganic nanoparticles (luminescent biosensors, generation of ROS, DNA damage, cell membrane integrity, electron microscopy, live/dead assays, Raman scattering, SDS-PAGE analysis of proteins, gene expression, etc.) in a bacterial system. Given the ability of bacteria to protect themselves from natural stresses and reach a ‘viable but nonculturable’ (VBNC) state, techniques alternative to traditional culturing are required to judge the antimicrobial properties of inorganic nanoparticles.
Silver nanoparticles

The antimicrobial nature of silver, and silver compounds, has been utilized for hundreds of years. The surge of antibiotics in the 1940s reduced the medicinal reliance on silver, but in recent years there has been a comeback in its uses for wound- and burn-dressings in a number of forms, including silver nanoparticles. Silver nanoparticles, in addition to silver ions, metallic silver, silver nitrate, silver sulfadiazine, and silver zeolite, typically encounter minimal bacterial resistance and possess strong bactericidal properties.\textsuperscript{87-90}

The antimicrobial effect of silver nanoparticles has been seen in Gram-positive bacteria, Gram-negative bacteria, and yeast.\textsuperscript{91-95} Silver nanoparticles are effective at preventing growth of bacteria on surfaces of agar plates, but are comparatively less-effective in liquid medium, due to aggregation of colloidal silver in the presence of high salts and other media components.\textsuperscript{94, 96-97} These studies suggest that the antimicrobial ability of silver nanoparticles is dependent upon surface oxidation (of the nanoparticle) and particle dispersion. The size and shape of silver nanoparticles also play a major role in their ability to interact with the bacterial surface and release silver ions (Ag\textsuperscript{+}) into solution. Given their enhanced surface area, smaller, spherical nanoparticles (1-10 nm) are more effective at attaching to the surface of a cell membrane and disturbing permeability and respiration than larger nanoparticles.\textsuperscript{96, 98-99} Thus, reducing the size and increasing the surface area of nanoparticles provides a greater number of reactive groups, Ag\textsuperscript{+} in this case, and is believed to enhance nanoparticle toxicity.\textsuperscript{100-102} Small nanoparticles penetrate the bacterial cell membrane and interact with the thiol groups of protein, preventing expression of ribosomal subunit proteins, deactivating the enzymes
and cellular components essential to ATP production, and by preventing DNA from replicating.

The mechanism of action of silver nanoparticles is not entirely understood, but current research has demonstrated that silver ions affect essential cellular components (cell membrane integrity, respiration, and ATP production). To date, there have been very few observations reported for microbial resistance against silver. Originally discovered in *Salmonella typhimurium*, and then later in *Pseudomonas aeruginosa, Candida albicans*, and the environmental isolate *Acinetobacter baumannii*, silver resistance has been the result of both intrinsic and acquired genes. The distinction between silver sensitivity and silver resistance is difficult to determine, however, because silver resistant phenotypes are not consistent. Silver resistance may be incurred by plasmids, specialized rapid-efflux pumps, or genetic mutations that are repaired after silver pressure has diminished.

Medical devices and wound dressings impregnated with silver nanoparticles have made a large impact on reducing infection in hospital settings; however, the toxicity of these compounds on humans is not fully understood. Cytotoxicity of silver nanoparticles on mammalian cells has been observed *in vitro*, demonstrating that silver nanoparticles may enhance the generation of reactive oxygen species and damage DNA. The potential for extensive DNA and cell damage is a precursor for carcinogenesis. Although the primary condition currently associated with silver is the cosmetic ailment known as argyria (irreversible bluish-gray discoloration of the skin), further research is required before relying on silver nanoparticles as an antimicrobial agent. A thorough
review of the effects of nanoparticles on the cell life cycle is provided by Mahmoudi et al.\textsuperscript{111}

**Titanium dioxide nanoparticles**

Titanium dioxide (TiO\textsubscript{2}) is a naturally-occurring compound that has been used extensively in cosmetics, sunscreen, and food additives because of its highly refractive qualities. Additionally, TiO\textsubscript{2} nanoparticles have been utilized for their antimicrobial properties. When photoactivated by UV or visible light, TiO\textsubscript{2} catalyzes the cleavage of water into hydrogen and oxygen, and produces reactive oxygen species (ROS) in solution.\textsuperscript{112} Currently, TiO\textsubscript{2} nanoparticles, which can be activated by weak UV light, are used in interior furnishings such as tiles and wallpaper in hospital rooms, air conditioning and purification units, wastewater/sewage purification systems, and pollution abatement strategies to reduce bacterial loadings.\textsuperscript{113}

Several studies have been performed to determine the antimicrobial efficiency of TiO\textsubscript{2} nanoparticles. After photoactivation, TiO\textsubscript{2} nanoparticles were highly toxic to *E. coli* and *P. aeruginosa*.\textsuperscript{114-117} The degree of toxicity was directly related to the cell wall of the microorganism, as *E. coli* (Gram-negative) was more susceptible than *S. aureus* and *E. faecalis* (Gram-positive), which were more susceptible than the fungi *C. albicans* and *A. niger*.\textsuperscript{118}

The mechanism of action responsible for killing microorganisms exposed to photoactivated TiO\textsubscript{2} is only partially understood. It has been theorized that the ROS generated through photoactivation are responsible for the antimicrobial efficacy of TiO\textsubscript{2}.\textsuperscript{119} ROS are able to damage the cell membrane and disrupt essential membrane-
bound proteins,\textsuperscript{120} in addition to creating single stranded or double stranded breaks in DNA, rendering it unable to be replicated.\textsuperscript{121-122} The antimicrobial activity of ROS varies between environments and experiments; therefore the exact mechanism of action is not fully understood nor agreed upon.\textsuperscript{123} Macrophages of mammalian immune systems rely heavily upon ROS to eliminate pathogens from the body. Therefore, microorganisms are exposed to ROS often, and have inherent defense mechanisms in place for protection. For example, \textit{Salmonella typhimurium} sequesters iron ions to protect their DNA from ROS, in addition to the expression of a Type III secretion system that prevents the host cell from reducing hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) to the more-deadly superoxide anion O\textsubscript{2} - .\textsuperscript{124, 125} Additionally, \textit{Salmonella enterica} possesses several antioxidant enzymes that detoxify ROS.\textsuperscript{126, 127} The inherent nature of photocatalysis and the creation of ROS limit the antimicrobial efficacy of titanium dioxide nanoparticles to aerobically grown organisms in water, air, or on surfaces.

\textbf{Gold nanoparticles}

Colloidal gold has been used by scientists and medical practitioners for well over a thousand years. In the present day, due to its exceptional ligand binding ability, spectroscopic detection, high contrast in electron microscopy, and general stability, gold nanoparticles are widely used in biological and chemical systems. Extensive reviews on the syntheses and applications of gold nanoparticles have been published.\textsuperscript{128-130}

Gold nanoparticles are readily taken up by host immune cells, thus enhancing their ability to deliver drugs to intracellular microbial pathogens.\textsuperscript{131} The exceptional binding of gold nanoparticles allows higher concentrations of complexed drugs to be
delivered to an affected area without releasing high levels of free, toxic drugs into the broader system. Gold nanoparticles lack the inherent antimicrobial effects noted for silver and titanium dioxide nanoparticles, therefore the simple presence of gold nanoparticles in solution with antibiotics is not enough to enhance the efficacy of antibiotics. However, antibiotics, when conjugated to gold nanoparticles confer an increased and more targeted local concentration (of antibiotics) and help destroy microorganisms more efficiently than antibiotics alone, while reducing levels of toxic drugs in the system. For example, conjugation of ampicillin, streptomycin, and kanamycin to gold nanoparticles decreased their minimum inhibitory concentrations (MIC) to the bacteria *Escherichia coli*, *Staphylococcus aureus*, and *Micrococcus luteus*. In addition to enhancing the efficacy of these drugs, conjugating the antibiotics to gold nanoparticles also made these drugs more stable and heat tolerant.

Conjugating antibodies to gold nanoparticles can allow for the targeting of specific microorganisms. Pissuwan *et al.* demonstrated that when antibodies were conjugated to gold nanoparticles, they selectively targeted and destroyed the parasitic protozoan *Toxoplasma gondii* after plasmonic heating. The *in vitro* experiment with antibody-conjugated gold nanoparticles killed approximately 80% of the parasite, whereas the antibody or gold nanoparticles alone did not kill a significant number of protozoa. Norman and colleagues used a similar approach to target the a multi-drug resistant strain of *Pseudomonas aeruginosa*. In their experiment, near-infrared irradiation was used to photothermally heat gold nanoparticles attached to *P. aeruginosa*, resulting in membrane disruption and 78% cell death. Additionally, vancomycin conjugated gold nanoparticles demonstrated activity against vancomycin-resistant
Enterococci, vancomycin-sensitive strains of Enterococci and other Gram-negative bacteria.

Carbon nanotubes

Carbon nanotubes are single-wall (SWNT) or multiwall (MWNT) cylinders of graphene, valued for their unique size- and structure-dependent properties. Due to their strength and thermal and electrical conductivity, carbon nanotubes have a wide range of commercial applications. The synthesis and use of commercial carbon nanotubes has been thoroughly reviewed. Recently, the biological toxicity of carbon nanotubes has become a pressing issue as humans and the environment come into more frequent contact with the nanomaterials. Studies have defined the major mechanisms of toxicity to eukaryote cells to be oxidative stress, agglomeration, physical interactions, nutrient sequestering, shading effects, and metal catalyst residues. The potential for stress to humans and the environment indicates that other living organisms, such as bacteria and fungi may also be susceptible to carbon nanotubes. Currently, the exact mechanism of toxicity of SWNT towards bacteria is unknown; however, several studies have confirmed that membrane damage is a common result of SWNT treatment. The role of oxidative stress is still debated.

One of the antibacterial first studies, conducted in 2007, showed that direct contact of carbon nanotubes with E. coli outer membranes caused irrecoverable damage and cellular lysis. Since then, multiple reports have confirmed that SWNT induce membrane damage and oxidative stress. A major challenge in using carbon nanotubes as antimicrobial agents is the widespread and broad toxicity of the nanomaterials.
Modified SWNT have also demonstrated exceptional antibacterial activity. Vecitis and colleagues showed a significant loss in *E. coli* viability due to both membrane damage and oxidative stress with an increasing fraction of metallic SWNT. They suggest that the electronic structure of SWNT contributed to oxidative stress, while the physical structure damaged cell membranes. The difficulty in utilizing SWNT lies in their ability to nonspecifically damage many cells. However, functionalized SWNT have demonstrated lower toxicity to human cells than pure SWNT, while maintaining antibacterial activity. To this point, a recent study has shown that surface functionalized SWNT have enhanced functionality against microbes (as demonstrated by loss of cell wall integrity) with decreased cytotoxicity of human cells. The study showed that aggregation and dispersity of the SWNT were the major contributors to toxicity. Similarly, SWNT/PLGA composites have been shown to possess antimicrobial activity against *E. coli* and *Staphylococcus epidermidis* upon contact. Contact-driven toxicity may be related to the ability of the tube ends to puncture cell membranes, as short SWNT had a high density of uncapped tube ends and were found to be more toxic than longer or capped SWNT. The role of SWNT/PLGA composites is significant in the development of films for biomedical implants that do not promote bacterial growth. The ability of SWNT to inhibit biofilm growth, which is often seen on biomedical implants, has been demonstrated in both Gram-negative and Gram-positive bacteria.

**Iron oxide nanoparticles**

Iron oxide nanoparticles with a diameter size of 50-100 nm were first applied in magnetic resonance imaging more than 20 years ago. Magnetic nanoparticles with a
diameter of 10 nm were used in cancer therapy due to the hyperthermia effect. For drug delivery applications, magnetic nanoparticles have been utilized to carry doxorubicin, human serum albumin, and cottonseed oil to for cancer treatment. In addition, surface functionalization was conducted on magnetic nanoparticles with ligands and polymers to load drugs. Recently, magnetic SiO$_2$ nanoparticles were used to kill pathogenic bacteria. As drug delivery vehicles, magnetic nanoparticles possess the unique advantage of guiding the nanoparticles to a desired location and keeping them localized at the site using an external magnet.

**Porous nanoparticles**

The future of nanomedicine and drug delivery rests upon the ability of nanoparticles to complex a high concentration of drugs, release those drugs in a controlled and timely manner, control breakdown of the drug-nanoparticle matrix, have easily manipulated surfaces, and be detectable *in vivo*. Porous nanoparticles, such as silicon, iron (III) carboxylate, and manganese oxide, are ideal carriers for drugs having the previously listed capabilities. Silica nanoparticles have been widely applied as carriers for delivery of enzymes, antibiotics, and DNA. The biocompatibility of silica nanoparticles makes them an especially ideal carrier for applications associated with the human body. As an important class of silica materials, mesoporous silicas have attracted huge interest since their initial synthesis by Mobil Corporation in 1992. Mesoporous silicas have been widely utilized in the fields of catalysis and biomedicine because of their uniquely large surface area, controllable particle size and pore size, uniform pore structure, and easy surface functionalization. Although not inherently antimicrobial, these
nanoparticles have the potential to be designed to carry antibiotics to multidrug resistant infections. Currently, researchers are focusing on utilizing porous nanoparticles to deliver anticancer drugs into tumors with incredible accuracy\textsuperscript{184-185}. Given the scope of the field of porous nanoparticles and drug delivery, further details will not be discussed here.

**Methods to determine efficacy of inorganic nanoparticles on bacteria**

Given the ability of bacteria to protect themselves from natural stresses and reach a ‘viable but nonculturable’ state, techniques alternative to traditional culturing are required to judge the antimicrobial properties of inorganic nanoparticles. Bacterial cell culture techniques are frequently used in conjunction with other methods, such as luminescent biosensors, detection of reactive oxygen species, cell membrane integrity determination, and electron microscopy.

For example, the toxicity of silver nanoparticles on the growth rate of *E. coli* is often determined by UV spectroscopy (OD 600 nm) of bacterial cultures.\textsuperscript{97} Many other studies rely on traditional agar plates to count colony forming units during or after nanoparticle exposure. As an alternative and additive to these methods, bioluminescent bacteria such as *Vibrio fischeri* and genetically modified *E. coli* have been used in nanoparticle ecotoxicological studies. The *V. fischeri* bioluminescent “Flash Assay” is useful for screening highly turbid nanomaterials that would otherwise confound UV spectroscopy measurements.\textsuperscript{186-189} In addition to bacterial biosensors that indicate the overall toxicity of inorganic nanoparticles, bacterial biosensors have also been developed to detect the presence of inorganic nanoparticles. Blinova and colleagues presented a study using zinc and copper sensitive strains of bioluminescent *E. coli*. In this study, the
bioluminescence of the genetically modified *E. coli* increased proportionally with the increase in available zinc or copper.\(^1\)\(^9\) As an alternative to biosensors, the physical appearance of bacteria during nanoparticle exposure has also been used to determine toxicity.

Using electron microscopy, the membrane organization of bacterial cells can be visualized to determine if the nanoparticle treatment induced cellular lysis or cell wall or cell membrane damage.\(^97,\)\(^136,\)\(^191\) Less powerful microscopic techniques are also used to determine cell viability after nanoparticle exposure. Bacterial membrane integrity can be assayed with a fluorescent LIVE/DEAD stain (Syto9 and propidium iodide), and visualized via confocal scanning laser microscopy.\(^136,\)\(^183,\)\(^192\) In this assay, cells with intact membranes take up Syto9, while cells with disrupted membranes (and considered dead) are stained with propidium iodide. Fluorescence stains can also be utilized to detect the burst of free radicals and reactive oxygen species from bacteria during nanoparticle-induced death. The oxidation of 2', 7'-dichlorofluorescin-diacetate (DCFH-DA) can be used to quantitatively determine the formation of ROS by bacteria under a microscope.\(^193^-^195\) These alternative techniques to growth studies are important in their ability to determine cell viability without relying on cell culture techniques.

