POLYMER-BASED NANOTHERAPEUTICS TO COMBAT DIFFICULT-TO-TREAT BACTERIAL INFECTIONS

Jessa Marie V. Makabenta

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POLYMER-BASED NANOTHERAPEUTICS TO COMBAT DIFFICULT-TO-TREAT BACTERIAL INFECTIONS

A Dissertation Presented

by

JESSA MARIE VALENZUELA MAKABENTA

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Department of Chemistry
POLYMER-BASED NANOTHERAPEUTICS TO COMBAT DIFFICULT-TO-TREAT BACTERIAL INFECTIONS

A Dissertation Presented
by
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DEDICATION

This work is dedicated to those who never tire of trying.

To my parents, Elma and Vicente, your love is the core of who I am.

To my siblings, Elvic, April and Vanessa, thank you for being the best support system in the world.
ACKNOWLEDGMENTS

This work was successfully completed because of the abundance of help and support I received from a lot of people.

To my thesis advisor, Prof. Vincent Rotello, my sincerest gratitude for the guidance and support throughout the years. Thank you for providing me plenty of encouragement and opportunities for growth, for your trust in my abilities and for giving me the freedom to explore research areas that I am interested in. In times of self-doubt, thank you for recognizing and reminding me that I am a capable scientist.

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It was such an incredible pleasure to work with the members of the Rotello group. I could not ask for a better set of people to work with daily, not only is the lab filled with talented scientists but also people who are compassionate and empathetic. I am immensely grateful to all the members for their significant contributions in this research, from brainstorming to assistance in experiments to manuscript preparations. Special thanks to the Avengerms, Ian, Ahmed and Jungmi, who I worked with a lot in almost all of my projects. I also want to sincerely thank the rest of the Antimicrobial subgroup, Ryan, Akash, Hassan and Elizabeth, and the research undergrads that I worked with. I also would like to acknowledge the contributions that Dave, Taewon, Rui, Ari, Rita, Aaro, Mingdi and Anil have made to this work.

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ABSTRACT

POLYMER-BASED NANOTHERAPEUTICS TO COMBAT DIFFICULT-TO-TREAT BACTERIAL INFECTIONS

SEPTEMBER 2023

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Directed by: Professor Vincent M. Rotello

The continuous emergence and spread of antibiotic-resistant bacteria are a global health emergency, debilitating the capability to prevent and cure various infectious diseases that were once treatable. Antibiotic therapy is further rendered ineffective due to biofilm formation and the ability of bacteria to thrive and colonize inside mammalian cells. Given the diminishing efficacy of available antibiotics combined with the scarcity of new therapeutics entering the antibiotic pipeline, innovative treatment strategies are urgently in demand. Nanomaterial-based strategies offer ‘outside of the box’ approach for the treatment of antibiotic-resistant bacterial infections. Nanomaterials feature tunable physicochemical properties that can be carefully modified to access multi-modal antimicrobial mechanisms novel to bacteria, allowing them to evade existing resistance mechanisms and have a higher barrier against resistance generation while maintaining high biocompatibility.

This research focused on leveraging poly(oxanorborneneimide)-based polymeric nanoparticles to develop different antimicrobial therapies effective against difficult-to-treat resistant bacterial infections, including wound biofilms and bacteria-induced peritonitis.
We fabricated different types of nanoparticles by varying their structural design and/or loading non-antibiotic therapeutics such as phytochemicals, siRNA or hydrophobic therapeutics. We then test these nanoparticles on *in vitro* and *in vivo* models to better understand their activity.

Antibiotic-resistant wound biofilm infections are a major global healthcare challenge. Chapters 2 to 7 discuss how we utilized and developed cationic antimicrobial polymeric nanoparticles (PNPs) as topical therapeutics against wound biofilm infections. Our strategies include use of PNPs 1) in combination therapy with antibiotics, 2) integration with hydrogel materials, and 3) as a nanocarrier for other therapeutics to achieve multi-modal treatment.

Intracellular pathogenic bacteria turn immune cells into a breeding ground for bacterial replication and reinfection, leading to challenging systemic infections including peritonitis. Chapters 8 and 9 focus on our strategy to address intracellular infections such as peritonitis using phytochemical-loaded polymeric nanoemulsions. The positively-charged polymer groups of the E-BNEs bind to the cell surface of macrophages, facilitating the entry of eugenol that then kills the intracellular bacteria.

Through this work, we identified appropriate strategies that allow us to afford nanotherapeutics that can breach the biofilm, as well as selectively interact and kill bacteria. So far, we have demonstrated that nanotherapeutics can do what antibiotics cannot: penetrate and destroy biofilms, and effectively eliminate intracellular bacteria both *in vitro* and *in vivo*. All these while maintaining safety to our cells and without resistance development observed. Polymer nanotherapeutics offer a promising alternative to antibiotics, alleviating challenges faced in the post-antibiotic era.
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CHAPTER 1

NANOMATERIAL-BASED THERAPEUTICS FOR ANTIBIOTIC-RESISTANT BACTERIAL INFECTIONS


1.1. Introduction

The emergence of antibiotic resistance in bacteria has resulted in the challenge of recalcitrant infections\textsuperscript{1,2}. Multidrug-resistant (MDR) bacteria are a global crisis, increasing morbidity and mortality of infected individuals and negatively impacting the clinical outcome of a wide range of groups, including those in intensive care units, undergoing surgery, transplantation, or cancer treatment\textsuperscript{2,3}. A 2017 report from the WHO Global Antimicrobial Surveillance System highlighted antibiotic resistance as a worldwide challenge\textsuperscript{4}. The estimated cost of treating antibiotic-resistant infections is substantial (~US$50,000 per individual), with an estimated US$20 billion societal cost annually\textsuperscript{5}. The use, and in some situations misuse, of antibiotics, combined with the scarcity of new therapeutics entering the antibiotic pipeline, further exacerbate this public health threat\textsuperscript{6}.

Planktonic (free-floating) bacteria are central players in multiple health threats, including sepsis\textsuperscript{3}. Infections associated with planktonic bacteria present acute threats and are rapidly becoming more challenging to treat due to rising rates of acquired antibiotic resistance. This challenge is amplified when bacteria form biofilms, which are associated with recurring and chronic bacterial infections\textsuperscript{7}. The ability of bacteria to protect themselves within biofilms complicates treatment of numerous infection-types, including chronic wounds, osteomyelitis, and infective endocarditi\textsuperscript{8}. Antibiotic resistance associated
with the biofilm state is distinct from acquired resistance, but can compound and exacerbate therapeutic challenges\textsuperscript{9}. Bacteria cells produce extracellular polymeric substance (EPS) that may serve as a barrier against host immune responses and some conventional antimicrobial agents\textsuperscript{7,9}. More importantly, biofilms exhibit a diversity of altered phenotypes, including slow growth rates, the presence of persister cells, and spatial and chemical heterogeneities that contribute to resistance to many available antibiotics\textsuperscript{10,11}.

Antibiotics are currently the main therapeutic strategy for treating both planktonic and biofilm infections\textsuperscript{12}. They target processes necessary for growth and/or survival of bacteria, including cell wall and cell membrane synthesis or maintenance, or the production of DNA, RNA or essential proteins. Many antibiotics are derived from products that have been deployed by microorganisms to combat one another for billions of years. The offensive molecules that have evolved throughout this warfare have generated defense responses; bacteria have developed the intrinsic ability to escape the activity of many traditional antibiotics\textsuperscript{13} (Figure 1.1). Eradicating MDR bacteria may require multiple or high dosages of antibiotic agents or the use of ‘last resort’ antibiotics\textsuperscript{12}. Adding to the therapeutic challenge, when bacteria are present in biofilms, biofilm-associated resistance becomes a compounding factor, oftentimes requiring physical removal of the biofilm through aggressive debridement, for example, accompanied by high doses of antibiotics\textsuperscript{14,15}. These strategies can result in long and expensive treatments, with the possibility of adverse effects and uncertain outcomes.
Figure 1.1. Antibiotic resistance mechanisms. Bacteria have multiple survival mechanisms that enable them to evade killing by antibiotics. The biofilm state itself confers resistance against antibiotics.