### 1.4 LINKING ANTIBIOTICS TO INORGANIC NANOPARTICLES:

**CHALLENGES IN SURFACE CHEMISTRY DESIGN**

The application of nanoparticles as drug delivery vehicles has attracted great attention in past decades. Nanoparticles possess unique properties, such as monodistribution of nanoparticle size, and thermal and magnetic properties.\(^196\) In addition,
owing to their unique physicochemical properties when compared to larger particles, provides the potential for nanoparticles to penetrate and reach areas of bio-membrane systems where dissolved molecules reach less-effectively. In the present section, we will explore how nanoparticle-based drug delivery vehicles can improve the solubility, pharmacokinetics and stability of free drugs.\textsuperscript{197}

**Surface functionalization**

Surface functionalization of nanoparticles is of great interest because of their potential applications in chemosensors, coatings, organic light-emitting devices (OLEDs) and biomedical engineering.\textsuperscript{198} In the biomedical field, surface functionalization plays a critical role in tailoring the properties of nanoparticles for enhanced binding capabilities for therapeutic delivery,\textsuperscript{199} selective recognition within biological systems,\textsuperscript{200} and improved cellular internalization.\textsuperscript{199}

**Charged moieties**

Positive charges, negative charges, and zwitterionic moieties have been functionalized onto nanoparticle surfaces. Cationic compounds have been considered important candidates for antimicrobial agents throughout the past twenty years. Among them, quaternary ammonium (QA)\textsuperscript{201} and phosphonium-based compounds\textsuperscript{202-203} are two main forms of these agents. QA compounds are the most important and commonly-used cationic agents used to kill bacteria. Dong and coworkers modified magnetic nanoparticles with poly(quaternary ammonium) (PQA) to kill \textit{E. coli}, which retained a 100\% biocidal efficiency over eight-cycles of usage of the nanoparticles.\textsuperscript{204} Tiller and
coworkers functionalized glass slides with poly(4-vinyl-N-alkylpyridinium bromide) to kill airborne bacteria on contact. The antimicrobial properties of QA compounds are likely ascribed to their interactions with bacterial cell membranes, which subsequently results in disruption of the membranes. Carmona-Ribeiro reviewed the specific functions of cationic materials when interacting with bacterial cell membranes and summarized several general steps for disruption of cell membranes. We refer interested readers to the literature for detailed information in this field.

QA compounds can also be used as drug delivery vehicles to load and release antibiotics. Lee et al. have demonstrated that MSN can be functionalized with surface positive charges to deliver an anionic anti-inflammatory drug, sulfasalazine, with controllable loading, and release by changing pH value. The positive charge surface was synthesized by a condensation reaction between trimethylammonium (TA)-silane and tetraethoxysilane (TEOS) of MSN. Sulfasalazine was loaded into the nanoparticle and remained in the framework of MSN under acidic conditions. It was then released by electrostatic repulsion from the gradually-formed negative surface charges that developed under neutral conditions (Figure 1.2).

Surface functionalization with negatively-charged compounds has also been widely investigated for antimicrobial or other biomedical applications. It is reported that positively charged nanoparticles demonstrate higher internalization in eukaryote cells, while negatively charged nanoparticles are taken up by diffusion. Surface attached anionic compounds can be employed as drug delivery vehicles to kill bacteria. Riffle and coworkers modified Fe$_3$O$_4$ nanoparticles with block copolymers PEO-$b$-PAA. The unattached segments of PAA provide thousands of anionic carboxylates which was used
to conjugate cationic aminoglycoside antibiotics via ionic complexation for therapeutic applications. The delivery vehicles can also be used to deliver moieties such as metal ions. Anionic poly(3-sulfopropyl- methacrylate) brushes were prepared on Si/SiO$_2$ surfaces and employed to complex silver ions inside the brushes. The surface-attached silver-containing brushes inhibited the growth of both Gram-negative and Gram-positive bacteria.

Zwitterionic materials (also called inner salts) with one pair or multiple pairs of positive and negative charges in their structures have also been anchored on a variety of surfaces. Surface attached zwitterionic materials were shown to be resistant to bacterial adhesion and biofilm formation. However, most of the applications of these surface attached zwitterionic moieties are still used in the antifouling field. These anchored zwitterionic materials are found to highly-resist to protein adsorption. The two main zwitterionic materials are based on sulfobetaine (SB) and carboxybetaine (CB). Thus, SB based sulfobetaine methacrylate and CB based carboxybetaine methacrylate materials have been widely investigated as antifouling materials.

Surface functionalization using different charge moieties can be characterized by a zeta potential test. This test reveals the surface electrical potential, which can be used to analyze their stability in solution. Generally, nanoparticles have been demonstrated to exhibit a stable dispersion in solution when the zeta potential is above ±30 mV. It is well known that the surface charge can inhibit aggregation of nanoparticles, thus surface modifications that introduce appropriate amounts of charges are an effective method to store nanoparticle suspensions.
Ligands and polymers

Relatively small molecules represent an operational class of materials that have been widely used to modify the surfaces of nanoparticles. They provide several advantages, such as low molecular weights, easy coordination onto nanoparticles, and easy processing conditions. Compared to macromolecules, the relatively smaller size of these molecules makes surface functionalizations with multiple ligands much easier. In the last section, charged small-molecule compounds were reviewed for surface modification. Thus, this section will only cover non-charged small-molecule ligands.

A wide range of small molecule ligands (SMLs) have been coated onto nanoparticles for applications in biosensing, diagnosis, and drug delivery. Those ligands can alter the nanoparticle’s stability, hydrophobic/hydrophilic properties, zeta potential, cytotoxicity, and the interactions with cells.

Small molecules provide a repulsive layer on particle surfaces, which can enhance the stability of nanoparticles in suspension and minimize nanoparticle aggregations. Two factors should be considered while choosing SMLs for nanoparticle stabilization: 1) the substrate particles, and 2) the dispersion solvent. Generally, silane SMLs are used to modify SiO$_2$ nanoparticles, thio SMLs are suitable to coat Au-nanoparticles and phosphate-based SMLs can be employed to functionalize iron oxide and TiO$_2$ nanoparticles. De and coworkers have summarized the surface functionalization of a variety of nanoparticles with corresponding SMLs. In all of the nanoparticle surface functionalizations, SMLs were bound to surfaces via chemical absorption or physical absorption (hydrophobic/ hydrophilic interactions). The dispersion solvents consisted of
organic solvents and water. Choosing appropriate solvents with a polarity close to that of the dispersion solvent is a necessary step while modifying particles.

Ligand exchange is a significant method to enhance the stability of nanoparticles in certain solvents. In this process, strongly bound ligands are typically used to replace weakly bound molecules to pursue firm surface attachment. For example, oleic acid, a significant and commonly used ligand for stabilizing metal oxide nanoparticles, is a weakly bound molecule and is generally exchanged with phosphate- and silane-based ligands\(^{235-237}\) for firmer attachment. Schadler \textit{et al.} reported using a phosphate-azide ligand to replace oleic acid on TiO\(_2\) and ITO surfaces,\(^{238}\) followed by further functionalization via “click chemistry” on the new ligand.

Generally, oleic acid or oleylamine are added to stabilize magnetic iron oxide nanoparticles in the preparation process. However, it limits the surface functionalization of particles and reduces the dispersion of the particles in hydrophilic media. Thus, ligand exchange is necessary for further applications of magnetic iron oxide nanoparticles. Bronstein and co-workers used N-(6-aminohexyl)-aminopropyltrimethoxysilane to replace oleic acid on iron oxide nanoparticles to stabilize the particles.\(^{239}\) Binder and co-workers employed 1,2-diols bearing \(\omega\)-azido or \(\omega\)-bromo ligands to replace octylamine or oleic acid on \(\gamma\)-Fe\(_2\)O\(_3\) nanoparticles followed by post-functionalization of the new ligand to obtain fluorescent properties.\(^{240}\) Sun and coworkers replaced oleylamine via ligand exchange to convert the nanoparticles from hydrophobic to hydrophilic for develop a stable dispersion in an aqueous environment.\(^{241}\) Hatton \textit{et al.} replaced oleic acid with various hydroxyl group containing ligands followed by post-functionalization for surface-initiated ATRP polymerizations.\(^{242}\)
Ligand exchange is also an important tool for surface modification of nanocrystals. Murray and co-workers used nitrosonium tetrafluoroborate to replace oleic acid or oleylamine on nanocrystals to stabilize the nanocrystals in various hydrophilic solvents and made the ligand exchange reversible.\textsuperscript{243} Talapin \textit{et al.} used metal chalcogenide complexes to exchange ligands on nanocrystals, an exchange that resulted in a hydrophilic property.\textsuperscript{244-245} In addition, surface functionalization of nanocrystals via ligand exchange has been widely explored in a variety of environments to obtain new properties.\textsuperscript{244-252}

Surface coating of polymers on particles impart new properties to the surfaces.\textsuperscript{253-254} The coatings can be used to further manipulate the nanoparticle’s stability in suspensions, the hydrophobic and hydrophilic properties, cytotoxicity, biocompatibility, and even interactions with cells. It also provides an additional platform to control the antibiotic loading and release on nanoparticles in drug delivery systems. In this section, a variety of common surface-modified polymers that were developed in recent years will be reviewed based on the class of the polymer.

Polymers containing cationic moieties have been coated onto surfaces to kill bacteria.\textsuperscript{255-257} The mechanism of the antimicrobial effect of cationic polymers has been discussed in the previous section. Generally, this class of polymer processes alkyl pyridinium or quaternary ammonium groups.\textsuperscript{258} Many quaternary ammonium based cationic polymers are prepared based on 2-dimethylaminoethyl methacrylate (DMAEMA).\textsuperscript{258-261} The surface charge densities (usually greater than $10^{15}$ groups/cm$^2$) of PDMAEMA brushes determine the effect of killing bacteria. The higher charge densities of the surface attached polymers, the better antimicrobial activities.\textsuperscript{260} Most PDMAEMA-
based QA compounds are prepared via the quaternization with alkyl halide, but viologen-
quaternized PDMAEMA demonstrated significantly increased antimicrobial activity
compared to alkyl halide-quaternized QA compounds due to the enhanced cationic
charged densities. Other quaternary ammonium based cationic polymer materials have
been developed in recent years. As a renewable material, rosin based polymers containing
multiple quaternary ammonium compounds have been developed to kill bacteria.
Alkyl pyridinium based polymers are usually prepared based on 4-vinyl pyridine.
Quaternized poly (vinylpyridine) brushes were coated on glass surfaces to kill Gram-
positive and negative bacteria with an effective charge density from $10^{12}$ to $10^{16}$
groups/cm$^2$. Klibanov and coworkers have developed surface-immobilized N-hexyl-
poly(vinylpyridine), N-Hexyl,N-methyl-polyethyleneimine (PEI), N-dodecyl,N-methyl-
PEI permanently microbicidal materials.

Poly(ethylene glycol) (PEG), a hydrophilic polymer, has been used to enhance the
water solubility of materials, and as such, is a significant polymeric material that is
widely-used in bioapplications owing to its unique properties of exceptional
biocompatibility and non-toxicity. Surface-coating with PEG can act as antifouling
materials, as it prevents protein absorption and minimizes cell attachment. Surface-
anchored PEGs have also been reported to prevent protein absorption, enhancing
circulation time, improving tumor targeting, and increasing stabilization in salt
solution.

Temperature-responsive poly(N-isopropylacrylamide) (pNIPAAm) is a unique
polymer that alters its conformation predictably in response to different temperature
ranges. Surface anchored PNIPAAm shrinks and generates pores on nanoparticles
allowing entrapped antibiotics and biomolecules to be released when the temperature is raised above the low critical solution temperature (LCST). The polymer swells and closes the surface pores to inhibit release when the temperature is below the LCST. These properties can be used to control drug release by adjusting the temperature. PNIPAAm has a LCST around 32 °C in water, which is perfect for broad applications of drug delivery and bio-separations. Yavuz et al. reported the controlled drug release of pNIPAAm-grafted Au-nanocage by adjusting the near-infrared laser to generate heat. The monomers NIPAAm and acrylic amide were polymerized by atom-transfer radical polymerization (ATRP) using a disulfide initiator (Figure 1.3). PDMAEMA is also a significant temperature-responsive polymer with a LCST around 45 °C in aqueous solutions while at pH 8.5. However, the PDMAEMA polymer is also pH-responsive due to the presence of multiple amino groups in its structures. Thus, the LCST is closely-associated with pH with a LCST of >50°C at pH 7.0 and no LCST at lower (i.e., acidic) pH.

Since pH responsive polymers usually contain ionizable groups in their structures, they can be protonated and deprotonated under different pH conditions. Generally, there are two classes of pH responsive polymers, namely acidic- and basic-polyelectrolyte (i.e., polyacids and polybases, respectively). Representatives of polyacids are poly(acrylic acid) (PAA) and poly(methacrylic acid) (PMAA). Both contain multiple carboxylic acids, which can be used to chemically- or physically-bind small molecules. Using this approach, they have been coated on nanoparticles to kill bacteria as antibiotic delivery materials. The representative of polybase is PDMAEMA. The main applications of PDMAEMA are preparing antimicrobial materials via quaternization.
However, it has also been immobilized on various surfaces for biomedical applications due to the pH responsive surfaces.\textsuperscript{219, 286-287}

**Polymer preparation by controlled radical polymerization**

Generally, surface functionalization of nanoparticles with polymers has been achieved using two methods, namely, “grafting from” and “grafting to” techniques.\textsuperscript{288} The “grafting from” technique provides a higher surface modification density than “grafting to” strategy due to the gradually increasing steric hindrance from already-grafted polymer chains that occurs in the “grafting to” technique. Controlled/Living radical polymerization (C/LRP) has been employed in the “grafting from” technique due to its powerful application in the synthesis of well-defined and advanced structure polymers, such as block copolymers, branch polymers, and star-shape polymers. Among all the C/LRP techniques, nitroxide-mediated polymerization (NMP),\textsuperscript{289} atom-transfer radical polymerization (ATRP),\textsuperscript{290-291} and reversible addition-fragmentation chain transfer polymerization (RAFT)\textsuperscript{292} have been excessively applied in the complex polymer structure synthesis.\textsuperscript{293-298} Surface functionalization of nanoparticles via controlled radical polymerization generates controlled chain length and polydispersity of polymers on surfaces.

The first report, to our knowledge, of surface functionalization using ATRP was in 1997 by Wirth and co-workers.\textsuperscript{299} Acrylamide was polymerized via ATRP on benzyl chloride-attached silica surfaces. Then, Matyjaszewski and co-workers significantly expanded grafting polymers from surfaces via ATRP.\textsuperscript{291, 300-301} The first report of surface functionalization using NMP was in 1999 by Hawker and Russell on silicon wafers.\textsuperscript{302}
Since the invention of RAFT in 1998, it was first used to modify surfaces by Tsuji et al. in 2001. They prepared the RAFT agent in-situ by conversion of a surface-supported ATRP initiator followed by surface-initiated RAFT polymerization of styrene. Brittain and co-workers employed azoundecylchlorosilane as an anchor-initiator to commence RAFT polymerization on silica particles. Brittain et al. also employed “click” reaction to anchor RAFT agents on silica particles to mediate the polymerization of styrene and methyl methacrylate. Benicewicz and colleagues employed a RAFT agent 4-cyanopentanoic acid dithiobenzoate (CPDB) to prepare a wide range of polymers from silica nanoparticle surfaces with a variety of graft densities of 0.01 - 0.68 chains/nm². Thus, CRP methods have been tremendously important techniques for the “grafting from” method to prepare polymer grafted nanoparticles.

CRP has also been widely applied in preparing a variety of free polymers, which are further used in “grafting to” strategies. Both “grafting to” and “grafting from” strategies have been demonstrated as effective methods to graft polymer brushes on surfaces, and have been reviewed by Benicewicz, Brittain, and Matyjaszewski. Normally, NMP require high reaction temperatures and ATRP generates residue copper or other metal after polymerization, which is extremely difficult to be completely removed. Thus, both of NMP and ATRP have not been widely applied on nanoparticle surfaces for biomedical applications. RAFT, generally employing mild reaction conditions without residue metal issues after polymerization, is adaptable to a variety of functional monomers. Due to the advantage of the RAFT technique, it has been widely applied for the surface functionalization of nanoparticles with lactose and peptides, and to deliver therapeutic agents and siRNA.
Controlling specificity in targeting bacterial cells

In recent years, and with increasing effort to understand the human microbiome (i.e. the bacterial communities inhabiting humans), it is now realized that the majority of bacteria inhabiting humans are non-pathogenic (i.e. commensal), and may even play essential roles in health.\(^{320}\) Most antibiotics, however, target general bacterial processes (e.g. cell wall formation, protein translation, DNA replication, etc. See Section 2), so even relatively ‘narrow-spectrum’ antibiotics have lethal effects on many commensal forms; which in turn, are thought to negatively affect human health. Therefore, a major challenge in the future development of antibiotics will be in designing approaches that target only the pathogenic (i.e. infection-causing) forms, while leaving the majority of commensal bacteria intact.

One approach for targeting specific bacteria has been the use of antibodies. Antibodies are produced by the immune response of a mouse, rabbit, or rat,\(^ {321}\) and can be raised against specific proteins (antigens) located on the surface of the bacterium. The antibody, after purification, is typically conjugated to a fluorophore for detection, and then used to identify (via its fluorescence) specific bacteria with the antigen signature amongst a plethora of other species/strains in a complex mixture.

Nanoparticles, when coupled with antibodies, have been used as carrier vehicles for highly-sensitive biodetection of specific bacterial pathogens.\(^ {322-324}\) NP-based approaches allow single-cell detection because a single NP contains many fluorophore molecules. Efficient and sensitive detection of bacteria is necessary for maintaining food quality, and in environmental and biomedical applications. Traditional detection techniques, such as viable colony counting\(^ {325}\) and polymerase chain reaction (PCR) to
detect pathogen genes, impose a high cost and are time expensive. Comparably, nanoparticles provide an excellent platform for sensitive and efficient detection because nanoparticle surfaces can be functionalized and quickly modified with specific antibodies to recognize biomarkers on bacteria.

The relatively large size (ca. 150 kDa) of many antibodies limits their ability to disperse under many biological conditions, especially when conjugated to nanoparticles. Antibodies are Y-shaped glycoprotein molecules, and consist of both a framework (FR) and hypervariable (HV) region. The tail of the HV region provides the antibody with specificity for the binding antigen (i.e. the binding domain). A small section of this tail (containing the binding domain) has been used to facilitate the development of 13 kDa single-domain antibodies (sdAbs) that can be conjugated to quantum dots (QD) (<12 nm dia.). The sdAbs can be conjugated to QDs in a highly-oriented manner to enhance binding efficiency and be used as ultra-small diagnostic nanoprobes. In general, antibody conjugated-nanoparticles have been used for highly sensitive detection of specific species within a complex mixture of bacteria.

Antibody conjugated-nanoparticles can also be designed as multifunctional platforms for targeted bacterial detection and destruction. When nanoparticles are equipped with magnetic properties (as discussed above), then conjugated with antibodies, they can be used for detection and/or separation of specific bacteria from a mixture of species. For example, antibody conjugated-magnetic nanoparticles, can separate the bacteria *E. coli* and *Salmonella typhimurium*, and allows a detection limit of $10^4 - 10^5$ cells/mL. Magnetic nanoparticles have also been applied as sensors to enhance interactions between vancomycin coatings on nanoparticles and D-alanyl-D-alanine in...
bacterial cells, with a corresponding detection limit of $10^3$ cells/mL. The antimicrobial enzyme lysostaphin was adsorbed on the surface of antibody conjugated-nanoparticles specific to the bacterium *Staphylococcus aureus*. Lysostaphin hydrolyzes the peptidoglycan linkages in the cell wall to lyse and kill the bacterium.

The unique size effect and emerging surface functionalization toolboxes make nanoparticles an excellent platform to efficiently and sensitively detect bacteria. As discussed earlier in this review (see Section 4.1), the surfaces of nanoparticles can be engineered with RAFTs at finely-controlled densities to tailor the accessibility of the conjugated moieties on the RAFTs. In this way, several different antibiotics can potentially be delivered by a single nanoparticle. This approach offers an exciting potential to increase the specificity of an antibiotic attack and to reduce destruction of helpful bacteria by antibiotics.

Finally, in order for nanoparticle-based approaches to operate efficiently, the nanoparticle-carrier must either penetrate the bacterial cell (as discussed below), or release its cargo at the cell surface. Approaches for the controlled release of conjugated molecules from nanoparticles has been developed using both pH-sensitive and infrared-sensitive ligands.

**Entry of nanoparticles into the cell**

Certain antibiotics, owing to their mechanism of action, require entry into the cytoplasm in order to kill or inhibit bacterial cells. Together, the cell membrane(s) and cell wall provide a protective barrier that restricts the movement of molecules and ions into and out of the cell, as well as maintains the structural stability of the cell. As
mentioned earlier, the outer membrane (OM) forms the outermost boundary with the extracellular environment of Gram-negative bacteria, while the cell wall accomplishes this in Gram-positive species (Figure 1.1). The OM and inner (plasma) membranes (IM) selectively allow smaller molecules (<600 Da) to diffuse through the lipid bilayer while preventing macromolecules larger than 1 kDa\textsuperscript{336} from permeating the membrane without active transport.

Many studies have shown that the interaction of some metallic nanoparticles with bacteria results in cell lysis (and death).\textsuperscript{188,337-338} In these studies, the nanoparticles used were relatively toxic and included ZnO, CuO, Ag, TiO\textsubscript{2}, and Al\textsubscript{2}O\textsubscript{3}. In some cases, nanoparticles were observed within lysed cells, suggesting that the nanoparticles were taken up by cells and cell death occurred either during or shortly after uptake.

It is now realized, however, that bacteria may also take up very small particles into their cellular cytoplasm, without apparent lethal effects. For example, quantum dots (<10 nm diameter) were shown to be taken up via a purine-dependent mechanism.\textsuperscript{339} Also, 10 nm diameter gold colloids have been shown to enter viable cells during the uptake of large proteins.\textsuperscript{340} Although examples exist, particle uptake by bacteria is contrary to our current understanding, and is not well understood. Very few studies have noted either direct or indirect uptake.

In order for non-lethal particle uptake to occur, the process will likely require active transport, and the ability of the bacterium to hydrolyze a small portion of the cell wall, and then reform the wall without significant loss of intracellular contents. The interior of a bacterial cell, however, has a high turgor pressure relative to the immediate outside environment. Successful entry into a bacterium without cell death presupposes
that the particle can cross through the cell membrane(s) and cell wall without lysis and significant loss of intracellular contents. How this might occur is currently unknown.

Some insight can be gained from the examination of the uptake of macromolecules into larger eukaryote cells.\textsuperscript{341-342} This process, called endocytosis, utilizes membrane vesicles to shuttle extracellular materials into the cell. There are two methods of endocytosis, classified as phagocytosis or pinocytosis.\textsuperscript{343-344} Pinocytosis includes macropinocytosis, clathrin-mediated endocytosis, and caveolae-mediated endocytosis, as shown in the Figure 1.4 and Table 1.1.\textsuperscript{345-346} These endocytosis pathways differ from each in the nature of the internalized materials, the size of vesicles, the type of cells, and the kinetics of the uptake process.\textsuperscript{345} However, endocytotic-like uptake by bacteria has only been shown in one case,\textsuperscript{340} and is not well understood at present.

Nanoparticles provide an excellent carrier to transport drugs\textsuperscript{347} and siRNA\textsuperscript{348} to the cytosol by endocytosis in eukaryote cells. Without the nanoparticle carrier, these molecules would not be able to diffuse through the lipid bilayer.

The interaction between nanoparticles and the cell membrane can be influenced by the size, shape, surface charges, and surface functional groups of nanoparticles. For example, Chithrani \textit{et al.} reported that 50-nm Au nanoparticles were more efficiently taken up by eukaryotic HeLa cells than 14-nm and 74-nm size Au nanoparticles.\textsuperscript{347-348} Spherical nanoparticles\textsuperscript{346} were reported to be internalized 500\% more than rod-shape nanoparticles in eukaryotes. The nanoparticles size and shape have been reported to influence the binding of receptors on the cell membrane.\textsuperscript{349} The surface coating on nanoparticles can be designed to inhibit protein absorption and minimize nonspecific interactions with cell membranes. For example, neutral ligands such as poly(ethylene
glycol) (PEG) and zwitterions have been used to reduce inefficient targeting and potential agglomeration of nanoparticles at the cell surface. Nanoparticles with surface negative charge moieties have been reported to have poor interactions with the cell membrane, which leads to very limited internalization. In contrast, cationic nanoparticles have been widely demonstrated to bind with negative moieties on cell membranes and thus facilitate the movement of substrates across the lipid bilayer. The mechanism of interaction between cationic nanoparticles and cell membranes was confirmed by AFM investigation, and showed that the positive moieties destabilize the membrane and cause the formation of pores in defective areas of the membrane. Thus, in eukaryote cells, the size, shape, surface charges, and surface functional groups of nanoparticles can influence the interaction between nanoparticles and the cell membrane. These factors will prove important when targeting intracellular bacterial pathogens.

**Drug loading**

In order to address drug delivery approaches to bacteria, it is useful to examine the techniques used to engineer nanoparticles for drug delivery. Generally, the two basic approaches used to attach drugs to nanoparticles include covalent and non-covalent binding. Both approaches possess specific advantages and disadvantages. Covalent binding offers a steady delivery platform, but usually requires pre-treatment of drugs. For example, Cheng and colleagues reported the synthesis of phthalocyanine-based photodynamic therapy (PDT) using drugs conjugated to gold nanoparticles. The drug release and PDT efficacy are affected by the chemical bond between the drug molecules and the Au surface. Specifically, the labile amino adsorption triggers the drug release into
HeLa cancer cells. In another study, Gu and co-workers reported the synthesis and improved activity of vancomycin-conjugated Au nanoparticles against vancomycin-resistant Enterococci (VRE) and Gram-negative bacteria. Covalent binding between drugs and nanoparticles provides a secure method of binding that lessens drug pre-release or leakage. Covalent binding also allows for the drug release speed to be controlled by adjusting the breakage rate of the covalent bond. Usually, slow release drug delivery strategies employ the covalent binding method.

In non-covalent binding strategies, potential drug pre-release or leakage can be avoided by using stimulus functionalities on the nanoparticle shell to block or cover the drug releasing pores. Baeza and co-workers reported the preparation of poly(ethyleneimine)-b-poly(N-isopropylacrylamide) (PEI/NIPAM) coated mesoporous silica nanoparticles (MSN) with encapsulated iron oxide nanocrystals to deliver multiple drugs simultaneously (Figure 1.5). The grafted block copolymer was designed to retain the drugs inside the NP through temperature-responsive control and by attaching proteins into the grafted polymer shell via intermolecular interactions. The use of specific functionalities to block nanoparticle pores is an efficient method to prevent drug pre-leakage. Non-covalent binding provides a straightforward method for drug delivery and decreases the risk of drug pre-leakage.

In addition to drug entrapment, other barriers have been designed to minimize drug pre-leakage in non-covalently bound nanoparticles. Chen et al. reported a new magnetic drug delivery system that utilizes doxorubicin (DOX)-associated Fe₃O₄ nanoparticles coated with a polyethylene glycol (PEG) modified porous silica shell (Fe₃O₄-DOX/pSiO₂–PEG) to treat tumors. The DOX-conjugated Fe₃O₄ nanoparticles
were embedded in a silica shell and coated with PEG chains (Figure 1.6). After etching the thick silica shell, the Fe₃O₄-DOX/pSiO₂–PEG was 150 nm in diameter. The porous silica shell presents a physical obstacle that decreases the dissociation rate of DOX from the nanoparticle core.

Like covalent binding, non-covalent binding provides a direct loading and release mechanism. However, drug pre-release or non-specific leakage is frequently noted. To counteract this problem, Mortera and co-workers prepared a mesoporous silica nanoparticle-based cage-like vehicle to deliver cysteine intracellularly.¹⁷⁸ The cysteine was encapsulated within the nanoparticle and only released when triggered by intracellular antioxidants. Similarly, Adeli et al. prepared a hybrid nanostructure with a gold core and a polyrotaxane shell.³⁵⁹ Both cisplatin and DOX were associated with the hybrid nanoparticles, and drug release controlled by photothermal explosion. Similarly, Rotello and co-workers reported the synthesis of monolayer-functionalized gold nanoparticles with a hydrophobic alkane thiol core and a hydrophilic shell.¹⁸⁵ The hydrophilic shell consisted of a tetra (ethylene glycol) (TEG) component, end-capped with a zwitterionic group. Hydrophobic drugs were entrapped in the hydrophobic region of the nanoparticle surface monolayer via nonspecific binding and released into cancer cells by membrane-induced diffusion.

**Controlled drug release**

A variety of release strategies of nanoparticle drug delivery vehicles have been reported, and are controlled by pH, light, temperature, enzymes, or magnetic fields.¹⁷⁹, ¹⁸⁵, ³⁵⁷, ³⁶⁰-³⁶³ The drug release strategies are directly dependent on the drug binding methods.
For those carriers with covalent binding between nanoparticles and drugs, a low pH solution will hydrolyze the covalent bond. For example, Zhang and co-workers prepared doxorubicin conjugated Fe₃O₄ encapsulated in thermo-responsive dextran-g-poly(N-isopropylacrylamide-co-N,N-dimethylacrylamide) and that conjugated to a 3-mercaptopropionic acid hydrazide-functionalized nanoparticle via an acid-labile hydrazone bond (Figure 1.7). At a temperature above the lower critical solution temperature (LCST), drug release was controlled in a mild buffer solution. Alternatively, UV (256 nm) and visible (530 nm) light was used to switch the confirmation of the polypeptide backbone of diarylethene-containing cyclic peptidomimics. Enhanced antimicrobial activity was seen with the ‘open’ form of the compound, and completely lost in the ‘closed’ form.

Carriers that are pH-responsive also provide a controlled drug release for non-covalently bound nanoparticle and drugs. He and co-workers reported a one-pot self-assembly strategy to prepare a MSN-based drug delivery vehicle whose release is stimulated by pH change (Figure 1.8).

Magnetic field, temperature, and light were used to stimulate the drug release from their corresponding vehicles. McGill and co-workers reported that magnetic nanoparticles can disrupt a viscous extracellular biopolymer under a low oscillating magnetic field (OMF) of 295-kHz and enhance the delivery of lambda DNA/HindIII. The OMF induced thermal activity of the nanoparticle can enhance its transportation in a viscous environment, as well as induce the breakage of DNA to trigger drug release. Urbina and co-workers prepared magnetite–poly(methylmethacrylate) (PMMA) and cobalt–PMMA nanoparticles with fluorescein isothiocyanate (FITC), and tested drug
release with magnetic and thermal control. Ren and co-workers reported the modification of Au (shell) - Au$_2$S (core) nanoparticle surface with 11-mercaptoundecanoic acid (MUA) to load the anti-tumor drug *cis*-platin. The loading of drug onto Au - Au$_2$S nanoparticles was pH-dependent and the drug release was triggered by near-infrared irradiation, which is not harmful to human tissues.

1.5 OVERCOMING THE BACTERIAL BARRIERS OF INFECTIONS:
NANOPARTICLES AS ANTIMICROBIAL DELIVERY VEHICLES

Using NPs as antimicrobial delivery vehicles (ADVs) offers an extraordinary potential to control bacterial infections. Most bacterial infections occur as attached *biofilms*, where cells are embedded within a protective matrix of extracellular polymers (EPS) that are secreted by the bacteria. Nanoparticles have shown a strong ability to complex to biofilms, and more specifically the EPS matrix of biofilms, both in natural environments and under laboratory conditions. In order to treat biofilm-based infections with NPs, their physical and chemical properties, primarily size and surface chemistry, must be designed to overcome several major hurdles within a biofilm. First, NPs must be able to penetrate the EPS matrix in order to reach and destroy the cells enveloped within a biofilm. The NP size will limit its ability to diffuse through the EPS, while the NP surface properties will dictate the amount of interaction between the EPS polymers and the NPs. Therefore, in order to inhibit a biofilm infection, the corona surrounding a NP must be tailored to facilitate diffusion through the biofilm EPS to resident bacterial cells. Although there is little research in this area, it has been noted that diffusion of NPs through biofilms is affected by the viscosity of the EPS, the
variability of cell density, the bulk fluid flow of water, and the external mass transfer resistance (stagnant liquid layer on surface of biofilm that slows penetration of solutes) in three dimensions. However, the movement of nanoparticles through EPS is a poorly understood phenomenon. This section will discuss how NPs are impacted by these factors, as related to bacterial biofilms.

The biofilm state: a primary hurdle for infection control

Many bacteria are now realized to occur as attached structured communities called biofilms, where cells are surrounded by a secreted matrix of extracellular polymeric substances (EPS). Biofilms have been recognized to play a major role in human health over the past 50 years, as the occurrence of bacterial infections has shifted from acute infections, such as cholera and diphtheria, to chronic infections, such as MRSA and *Pseudomonas aeruginosa*. Chronic bacterial infections are generally found in the biofilm state, allowing them to evade the immune system and persist through antibiotic treatment. Such biofilm-based diseases are common and include infectious kidney stones, bacterial endocarditis, cystic fibrosis airway infections, periodontitis, and indwelling medical device infections. A major difficulty in treating chronic bacterial infections lies in the biofilm matrix (i.e. EPS) in which the bacteria reside. Antibiotics that are able to eliminate planktonic forms of bacteria may require 100- to 1000-fold higher concentrations to defy the heterogeneous complexity of a biofilm matrix, and even then the infection often will persist. Biofilm-associated cells possess a number of special adaptations, collectively called ‘insurance effects’, which serve to enhance their survival and persistence against stressors such as antibiotics.
As seen in Figure 1.9, the biofilm matrix provides nutrients, protection, and hydration to a dense community of bacteria. A significant amount of research has been devoted to understanding the role and development of the biofilm matrix (for a thorough review, see Flemming & Wingender). In response to environmental conditions, the integrity and function of the biofilm changes to ensure survival and persistence of the bacteria. The complexity of the matrix is due to its many components, which include proteins, lipids, polysaccharides, glycoproteins, glycolipids, membrane vesicles, nucleic acids, ions, and, of greatest abundance, water. EPS also contains many sorption sites for potential interactions with nanoparticles and charged molecules. The plasticity of the biofilm matrix allows it to fulfill the roles of protector, electron donor/acceptor, anchor, and retainer of nutrients, enzymes, energy, and genetic material under dynamic conditions. A recent study has suggested that the EPS of the biofilm matrix contributes to an osmotic gradient in the matrix and drives biofilm spreading. In addition to a complex composition, biofilms also exhibit heterogeneity in bacterial species and gene expression.