Nanomaterials (NMs) are organic, inorganic or hybrid particles that are generally ≤100 nm in size; however, particles ≤500 nm in size are also considered as nanomaterials\textsuperscript{16}. They can be in different forms including nanoparticles, nanowires and nanorods. Nanomaterials have an almost unlimited range of structures and morphologies, from rods to pyramids to fibrous networks to spheres, with hollow or solid interiors bearing rough or smooth surfaces. Materials in the nanoscale realm possess distinctive physicochemical characteristics, including size, shape and surface, compared to their bulk counterparts\textsuperscript{17}. The unique properties of nanomaterials have revolutionized many technologies and industries, including medicine. Being comparable in size to biomolecules and bacterial intracellular structures, nanomaterials can be engineered as new therapeutic modalities.
Nanomaterials access antimicrobial modalities that are novel to bacteria, and thus are not in their natural defensive arsenal. Recent advances in nanomaterial-based systems provide new opportunities to address MDR planktonic as well as biofilm infections, acting either as inherent therapeutics or nanocarriers for antimicrobial agents\textsuperscript{18}. The unique physicochemical properties of nanomaterials, such as size, shape, and surface chemistry, influence their therapeutic activity\textsuperscript{19}. The sizes and shapes of different nanomaterials are similar to bacterial biomolecules, affording a variety of interactions that can be regulated through surface functionalization. High surface to volume ratios and multivalent interactions are important for creating antibacterial NMs\textsuperscript{18,19}. Nanomaterials can evade existing resistance mechanisms and may be less prone to select for resistance than conventional antibiotics\textsuperscript{20}. Moreover, nanomaterials have the ability to eradicate bacteria in biofilms\textsuperscript{19}. Taken together, nanotechnology provides a new toolkit for the creation of treatment strategies for MDR infections.

1.2 Mechanisms against planktonic bacteria

The array of sizes and shapes adopted by nanomaterials offers unique capabilities for targeting bacteria\textsuperscript{21} (Figure 1.2). Nanomaterials can employ multiple bactericidal mechanisms, including direct cell wall and/or membrane damage, generation of reactive oxygen species (ROS), and binding to intracellular components. Most antibiotics target cell walls or membranes, or disrupt intracellular processes. Nanomaterials can target these cellular features, albeit in different ways, and offer advantages in combating antibiotic-resistant pathogens relative to small molecule drugs (TABLE 1.1). Further, nanomaterials can be used as nanocarriers for delivery of therapeutic agents\textsuperscript{21,22}. The mechanisms employed by nanomaterials arise from their unique physicochemical properties, in
particular multivalent interactions with bacterial cells. Van der Waals forces, receptor-ligand interactions, hydrophobic interactions and electrostatic attractions have roles in NM–bacteria interfaces\textsuperscript{23}.

\textbf{Figure 1.2.} Nanomaterials and their antimicrobial mechanisms. Bacteria typically have diameters ranging 0.2–10 µm. Varying nanomaterials and preparation methods provide a wide range of particle sizes (2–500 nm) that facilitate maximal contact and strong interactions with bacterial membranes. Nanomaterials display a variety of bactericidal mechanisms. Electrostatic interactions of NMs with the negatively charged groups present on bacterial surfaces results in membrane damage and cytoplasmic leakage. NMs can bind various intracellular components, such as ribosomes, proteins and/or DNA, disrupting their functions. NMs with catalytic activities increase the production of reactive oxygen species (ROS), such as hydroxyl radicals and superoxides, causing oxidative cellular stress. Nanomaterials can also be used for delivery of therapeutic agents; some nanomaterials readily enter bacterial cells through membrane fusion, facilitating delivery of their cargo.
1.2.1. Cell wall and membrane disruption

The bacterial cell envelope has evolved to serve as a physical barrier towards antimicrobials. Teichoic acids, present in the cell wall of Gram-positive bacteria, and lipopolysaccharide, found in the outer membrane of Gram-negative bacteria, have phosphate groups that render bacterial surfaces negatively charged. This highly polar environment limits penetration of hydrophobic antimicrobials across membranes, compromising their activity against bacteria\(^{23}\).

Bacterial cell surfaces are more negatively charged than are those of mammalian cells, facilitating preferential electrostatic interactions with positively charged materials\(^{24}\). The charge density and hydrophobicity of the NM surface are important factors in designing NMs to selectively disrupt bacterial membranes\(^{25,26,27}\). Highly cationic nanomaterials can bind to the surface of mammalian cells, as can NMs with overly hydrophobic surfaces, reducing selectivity. Cationic nanomaterials with good amphiphilic balance, the optimized balance between hydrophobicity and cationic charge, can provide potent antimicrobial effects with low levels of hemolysis and cytotoxicity\(^{25}\).

A range of nanomaterial-based strategies focus on targeting the negatively charged surface of planktonic bacteria\(^{25,26,28,29}\). One study synthesized biodegradable cationic and amphiphilic polycarbonates that self-assemble into cationic micellar nanoparticles (NPs), killing methicillin-resistant \textit{S. aureus} (MRSA). These polymeric NPs interact with bacteria through electrostatic interactions, resulting in disruption of the membranes and cell lysis\(^{30}\). ‘Nanoknives’, materials with sharp-pointed edges, are particularly effective in compromising bacterial membrane integrity. In one study, single-walled carbon nanotubes and graphene oxide ruptured the cell surface of \textit{Ralstonia solanacearum}, leading to
cytoplasmic leakage and bacterial death\textsuperscript{31}. The ability of bacteria to develop resistance against therapeutics that damage the cell envelope is likely to be limited, making these strategies promising for long-term use with minimal risk of emergence of bacterial resistance\textsuperscript{29,32}.

Table 1.1. Overcoming resistance mechanisms

<table>
<thead>
<tr>
<th>Mechanism of action</th>
<th>Antibiotics</th>
<th>Potential ways nanomaterials evade resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Class</td>
<td>Select resistance mechanism(s)</td>
</tr>
<tr>
<td>Cell wall or membrane</td>
<td>β-lactams</td>
<td>Drug modifying enzymes\textsuperscript{33}</td>
</tr>
<tr>
<td>disruption</td>
<td>Glycopeptides</td>
<td>Binding site modifications\textsuperscript{34}</td>
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<td></td>
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<td>Porin changes\textsuperscript{35}</td>
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<tr>
<td></td>
<td>Peptide antibiotics</td>
<td>Outer membrane modifications\textsuperscript{37}</td>
</tr>
<tr>
<td>Damage to intracellular</td>
<td>Aminoglycosides</td>
<td>Drug modifying enzymes\textsuperscript{35}</td>
</tr>
<tr>
<td>components</td>
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<td></td>
<td>Macrolides</td>
<td>Efflux pumps\textsuperscript{40}</td>
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<td></td>
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<td>Binding site modifications\textsuperscript{35}</td>
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<td></td>
<td>Quinolones</td>
<td>Binding site modifications\textsuperscript{35}, Porin changes\textsuperscript{35}</td>
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Physical damage to the cell envelope limits development of resistance; Flexible design space and unique physico-chemical properties can be used to maximize disruptive interactions\textsuperscript{18}.
1.2.2. Generation of reactive oxygen species

Reactive oxygen species (ROS) are byproducts of cellular oxidative metabolic processes that affect cell differentiation, signaling, survival and cell death. Accumulation of excessive ROS results in lethal oxidative stress. ROS can damage cells through multiple mechanisms, in particular through reaction of superoxide and hydroxyl radicals with thiols in proteins, deactivating membrane receptors. There are several mechanisms by which NPs generate ROS: direct ROS production from the NP surface or from leached ions; interaction with intracellular organelles; and oxidation through interaction with redox active biomolecules, including NADPH oxidase. Some metal-based NPs employ ROS generation as their major antibacterial mechanism (reviewed in refs.) due to their inherent photocatalytic activity (that is, photodynamic therapy).

An example of ROS-based antibacterial activity is the release of free copper (Cu\(^+\)) ions from copper iodide (CuI) NPs, generating ROS and damaging bacterial DNA and intracellular proteins of *Escherichia coli* and *Bacillus subtilis*. Silver–zinc oxide nanocomposites likewise exhibited antibacterial activity against *S. aureus* and antibiotic-resistant *E. coli*, ascribed to potent ROS generation and release of silver (Ag\(^+\)) and zinc (Zn\(^{2+}\)) ions. These combined processes then generated a cascade of bactericidal effects, including damaged cell membranes, protein dysfunction, inhibition of DNA replication and leakage of intracellular contents. Silver and other Fenton-inactive metals increase ROS in bacteria through their ability to disrupt cellular donor ligands coordinating with iron, such as cysteine, and to induce release of Fe from [4Fe-4S] clusters; this Fe release then increases ROS formation.
Gold NPs (AuNPs) have also shown enzyme-like activities\(^{49}\). Mesoporous silica can provide support and enhance the stability and catalytic-activity of the AuNPs\(^{50}\). AuNPs bound on the surface of bifunctionalized mesoporous silica nanoparticles (MSN) (MSN–AuNPs) have been shown to display peroxidase- and oxidase-like activities, killing both Gram-positive and Gram-negative bacteria, dual enzyme-like activity which increases efficiency of ROS production, imparting oxidative stress to bacteria\(^{51}\).