While occasionally biofilms can consist of a clonal population of one bacterial species, they most often occur as a population of many, diverse bacterial species. Under both circumstances, gene expression varies throughout the biofilm in response to environmental conditions, resulting in localized areas of specific cell activity. For example, the bacterium *P. aeruginosa* modifies gene expression to control alginate production and motility in response to biofilm aggregation. Even within the EPS itself there is much heterogeneity in its physical structure owing to the existence of microdomains, which may contribute to diffusive transfer. Specific extracellular
polysaccharides (e.g. PsI) are now related to attachment and biofilm formation in *P. aeruginosa*.\(^{392-393}\) Similarly, *Staphylococcus aureus* has been shown to regulate key structural biofilm components via gene regulation throughout biofilm development.\(^{394}\)

The ability to display multiple spatiotemporal phenotypes throughout biofilm formation is a survival technique used by bacteria to colonize surfaces and establish an infection. The microenvironment created by the gradient of matrix components throughout the biofilm allows bacterial cells to thrive and persist under adverse conditions once the biofilm has been established. An excellent review of biofilm initiation and development is provided by Hall-Stoodley *et al.*\(^{370}\)

**Challenges for inhibition of biofilms using engineered nanoparticles**

It is reasonable to assume that diffusion is a requirement for substantial biofilm growth (i.e. how else can nutrients (to cells) and wastes (from cells) move within a biofilm?). Using time-lapsed confocal scanning laser microscopy, the time of penetration for a 10 kDa molecule to reach the center of a cell cluster was estimated to be 3 minutes.\(^{395}\) The inherent complexity of a bacterial biofilm makes it easy to underestimate nanoparticle mobility and difficult to standardize diffusion coefficients. Diffusion models must consider the 1) viscosity of the EPS, 2) the variability of cell density, 3) the bulk fluid flow of water, 4) interactions of the solute with the EPS (*i.e.* diffusion reaction constraints), 5) the sizes (and volumes) of water spaces between EPS molecules, and 6) the external mass transfer resistance (stagnant liquid on top layer of biofilm that slows penetration of solutes) in three dimensions.
The EPS matrix hurdle: a physical barrier to nanoparticle diffusion

Both simple and complex models have been developed to determine the rate at which molecules and/or nanoparticles of a particular size can permeate through and diffuse within a biofilm of a particular viscosity and composition. The rapidly developing field of nanotechnology offers an alternative method to treating biofilm infections, and functionalized nanoparticles have the potential to efficiently deliver antimicrobial compounds to microbial cells within a biofilm.

Diffusion through the EPS matrix is a complex process. Currently, it is thought that small molecules diffuse through both pure water and EPS at approximately the same rate. The EPS matrix consists of mostly free water that is immobilized within pore spaces between a framework of polymeric molecules. Given that small molecules and nanoparticles often move freely through the water, the limiting factor in their movement through biofilms is directly related to interactions with the EPS matrix polymers. Many diffusion rates of small molecules have been measured in various types of biofilms with values ranging from 0.9 to 0.2, when compared with diffusion through pure water. Using our knowledge of small molecules in biofilms, the movement of nanoparticles in biofilms can be studied.

Currently, a key criterion in the design of nanoparticles intended to penetrate a bacterial biofilm is the size and charge of the nanoparticle. Recent studies have shown that nanoparticles are able to diffuse through a biofilm; however, the rate of diffusion has been directly related to the size of the nanoparticle. As such, a final hurdle involves the design of nanoparticle size and surface properties that will facilitate their penetration into a biofilm matrix.
Several publications have suggested that the connectivity and size of pores within a biofilm influences the speed and penetration of nanoparticles. Hindrances such as a porous biofilm, local accumulation of nanoparticles on bacterial cells and large macromolecules, the nonspecific adsorption of nanoparticles to freely diffusing species, abiotic particles, and gas all play a role in preventing efficient diffusion of nanoparticles. Studies have been conducted to show that larger nanoparticles diffuse slower through biofilms because they get trapped in pores, cell aggregates, and the general biofilm matrix more than their smaller counterparts. Peulen et al. demonstrated that 57 nm, 92 nm, and 135 nm nanoparticles had little success with penetration in a dense biofilm. They concluded that the effective pore size in loose biofilms is approximately 50 nm, which translates to efficient diffusion for nanoparticles near 10 nm in diameter. Hidalgo et al. showed that nanoparticles of up to 70 nm could penetrate a bacterial biofilm; however, they also showed that nanoparticles measuring less than 30 nm were most effective at homogenously filtering through the matrix. Steric hindrance by the biofilm matrix does not play a major role in nanoparticle diffusion, and therefore does not influence the rate at which nanoparticles diffuse through a biofilm. Currently, a key criterion for designing nanoparticles that will effectively penetrate and target bacterial cells within a heterogeneous biofilm will be size manipulation.

**Enhanced antibiotic resistance in biofilms: physiological and genetic**

The phenotypic, genotypic, and physical complexities of a bacterial biofilm present many challenges to modern medicine. In general, bacterial antibiotic resistance
occurs as a consequence of genetic mutation, acquisition of antibiotic resistance genes, and/or horizontal gene transfer. Antibiotic resistance and antibiotic resistance genes have been present in bacterial genomes throughout their evolution. A study from 2004 showed that serine β-lactamase (an enzyme capable of inactivating β-lactam antibiotics) genes originated nearly 2 billion years ago, and have been present on plasmids for millions of years. Most antibiotic producing organisms carry genes that encode antibiotic resistance to ensure self-protection, such genes are usually found in the same gene cluster as the antibiotic synthesis genes. A thorough review of the presence and role of antibiotic resistance genes in natural environments is provided by Allen et al.

In addition to genomic manipulations, mechanisms of antibiotic resistance within a biofilm are also related to restricted antibiotic penetration, decreased bacterial growth rates and metabolism, quorum sensing and induction of a biofilm-specific phenotype, induction of stress response genes, and an increase in the expression of efflux pumps. These mechanisms are referred to as non-inherited resistance, and are intrinsic phenotypic characteristics of bacteria in a biofilm or structural obstructions of the biofilm matrix. A detailed analysis of antibiotic resistance in biofilms has been reviewed by Høiby et al.

Kirby et al. developed a method to assess the contribution of the physical structure to the phenotypic resistance of biofilms, without genetic or chemical methods that could be confounded by pleiotropic effects. Their study demonstrated that during high cell densities, planktonic cultures exhibited similar levels of antibiotic resistance as biofilm cells; however, the cells released from biofilms were individually more
susceptible to antibiotics that target the cell membrane components (colistin) or depend on membrane function for uptake (gentamicin, streptomycin) than their planktonic counterparts. They suggest that the cell membrane of biofilm bacteria may be more sensitive to antibiotics than the cell membranes of planktonic bacteria. Their results indicate the both cell membrane physiology and the structure of the EPS matrix plays a role in antibiotic resistance within biofilms.

The diverse nature of biofilms also enhances resistance to antibiotics, as seen when mixed biofilms of *Candida albicans* and *S. aureus* more effectively resist vancomycin than either as mono-biofilms or mono-planktonic cultures. Social interactions are essential for successful multicellular complexity. The ability of bacteria to interact within a biofilm allows the cells to maintain a more-stable environment that confers antibiotic resistance via horizontal gene transfer, the sharing of common resources, and the regulation of core sets of genes. The physiological heterogeneity of a bacterial biofilm makes it extremely pliant and adaptable, and incredibly difficult to combat and eliminate. However, this complexity also offers a variety of targets for researchers to exploit.

A recent review by Yang *et al.* discussed current approaches that are used to eliminate bacterial biofilms. The review focused on biochemical approaches such as antimicrobial agents and peptides, physiochemical approaches such as modifying industrial surfaces with anti-adhesive and microbicidal agents, biological approaches such as inhibiting biofilm formation with the use of natural products found in mixed culture biofilms, and approaches that directly interfere with structural development and differentiation within a biofilm such as eliminating EPS production or inhibiting the
ability of bacteria to socially interact via quorum sensing inhibition. In an effort to combine these many approaches, the development of functionalized nanoparticles has been investigated.

While the formulation of nanoparticles is well established and continues to be improved upon, their applications to target bacteria and the exact mechanisms of action are less well understood. A recent study by Decho, Benicewicz, and colleagues found that when complexed to SiO$_2$ nanoparticles, the common antibiotic penicillin-G is effective in killing penicillin-resistant strains of bacterial pathogens, including strains of methicillin resistant \textit{Staphylococcus aureus} (MRSA), at low total concentrations. The authors termed this effect the ‘grenade hypothesis’ and postulated that each nanoparticle delivers a concentrated package of antibiotic to a given cells, perhaps overwhelming its resistance mechanism (\textit{e.g.} beta-lactamase enzymes), which are normally used in defense against the antibiotic. While this hypothesis remains to be confirmed, the use of nanoparticles as antibiotic-delivery vehicles (ADVs) is an emerging area of exploration.

\textbf{1.6 CONCLUSION}

Nanoparticle-based delivery of antibiotics is being proposed, and developed, as a highly-efficient means to deliver antibiotics and target bacterial infections. It offers the possibility to deliver high concentrations of antibiotics, and thus overwhelm bacterial antibiotic resistance strategies. The future efficacy of nanoparticle-based delivery of antibiotics rests upon the ability of nanoparticles to localize a high-concentration of drugs, reach a pre-determined target with high specificity and efficiency, and release those drugs in a controlled and timely manner without toxic effects to \textit{in vivo} host cells.
Table 1.1 Description of major endocytosis pathways. Reprinted with permission.

<table>
<thead>
<tr>
<th>Type of endocytosis</th>
<th>Brief description</th>
<th>Size of vesicle formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Phagocytosis</td>
<td>Internalization of solid particles such as bacteria and yeast by specialized cells.</td>
<td>Dependent on the particles being engulfed. (Below)</td>
</tr>
<tr>
<td>2) Pinocytosis</td>
<td>Fluid-phase uptake of extracellular molecules. Multiple pinocytic pathways are possible (below).</td>
<td>&gt;1 μm</td>
</tr>
<tr>
<td>3) Macropinocytosis</td>
<td>Trapping of large fluid pockets by formation and enclosure of membrane protrusions.</td>
<td>≈120 nm</td>
</tr>
<tr>
<td>4) Clathrin-mediated endocytosis</td>
<td>Concentration of transmembrane receptors and bound ligands in “coated pits” on the plasma membrane formed by the assembly of cytosolic proteins, the main assembly unit being clathrin.</td>
<td>≈50–60 nm</td>
</tr>
<tr>
<td>5) Caveolae-mediated endocytosis</td>
<td>Flask-shaped invaginations in the plasma membrane that mediate uptake of extracellular molecules into the cell by specific receptor binding.</td>
<td></td>
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</tbody>
</table>

Figure 1.1 Diagram of cell walls of Gram-positive and Gram-negative bacteria. Download for free at http://cnx.org/contents/9e7c7540-5794-4c31-917d-fce7e50ea6dd@11@11.
Figure 1.2 A schematic illustration showing the loading and release mechanism of an anionic drug in TA modified MSN (a) Before the drug is adsorbed, (b) after drug adsorption, (c) electrostatic repulsion triggering drug release, and (d) ion-exchange triggered drug release. Reprinted with permission.
Figure 1.3 (a) A schematic illustration showing the controlled drug release of a pNIPAAm-grafted Au nanocage by adjusting a near-infrared laser to generate heat and release the entrapped contents; (b) Atom-transfer radical polymerization (ATRP) of NIPAAm and acrylic amide (AAm) monomers. Reprinted with permission. 276
Figure 1.4 Major endocytosis pathways. Reprinted with permission.\textsuperscript{345}

Figure 1.5 The schematic illustration of multidrug release by MSN-polymer nanocomposite. Reprinted with permission.\textsuperscript{184}
Figure 1.6 The schematic illustration of the synthesis of DOX-associated Fe$_3$O$_4$ nanoparticles coated with a PEG modified porous silica shell. Reprinted with permission.\textsuperscript{358}
Figure 1.7 The schematic illustration of the synthesis of core-shell drug delivery vehicle. Reprinted with permission.

Figure 1.8 A schematic illustration showing the one-pot self-assembly strategy for synthesis of drugs@micelles@MSNs. Reprinted with permission.
Figure 1.9 A biofilm is composed of attached microbial cells encased within a matrix of extracellular polymeric secretions (EPS), which surround and protect cells. The EPS matrix is typically composed of polysaccharides, proteins, lipids, and extracellular DNA (eDNA). Localized within the EPS matrix (see figure inset) are extracellular enzymes (e-Enzymes). Also present are small pieces of DNA carrying specific genes, called plasmids. Both e-Enzymes and plasmids are protected against degradation with the biofilm. Finally, bacteria release signal molecules for a process of chemical communication called quorum sensing. A biofilm may extend from just a few to hundreds of micrometers above a surface, but is equipped with many inherent adaptations that are not present in planktonic cells. Reprinted with permission.
CHAPTER 2

FUNCTIONALIZED NANO Particles SILENCE BACTERIAL COMMUNICATION

Excessive use of antibiotics has resulted in an increased rate of bacterial resistance and poses a significant public health threat. As a result, non-cytotoxic methods of controlling bacterial infections, such as interference in bacterial communication (i.e. quorum sensing), are necessary to avoid resistance against antimicrobial therapies. Quorum sensing (QS) is a chemical signaling scheme used by bacteria to communicate and coordinate their activities within a biofilm. Figure 2.1 displays a common structure of a chemical signal used by bacteria during communication. QS regulates the features that make bacteria dangerous, such as biofilm formation, virulence factor production, and uptake of antibiotic resistant genes.

The goal of this chapter was to develop functionalized nanoparticles that will weaken the communication network of bacteria, rather than target the viability of cells, in order to fight pathogenic infections while limiting the risk of antibiotic resistance. To accomplish this I used functionalized silicon dioxide nanoparticles (Si-NPs) to quench the small, diffusible signal molecules (e.g. Figure 2.1) that bacteria rely on to communicate and coordinate their activities. The functionalized Si-NPs are added to bacterial cultures and bind the chemical signals as they diffuse between cells.
Sequestering these signals via functionalized nanoparticles and subsequently blocking bacterial communication can potentially prevent activities such as pathogenesis, adhesion, and biofilm formation. This chapter describes the growth and transcription studies used to demonstrate that this anti-virulence technology controls bacterial infections by directly influencing bacterial communication and subsequent activities regulated by quorum sensing.

Inspired by developments in the field of drug delivery, nanoparticles have been designed to carry compounds that will quench signal molecules. Removal of signals from the immediate bacterial environment prevents the molecule from reaching its cognate receptor, thus inhibiting the signal/receptor interaction, and interfering with down-stream regulation. In theory, this process will quench signal molecules and “turn off” quorum sensing and silence bacterial communication. β-cyclodextrin (β-CD) is a compound that nonspecifically binds N-acyl-L-homoserine lactone molecules, a common class of signal molecules produced by Gram-negative bacteria. β-CD consists of seven glucopyranose units bound by α-1,4-glycosidic linkages (Figure 2.2).

This molecule takes the shape of a truncated cone with a hydrophilic exterior and a slightly hydrophobic interior. β-CD was chosen as a model compound because the formation of a CD complex can often be detected and because the reactivity of a guest molecule in a CD complex is often modified. In many cases, the CD accelerates the various reactions associated with the guest molecule and modifies the reaction pathway. Herein we present a new approach to disrupting bacterial quorum sensing with silicon dioxide nanoparticles that have been surface-functionalized with β-CDs.
2.1 CHARACTERIZATION OF MONOLAYER NANOPARTICLES

The unique properties of surface-functionalized nanoparticles make them ideal for targeting the multiple avenues of bacterial defenses and weakening an infection. Surface functionalization plays a critical role in tailoring the properties of nanoparticles via well-developed surface chemistry.\textsuperscript{254,447} A method has recently been developed for the synthesis of carboxylic acid-coated silicon dioxide nanoparticles with a controllable functionalized surface.\textsuperscript{284-285} Using this method, monolayer β-CD coated fluorescent silicon dioxide nanoparticles were synthesized via a coupling reaction between the hydroxyl groups of β-CD and a monolayer dye-labeled carboxylic acid coated silicon dioxide nanoparticle. A representation is seen in Figure 2.3.