1.2.3. Damage to intracellular components

Cellular homoeostasis and intracellular signaling pathways are central to the function and survival of bacteria. Nanomaterials can be engineered to interfere with these processes, ultimately leading to cell death. These disruptions include alteration in gene expression, protein synthesis or DNA damage\(^{52,53}\). As an example, AuNPs were functionalized with 4,6-diamino-2-pyrimidinethiol (DAPT), an analogue of 2-pyrimidinethiol (found in \textit{E. coli}), to generate pyrimidine-capped AuNPs (Au–DAPT)\(^{54}\). These NPs inhibited proliferation of MDR strains of \textit{E. coli} and \textit{P. aeruginosa}. Mechanisms of action of Au–DAPT were elucidated through the following: gel electrophoresis showing the ability of NPs to bind bacterial DNA; electron microscopy images displaying leakage of nucleic acids and Au–DAPT binding to ribosomes and chromosomes; an \textit{E. coli}-free transcription/translation system demonstrating protein synthesis inhibition; and colorimetric assays showing selective chelation of Mg\(^{2+}\), destabilizing the cell membrane. Similarly, polymer-coated silver NPs killed \textit{E. coli} cells by inhibiting both the Krebs cycle and amino acid metabolism\(^{55}\). Polymers were used to modify the surface of silver NPs (AgNPs) to increase interactions with bacterial cells. The mechanism of action was confirmed by observing downregulation of the expression of
genes associated with tricarboxylic acid cycle (aceF and frdB) and amino acid metabolism (gadB, metL and argC), ultimately leading to cell death.

1.2.4. Delivery of therapeutic agents

Several therapeutics incorporating nanotechnology called— liposomal nanoformulations in particular — have been FDA-approved and made available for clinical use to treat different diseases, including cancer\textsuperscript{56}. Similarly, NMs may be used as carriers for delivery of antimicrobial agents\textsuperscript{57}. Therapeutics can be encapsulated inside NMs or bound to their surfaces\textsuperscript{58,59}. NMs offer protection of these agents against enzymes and molecules that might otherwise degrade them. This protection can increase therapeutic efficiency of a drug, resulting in lower dosage requirements to augment therapeutic effects and reduce host toxicity\textsuperscript{60}. The use of delivery systems can also enhance stability, solubility and biocompatibility of otherwise pharmacologically challenging antibiotics. Use of nanocarriers can minimize selection of resistance through delivery of therapeutics that elicit multiple mechanisms of action, and through targeted release of cargo, preventing exposure of bacteria to sub-inhibitory doses of the drug\textsuperscript{61,62}. For instance, the antibiotic gentamicin loaded into poly(lactide-co-glycolide) NPs exhibited improved antimicrobial activity against \textit{P. aeruginosa in vitro} and \textit{in vivo}. Subsequently, levofloxacin loaded into silver core-embedded mesoporous silica nanoparticles (Ag@MSNs@LEVO) afforded an effective treatment of MDR isolates of \textit{E. coli}; the combination of antibiotic levofloxacin with Ag@MSNs yielded a synergistic antimicrobial effect. The silver component of the system not only functioned as a carrier but also imparted antimicrobial effects through silver ion generation. In an \textit{in vivo} murine peritonitis model, treatment with Ag@MSNs@LEVO reduced bacterial burden by three orders of magnitude, with
concomitant reduction of damage to the spleen and peritoneum, with no toxicity observed\textsuperscript{63}. In a related approach, ampicillin was attached to the surface of AuNPs and AgNPs, yielding broad-spectrum bactericidal agents that evade resistance mechanisms of MDR strains of \textit{P. aeruginosa} and \textit{Enterobacter aerogenes}, and of MRSA\textsuperscript{59}.

Therapeutic selectivity and enhancement of delivery efficiency can be achieved through release of drug in response to specific stimuli\textsuperscript{62,64}. Bacterial infection sites are weakly acidic and that can be targeted\textsuperscript{62,65,66}. For example, vancomycin was encapsulated in a pH-responsive, surface charge-switching triblock copolymer poly(D,L-lactic-co-glycolic acid)-b-poly(L-histidine)-b-poly-(ethylene glycol) (PLGA–PLH–PEG). Therapeutic cargo was released only upon interaction with the acidic infection site, providing a target for vancomycin delivery. PLGA was chosen due to its low toxicity and ease of surface fine tuning; PEG reduced off-target interactions, prolonging circulation time; and PLH provided the charge-switchable characteristic of the polymer. The selective protonation of the imidazole groups of PLH at weakly acidic conditions allowed for a stimuli-responsive effect. Biomaterials can also provide charge-switching behavior, with pH-triggered release of vancomycin achieved using chitosan NPs. Further, bacterial toxins can be used as a trigger for release of antimicrobials. Lecithin, a phospholipid, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-poly(ethylene glycol) (DSPE-PEG3400) were used to coat a mixture of fatty acids, forming liposome-based nanoreactors that release calcium peroxide and rifampin in the presence of \textit{α}-toxin, a pore-forming toxin produced by \textit{S. aureus}\textsuperscript{67}. This strategy selectively targeted pathogenic bacteria as demonstrated by its antimicrobial activity against MRSA and minimal effect on non-pathogenic \textit{B. subtilis}. 
Overall, nanomaterials provide multiple bactericidal pathways to combat bacteria and evade antibiotic resistance mechanisms. Appropriate engineering of size, shape and surface properties provides a broad design space for novel antimicrobial agents.

1.3. Combating planktonic bacteria

Drug resistant hospital-acquired (nosocomial) infections are challenging to treat. A group of pathogens comprised of *Enterococcus faecium*, *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter* species — collectively termed as ‘ESKAPE’ pathogens — is responsible for the majority of nosocomial infections, complicating the conditions of patients that are often immunocompromised\(^2,3,6\). Approaches for treating infections caused by these pathogens are limited due to the rapid rate of resistance development, even against ‘last resort’ antibiotics\(^6\). In this regard, nanomaterials can provide a lifeline for therapeutic design, as there is limited to no resistance development observed with nanomaterial-based strategies\(^20,30,32,68\).

Numerous studies have explored the utility of nanomaterials against the ‘ESKAPE’ pathogens (Figure 1.3)\(^69,70,71,72\). One study reported the activity of structurally nanoengineered antimicrobial peptide polymers (SNAPPs) against MDR Gram-negative ESKAPE pathogens, *in vitro* and in an *in vivo* murine peritonitis model\(^73\). Researchers designed artificial antimicrobial peptide (AMP)-inspired peptide polymer NPs consisting of lysine and valine residues that self-assemble into star-shaped unimolecular structures, mimicking AMPs. SNAPPs elicit multiple proposed bactericidal mechanisms, including damage to outer and inner cell membranes, disruption of ion efflux or influx regulation and induction of an apoptotic-like cell death pathway. The proposed multimodal antimicrobial
activity of SNAPPs renders the barrier to resistance high. Comparing the concentration that results in death of 50% mammalian cell population [that is, the half maximal inhibitory concentration (IC$_{50}$)] and concentration which kills half of bacterial isolates [minimum bactericidal concentration (MBC$_{50}$)], SNAPPs had a therapeutic index higher than colistin, a drug of last resort for MDR Gram-negative bacillary infections. Further, MDR A. baumannii did not acquire resistance towards SNAPPs after multiple passages in sub-inhibitory concentrations. Liposome-based NPs are another promising system, restoring potency of the antibiotics cefepime, imipenem and ceftazidime against MDR P. aeruginosa$^{74}$, chloramphenicol against MRSA$^{75}$ and amikacin against K. pneumoniae$^{76}$ through efficient drug delivery. Similarly, delivery of AMPs was achieved with the use of PLGA NPs, providing a successful treatment strategy for P. aeruginosa lung infection in an in vivo murine model$^{77}$. 
Figure 1.3. Examples of nanomaterial-based strategies used to combat bacterial infections. Nanomaterial-based strategies can be used to combat planktonic bacterial infections (parts a and b), intracellular infections (parts c and d) and biofilm infections (parts e and f). Image in part a reproduced, with permission, from REF 73 © (2016) Macmillan Publishers Limited, part of Springer Nature. Image in part b reproduced, with permission, from REF 74 © (2019) American Chemical Society. Image in part c reproduced, with permission, from REF 8888 © (2016) Elsevier Ltd. Image in parts d and f reproduced, with permission, from REF 84 and 130, respectively © (2018) American Chemical Society. Image in part e reproduced, with permission, from REF. 122 © (2018) Nature Communications. All rights reserved.
1.4. Combating intracellular bacteria

Bacteria can reside within mammalian cells, giving rise to recurring systemic infections\textsuperscript{78}. For example, \textit{Salmonella enterica} subsp. \textit{enterica} serovar Typhimurium is a common facultative intracellular pathogen that causes life-threatening food-borne infections in millions of people worldwide each year \textsuperscript{79}. \textit{Salmonella} species can survive and replicate inside host cells, including macrophages. Intracellular localization of bacteria adds a level of complexity to treatment, because many antibiotics have limited ability to cross mammalian cell membranes and can also be actively exported out by the host cell\textsuperscript{80,81}. Nanomaterials can mitigate this challenge through their ability to penetrate eukaryotic cells, as well as through their high drug loading capacity (Figure 1.3).