The carboxylic acid coated nanoparticles were prepared based on a ring opening reaction between succinic anhydride and amino-functionalized silica nanoparticles with a variety of surface graft densities ranging from 0.24-0.65 groups/nm\textsuperscript{2}. Thus, the graft density of β-CD functionalized nanoparticles can be tailored by varying the feed ratio between bare silica nanoparticles and amino-silane compound. The as-synthesized β-CD functionalized nanoparticles were purified via dialysis to remove un-reacted free β-CD molecules, as confirmed by \textsuperscript{1}H NMR (Figure 2.4). Additionally, thermogravimetric analysis (TGA) confirmed that the monolayer β-CD accounted for 2.78\% of the total weight for particles having a surface graft density of 0.24 groups/nm\textsuperscript{2} (Figure 2.5). The core diameter of the nanoparticles was either 15 nm or 50 nm.
2.2 CHARACTERIZATION OF POLYMER NANOPARTICLES

In addition to monolayer β-CD coated nanoparticles, polymer coated nanoparticles were also tested. Polymer grafted silica nanoparticles containing β-CD side groups were prepared via the condensation reaction between the grafted poly(methacrylic acid) and the hydroxyl groups on β-CD. A schematic representation is seen in Figure 2.6.

The dye-labeled poly(methacrylic acid) grafted silica nanoparticles were prepared by direct surface-initiated RAFT polymerization of methacrylic acid on dye-labeled silica nanoparticles. Thus, the carboxylic acid loading can be controlled by tailoring the surface grafted poly(methacrylic acid) brushes length as well as the graft densities. TGA data showed that the surface polymer supported chains with multiple β-CD accounted for 61.7% by weight for particles that have a PMAA brush density of 0.18 chains/nm² and molecular weight of 54,900 mol/g (Figure 2.7). The β-CD side chain based polymer grafted nanoparticles showed strong fluorescence under UV light, even after multiple-step surface chemical modifications (Figure 2.8).

2.3 NMR DETECTS BINDING OF HSLS AND β-CYCLODEXTRIN

The NMR diffusion measurements were used to provide insight into the binding of N-octanoyl-L-homoserine lactone (C8-HSL) to both α-cyclodextrin and β-cyclodextrin. The data for various concentrations of C8-HSL and CD are summarized in Table 2.1. The observed diffusion coefficients ($D$) are the average values that were determined for each $^1$H resonance arising from the two compounds. While the $D$ of the cyclodextrins in each solution remains unchanged (within experimental error), the $D$ of C8-HSL decreases towards the $D$ of CD as the ratio of CD to HSL is increased. In the
case of a large excess of CD, the equilibrium of C8-HSL shifts to the bound form and the $D$ of C8-HSL is the same as the CD. This indicates that all of the C8-HSL is in the bound form.

It is important to note that NMR diffusion data for the HSL can be fit with a single exponential function and there is no evidence of two diffusing forms, i.e., free and bound. Therefore, the data suggests that during the timeframe of the NMR experiments (100s msec) the HSL is rapidly moving between free solution and bound into the CD and an equilibrium of the two forms exists.

The observed diffusion coefficient ($D_o$) for the C8-HSL in these mixtures is a weighted average between the free diffusion coefficient ($D_f$) and that of the bound from ($D_b$)448

$$D_o = F_f D_f + F_b D_b$$

(1)

where $F_f$ is the fraction of C8-HSL that is free and $F_b$ is the fraction that is bound. The dissociation constant can be calculated from the measured diffusion coefficient and the starting concentrations of the two species by:

$$K_d = \frac{[CD]}{D_o - D_f} + \frac{[C8-HSL]}{D_b - D_f}$$

(2)

Since diffusion coefficients are sensitive to variations of temperature and sample viscosity, comparisons across samples are difficult. However, the observed diffusion
coefficients \((D_o)\) can be corrected using the diffusion of water in each sample and comparing that to a standard value. For this study we corrected each \(D_o\) with that of the \(H_2O\) measured in the solution of only C8-HSL.

\[
D_{corr} = D_o \frac{D_{H_2O}}{D_{H_2O-C8-HSL}}
\]  

(3)

The calculated values of \(K_d\) for each of the solution are included in Table 2.1. The three values for the \(\beta\)-CD are in close agreement with each other (average = 1.50 x 10^{-3} M). This value is 7.5 times higher than that of the \(\alpha\)-CD. Expressed differently, C8-HSL binds 7.5 times stronger in the \(\alpha\)-CD than the \(\beta\)-CD \((K_a = 1/K_d)\). The smaller interior of the \(\alpha\)-CD is a better fit for the C8-HSL. This procedure was also used to determine the diffusion coefficient for 3OC6-HSL and \(\beta\)-CD; however, initial attempts were unsuccessful and did not indicate binding.

The binding strength between \(\beta\)-CD and HSLs was determined, although \(\beta\)-CD was strictly used as a model binding agent and will eventually be replaced with a highly-specific compound. NMR diffusion experiments demonstrated that both C8-HSL (produced by \(V. fischeri\)) and C6-HSL (produced by \(Pseudomonas aeruginosa\)) could bind to \(\beta\)-CD in a 1:1 ratio; however, there was no evidence that 3OC6-HSL (also produced by \(V. fischeri\)) formed a complex with \(\beta\)-CD. Further calculations revealed the binding and dissociation constants of the experimental complexes. As seen in Table 2.2, there was weak binding between the HSLs and \(\beta\)-CD with 17% of C6-HSL and 35% of C8-HSL bound to \(\beta\)-CD at any one time in the 1:1 solution. After demonstrating that the formation of a \(\beta\)-CD/HSL complex was possible, we sought to quantify the quenching
ability of β-CD in vitro. Using growth and luminescence studies we observed the influence of β-CD on V. fischeri bioluminescence.

2.4 β-CYCLODEXTRIN QUENCHES HSLs IN VITRO

To test the quorum quenching function of these nanoparticles, the marine bacterium Vibrio fischeri was employed as a model. V. fischeri relies on N-acyl-L-homoserine lactone molecules to trigger coordinated activities such as colonization and bioluminescence in a threshold dependent manner. Specifically, V. fischeri synthesizes and responds to N-3-oxo-hexanoyl-L-homoserine lactone (3OC6-HSL) and N-octanoyl-L-homoserine lactone (C8-HSL) via the lux operon, which is also responsible for the proteins that synthesize luminescent luciferase. This quorum sensing function allows V. fischeri to establish a symbiotic relationship with the Hawaiian bobtail squid (Euprymna scolopes) in their natural ocean habitat.

Environmental conditions are essential for the proper functioning of V. fischeri bioluminescence. A symbiotic culture produces approximately 1000-fold brighter bioluminescence and more 3OC6-HSL than cultured cells at the same density. Therefore, HSLs are added to the cultures in vitro to induce quorum sensing and visible bioluminescence. Figure 2.10 portrays V. fischeri cultures induced with high levels of 3OC6-HSL and C8-HSL.

V. fischeri cultures grown in the presence of 2 μM 3OC6-HSL exhibited strong bioluminescence and normal growth. When treated with concentrations of free β-CD ranging from 250 nM to 7 mM, the growth study showed that exposure to 2 mM β-CD resulted in the most significant decrease in bioluminescence (Figure 2.11). A preliminary
analysis of β-CD functionalized nanoparticles with 2 μM 3OC6-HSL demonstrated that β-CD was significantly more effective at dimming bioluminescence of *V. fischeri* when functionalized as a monolayer to a nanoparticle than it was as a free compound (diagram in Figure 2.12, results in Figure 2.13). In contrast, the β-CD polymer coated nanoparticles did not have a significantly different effect than free β-CD on luminescence (Figure 2.14). Because 2 μM 3OC6-HSL exceeds the expected HSL production of symbiotic *V. fischeri* cultures, and does not induce the *lux* operon like C8-HSL, we altered the HSL conditions to mimic the natural environment.

Growth experiments were repeated with *V. fischeri* cultured with 125 nM 3OC6-HSL and 0.25 nM C8-HSL. Optical density and bioluminescence were monitored throughout growth to determine the impact of free and functionalized β-CD. All cultures were grown in defined minimal medium, and therefore were not in a rich nutrient environment. Additional growth studies indicated that the bacteria did not thrive on β-CD or the various Si-NPs. Also, when all carbon sources in the defined minimal medium were replaced with β-CD, no growth occurred. Cultures grown without the addition of HSLs produced negligible levels of bioluminescence. Therefore, β-CD, with or without NPs, did not influence the growth or health of the cells.

*V. fischeri* cultures grown in the presence of 125 nM 3OC6-HSL and 0.25 nM C8-HSL produced measureable bioluminescence; although, bioluminescence in general was lower compared to the cultures treated with 2 μM 3OC6-HSL (as indicated by the different scales in Figure 2.13 and Figure 2.15). Our study found that at environmentally relevant levels of HSLs, bioluminescence was significantly reduced *in vitro* by β-CD. By functionalizing β-CD to 50 nm NPs, 133 nM β-CD was able to produce the same result as
a 2x higher concentration of free 250 nM β-CD (Figure 2.15). We found that 15 nm Si-NPs with or without β-CD decreased bioluminescence to the same extent, and thus may not have a significant role in dimming luminescence.

The long half-life (relative to a 12-hour growth experiment) of luciferase likely contributed to the uncertainty in discerning subtle changes in quorum sensing over time.

To further examine the role of functionalized and free β-CD in *V. fischeri* quorum sensing, we then examined changes in transcription of the *lux* operon during exposure to β-CD and β-CD functionalized Si-NPs.

### 2.5 TRANSCRIPTION OF THE *lux* OPERON DURING NANOPARTICLE EXPOSURE

Bioluminescence in *V. fischeri* is generated by the *lux* operon and is activated by 3OC6-HSL and C8-HSL. Transcription of the *lux* operon results in the production of luciferase, the enzyme responsible for bioluminescence. In this study, the fold change in *luxA* and *luxR* transcription was quantified to determine the activity of the *lux* operon during exposure to β-CD. LuxA forms the alpha subunit of luciferase and was used to monitor bacterial luminescence. LuxR is a receptor for both 3OC6-HSL and C8-HSL, and initiates the *lux* operon, and was used to monitor signal production. Table 2.3 summarizes the fold-changes calculated from the C_t values generated by qPCR with the Livak method (ΔΔC_t) of both HSL-treated cultures.

The quantity of transcripts produced by untreated cultures (controls) and cultures treated with 250 nM β-CD were not markedly different, indicating that low concentrations of free β-CD are ineffective in down-regulating *luxA* and *luxR* at
environmental levels of HSLs (data not shown). The addition of much higher (i.e. 2 mM) β-CD significantly reduced the expression of luxA and luxR transcripts, as indicated by the calculated fold changes. In an attempt to more-efficiently deliver β-CD to the bacterial cell, low concentrations of β-CD were delivered via Si-NPs. As Table 2.3 indicates, both the functionalized and non-functionalized 15 nm Si-NPs reduced the expression of luxA and luxR transcripts to a similar extent. The data agree with the growth study, indicating that the influence of 15 nm Si-NPs and 155 nM β-CD functionalized 15 nm Si-NPs is indistinguishable. Conversely, treatment with the larger nanoparticles (i.e. functionalized and non-functionalized 50 nm Si-NPs) resulted in significantly different fold changes. The 133 nM β-CD, when bound to functionalized 50 nm Si-NPs, resulted in the greatest down-regulation of luxA and luxR transcripts out of all treatments, demonstrating the strong utility of this size of functionalized silica nanoparticles. The discontinuity of the bioluminescence measurements between the growth and transcription studies was likely influenced by the relatively long half-life of bioluminescence, which contributed to an inability to discern differences using bioluminescence during growth.

The growth and transcription studies indicate that the 15 nm and 50 nm Si-NPs resulted in different levels of bioluminescence, although both Si-NPs were synthesized using the same method. Based on the nanoparticle synthesis methods and TGA analyses, we determined that the 15 nm Si-NPs carry 0.06 μmol β-CD / mg Si-NP complex, while the 50 nm Si-NPs carry 0.15 μmol β-CD /mg Si-NP complex, indicating that the surface densities of the two Si-NPs are different. Additionally, the surface area of the 50 nm Si-NPs is more than ten times the surface area of the 15 nm Si-NPs, which may have
provided for more space between individual β-CD molecules and allowed for more efficient binding with the HSLs. Given that a 50 nm Si-NP can carry 1.7 times more β-CD than a 15 nm Si-NP, a higher dose of 15 nm Si-NPs was used to treat the samples to achieve comparable levels of β-CD. Three major possibilities exist to explain this. First, the higher concentration of Si-NPs aggregated in solution, making more of the functional groups unavailable on this size Si-NP. Alternatively, the β-CD may have been too dilute among the 15 nm Si-NPs and therefore did not act differently than dissolved β-CD. Lastly, it is possible that the 15 nm Si-NPs behave differently than 50 nm Si-NPs in an aqueous solution. It has been shown that the surface modifications of silica affect the dispersion of Si-NPs, and that smaller-sized Si-NPs have unique physicochemical properties and optical absorption characteristics. Therefore, smaller nanoparticles with a low surface density may behave differently than larger nanoparticles with a higher surface density. In summary, it is likely that the β-CD dose carried by the 15 nm Si-NPs behaved differently than the 50 nm Si-NPs, and therefore was unable to impact quorum sensing more significantly than free β-CD. The larger 50 nm Si-NPs, in contrast, were able to carry a higher dose of β-CD on fewer nanoparticles, and directly influenced quorum sensing in V. fischeri.

2.6 CONCLUSION

In this study, we have demonstrated that functionalized silica nanoparticles can be used to enhance the role of quenching agents in vitro. Specifically, we demonstrated that β-CD is able to bind HSLs and down-regulate bacterial quorum sensing genes. We have
found that the quenching ability of β-CD was much greater on functionalized 50 nm Si-NPs, and provides a strong model for future designs.

The growth experiment was conducted with several forms of cyclodextrin to determine an efficient binding agent for HSLs. We found that α-cyclodextrin and 2-hydroxypropyl-β-cyclodextrin did not silence bacterial communication any more or less efficiently than β-cyclodextrin. However, we found that both 125 nM and 250 nM methyl-β-cyclodextrin dimmed luminescence \textit{in vitro} by 31.8\% and 48.9\%, respectively, while 250 nM β-cyclodextrin dimmed luminescence by 16.7\% (calculated from data in Figure 2.15). When functionalized to a nanoparticle, lower levels (133 nM) of β-CD dimmed luminescence by 33.3\%. We are currently designing nanoparticles to carry low levels of methyl-β-cyclodextrin in an effort to enhance the potency of the Si-NP complex. In addition to more specific binding agents, we are also pursuing bacterial receptor proteins that will bind signal molecules with high species specificity.

The importance of nanoparticle therapies is imperative to the treatment of biofilm infections. One of the most significant challenges in the treatment of chronic infections is delivering effective drugs to bacterial biofilms. Biofilms are surface-associated bacterial communities living in a highly organized structure at a liquid interface,\textsuperscript{459-460} and have been estimated to be responsible for 80\% of hospital-acquired infections.\textsuperscript{460} The protective nature of bacterial biofilms makes them able to physically limit antibiotic penetration,\textsuperscript{419, 421, 423, 461} quickly regulate multidrug efflux pumps\textsuperscript{431, 460} and stress response genes,\textsuperscript{462-463} induce a biofilm-specific phenotype,\textsuperscript{423-424, 426} and easily trade and enhance antibiotic resistance genes among their bacterial members.\textsuperscript{435, 464} Here, with the advent of nanotechnology, we have demonstrated a unique ability (and tool) to
specifically target bacterial communication, which may be used to penetrate infectious biofilms and eliminate infections.

2.7 METHODS

β-CD ANALYSIS

Instrumentation

NMR diffusion measurements were performed on a Varian Mercury/VX 400 spectrometer operating at 400.273 MHz (¹H). Pulsed-field gradient spin echoes with varying gradient intensity were collected with the Doneshot pulse sequence that was included with VNMRJ 2.2D software.⁴⁶⁵ The standard Varian Performa I pulsed field gradient amplifier and 5 mm broadband probe were capable of producing a maximum of 20 gauss/cm field gradients. Spectra were taken at ambient temperature with bipolar gradient pulses of 4 ms and a diffusion delay of 100 ms. All data processing was done with the Varian DOSY software package.

GROWTH STUDIES

Bacteria and Growth Media

V. fischeri JB10 is a derivative of the ES114 strain that contains a chromosomal gfp reporter within the lux operon, resulting in luxI-gfp-CDABEG.⁴⁶⁶ The strain was prepared and obtained from Professor Eric Stabb (Univ. Georgia, Athens, GA). V. fischeri JB10 was cultured from a glycerol stock and grown in marine broth (Difco 2216) to exponential phase. The cells were then transferred to defined minimal medium⁴⁵⁶ for an
optical density of approximately 0.1 (Abs$_{600\text{nm}}$) for the growth and RNA extraction experiments.