In one example of nanomaterial-based treatment of intracellular infections, enrofloxacin-loaded docosanoic acid solid lipid nanoparticles (SLNs) increased intracellular accumulation of enrofloxacin up to $\sim$40-fold and enhanced \textit{Salmonella enterica} subsp. \textit{enterica} serovar Typhimurium killing inside macrophages\textsuperscript{82}. In another approach, colistin, a poorly permeable antibiotic, was formulated into liposomes functionalized with a bacterial-derived protein to promote internalization into eukaryotic cells, providing therapeutics with high oral bioavailability\textsuperscript{83}. In another strategy, gentamicin was loaded into MSN with bacterial toxin-responsive lipid bilayer surface shells. Functionalization of the MSN surface shell with bacteria-targeting peptide ubiquicidin (UBI\textsubscript{29-41}) allowed targeted treatment of intracellular \textit{S. aureus}\textsuperscript{84}.

\textit{Mycobacterium tuberculosis}, the causative agent of tuberculosis, is another example of an intracellular pathogen that survives within host macrophages\textsuperscript{85}. Several studies have demonstrated the activity of nanomaterials against intracellular...
Mycobacterium species. One study reported a library of cationic star-shaped polycarbonate nanostructures with wide-spectrum antimicrobial activity and low rates of haemolysis\textsuperscript{86}. Mannose-functionalization of the polycarbonates allowed mannose receptor-mediated entry of the nanostructures into the macrophages, consequently restricting the growth of intracellular Mycobacterium bovis BCG. In another study, biodegradable multimetallic microparticles (MMPs), consisting of AgNPs and zinc oxide nanoparticles (ZnONPs) encapsulated within PLGA polymer, were used to deliver the antituberculosis drug rifampin into M. tuberculosis-infected alveolar macrophages, demonstrated through an in vitro intracellular infection model\textsuperscript{87}. The ability of AgNPs and ZnONPs to interact with and compromise bacterial membrane stability furthered the antimicrobial effects of the system.

Nanomaterial-based strategies to combat other intracellular pathogens have also been developed. For example, AuNP–DNA aptamer conjugates loaded with antimicrobial peptides showed activity against intracellular Salmonella enterica subsp. enterica serovar Typhimurium\textsuperscript{88} and Vibrio vulnificus\textsuperscript{89} in in vivo murine infection models. In another example, gentamicin-loaded AuNPs decorated with phosphatidylcholine eradicated intracellular Listeria monocytogenes and P. aeruginosa in infected macrophages\textsuperscript{57}.

1.5. Therapeutic strategies against biofilms

MDR biofilm infections present a particularly difficult therapeutic challenge\textsuperscript{90}. The matrix composed of EPS may provide a barrier to some cellular and small molecule (for example, antibiotic) assaults. Bacteria embedded within the matrix are capable of synergistic interactions, cell-to-cell communications and the transfer of resistance
Furthermore, the deeper layers of the matrix have low oxygen and nutrient supply, inducing formation of dormant persister cells, which promote antimicrobial tolerance and resistance\textsuperscript{90, 91} (Figure 1.1).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Nanomaterial properties and design elements. Thoughtful engineering of nanomaterial surfaces modulates interactions between NPs and bacteria. The interface between NPs and bacteria is characterized by hydrophobic and electrostatic interactions and van der Waals forces that can be modulated by tuning nanomaterial properties. Tuning the size, surface and shape of a nanomaterial can maximize antibacterial activity, biofilm penetration, biocompatibility, biodistribution and the therapeutic index. COOH-QD, carboxyl-functionalized quantum dot; hexyl-QD, dimethylhexyl ammonium-functionalized quantum dot; PEG-QD, polyethylene glycol-functionalized quantum dot; TTMA-QD, trimethyl ammonium-functionalized quantum dot. Figure adapted with permission from ref.132, The Royal Society of Chemistry.}
\end{figure}

Overcoming the physical barrier presented by biofilms is needed to combat biofilms. The EPS is comprised of biopolymers including nucleic acids, proteins and polysaccharides that provide a three-dimensional protective scaffold for bacteria. The matrix is rich in negatively charged components and hydrophobic groups, with pores filled with water facilitating the transport of nutrients\textsuperscript{10}. Tuning surface functionality and design of NMs can facilitate biofilm penetration\textsuperscript{92, 93} (Figure 1.4). Size and electrostatic
interactions are important factors influencing the biofilm penetration profile of nanomaterials. Generally, uncharged NPs with sizes <350 nm have greater mobility across pores within biofilms, whereas cationic NPs have good distribution throughout the matrix\textsuperscript{61, 94, 95, 96}.

### 1.5.1 Targeting resident pathogens

Upon biofilm penetration, nanomaterials can interact with bacteria and exert the therapeutic mechanisms discussed above for planktonic bacteria (Figure 1.5a). For instance, the efficient biofilm penetration profile and bacterial membrane-damaging activity of poly(oxanorborneneimide)-based cationic polymeric NPs eradicated MDR biofilms of \textit{P. aeruginosa}, \textit{Enterobacter cloacae} complex and MRSA\textsuperscript{94}. In another approach, the use of stimuli-responsive NPs led to the activation of bactericidal effects in a spatio-temporally controlled manner. pH-responsive silver nanoantibiotics (rAgNAs) were developed using self-assembled silver nanoclusters and the charge-switchable ligand of poly(ethyleneglycol)-poly(aminopropyl imidazole-aspartate)-polyalanine (PEG–PSB–PALA)\textsuperscript{97}. Protonation of the imidazole groups in the low-pH microenvironment of biofilms induced disassembly of rAgNAs due to electrostatic repulsion with silver ions. Disassembly into smaller Ag nanoclusters enabled biofilm penetration, killing deeply embedded MRSA cells. Similarly, application of an external magnetic field facilitated biofilm penetration by AgNPs\textsuperscript{98}; superparamagnetic iron oxide nanoparticles were coated with silver rings and the generated magnetic field allowed biofilm penetration, with silver conferring antibacterial activity.
Figure 1.5. Eradicating biofilms using nanoparticles. Biofilms are comprised of cells with phenotypic heterogeneity embedded across the 3D-matrix of their self-secreted extracellular polymeric substance (EPS). The ability of nanoparticles (NPs) to penetrate throughout the matrix allows them to interact with cells, both susceptible (blue) and resistant (red), entrenched within the EPS (part a) and/or initiate disruptive interactions with the matrix that weaken physicochemical interactions responsible for keeping the 3D structure of biofilms intact (part b). NPs can then either exert their inherent antimicrobial action or deliver therapeutic agents, such as antibiotics or essential oils, to kill the bacteria within the biofilms. NPs can alternatively deliver EPS-degrading enzymes that promote dispersion of biofilms, facilitating their disruption.

Nanomaterials can also deliver therapeutics to bacterial cells embedded within the EPS matrix. For example, although the potent antimicrobial carvacrol oil, an essential oil found in oregano and thyme, poorly penetrates biofilms, one study utilized carvacrol to eradicate biofilms using biodegradable oil-in-water crosslinked polymeric nanocomposites (X-BNCs). X-BNCs eliminated MDR biofilms of both Gram-negative and -positive
bacteria while maintaining minimal cytotoxicity towards mammalian cells\textsuperscript{32}. The polymer scaffold (PONI-GMT) contained guanidinium, maleimide and tetraethylene glycol monomethyl ether groups. The cationic property of the nanocomposite was attributed to guanidinium. The presence of maleimide groups provided crosslinking sites and imparted biodegradation points onto the nanocomposite while the tetraethylene glycol monomethyl ether conferred hydrophilicity to the assembly. Careful design of the polymer increased solubility, stability, biodegradability, and antimicrobial potency of carvacrol oil, while assisting its penetration of the biofilm matrix. Similarly, nanoscale liposomes delivered the antibiotic amikacin through size-dependent biofilm penetration, as a strategy to treat chronic \textit{P. aeruginosa} biofilm lung infections\textsuperscript{99}, an approach that is currently in phase III clinical trials.

\textbf{1.5.2 Disrupting the EPS matrix}

Beyond killing bacteria, the EPS matrix can be disrupted for the purpose of treating biofilms\textsuperscript{100}. EPS scaffold remaining after treatment can be inhabited and populated by other microorganisms. Different NM-based approaches can be employed to disperse EPS matrix, including mechanical disruption and delivery of matrix-degrading enzymes (e.g., DNase, hydrolase, protease) (Figure 1.5b). For instance, poly(lactic-co-glycolic acid) NPs loaded with ciprofloxacin were functionalized with DNase I to target \textit{P. aeruginosa} biofilms\textsuperscript{101}. DNase I degraded eDNA, rendering the 3D network fragile and susceptible to ciprofloxacin. Similarly, AuNPs functionalized with proteinase K dispersed \textit{Pseudomonas fluorescens} biofilms\textsuperscript{102}. Alternatively, magnetic iron oxide nanoparticles (MNPs) disrupted MRSA biofilms with the application of direct current and alternating current magnetic fields\textsuperscript{103}. Application of a rotating direct current magnetic field mechanically damaged the
biofilm matrix. MNPs traversing across the 3D network acted as ‘shield breakers’, destroying biofilms through static friction. Exposure of MNPs to an alternating current magnetic field resulted in a localized increase in temperature that dispersed embedded cells. As mechanisms of action of these MNPs do not include killing of bacteria, this system offers a long-term anti-biofilm strategy that may escape resistance development.

A promising strategy for targeting biofilm growth is interruption of bacterial communication systems essential for coordinated activities, including colonization and biofilm development. Bacteria communicate through quorum sensing, a process that can be sabotaged to prevent formation of biofilms or induce their dispersion\textsuperscript{104,105,106}. One study demonstrated that hampering quorum sensing can silence bacterial communication\textsuperscript{107}. Silicon dioxide NPs (SiNPs) decorated with β-cyclodextrin (β-CD) blocked communication between \textit{Vibrio fischeri} cells. \textit{V. fischeri} exhibits bioluminescence controlled by population density, which can be monitored through the quorum sensing signaling molecule acylhomoserine lactone (HSL). The β-CD group of SiNPs binds to HSL, quenching its activity. As a result, the luminous output of \textit{V. fischeri} was reduced. Further, downregulation of luminescence genes, \textit{luxA} and \textit{luxR}, was observed. Other studies have demonstrated inhibition of biofilm formation and virulence factors by deactivating quorum sensing molecules using liposome-based NPs\textsuperscript{108}, chitosan nanoparticles\textsuperscript{109,110} and metal-based nanoparticles\textsuperscript{111,112}.

Nanomaterial penetration profiles predict success of biofilm elimination. Size and amphiphilicity mainly influence NP distribution across the biofilm. The exact interactions of NPs with the EPS depend on the type of biofilm, which varies by species and in some
cases strain of bacteria. Controllable parameters of nanomaterials provide a flexible toolkit to address the diversity of biofilm infections.

1.6 Combating biofilm infections

The number of biofilm-related infections continues to grow year by year\textsuperscript{113,114}. Bacteria can form biofilms in and on tissues and organs, including on skin, in the oral cavity, and on linings of gastrointestinal and respiratory tracts\textsuperscript{8, 90}. Biofilms largely contribute to chronic and persistent infections. With advances in the understanding of medical biofilms, nanotherapeutic strategies have emerged to potentially address biofilm infections.

1.6.1 Oral biofilms

The oral cavity is a major site for biofilms; \textit{Streptococcus mutans} is a common oral biofilm pathogen. The acidic microenvironment of dental biofilms (that is, plaque) results in destruction of tooth enamel, causing dental caries\textsuperscript{115,116}. NP-based strategies have been used to address oral biofilm-associated infections, taking advantage of the highly acidic oral biofilm microenvironment. Liposomes coated with the quaternary ammonium-modified chitosan have been used to deliver the antibiotic doxycycline to \textit{Porphyromonas gingivalis} oral biofilms\textsuperscript{117}. The residual amines of chitosan provide pH-responsive groups that are protonated under acidic conditions, providing pH-dependent activity. Similarly, nanocarriers fabricated with pH-responsive block copolymers that can bind to negatively charged hydroxyapatite have been used to deliver farnesol\textsuperscript{118} and chlorhexidine\textsuperscript{119} for treatment of dental caries. NPs that induce ROS production and EPS matrix degradation are also being investigated for oral biofilm treatment. For instance, catalytic NPs (CAT-
NPs) consisting of biocompatible Fe$_3$O$_4$ were utilized to catalyze in situ generation of free radicals from H$_2$O$_2$, resulting in a reduction of S. mutans biofilms$^{120}$. Coating iron oxide NPs with FDA-approved polymers, such as dextran, increased their stability in aqueous formulation and enhanced biocompatibility with oral soft tissues$^{121}$. The iron-supplying nanotherapeutic ferumoxytol was ‘reinvented’ from an iron deficiency drug into a topical oral biofilm therapeutic$^{122}$. This FDA-approved iron-based nanoparticle possesses a pH-dependent peroxidase-like property that provides localized catalytic activity (Figure 1.3e). This work demonstrated that ferumoxytol diffuses within biofilm matrices and generates free radicals from H$_2$O$_2$, resulting in in situ bacterial death and EPS degradation. Both a human-derived ex vivo model and an in vivo rodent dental caries model revealed efficacy in preventing acid damage of enamel and suppression of dental caries without altering the oral microbiota, and safety towards gingival and mucosal tissues.

1.6.2 Wound biofilms

Wound infections affect ~300 million people worldwide, with treatment costs estimated as high as $25 billion in the US alone$^{123,124}$. In these infections, necrotic tissue fosters attachment of bacteria and provides nutrients that enhance bacterial proliferation and biofilm formation, which impedes wound healing by inhibiting re-epithelialization and prolonging inflammation$^{15,113,125}$. Silver NPs incorporated in hydrogels or in wound wraps are used to treat wound infections$^{126}$. Other types of NPs have also been increasingly studied for the treatment of biofilm-infected wounds$^{127,128}$. For example, copper particles incorporated into biodegradable nanofibers prevented formation of and eradicated preformed biofilms of P. aeruginosa and S. aureus. Further in vitro and in vivo studies are underway to demonstrate the applicability of this strategy for wound dressings$^{129}$. Another
strategy utilizes the amphiphilic core-shell polymeric NP, DA95B5, which removes preformed biofilms of MRSA through nanoscale bacterial ‘debridement’\textsuperscript{130} (Figure 1.3f). DA95B5 can diffuse through the EPS, disrupting biofilms by weakening attachment of bacteria to the matrix. An \textit{in vivo} murine excisional wound biofilm model demonstrated effective dispersal of MRSA biofilms. DA95B5-soaked hydrogel pad dressings reduced bacterial counts in mice by up to \textasciitilde 4 log. Notably, the NP exhibited minimal \textit{in vitro} eukaryotic cell lysis and low \textit{in vivo} toxicity. Combination of these NPs with molecules that accelerate wound healing, including growth factors, anti-inflammatory molecules and extracellular matrix (ECM) mimics may further NP-based strategies for the treatment of wound infections. As an example, a pH-responsive antimicrobial nanofiber network, formed by the self-assembly of the octapeptide IKFQFHFD, was incorporated into a hydrogel and loaded with cypate and proline\textsuperscript{131}. The octapeptide possesses inherent antimicrobial property through cell wall and membrane disruption; cypate is a photothermal drug that is thought to disrupt EPS matrix; and procollagen component proline is added to aid in collagen and ECM matrix reformation. The hydrogel eradicated MRSA biofilms and facilitated healing in chronic wounds as demonstrated in an \textit{in vivo} diabetic mice model.