**Bioluminescence response during exposure to nanoparticles**

*V. fischeri* JB10 was grown in marine broth to exponential phase and then transferred (0.5%) to a defined minimal medium. For growth experiments, 96-well plates were incubated at 28°C in a Victor X Multilabel plate reader for 24 hours. Optical density was measured at 600 nm and GFP fluorescence was measured with a 485/20 nm excitation filter and a 528/20 nm emission filter. Bioluminescence was also measured. All measurements were conducted every two hours for 24 hours following 10 seconds of vigorous shaking. The total volume of each well was 200 µL, which included either 250 nM β-cyclodextrin, 2 mM β-cyclodextrin, 15 nm SiO$_2$ nanoparticles (2.75 x 10$^{-3}$ mg/mL NPs), 15 nm SiO$_2$ nanoparticles functionalized with 155 nM β-cyclodextrin (2.75 x 10$^{-3}$ mg/mL NPs), 50 nm SiO$_2$ nanoparticles (8.9 x 10$^{-4}$ mg/mL NPs), or 50 nm SiO$_2$ nanoparticles functionalized with 133 nM β-cyclodextrin (8.9 x 10$^{-4}$ mg/mL NPs). Cultures also included 125 nM 3OC6-HSL, 0.25 nM C8-HSL, or both 125 nM 3OC6-HSL and 0.25 nM C8-HSL. All treatments were added at time zero. A growth experiment was also performed with 600 nm SiO$_2$ nanoparticles (data not shown, no cell growth occurred).
QUANTITATIVE PCR AND TRANSCRIPT ANALYSIS

*lux* transcription during exposure to nanoparticles

*V. fischeri* JB10 was grown in marine broth to exponential phase and then transferred (1%) to a defined minimal medium. Cultures were grown at 28°C and 200 rpm in 250 mL shake flasks with a working volume of 50 mL for 8 hours. Treatments were added at 8 hours and exposure/growth continued for 4 hours. At 12 hours of total growth, 2 mL of cells were harvested by centrifugation at 2000 x g (5 min). Cells were treated with 2 mg/mL lysozyme in TE buffer for 10 minutes and then homogenized with a needle and syringe. RNA was extracted with PureLink RNA Mini Kit (Ambion) and on-column DNA digestion was performed with PureLink PCR Micro Kit (Invitrogen). qPCR was performed on a BioRad CFX96 Real Time System with a C1000 Thermal Cycler. Data was analyzed by the Livak method (\(\Delta\Delta C_t\)) and fold changes were determined by using \(2^{-\Delta\Delta C_t}\).

Primers were designed for *luxR* and *luxA* with NCBI’s Primer-BLAST. Primers for 16srRNA were found in the literature. All primers were ordered from Integrated DNA Technologies (Coralville, Iowa) and are listed in Table 3.4. Genomic DNA from *V. fischeri* JB10 was amplified with the primers and sequenced by Selah Genomics (Columbia, SC) to check the accuracy of the primers.

NANOPARTICLE SYNTHESIS

Materials

All chemicals were obtained from Fisher or Acros and used as received unless otherwise specified. Trimethylsilyldiazomethane (2.0 M in hexanes) was obtained from
TCI. 4-Cyanopentanoic acid dithiobenzoate (CPDB) anchored silica nanoparticles were prepared according to the literature.\textsuperscript{285,314} 3-aminopropyldimethyl-ethoxysilane was obtained from Gelest, Inc. (Morrisville, PA). Methacrylic acid (99.5%, Acros) were purified by passing through an activated neutral alumina column. AIBN was recrystallized from methanol before use.

**Instrumentation**

\(^1\)H NMR was conducted using CD\(_3\)OD as the solvent with a Varian Mercury/VX 400 spectrometer. Molecular weights and PDI were determined using gel permeation chromatography (GPC) equipped with a 515 HPLC pump, a 2410 refractive index detector, and three Styragel columns. The columns consisted of HR1, HR3 and HR4 in the effective molecular weight ranges of 100-5000, 500-30000, and 5000-500000, respectively. The GPC used THF as eluent at 30 °C and a flow rate of 1.0 mL/min, and was calibrated with poly(methyl methacrylate) standards obtained from Polymer Laboratories. Samples were filtered through microfilters with a pore size of 0.2 μm before injection. Infrared spectra were determined with a BioRad Excalibur FTS3000 spectrometer. UV-vis spectra were measured with a Perkin-Elmer Lambda 4C UV-vis spectrophotometer. Infrared spectra were recorded with a PerkinElmer Spectrum 100 spectrometer. Thermogravimetric analysis (TGA) was conducted using a SDT Q600 TGA system (TA Instruments) with a temperature ramping from 25 °C to 900 °C at a rate of 10 °C/min under nitrogen.
**Preparation of monolayer dye-labeled β-CD functionalized silica nanoparticles**

A DMF solution of β-CD (70.56 mg, 62.16 μmol), N,N'-dicyclohexyl carbodiimide (DCC, 10.3 mg, 49.73 μmol) and 4-dimethylaminopyridine (DMAP, 0.5063 mg, 4.144 μmol) were added to a 15 mL DMF solution of dye-labeled carboxylic acid-functionalized silica nanoparticles (graft density: 0.24 groups/nm², 0.7281g). The reaction was stirred at room temperature overnight. The reaction solution was then poured into 200 mL ethyl ether followed by centrifugation at 3000 rpm for 5 min. The recovered particles were then redispersed in 20 mL of ethanol and subjected to dialysis process to further remove impurities. The dye-labeled β-CD coated silica nanoparticles were finally dissolved in ethanol/water mixture solvents for further use.²⁸⁴

**Preparation of polymer dye-labeled β-CD grafted silica nanoparticles**

A DMF solution of β-CD (3.711 g, 3.27 mmol), N,N'-Dicyclohexylcarbodiimide (DCC, 0.54 g, 2.616 mmol) and 4-Dimethylaminopyridine (DMAP, 26.6 mg, 0.218 mmol) were added to a 10 mL dry DMF solution of dye-labeled poly(methacrylic acid) grafted silica nanoparticles (graft density: 0.30 chains/nm², 252 mg). The reaction was stirred at room temperature for overnight. Then the reaction solution was then poured into 200 mL ethyl ether followed by centrifugation at 3000 rpm for 5 min. The recovered particles were then redispersed in 20 mL of ethanol and subjected to dialysis process to further remove impurities. The dye-labeled poly(β-CD) grafted silica nanoparticles were finally dissolved in water for further use. This method is based on the work of Wang & Benicewicz.²⁸⁵
Table 2.1 Measured and corrected diffusion coefficients\((D)\) for various mixtures of C8-HSL and cyclodextrin. The dissociation constant\((K_d)\) is calculated from equation 2. All solutions were prepared in D\(_2\)O.

<table>
<thead>
<tr>
<th>Solution composition</th>
<th>Measured (D) (x10^{-10}) m(^2)s(^{-1})</th>
<th>Corrected (D) (x10^{-10}) m(^2)s(^{-1})</th>
<th>(K_d) (10^{-3} M)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C8-HSL</td>
<td>(\alpha/\beta)-CD</td>
<td>H(_2)O</td>
</tr>
<tr>
<td>10 mM C8-HSL</td>
<td>3.55</td>
<td>13.33</td>
<td></td>
</tr>
<tr>
<td>5.3 mM C8-HSL + 23 mM (\alpha)-CD</td>
<td>2.00</td>
<td>2.00</td>
<td>13.62</td>
</tr>
<tr>
<td>8 mM C8-HSL + 8 mM (\alpha)-CD</td>
<td>2.24</td>
<td>2.04</td>
<td>12.68</td>
</tr>
<tr>
<td>2 mM C8-HSL + 2 mM (\beta)-CD</td>
<td>2.79</td>
<td>1.98</td>
<td>12.79</td>
</tr>
<tr>
<td>3 mM C8-HSL + 2 mM (\beta)-CD</td>
<td>2.88</td>
<td>1.92</td>
<td>12.89</td>
</tr>
<tr>
<td>4 mM C8-HSL + 2 mM (\beta)-CD</td>
<td>2.97</td>
<td>1.93</td>
<td>13.04</td>
</tr>
</tbody>
</table>

Table 2.2 Binding strength of \(\beta\)-CD and C6-HSL or C8-HSL as determined by NMR.

<table>
<thead>
<tr>
<th></th>
<th>C6-HSL</th>
<th>C8-HSL</th>
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</thead>
<tbody>
<tr>
<td>Binding constant</td>
<td>0.11</td>
<td>0.69</td>
</tr>
<tr>
<td>Dissociation constant</td>
<td>9.05</td>
<td>1.44</td>
</tr>
<tr>
<td>Percent bound</td>
<td>17%</td>
<td>35%</td>
</tr>
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</table>
Table 2.3 Calculated fold change of transcription by *V. fischeri* after treatment exposures to 125 nM 3OC6- and 0.25 nM C8-HSLs. Negative values indicate down-regulation.

<table>
<thead>
<tr>
<th>Treatment Condition</th>
<th>luxR</th>
<th>luxA</th>
</tr>
</thead>
<tbody>
<tr>
<td>no treatment (control)</td>
<td>-1.4</td>
<td>4.8</td>
</tr>
<tr>
<td>250 nM β-CD</td>
<td>-1.7</td>
<td>1.7</td>
</tr>
<tr>
<td>2 mM β-CD</td>
<td>-97.8</td>
<td>-39.1</td>
</tr>
<tr>
<td>15 nm bare-NPs</td>
<td>-250.4</td>
<td>-432.9</td>
</tr>
<tr>
<td>15 nm 155 nM β-CD-NPs</td>
<td>-245.7</td>
<td>-629.77</td>
</tr>
<tr>
<td>50 nm bare-NPs</td>
<td>-1723.4</td>
<td>-365.8</td>
</tr>
<tr>
<td>50 nm 133 nM β-CD-NPs</td>
<td>-2125.5</td>
<td>-2171.1</td>
</tr>
</tbody>
</table>

Table 2.4 Primer sequences used in qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
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<tbody>
<tr>
<td>luxA</td>
<td>ATCCCCATCTTCTGTAACGG</td>
<td>ACAGAACATGGCCACGACAT</td>
</tr>
<tr>
<td>luxR</td>
<td>CGTGGCGGAGTGAAGGAAAA</td>
<td>TGGCGCCAGTTAAAAATTGCT</td>
</tr>
<tr>
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<td>CTACCTTGTACGACTTCACC</td>
</tr>
</tbody>
</table>
Figure 2.1 *N*-acyl homoserine lactone, a chemical signal used by bacteria during quorum sensing.

Figure 2.2 β-cyclodextrin chemical (A) and toroidal (B) structure.

Figure 2.3 Schematic representation of monolayer β-CD coated fluorescent silicon dioxide nanoparticles. The black center sphere is the silicon dioxide core, and the white outside rings represent the β-CD.
Figure 2.4 \textsuperscript{1}H NMR spectra of the as-synthesized $\beta$-CD coated silica nanoparticles. (a) before dialysis; (b) after dialysis.
Figure 2.5 TGA of (a) dye-labeled monolayer carboxylic acid coated silica nanoparticles (graft density: 0.18 groups/nm$^2$); (b) dye-labeled monolayer β-CD coated silica nanoparticles.
Figure 2.6 Schematic representation of a polymer grafted silica nanoparticle with β-CD side group. Gray center sphere represents the silica core and the white rings enlarged to the right represent β-CD.
Figure 2.7 TGA of (a) dye-labeled poly(methacrylic acid) grafted silica nanoparticles (graft density: 0.18 groups/nm$^2$); (b) dye-labeled poly(β-CD) grafted silica nanoparticles.
Figure 2.8 Photograph of dye-labeled poly(β-CD) grafted silica nanoparticles in DMSO.

Figure 2.9 Acyl homoserine lactone molecules synthesized and recognized by *V. fischeri*. 
Figure 2.10 *V. fischeri* JB10 cultures in marine broth. Bioluminescence induced with 3OC6-HSL and C8-HSL.
Figure 2.11 Maximum relative bioluminescence per OD (600 nm) of *V. fischeri* during exposure to β-CD and 2 μM 3OC6-HSL. Error bars represent standard errors of the mean (n=3). Asterisk indicates significance (P≤ 0.05).
Figure 2.12 Schematic of nanoparticle-based silencing of bacterial quorum sensing. (a) Diagram of *V. fischeri* *lux* operon during quorum sensing (QS). Triangles represent 3OC6-HSL. LuxR/3OC6-HSL complex initiates bilateral transcription of *lux* operon. LuxI produces 3OC6-HSL. Inset: *V. fischeri* culture flask luminescing after treatment with HSLs. (b) Diagram of *V. fischeri* during nanoparticle treatment. Binding of HSLs by Si-NPs quenches QS and subsequent gene expression in *lux*.
Figure 2.13 Changes in bioluminescence by *V. fischeri* during exposures to 2 μM 3OC6-HSL, with either β-CD or β-CD functionalized Si-NPs. Error bars represent standard error of the mean (n=3). Asterisks indicate significance (P≤ 0.05).
Figure 2.14 Maximum relative bioluminescence per OD (600 nm) of *V. fischeri* during exposure to 2 μM 3OC6-HSL and β-CD or β-CD functionalized 15 nm polymer nanoparticles. Error bars represent standard error (n=3). Asterisks indicate significance (P≤ 0.05).
Figure 2.15 Mean relative bioluminescence per OD (600 nm) of *V. fischeri* during exposure to 125 nM 3OC6-HSL and 0.25 nM C8-HSL treated with 250 nM β-CD, bare 15 nm Si-NPs, 155 nM β-CD functionalized 15 nm Si-NPs, bare 50 nm Si-NPs, or 133 nM β-CD functionalized 50 nm Si-NPs. Error bars represent standard error of the mean (n=3). Asterisks indicate significance (P≤ 0.05).
CHAPTER 3

EXTRACELLULAR GLASS WITHIN EPS MATRIX IS A PROTECTIVE STRATEGY FOR BIOFILMS AGAINST DESICCATION

Water-limitation and desiccation are common stressors encountered by microbial communities at the fringes of aquatic and extreme environments. Microbes in these environments are localized within the protective confines of biofilms surrounded in a thick matrix of extracellular polymeric substances (EPS). Remarkably, desiccated biofilms are able to rapidly resume cellular and extracellular activities upon rehydration. How this process occurs, however, has remained elusive and constitutes a significant gap in understanding resiliency in microbial cells.

Here we demonstrate a key step in this process: formation of an EPS glass within the anhydrophilic microbial mats of Bahamian salt-ponds. EPS glass formation was determined using differential scanning calorimetry (DSC). The functional integrity of key extracellular molecules, called acylhomoserine lactones (AHLs), used in chemical-signaling and diffusion-sensing, was preserved by vitrification in EPS glass during desiccation, and allowed rapid-release and resumption of activity following rehydration. AHLs were recoverable from dried mats stored for over seven years, and upon rehydration AHLs were determined to be intact and functional using mass spectrometry.
(MS) and signaling bioassays, respectively. Glasses were formed in vitro using extracted components of natural EPS. The disaccharide α-α-trehalose, an abundant component of natural mat EPS, likely contributes in situ to glass formation and AHL stabilization. Under laboratory conditions, AHLs were stabilized by trehalose-glasses. Other EPS components, however, may additionally contribute to the natural glass as well. Solid-state nuclear magnetic resonance (NMR) showed that trehalose stabilizes but does not directly complex with AHLs during desiccation. The results show that preservation of AHLs during desiccation provides microbial communities with the capability to resume signaling and diffusion-sensing activities soon after rehydration. It is proposed that glass formation may play roles in the broader resiliency of microbial life in many environments.

Microorganisms frequently encounter water-limitation and survive desiccation in many environments on the Earth’s surface. These environments exist on the fringes of aquatic environments and in extreme environments, such as desert crusts, that have been estimated to cover approximately 25% of the Earth’s terrestrial surface. Survival in these environments is often localized within the protective confines of biofilms where microbial cells are surrounded by a thick matrix of EPS.

Desiccation, defined here as the progressive loss of water, is a common stressor that occurs with varying magnitude in coastal oceans, as rock varnish, in hypersaline ponds, in the far reaches of desert crusts, terrestrial soils, intermittent freshwater ponds and streams, and even the Antarctic Dry valleys, and in the fouling communities of sidewalks, buildings, statues, and roof shingles. The extent of dehydration (to cells) varies from environments where water is present much of the time, to environments
where water is intermittently present for only extremely brief periods (e.g., a few hours per year). Therefore, the ability of microbial communities to adapt to water-limitation, or even complete anhydrobiosis, is likely to be a very widespread process both in the present day and throughout the history of life.

Microbial mats, located within the hypersaline ponds of San Salvador Island, Bahamas, and other such environments, face a multitude of stresses such as diel changes in temperatures, UV-irradiation, and rapid (minutes) to intermittent (seasonal) osmotic challenges. They typically experience a wide range of salinities (10 to 340 ppt), intense irradiance (> 2200 µE m\(^{-2}\) s\(^{-1}\)), high temperatures (> 40°C), and chronic nutrient depletion.\(^{471}\) The mats form well-defined horizontal layers that have previously been described.\(^{471-474}\) Over an annual cycle, seasonally driven wet/dry conditions typically result in a slow evaporation of water in ponds. Highest temperatures and salinities may reach greater than 44°C and 340 ppt, respectively, during summer, and may be followed by partial to complete desiccation. Sudden heavy rainfalls during July and August commence the ‘wet season’ and contribute large pulses of freshwater to ponds. Thus, the surface microbial mat communities may experience rapid shifts in ionic concentrations (300 ppt shift) over a relatively short time interval (e.g. minutes to hours), yet they remain metabolically active, and appear well-adapted to these environmental fluctuations.