1.7. References


109. Omwenga, E. O., Hensel, A., Shitandi, A. & Goycoolea, F. M. Chitosan nanoencapsulation of flavonoids enhances their quorum sensing and biofilm formation


2.1 Introduction

Antibiotic-resistant bacteria causes more than 2 million cases of infections and 23,000 deaths each year in US alone.⁰ Worldwide annual death toll due to multi-drug resistant (MDR) bacteria increases to 700,000 and is expected to reach 10 million by the year 2050.² The ‘ESKAPE’ (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter species) pathogens pose the biggest threat to global health due to their multi-drug resistance.³ In particular, infections caused by Gram-negative species of ‘ESKAPE’ pathogens show increased resistance due to an additional highly impermeable outer membrane barrier.⁴ Threat posed by MDR bacteria is further aggravated by their ability to form bacterial biofilms, rendering infections refractory to both traditional antimicrobial therapies and host immune response.⁵ Biofilm-associated infections can frequently occur on medical implants, indwelling devices and wounds.⁶ Conventional strategies to treat these intractable infections involve high dosage treatment with last resort antibiotics such as colistin and carbapenems, increasing the risk of neurotoxicity and nephrotoxicity.⁷ Rigorous antibiotic therapy is often followed by surgical debridement of infected tissue, resulting in low-patient compliance and excessive healthcare costs.⁸ A significant decline
in the number of approved antibiotics against MDR bacteria, with no new antibiotic
developed against Gram-negative bacteria in the last fifty years, has contributed to the urgency for developing novel antimicrobial therapies.\textsuperscript{9}

Antibiotic cocktails targeting multiple pathways in pathogens have demonstrated increased antimicrobial efficacy.\textsuperscript{10} However, this strategy is associated with increased risk of antibiotic-resistance development. Moreover, antibiotic combination therapies often fail to treat MDR Gram-negative pathogens due to limited penetration of antibiotics inside the cells.\textsuperscript{11} Combination therapies utilizing antibiotics with membrane-sensitizing adjuvants have shown high efficacy in treating planktonic Gram-negative infections.\textsuperscript{12} However, these small-molecule based therapies fail to treat biofilm-associated infections due to their inability to penetrate Extracellular Polymeric Substance (EPS) matrix of biofilms.\textsuperscript{13}

Synthetic macromolecules such as nanoparticles and polymers have demonstrated ability to strongly bind and destabilize the bacterial outer membrane.\textsuperscript{14} In addition, amphiphilic polymers exhibited excellent potential in penetrating biofilm matrix.\textsuperscript{15} We hypothesized that combining the membrane-sensitizing and penetration-ability of polymers with the selective activity of antibiotics could offer enhanced efficacy in combating MDR bacterial and biofilm infections. Here, we report a combination therapy using engineered polymeric nanoparticles (PNPs) with colistin against resistant bacterial species. We observed 16- to 32-fold decrease in the colistin dosage required to combat planktonic and biofilm bacteria in combination therapy as compared to colistin alone. The observed synergy can be attributed to enhanced bacterial membrane permeability when the antibiotic was used in combination with PNPs. We further determined that antibiotic accumulation increases about 4-fold inside the biofilms in presence of PNPs, contributing to the enhanced
efficacy. Overall, this combination therapy illustrates the ability of functionalized polymers to enhance the potency of antibiotics against resistant bacterial infections, while minimizing the side-effects associated with high dosages of therapeutics.

2.2 Results and Discussion

2.2.1. Generation and characterization of polymeric nanoparticles

We have recently reported that distribution of cationic and hydrophobic moieties on a polymer plays a critical role in determining the antimicrobial efficacy of membrane-disrupting polymers.\textsuperscript{15b} We have designed a library of polymers by varying the hydrophobicity of the cationic headgroups and changing the alkyl chain length bridging the headgroup with polymer backbone, to systematically probe the bacterial membrane permeability of the polymers (Figure 2.1a). We observed that the polymers with an 11-carbon alkyl chain bridge self-assembled to form cationic polymeric nanoparticles (PNPs) with a size \(\sim 15\) nm, as shown by transmission electron microscopy (TEM) in Figure 2.1c and Figure A1. On the other hand, polymers with smaller alkyl chain (2 and 6) bridge do not self-assemble into PNPs.

2.2.2 Membrane disrupting ability of polymers and combination therapy with antibiotic

Next, we screened the membrane perturbation ability of polymers (P1-P9) against Uropathogenic clinical isolate of \textit{E. coli} using N-phenyl-1-napthylamine (NPN) uptake assays.\textsuperscript{16} We observed that membrane permeation ability of the polymers increases with the increase in the overall hydrophobicity of the structure. However, increasing the length of alkyl chain bridging the polymer backbone to cationic headgroup has a stronger effect
in membrane-sensitizing ability of polymers, as compared to increasing the hydrophobicity of the cationic headgroup alone (Figure 2.1d). A strong structure-activity relationship was observed with the most hydrophobic polymers (P6-P9) demonstrating highest membrane perturbation activity against bacteria (Figure 2.1b).

Figure 2.1 Molecular structures and characterization of oxanorbornene polymer derivatives a) Molecular structures of oxanorbornene polymer derivatives. Log P represents the calculated hydrophobic values of each monomer. (b) Membrane permeability induced by different polymer derivatives measured as (%) uptake of N-phenyl-1-napthylamine (NPN) plotted vs overall hydrophobicity of the polymer derivatives. (c) Schematic representation showing self-assembly of polymer derivatives (n=9) into polymeric nanoparticles. Characterization of polymer nanoparticles (P7) using TEM. (d) Bar graphs demonstrating membrane disruption as a function of polymer nanoparticles with different alkyl chain length bridging polymer backbone and cationic headgroup. Values are expressed as mean ± standard deviation of ≥ 3 replicates.
After establishing the membrane perturbing ability of the polymers, we tested these polymers (P4-P9) for synergistic therapy in combination with colistin antibiotics against bacteria. We evaluated the minimal inhibitory concentrations (MIC) for polymers and colistin using broth dilution methods as reported in Figure 2.2. Next, we performed checkerboard titrations for varied combinations of polymers and colistin and evaluated their FICI (Fractional Inhibitory Concentration Index) scores. A FICI score of \( \leq 0.5 \) is defined as a synergistic interaction, whereas an additive interaction has FICI score between 0.5 and 4. Interactions are additive if the effect of the combination is approximately the independent contribution of the individual components. In contrast, synergistic interactions are when the combined effect is greater than that of the individual components administered individually. Polymers (P7–P9) with higher membrane-sensitizing ability exhibited synergistic response in combination with colistin antibiotic (FICI scores ranging from 0.375 – 0.5) as shown in Figure 2.2. Moreover, an 8- to 16-fold reduction in colistin dosage was observed when used in combination with P7-P9 (Table A1). While polymers (P4-P6) with lesser membrane permeation ability showed additive response (0.5 < FICI < 1). We further investigated the cytotoxicity of the most potent polymers (P7-P9) by performing cytotoxicity assays on human fibroblast cell line. We determined the IC\(_{50}\) (half-maximal inhibitory concentration) of the cells to calculate therapeutic selectivity of polymers (ability to kill bacteria while causing minimal toxicity to mammalian cells). Least hydrophobic polymer P7 demonstrated an IC\(_{50}\) of \(~22~\)µM, providing a therapeutic selectivity (IC\(_{50}/\)MIC) of ~360. While polymer P8 and P9 demonstrated an IC\(_{50}\) ~ 20 and 2.5 µM, generating a therapeutic selectivity of ~160 and ~20, respectively.
Figure 2.2. Checkerboard broth microdilution assays between colistin and polymer derivatives (a) P7, (b) P8 and (c) P9 against uropathogenic E. coli (CD-2). Dark cells represent higher bacterial cell density. Checkerboard data are representative of ≥2 biological replicates. (d) Table showing Minimum inhibitory concentrations (MICs) of colistin and different polymer derivatives. FIC indices were calculated using checkerboard broth microdilution assays as described in the methods section. (e) Cell viability of 3T3 fibroblast cells after treatment with PNPs. Values are expressed as mean ± standard deviation of at ≥3 replicates.

After establishing synergistic interaction between PNPs and colistin antibiotic against E. coli, we tested PNP-colistin combination against multiple uropathogenic clinical isolates to determine their broad-spectrum applicability. P7 PNPs showed synergistic effect against Gram-negative clinical isolates of P. aeruginosa, E. cloacae complex, MDR E. coli and Acinetobacter species (Figure 2.3), yielding up to 16-fold reduction in colistin dosage to combat the resistant bacteria. Similarly, other analogues of PNPs (P8) also demonstrated synergistic response with colistin against Gram-negative strains of P. aeruginosa (Figure A2). On the other hand, PNP-colistin combination tested against Gram-positive strains (methicillin-resistant S. aureus, B. subtilis and S. epidermidis) exhibited additive
interactions (Figure A3). These results indicate that using membrane-sensitizing polymeric nanoparticles can be used as a general strategy to generate synergistic antimicrobial therapy against Gram-negative MDR bacteria.

**Figure 2.3.** Checkerboard broth microdilution assays between colistin and P7 PNPs against uropathogenic (a) *P. aeruginosa* (CD-1006), (b) *En. cloacae* complex (CD-1412), (c) MDR *E. coli* (CD-549), (d) *Acinetobacter* species (CD-575). Checkerboard data are representative of at ≥2 biological replicates. (e) Table showing MICs (Minimum Inhibitory Concentration) and FICI (Fractional Inhibitory Concentration) scores obtained for PNP-colistin combination against different strains of bacteria. Change in bacteria membrane permeability assayed by (f) crystal violet uptake and (g) zeta potential in presence of PNP, colistin and PNP-colistin combination. Values are expressed as mean ± standard deviation of at least three replicates. *p < 0.05, ***p < 0.001.

We hypothesized that PNP-colistin combination disrupted Gram-negative bacterial membranes at sub-inhibitory dosages, owing to the strong cationic and hydrophobic nature
of the PNPs.\textsuperscript{15b} Our claims were supported by staining assays using membrane impermeable crystal violet (CV) dye where PNP-colistin combination showed increased CV accumulation inside cells as compared to PNPs and colistin alone (Figure 2.3f).\textsuperscript{20} Additionally, bacterial membrane disruption was further monitored by measuring the zeta potential of bacterial surface. Bacteria treated with PNP-colistin combination (at sub-lethal dosages) showed sharp shift towards neutral charge as compared to the controls, indicating increased membrane disruption and decreased bacterial viability (Figure 2.3g, Figure A4).\textsuperscript{20a,21}

\textbf{2.2.3 Combination therapy for penetration and treatment of biofilms}

After establishing the ability of PNP-colistin combination against planktonic “superbugs”, we investigated the combination against resistant biofilms. Biofilms are three-dimensional micro-colonies of bacteria embedded inside an extra polymeric substance (EPS) matrix that prevents the penetration of antibiotics inside the biofilms.\textsuperscript{5,6,7} Limited biofilm penetration plays a major role in rendering antibiotics ineffective against biofilm-associated infections. On the other hand, amphiphilic PNPs have shown excellent ability to penetrate biofilms. We hypothesized that using colistin in combination with PNPs would be able to enhance the penetration and accumulation of colistin inside the biofilms, thereby increasing the overall therapeutic effect of the combination therapy.\textsuperscript{22} We treated DsRed-expressing \textit{E. coli} biofilm with Rhodamine Green-tagged colistin in presence and absence of PNPs and examined using confocal microscopy. As shown in Figure 2.4, antibiotic accumulation inside biofilms increased by ~4-fold in presence of polymers as compared to the controls. Furthermore, fluorescent-tagged colistin was homogenously distributed throughout the biofilms when used in combination with PNPs, whereas in
absence of PNPs colistin was confined to the top layer of the biofilm. These results demonstrate that cationic PNPs can increase the accumulation of antibiotics inside the biofilms.

Figure 2.4  Biofilm penetration profile and antibiofilm activity of PNPs (a) Representative 3D projection of confocal images stacks of DsRed (Red Fluorescent Protein) expressing E. coli DH5α biofilm after 1-hour treatment with Rhodamine Green-tagged colistin (1 mg. L\(^{-1}\)) in presence and absence of PNP. The panels are projection at 90° angle turning along X axis. Scale bars are 20 μm. (b) Integrated intensity of Rhodamine Green and DsRed biofilm where 0 μm represents the top layer and ~8 μm the bottom layer. Checkerboard broth microdilution assays between colistin and P7 PNPs against uropathogenic biofilm (c) P. aeruginosa (CD-1006), (d) E. coli (CD-2). Checkerboard data are representative of ≥2 biological replicates. (e) Table showing MBECs (Minimum Biofilm Eradication Concentration) and FICI (Fractional Inhibitory Concentration) scores obtained for PNP-colistin combination against biofilms.
Next, we investigated the therapeutic efficacy of the PNP-colistin combination against biofilms. We evaluated minimum biofilm inhibition concentration (MBIC) and minimum biofilm eradication concentration (MBEC) for PNPs and colistin using broth dilution methods as reported in Figure 2.4. We then performed checkerboard titrations using PNP-colistin combination against biofilms and evaluated the FICI (Fractional Inhibitory Concentration Index) scores to evaluate the efficacy of combinations. FICI scores for PNP-colistin combinations demonstrated synergistic effect as compared to the FICI scores for the individual components, with ~32-fold decrease in colistin dosage. Similar checkerboard studies performed using colistin with other PNP analogues (P8) also showed synergistic effect against biofilms (Figure A5). These results further indicate that using cationic and hydrophobic PNPs can be used a general strategy to increase the accumulation of antibiotics inside the biofilms, thereby increasing their potency.

2.3 Conclusion

We have designed bacterial membrane-sensitizing and biofilm penetrating polymeric nanoparticles that exhibit synergistic interaction with last-resort antibiotic colistin. The bacterial membrane permeability of these polymeric nanoparticles can be regulated by incorporating hydrophobic moieties in the polymer structure. PNPs can enhance the potency of colistin up to 16-fold, owing to the increased susceptibility of bacterial membrane to the polymers. Moreover, polymeric nanoparticles enhance the accumulation of antibiotics inside the biofilms, resulting in synergistic effect of PNP-colistin combination in eradicating biofilms. PNPs render biofilms susceptible to colistin and reduce the antibiotic dosage by 32-fold as compared to antibiotic alone. Taken together, strong membrane permeability and biofilm penetration ability of PNPs make
them promising candidates to enhance the efficacy of standard antibiotic therapies while circumventing the concerns associated with high antibiotic dosage. Moreover, combination therapies using PNPs have the potential to rejuvenate antibiotics that are rendered ineffective due to antibiotic-resistance.

2.4 Experimental Section

2.4.1 Membrane permeability assay using N-Phenyl-1-naphthylamine (NPN) assay

NPN assays were performed using previously established protocols. Briefly, bacteria were grown overnight in LB media at 37 °C and 275 rpm until reached stationary phase. The bacterial cells were then harvested and washed using 0.85% NaCl solution three times and then resuspended in PBS. Concentration of bacterial cells was determined by measuring the optical density at 600 nm. 100 μL (0.1 OD) bacterial solution was added to 50 μL of test materials in a black 96-well plate. After a 30-minute incubation at room temperature, 50 μL of 40 μM NPN was added followed by fluorescence measurement (excitation= 350 nm; emission=420 nm) using a Molecular Devices SpectraMax M2. Cells without treatment served as the negative control while 100 mg/L colistin was used as the positive control. %NPN uptake was calculated as follows:

\[
%\text{NPN uptake} = \frac{\text{fluorescence sample} - \text{fluorescence untreated}}{\text{fluorescence positive control} - \text{fluorescence untreated}} \times 100
\]

2.4.2 Determination of Minimum Inhibitory Concentrations (MICs)

MIC is defined as the lowest concentration of an antimicrobial agent required to inhibit the growth of bacteria overnight as observed from the naked eye. Bacteria cell were grown using the protocol described above. Next, bacterial solutions with
concentrations of $1 \times 10^6$ cells/mL were prepared in M9 media. 50 µL of prepared bacteria solution were mixed with 50 µL of polymer/antibiotic prepared in M9 media in a 96-well clear plate resulting in final bacterial concentration of $5 \times 10^5$ cells/mL. Polymers were tested with half-fold variations in concentrations as per the standard protocols in concentration ranging from 64,000 nM – 4 nM. A sterile control group with no bacterial cells present and growth control group without addition of any polymers were carried out at the same time. The prepared 96-well plates were incubated for 16 hours. The experiments were performed in triplicates with two individual runs performed on different days.