One major adaptation that bacterial communities rely on to develop and survive is quorum sensing. Quorum sensing is a communication mechanism that relies on chemical signals to regulate essential microbial processes at high cell densities.\(^{475}\) The complex communities within microbial mats utilize quorum sensing to coordinate gene expression.\(^{475}\) A common group of chemical signals used by Gram-negative bacteria
during quorum sensing are the acylhomoserine lactones (AHLs). AHLs contain a lactone ring and a long acyl chain, and exist outside of the cells during transit. The length of the acyl chain is essential to an AHL’s molecular stability, especially under extreme conditions such as high or low pH.\textsuperscript{476-477} While, AHLs are subject to extreme environmental conditions,\textsuperscript{478} the EPS may protect and facilitate the transfer of AHLs among cells.\textsuperscript{479-480} The persistence of AHLs within an anhydrophilic microbial mat is important to understanding the persistence of microbial communities under extreme environmental conditions.

EPS are a common feature of microbial mats, and are especially apparent in hypersaline systems.\textsuperscript{481} The EPS contribute a hydrated, but relatively permeable, extracellular microenvironment for cells.\textsuperscript{482} This extracellular matrix is thought to stabilize cells against rapid fluctuations in ions and nutrients,\textsuperscript{483} and has been shown to aid in the desiccation-tolerance of soil bacteria.\textsuperscript{484-485} During the drying process, the EPS typically condenses as salinity of the overlying water increases. When salinity reaches 180-200 g/L the EPS forms a tough, ‘leathery’ surface consistency. This corresponds to the greatly reduced ability of ions in the overlying water to exchange with the inner mat pore-water (Decho, personal communication). Finally, the EPS forms a hard, plastic-like material that exhibit the properties of an organic ‘glass’ state. Glasses are often formed by simple disaccharide sugars, and even polymeric molecules.\textsuperscript{486} They are extensively used in dried foods to preserve taste, flavor, and shelf life. The glass state preserves important molecules by preventing denaturation in the absence of water.\textsuperscript{487} Interestingly, upon the addition of water, these communities of cells, can exhibit a relatively rapid (e.g., minutes to hours) recovery from anhydrobiosis.
At the level of the individual microbial cell(s), previous work has shown that osmotic stress may occur through the net loss of water (from cells) and/or from increased ionic changes.\textsuperscript{482} As salt stress increases, halophilic or halotolerant microorganisms have evolved two basic intracellular pathways in order to compensate: a KCl-type and a compatible-solute type of osmoadaptation.\textsuperscript{488-489} Bacteria utilize a range of major osmolytes (e.g. glycerol, glycine-betaine, trehalose, glycine, B-glutamate and proline) to compensate for increasing osmotic challenges.\textsuperscript{482, 490-491}

An organic glass provides a matrix of very high-viscosity, which retards damaging chemical reactions and deleterious physical events, such as fusion and crystal formation,\textsuperscript{486} and may stop all chemical reactions that require molecular diffusion.\textsuperscript{492} Trehalose has a relatively high glass-transition temperature ($T_g = 85\text{-}120^\circ\text{C}$) when compared to other sugars,\textsuperscript{493} and therefore forms a type of amorphous solid with high viscosity, at high temperatures.

Previous studies have shown that trehalose is commonly utilized intracellularly by soil bacteria during periods of drought\textsuperscript{494} in order to maintain membrane stability\textsuperscript{495} and preserve the structure of proteins.\textsuperscript{496} Trehalose is a non-reducing disaccharide that consists of two glucose molecules linked by an $\alpha, \alpha$-1,1-linkage ($\alpha$-D-glucopyranosyl $\alpha$-D-glucopyranosyl), and is often found in EPS.\textsuperscript{494} The very low hydration adjacent to the glycosidic oxygen imparts conformational rigidity that allows trehalose to act as a sugar template for stable interactions with hydrogen-bonded water molecules.\textsuperscript{497}

In the present study, we have focused specifically on the role of trehalose as an extracellular bioprotectant during desiccation, as it is a major component of anhydrophilic microbial mats.\textsuperscript{471, 474} A fundamental examination was conducted to
understand the role of glass formation in stabilizing certain quorum sensing signals, called acylhomoserine lactones (AHLs), during desiccation under natural conditions. To determine the ability of trehalose to protect constituents of microbial mats, we additionally studied its role as a bioprotectant of the enzyme acylase. We hypothesized that trehalose and other components of EPS may contribute to the enhanced persistence of signals and other key extracellular molecules within resident microbial communities.

3.1 RESULTS

Bacteria and other microbial cells in a wide range of environments often undergo reduced water-availability and desiccation. Herein is an examination of the microbial mats of hypersaline ponds, which are characterized by a highly diverse and structured community of bacteria and archaea within an abundant EPS matrix. These microbial communities rapidly regain activity when rehydrated, even after prolonged desiccation. EPS plays an important role in this process. As desiccation ensues, the EPS and associated cells on the mat surface condense to form an impervious, leathery-like layer, and eventually, a hard, plastic-like layer. The properties of dried mat EPS were examined here to understand their role in microbial survival during desiccation.

Analysis of EPS in Salt Pond microbial mat

As seen in Figure 1, the desiccation of Salt Pond over time resulted in the precipitation of salts on the surface of the mat. Previous studies have shown that the salinity and temperature of Salt Pond typically increases throughout the dry season. Transition of the surface mats from wet to dry occurs in several steps that are closely
related to seasonal patterns in precipitation and temperature. During the wet season (approx. October-March), active microbial growth occurs in the Salt Pond surface mats (Figure 3.1A). As the dry season commences, net loss of water, due to net evaporation and higher surface temperatures, result in progressively higher salinities within Salt Pond (Figure 3.1B). At high salinities (approximately 170 – 340 ppt), various salts begin to come out of solution and co-precipitate on the mat surface. During this period, occasional brief rains and wind slowly remove much of the evaporites from the surface, leaving a coherent, leathery EPS surface (Figure 3.1C). Examinations with scanning electron microscopy (SEM) showed that the surface consists of a continuous crystalline/amorphous structure of primarily halite, with lesser amounts of gypsum, anhydrite, and other salts (Figure 3.2). Transmission electron microscopy (TEM) revealed thick EPS capsules surrounding desiccated cells (Figure 3.3).

**Salt Pond EPS has a high abundance of glucose**

EPS plays a critical role in providing structure and protection to the microorganisms within a microbial mat (Figure 3.3). Figure 3.4 shows a vertical cross section taken from a hydrated microbial mat in Salt Pond. The distinct colors of each layer are due to the major constituent of each. The golden polymer layer (L1) is abundant in diatoms, the green layer (L2) is abundant in cyanobacteria, and the purple/red layer (L3) is abundant in sulfur and nonsulfur purple bacteria. Although dominated by certain species, each layer contains a complex mixture of microorganisms. While each layer contains EPS, the polymer layer (L1) contains the highest abundance of EPS. Samples were collected throughout a standard vertical transect at stations in the Salt Pond.
to measure EPS abundance, extending from the high-water region of the pond (infrequently covered by water) to the low-water region (covered most of the year). At each sample site, the uppermost layer of the microbial mat contained the highest levels of EPS (Figure 3.5). The abundance of EPS on the surface of the mat and surrounding individual cells may bestow some protection against the extreme environment to the underlying mat and its resident microbes, respectively. To better understand the role of EPS during desiccation, we investigated the composition of the EPS samples, and performed laboratory tests to analyze the carbohydrate components.

EPS samples, collected from the Salt Pond microbial mat, were analyzed via gas chromatography/mass spectrometry (GC/MS). The GC/MS analysis revealed the presence of sugar monomers, including the neutral sugars glucose, galactose, mannose, xylose, the 6-deoxyhexoses fucose, rhamnose, and arabinose, the uronic acids glucoronate and galacturonate, and N-acetyl-D-glucosamine (Table 3.1). The relative enrichment of deoxy-sugars has been shown to increase the hydrophobic nature of some EPS. Methylation, detected in only trace amounts, can influence the conformational and physical characteristics of the EPS and their hydrophobicity. In both the hydrated and desiccated samples, glucose was found to have the highest mole percentage of the total mass. Glucose is often a dominant sugar in EPS. The high concentration of glucose may indicate the presence of trehalose, but could not be distinguished in these analyses. Trehalose is an \(\alpha,\alpha\)-1,1-linked disaccharide of glucose, and has been shown to play a role in osmoprotection. Osmoprotection is crucial to the survival of microorganisms in extreme environments, such as the high salinity and high temperature environment found in Salt Pond.
Trehalose forms glass in EPS during desiccation

To study the ability of EPS to form a glass in the microbial mat, and to demonstrate the role of EPS as an osmoprotectant, we isolated EPS from Salt Pond. The sample was dried and analyzed via differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). Trehalose dihydrate and anhydrous trehalose were also dried and used as standards. DSC revealed that both trehalose dihydrate (Figure 3.6) and trehalose anhydrous (Figure 3.7) resulted in phase transitions at 85°C, indicating the presence of a glass. DSC also detected phase transitions at the melting points of trehalose dihydrate and trehalose anhydrous, as was expected. The DSC analysis of the Salt Pond extract revealed that the EPS sample contained several compounds, many of which experienced phase transitions (Figure 3.8). Furthermore, TGA confirmed the presence of several compounds in the EPS sample, including trehalose dihydrate (Figure 3.9), as seen in Figure 3.10. The DSC and TGA results verified that the EPS sample contained trehalose, and that both trehalose dihydrate and anhydrous trehalose are able to form a glass.

C6-AHL subject to degradation in salt water

The common bacterial quorum sensing signal molecule, N-hexanoyl-L-homoserine lactone (C6-AHL), is frequently found in microbial mats. To investigate the heat tolerance of C6-AHL, it was tested in solution at varying temperatures. The results indicated that C6-AHL retained activity at 25, 35, and 45°C. Activity was not detected after exposure to 55 or 65°C. Signal activity was determined by the presence of pigmentation in the biosensor C. violaceum CV026. The microbial mat presents a complex environment to signal molecules, and therefore the signal molecules may utilize
several other components to maintain activity at high temperatures. To explore the role of salt in the preservation of C6-AHL, salt water samples from Salt Pond were added to the experimental solution.

Long term (3 days) exposure to 55°C in high salt solutions was tested to determine if the natural salt water environment provided protective elements to the signal molecule. Water samples collected from Salt Pond in San Salvador, Bahamas were used. The C6-AHL was dissolved in small volumes of salt water and dried over the course of three days. The initial salt concentrations were 280 ppt, 140 ppt, 70 ppt, and 35 ppt. Sodium chloride (NaCl) was used as the control. C6-AHL dried in 28 ppt (normal salt concentration of ocean) Salt Pond water retained activity. However, C6-AHL did not retain any activity after desiccation at 55°C in solutions of 280 ppt, 140 ppt, 70 ppt, or 35 ppt Salt Pond water. A qualitative analysis of the water sample from Salt Pond was performed via ICP-MS. The analysis showed that the sample contained numerous trace metals, including calcium, manganese, and magnesium. Interestingly, C6-AHL dissolved in 280 ppt Salt Pond water and stored at room temperature also lost activity after three days.

This experiment was repeated with gentle heating over a longer time period to better mimic the microbial mat environment. After heating at 35°C to dryness (five days), neither the Salt Pond water samples nor the pure NaCl samples retained C6-AHL activity. To ensure that the biosensor was providing accurate results, we used the parent strain of *C. violaceum* to test for quorum sensing inhibitors. Neither the high salt solutions of Salt Pond water or NaCl inhibited quorum sensing in *C. violaceum*. These results indicate that C6-AHL activity is dependent on both the temperature and duration of heating. Given the
complexity of a microbial mat, we next explored the role of trehalose as an osmoprotectant during desiccation.

**Trehalose provides protection to C6-AHL during desiccation**

To investigate the heat tolerance of C6-AHL in the presence of trehalose, samples were subjected to high heat. Solutions of C6-AHL and trehalose were heated at either 50°C or 60°C for five days. The 60°C heat treatment was followed by exposure to 100°C for 15 minutes to ensure the formation of a trehalose glass. Both experiments were conducted at pH 7.5 and pH 11, two conditions commonly found in microbial mats. A third component, the enzyme acylase, was also added to the solution to determine if trehalose could provide protection to an enzyme during desiccation.

For the experiment conducted at 50°C, the results indicated that activity of C6-AHL was maintained after trehalose glass formation with and without acylase at both pH 7.5 and pH 11. Signal activity was determined by the biosensor *C. violaceum* CV026, as seen in Table 3.2. These results suggested that C6-AHL is tolerant of long term exposure to 50°C in the dehydrated state in the presence of trehalose. Interestingly, C6-AHL was also tolerant of the pH 11 environment in the presence of trehalose although, typically, high pH environments destabilize homoserine lactones. Under these conditions, acylase activity was not preserved.

For the experiment conducted at 60°C and 100°C, the results indicated that C6-AHL tolerated heat treatment in the presence of trehalose at pH 7.5. However, C6-AHL activity was not preserved in the presence of trehalose at pH 11. And, once again under both conditions, acylase activity was not preserved. It is likely that the acylase was
activated at pH 11, and destroyed the C6-AHL before being degraded by heat. The data is presented in Table 3.2.

To verify the formation of a trehalose glass and to determine if any structural changes occurred in the sample, solid state NMR spectroscopy was performed. As before, the samples were subjected to heat treatment at 60°C for five days followed by 100°C for 15 minutes at pH 7.5. The higher temperature conditions were used in this experiment to ensure that all residual water was removed from the samples before analysis. The NMR spectra of both heat-treated and untreated trehalose and trehalose with C6-AHL can be seen in Figure 3.11. The spectra indicate that the structure of C6-AHL was not altered by heat treatment with (Figure 3.11B) or without (Figure 3.11A) trehalose present. These results correspond with the biosensor data presented in Table 3.2. As expected, the structure of trehalose was altered by heat treatment, as can be seen by comparing Figure 3.11C and Figure 3.11D. The heat treatment of trehalose resulted in broad, unresolved resonances due to the highly disordered molecules and amorphous state of the glass (Figure 3.11C). The untreated trehalose samples appear highly crystalline in structure (Figure 3.11D). In the presence of C6-AHL, the trehalose spectra indicated a glass structure (Figure 3.11B). These results suggest that the glassy state of the trehalose is not influenced by the presence of C6-AHL. Also, the heat treatment does not change the resonances or the structure of C6-AHL in the presence or absence of trehalose. The spectra of heat-treated C6-AHL alone (data not shown) was identical to the untreated C6-AHL spectra (Figure 3.11A) and the spectra of the heat-treated trehalose with C6-AHL (Figure 3.11B). Ultimately, this experiment demonstrated that trehalose forms a glass at high temperatures, the structure of which is not influenced by C6-AHL.
Together, the C6-AHL assay and the solid state $^{13}$C NMR spectroscopy illustrate that C6-AHL alone was tolerant of the 50°C treatment, but not the 60°C / 100°C treatment. The fact that C6-AHL activity was preserved at the higher treatment in the presence of trehalose suggests that trehalose plays a role as an osmoprotectant. Given that our model relies on a simple system, and lacks the complexity of an environmental microbial mat, we further studied the role of divalent cations during glass formation.

**Divalent cations stabilize enzyme during desiccation**

To further analyze the activity of acylase during glass formation, and to determine if its activity is being preserved in the model system, an enzymatic assay was performed after heat treatment. The initial glass samples containing acylase, described in Table 3.2, did not have any enzymatic activity after glass formation. It is known that some enzymes within microbial mats are stabilized by interactions with carbohydrates. However, given that our system simply mimics a microbial mat, and is lacking several of the key components therein, we added a variety of divalent cations in an attempt to further stabilize acylase.

The study found that the presence of manganese at pH 11 during heat treatment resulted in high enzymatic activity. At pH 7.5, calcium was most effective at preserving enzymatic activity. As seen in Table 3.3, we looked at glucose, trehalose, and dextran, and the dissolved cations manganese, magnesium, and calcium. In each case, we found that dissolved cation alone with acylase was not enough to preserve activity (data not shown). However, in some cases the addition of glucose, trehalose, or dextran during heat treatment aided in preserving the activity of acylase. The results of the glass formation
experiment and the acylase analysis indicated that several interactions within a microbial mat may be responsible for preserving the integrity of these compounds.

**C6-AHL loses activity in high salt solutions**

In an effort to mimic the natural microbial environment, the heat tolerance of C6-AHL was tested in a high salt solution. C6-AHL was dissolved and then dried in salt water collected from Salt Pond (280 ppt)

Trehalose is a common constituent of microbial mats, and is believed to provide some protection to small molecules during desiccation. To determine if the presence of trehalose aided in the retention of C6-AHL activity during exposure to 55° C, it was added to the experimental solution of salt water and C6-AHL. After drying for three days at 55° C in the presence of Salt Pond water, NaCl, or deionized water, C6-AHL was inactive.

Once again, the experiment was repeated with gentle heating over a longer time period to better mimic the microbial mat environment. The samples were heated at 35° C to dryness over the course of five days. The samples were then heated briefly at 55° C to remove residual water and force the trehalose to transition to a glass. C6-AHL did not retain activity under these conditions (in water, in salt water, or in NaCl) with or without trehalose present. Surprisingly, the trehalose did not act as an osmoprotectant in this case. These results indicate that the complex microbial mat ecosystem requires other compounds in addition to trehalose to preserve quorum sensing signal molecules.
3.2 DISCUSSION

Microorganisms have evolved to survive in extreme environments through a number of adaptations. Microbial mats consist of a structured, laminated community that allows microorganisms to survive and thrive in extreme environments. The hypersaline mat system in Salt Pond on San Salvador Island, Bahamas, experiences dramatic seasonal shifts in precipitation, ionic concentrations, and water availability. These shifts create an extreme environment for the resident microorganisms. Annual rainfall on San Salvador Island is approximately 100 cm, and evaporation exceeds precipitation most years. Rainfall is dependent on tropical storms, hurricanes, and related cyclical weather patterns. Consequently, the majority of precipitation occurs during the months of September and October. After the onset of the dry season, large areas of the mats become and remain desiccated for much of the year. The water level, salinity, and extent of desiccation for Salt Pond mats are therefore, closely linked to rainfall. Salinity is largely dominated by univalent ions (e.g. $\text{Na}^+$ and $\text{Cl}^-$) and a wide variety of trace metals. When compared to mats in more temperate climates, the San Salvador mats exhibit lower CO$_2$ and N$_2$ fixation. The extreme temperature and salinity during the dry season creates a hypersaline environment in the microbial mat. Average temperatures reached above 40°C, with the salinity steadily increasing to 300 ppt throughout the dry season. There are several key components within microbial mats that allow microorganisms to respond to these extreme conditions and survive.

Foremost, we theorize that the abundance of EPS in the microbial mat acts as a physical shield for the lower layers. The image of the dried Salt Pond mat (Figure 3.1) shows the thick upper crust of salt that protects the surface of the microbial mat. SEM
imaging confirmed that the upper layer of the microbial mat contained crystalline and amorphous polysaccharides, both of which provide a physical barrier to UV radiation and oxidative damage. Furthermore, we found that EPS was most abundant in the top most layer of the mat (Figure 3.5). The EPS of the Salt Pond microbial mat contained a wide variety of monosaccharides, most notably glucose. We suggest that the high concentration of glucose may be inclusive of a high trehalose concentration. Of the polysaccharides, trehalose forms the most stable glass, and is often used by the food industry as a preservative and stabilizer.

We next investigated the ability of divalent cations to stabilize enzymes during desiccation. We found that in the presence of glucose, trehalose, and dextran, calcium and manganese enhanced the stability of acylase during heat treatment. Desiccation increases potential for UV damage and oxidative damage; therefore we investigated the potential protective elements within the microbial mat. Trehalose is a $\alpha,\alpha-1,1$-linked glucose disaccharide that often provides osmoprotection and stability to small molecules. Trehalose is a major component of EPS, and is able to form a glass at high temperatures. We have observed biological activity in previously desiccated microbial mats after periods of rainfall. We theorize that trehalose plays a role in preserving microorganisms and small molecules during desiccation and throughout the dry season. Here we have provided evidence that trehalose protects the quorum sensing signal molecule C6-AHL at high temperatures. Trehalose is able to form a glass at temperatures above 55°C, the structure of which does not modify the structure of C6-AHL. Normally, C6-AHL would be degraded by high temperatures. Interestingly, we could not demonstrate the protective properties of trehalose in the presence of high salts. We theorize that several components
may be responsible for the preservation and osmoprotection of C6-AHL during desiccation.

A second compound, acylase, was also investigated in the presence of trehalose. We found that acylase was not able to withstand high temperatures, and was not aided by the presence of trehalose. However, acylase activity was improved when the enzyme was dried in the presence of divalent cations, specifically calcium and manganese. Salt Pond contains both of these cations in abundance, and is frequently flooded with sea water, where these cations are also present. The polysaccharides of EPS are known to retain and stabilize enzymes. Several trace metals are found in Salt Pond water, and may contribute to the formation of a protective layer of EPS.

3.3 CONCLUSION

This study showed that the formation of an organic glass occurred during desiccation of EPS at the Salt Pond microbial mat. Formation of the glass preserved the integrity and viability of cells, and the activities of important extracellular signal molecules for several years. Chemical communication is a crucial process guiding interactions among bacteria and archaea in hydrated conditions. AHLs were preserved in the extracellular microbial glass during dehydration of natural mats and were able to commence signaling upon rehydration. Under laboratory conditions, AHLs were protected in the presence of trehalose, a glass-forming disaccharide, which is abundant in the EPS of natural mats. Other EPS components may have also contributed the AHL preservation. Using solid state NMR, trehalose was shown to undergo a structural change and prevent denaturation of AHLs during desiccation. The trehalose/AHL complex
‘dissolved’ upon rehydration and allowed AHLs to resume signaling activity. It is proposed that glass formation has implications for the broader resiliency of microbial life in many environments.

Overall, this study suggests that trehalose glass may play an important role in preserving the integrity of quorum sensing signals and bacterial enzymes in a high salinity anhydrophobic microbial mat. Further investigations of environmental samples and higher complexity laboratory models are needed to fully understand the role of trehalose as an osmoprotectant.

3.4 MATERIALS AND METHODS

Sample Collection: Natural Mats
All samples were collected from Salt Pond, located near the northeastern end of San Salvador Island, Bahamas (24°05’ N, 74°30’ W). Intact mat samples (6 x 6 cm) were collected to a depth of approximately 2 cm and then returned to the Grice Research Center laboratory on San Salvador Island for further processing. For EPS analyses, the samples were sectioned into 1 mm thick slices under a dissecting microscope using sterile razor blades. The samples were transferred to 50 mL tubes and extracted (within 1 hour of collection) in the laboratory. The EPS sample was extracted with ethanol and dried at 35°C for several days.

Glass Formation
Solutions of 0.2 mg of glucose, trehalose, or dextran were prepared in sterile deionized water and incubated in open-top glass tubes at 50°C for 5 days on a heating block. Some
samples contained 0.1 mg $N$-hexanoyl-$L$-homoserine lactone (Cayman Chemical, Ann Arbor, MI) and/or 200 units acylase I from porcine kidney (Sigma-Aldrich). The first experiment was conducted at 50°C for five days to mimic the environment of a natural microbial mat. The second experiment was conducted at 60°C for five days followed by 15 minutes at 100°C to ensure the formation of a glass.

**Solid State NMR Spectroscopy**

Solid state NMR spectroscopy was conducted on samples of treated (glass) and untreated (control) trehalose and C6-AHL. Samples were incubated at 60°C for five days, followed by 15 minutes at 100°C. The pH of the samples was 7.5. Solid state $^{13}$C CP-MAS spectra were collected on a Bruker Avance III-HD 500 MHz spectrometer fitted with a 1.9 mm MAS probe. The spectra were collected at ambient temperature with sample rotation rate of 20 kHz. 1.5 msec contact time with linear ramping on the $^1$H channel and 62.5 kHz field on the $^{13}$C channel were used for cross polarization. $^1$H dipolar decoupling was performed with SPINAL64 modulation and 145 kHz field strength. Free induction decays were collected with a 27 msec acquisition time over a 300 ppm spectra width with a relaxation delay of 1.5s.

**Quorum Sensing Signal Detection**

*Chromobacterium violaceum* CV026 (provided by Professor Robert McLean of Texas Tech University) was used to detect $N$-hexanoyl-$L$-homoserine lactone (C6-AHL). Glass samples were resuspended in 50 μL sterile deionized water and allowed to rehydrate at room temperature for 10 minutes. 10 μL of this solution was applied to a sterile paper
disk on Luria Bertram (30 g/L) agar plates inoculated with \textit{C. violaceum} CV026. Plates were incubated at 30°C overnight. The production of a violet pigment by the bacteria indicated the presence of C6-AHL.

**Temperature tolerance of C6-AHL**

5 µM solutions of C6-AHL in water were subjected to 30 minutes of heat at 25, 35, 45, 55, and 65°C. Signal activity was determined by the biosensor \textit{C. violaceum} CV026. The C6-AHL was dissolved in small volumes of Salt Pond water and dried over the course of three days at both 35 and 55°C. The initial salt concentrations were 280 ppt, 140 ppt, 70 ppt, and 35 ppt. Pure NaCl was used as the control in the same concentrations. The experiment was repeated with 1 mM trehalose added to the solutions.

**Acylase I Enzymatic Assay**

Acylase I was detected by spectrophotometric analysis according to Mitz & Schlueter (1958). Briefly, the decrease in absorbance at 238 nm was observed every minute for five minutes for solutions containing the treated (in glass) or pure (control) acylase I and 100 mM potassium phosphate and 14 nM N-acetyl-L-methionine (Sigma-Aldrich). The number of units/mL enzyme was determined by \( (\Delta A_{238\text{nm}}/\text{min Test} - \Delta A_{238\text{nm}}/\text{min Blank}) \times 60 \times (3 \times \text{dilution factor}) / (0.019 \times 0.1) \).

**Glass Experiment with Cations**

Divalent cations were added to solutions of glucose, trehalose, or dextran at pH 7.5 or pH 11. Samples were comprised of 0.2 mg sugar, 200 units acylase I, and 0.1 mg dissolved
cation from either MnCl$_2$$\cdot$4H$_2$O, CaCl$_2$, NaCl, or MgCl$_2$$\cdot$6H$_2$O. Samples were heated in open-top glass tubes at 50°C for five days. After incubation, samples were resuspended in deionized water and acylase activity was determined spectrophotometrically with the aforementioned assay.

**DSC, TGA, and ICP-MS**

DSC was performed with a Q2000 Differential Scanning Calorimeter (TA Instruments, USA). Approximately 10 mg of sample was loaded into non-hermetic aluminum Tzero pans. An empty aluminum pan was used as a reference for all experiments. The heating rate was 10° C/min to 300° C under nitrogen flow. TGA characterization was performed with a Q500 Thermogravimetric Analyzer (TA Instruments, USA). Approximately 10 mg of sample was heated at a rate of 10 °C/min to 950 °C under nitrogen flow. Data were recorded by the instrument software. A qualitative trace metal analysis of water from Salt Pond was performed via inductively coupled plasma (ICP) – quadrupole mass spectrometry on a Thermo Finnigan Element XR ICP-MS.
Table 3.1 Composition of sugar monomers within EPS extracted from natural mats. Carbohydrate monomer composition of EPS isolated from the upper layer of microbial mat as determined by GC/MS. Values represent mole percentage of total mass. N=2

<table>
<thead>
<tr>
<th>Monomer</th>
<th>Hydrated</th>
<th>Desiccated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arabinose</td>
<td>4.2</td>
<td>2.1</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>7.6</td>
<td>11.8</td>
</tr>
<tr>
<td>Fucose</td>
<td>10.6</td>
<td>4.9</td>
</tr>
<tr>
<td>Xylose</td>
<td>16.3</td>
<td>13.3</td>
</tr>
<tr>
<td>Glucuronate</td>
<td>7.6</td>
<td>5.5</td>
</tr>
<tr>
<td>Galacturonate</td>
<td>4.7</td>
<td>2.5</td>
</tr>
<tr>
<td>Mannose</td>
<td>9.3</td>
<td>13.7</td>
</tr>
<tr>
<td>Galactose</td>
<td>8.9</td>
<td>17.5</td>
</tr>
<tr>
<td>Glucose</td>
<td>24.4</td>
<td>28.7</td>
</tr>
<tr>
<td>N-acetyl glucosamine</td>
<td>6.2</td>
<td>--</td>
</tr>
<tr>
<td>Unknown</td>
<td>0.2</td>
<td>--</td>
</tr>
<tr>
<td>Mass % of Carbohydrate of EPS</td>
<td>28%</td>
<td>31.4%</td>
</tr>
</tbody>
</table>
Table 3.2 C6-AHL activity in trehalose as determined by pigmentation response of *C. violaceum* CV026. ‘Yes’ indicates the presence of a violet pigment during bacterial growth, which is produced in response to C6-AHL. ‘No’ indicates the absence of a response. Samples were incubated at 50°C for five days or 60°C for five days followed by 100°C for 15 minutes.

<table>
<thead>
<tr>
<th></th>
<th>50°C</th>
<th>60°C/100°C</th>
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</thead>
<tbody>
<tr>
<td>pH 7.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C6-AHL</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>C6-AHL + Acylase I</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>pH 11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C6-AHL</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>C6-AHL + Acylase I</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 3.3 Acylase activity in sugar after heat treatment at 50°C for five days at pH 7.5 and pH 11.

<table>
<thead>
<tr>
<th></th>
<th>Glucose</th>
<th>Trehalose</th>
<th>Dextran</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mn</td>
<td>None</td>
<td>None</td>
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</tr>
<tr>
<td>Mg</td>
<td>None</td>
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<tr>
<td>Ca</td>
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<tr>
<td>pH 11</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mn</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Mg</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Ca</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>
Figure 3.1 Natural microbial mats collected from Salt Pond on San Salvador Island, Bahamas showing wet- (A) and dry- (B, C) season sections of mat surface. Note underlying layer abundant in cyanobacteria during wet season (A). During early dry-season, evaporites, including gypsum and halite, begin to appear as salinity increases (B), and eventually encrust the dry surface mat (C).
Figure 3.2 SEM of dried surface layer of microbial mat. Sample consists of a continuous crystalline/amorphous structure of primarily halite, with lesser amounts of gypsum, anhydrite, and other salts.

Figure 3.3 TEM of dry, natural microbial mats showing intact cells surrounded by a dense capsular layer of EPS.
Figure 3.4 Vertical cross-section of microbial mat in Salt Pond showing distinct layering of microbial groups. The uppermost surface Layer (L1) is the layer containing abundant EPS, diatoms, archaeb, as well as salt precipitates (during dehydration); Layer 2 (L2) is the dense green ‘cyanobacteria-rich’ layer; Layer 3 (L3) is a layer dominated by purple sulfur bacteria.

Figure 3.5 Abundances of EPS isolated from surface layers of hypersaline mat in Salt Pond, San Salvador Island, Bahamas. Surface Layer (L1) is the layer containing the salt precipitate and polymers; Layer 2 (L2) is the dense green ‘cyanobacteria’ layer; Layer 3 (L3) is the layer dominated by purple sulfur bacteria.
Figure 3.6 DSC thermogram recorded upon heating of trehalose dihydrate at rate of 10°C/minute. The sample was dried for five days at 35°C prior to analysis. The small shoulder at 85°C indicates a discontinuity in heat flow as the sample transitioned to the glassy state and absorbed heat ($T_g$). The large peak corresponds with the melting point of trehalose dihydrate (97°C).
Figure 3.7 DSC thermogram recorded upon heating of trehalose anhydrous at rate of 10°C/minute. The sample was dried for five days at 35°C prior to analysis. The small shoulder at 85°C indicates a discontinuity in heat flow as the sample transitioned to the glassy state and absorbed heat ($T_g$). The larger peak corresponds with the melting point of trehalose anhydrous (203°C).
Figure 3.8 DSC thermogram recorded upon heating of Salt Pond EPS at rate of 10°C/minute. The sample was dried for five days at 35°C prior to analysis. The multiple shoulders and peaks indicate the transition temperatures ($T_g$) of multiple compounds. Specifically, the $T_g$ of trehalose can be seen at 85°C.
Figure 3.9 TGA thermogram of trehalose dihydrate recorded upon heating at rate of 10°C/minute. The sample was dried for five days at 35°C prior to analysis. The green line represents weight loss occurred. The blue line represents the first derivative of the weight loss curve. The inflection point, the greatest rate of change on the weight loss curve (green), is indicated by the peak of the first derivative curve (blue).
Figure 3.10 TGA thermogram of Salt Pond EPS recorded upon heating at rate of 10°C/minute. The sample was dried for five days at 35°C prior to analysis. The small peak at 300°C corresponds with the peak expected for pure trehalose dihydrate. The blue line represents the first derivative of the weight loss curve. The inflection point, the greatest rate of change on the weight loss curve (green), is indicated by the peaks of the first derivative curve (blue).
Figure 3.11 Solid state $^{13}$C NMR spectra of (a) untreated C6-AHL, (b) heat-treated C6-AHL with trehalose, (c) heat-treated trehalose, and (d) untreated trehalose.
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