2.4.3 Checkerboard titrations for combination therapy

We performed two-dimensional checkerboard titrations using micro-dilution method to determine the synergy between antibiotics and polymers. The concentration of Polymers and colistin were varied using 2-fold serial dilutions. The wells without any visual growth were considered as a combination that inhibits bacterial growth. For the colistin-polymer combinations, concentrations of the components were varied according to their MIC against the respective bacterial strains. The checkerboard titrations were performed in a set of three independent plates and repeated on different days. A schematic for a checkerboard titration plate is given in Figure A6. Fractional Inhibitory Concentration Index (FICI) for Colistin-polymer combination was calculated using FICs of colistin and polymer independently using the following equation:

\[
\text{FIC}_C = \frac{(\text{MIC of colistin and polymer combination})}{(\text{MIC of colistin alone})}
\]
\[
\text{FIC}_P = \frac{(\text{MIC of colistin and polymer combination})}{(\text{MIC of polymer alone})}
\]
\[
\text{FICI} = \text{FIC}_C + \text{FIC}_P
\]
FICI values ≤ 0.5 corresponds to synergistic combination, whereas FICI values between >0.5 and 4.0 indicates additive effect. FICI values > 4.0 respond to antagonistic effect.\textsuperscript{18b}

\subsection*{2.4.4 Mammalian cell viability assay}

Cell viability studies performed using the previously established protocols.\textsuperscript{13c}\textsuperscript{18b} Briefly, 20,000 NIH 3T3 Fibroblast cells (ATCC CRL-1658) were cultured in Dulbecco’s modified Eagle medium (DMEM, ATCC 30-2002) with 1% antibiotics and 10% bovine calf serum in a humidified atmosphere of 5% CO\textsubscript{2} at 37 °C for 48 hours. Media was replaced after 24 hours and the cells were washed (one-time) with phosphate-buffered saline (PBS) before incubation with polymers. Polymer solution were prepared in 10% serum containing media (pre-warmed) and incubated with cells in a 96-well plate for 24 hours in a humidified atmosphere at 37 °C. Alamar Blue assays were performed to assess the cell viability as per the established protocol of Invitrogen Biosource (manufacturer). Red fluorescence resulting upon the reduction of alamar blue agent was quantified using a Spectromax M5 microplate reader (Ex: 560 nm, Em: 590 nm) and used to determine cell viability. Cells incubated with no polymers were considered as 100% viable. Each experiment was performed in triplicates and repeated on two different days.

\subsection*{2.4.5 Membrane penetration using crystal violet assay}

Bacteria cells were cultured, and their concentrations were measured using the methodology reported above. Crystal violet assay were performed using the previously reported protocols.\textsuperscript{20} Briefly, 0.1 OD bacterial solution was prepared in phosphate-buffered saline (PBS) solution then, incubated with the test material for 30 minutes at 37 °C. Untreated cell which served as the negative control was prepared similarly without
treatment. The cells were harvested by centrifugation at 9300×g for 5 minutes at 4 °C followed by redispersion in PBS with 5 μg/mL crystal violet. After incubation at 37 °C for 10 minutes, the bacterial cell solution was centrifuged at 13,400×g for 15 min. The resulting pellet was resuspended in 80:20 ethanol: acetone and the OD of the solution was measured at 590 nm using a Molecular Devices SpectraMax M2. OD value from the normal untreated cell was used as blank while the OD value of crystal violet solution was considered as 100%. The percentage of crystal violet uptake was expressed as follows:

\[
\%CV\ uptake = \left( \frac{OD_{sample} - OD_{blank}}{OD_{CV\ only} - OD_{blank}} \right) \times 100
\]

2.4.6 Monitoring zeta potential of bacterial membrane

Zeta potential for bacteria membrane was monitored using previously reported protocol. Briefly, bacteria were cultured and harvested as per the above-mentioned protocols. Next, 0.01 OD of bacteria cells in phosphate buffer (PB) solution (5 mM, pH=7.4) was incubated with the test materials (colistin only, polymer only and their combinations) at 37 °C for 15 minutes. The cells were harvested by centrifugation (7000×g for 5 minutes, 4 °C), then the resulting pellets were resuspended in PB. Solutions were then subjected to zeta potential measurements using Zetasizer Nano ZS. Untreated bacteria were used as the negative control.

2.4.7 Biofilm formation and penetration studies using confocal microscopy

DsRed-expressing bacteria were inoculated in lysogeny broth (LB) medium at 37 °C until stationary phase. The cultures were then harvested by centrifugation and washed with 0.85% sodium chloride solution three times. Concentrations of resuspended bacterial
solution were determined by optical density measured at 600 nm. $10^8$ bacterial cells/mL of DsRed (fluorescent protein) expressing *E. coli*, supplemented with 1 mM of IPTG ((isopropyl β-D-1-thiogalactopyranoside), were seeded (2 mL in M9 media) in a confocal dish and were allowed to grow. After 3 days media was replaced by a combination of 1 mg. L$^{-1}$ of Rhodamine Green-Colistin and P7 PNPs (150 nM) and incubated for 1 hour. Biofilm samples incubated with only Rhodamine Green-Colistin (1 mg. L$^{-1}$) were used as control. The cells were then washed with PBS three times. Confocal microscopy images were obtained on a Zeiss LSM 510 Meta microscope by using a 63× objective. The settings of the confocal microscope were as follows: green channel: $\lambda_{ex}$=488 nm and $\lambda_{em}$=BP 505-530 nm; red channel: $\lambda_{ex}$=543 nm and $\lambda_{em}$=LP 650 nm. Emission filters: BP=band pass, LP=high pass.

2.4.8 Determination of Minimum Biofilm Eradication Concentration (MBEC)

MBEC is defined as the minimum concentration of an antimicrobial agent at which there is no bacteria (biofilm) growth. We used previously established protocols to determine the MBECs for the polymers and antibiotics. Briefly, bacterial cells from an overnight culture were diluted to 1/5th using tryptic soy broth (TSB) and incubated at 275 rpm, 37 °C until they reach mid-log phase. 150 μL of bacteria solution was added to each row of a 96-well microtiter plate with pegged lid. Biofilms were cultured by incubating the plate for 6 hours in an incubator-shaker at 37 °C at 50 rpm. Then, the pegged lid was washed with 200 μL PBS for 30 seconds and transferred to a plate containing the test material prepared in a separate 96-well plate using M9 minimal media. The plate was incubated at 37 °C for 24 hours. Then, the biofilms on the peg-lid were washed with PBS
and transferred to a new plate containing only M9 minimal media. The plate was further incubated at 37 °C to determine the Minimum Biofilm Eradication Concentration (MBEC).

2.4.9 Checkerboard titration for synergy testing: Eradication of biofilms

Two-dimensional checkerboard titrations similar as described above were used testing synergy against biofilms. The concentration of Polymers and colistin were varied using 2-fold serial dilutions and MBEC was determined using the above-mentioned protocol. The 96-well plates for the combinations were prepared using the layout described in Figure A6. The wells without any visual growth were considered as a combination that eliminates biofilm formation. For colistin-polymer combinations, concentrations of the components were varied according to their MBEC against the respective bacterial strains.

2.4.10 Statistical Analysis

The data in checkerboard experiments are representative of at least two biological replicates. For all membrane permeability and fibroblast viability experiments, data are presented as mean ± standard deviation of at least three replicates. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test. A p value < 0.05 is considered as statistically significant. GraphPad Prism was used to perform statistical analyses.

2.5. References


CHAPTER 3

POLYMERIC NANOPARTICLES ACTIVE AGAINST DUAL-SPECIES BACTERIAL BIOFILMS

Adapted with permission from “Makabenta, J.M.V. †; Park, J. †; Li, C-H.; Chattopadhyay, A.N.; Nabawy, A.; Landis, R.F.; Gupta, A.; Schmidt-Malan, S.; Patel, R.; Rotello, V.M. Molecules 2021, 26 (16), 4958.” Copyright (2021) MDPI.

3.1 Introduction

Biofilm infections are a serious health concern due to their high antibiotic tolerance. In the United States alone, approximately 1.7 million hospital-acquired-infections are associated with biofilms each year, resulting in ~11 billion USD annual economic burden. Biofilms are communities of bacterial cells embedded in a self-secreted extracellular polymeric substance (EPS) matrix. The complex nature of biofilms promotes development of drug resistance and its spread. The matrix protects resident bacteria against antimicrobial agents and host immune response. Spatial and chemical heterogeneity across the matrix results in a diversity of phenotypic changes. Furthermore, scarce nutrient and oxygen supply in the inner layers of the matrix induce the formation of dormant and drug-tolerant persister cells. Taking all these challenges together, biofilms pose tougher therapeutic challenges than their planktonic counterparts.

Most clinically relevant biofilm infections, such as diabetic foot ulcers, implant-associated infections and pneumonia in cystic fibrosis patients, are composed of multiple bacterial species. Interspecies interactions promote co-aggregation, metabolic cooperation and transfer of resistance genes. Polymicrobial infections play roles in chronic and recurring infections. Multi-species biofilms can exacerbate resistance to antibiotics: different species can exhibit distinct drug susceptibilities that allow biofilms to
overcome antibiotic treatment. As interactions among different species diversify, the composition of the EPS matrix becomes more heterogenous. Polymicrobial biofilms exhibit increased biomass, providing a barrier that further impedes and deactivates antibiotics. Current strategies to combat multi-species biofilms involve high dosages of combination antibiotic regimens or invasive methods of biofilm removal. These approaches entail risk, high treatment costs and low patient compliance. The lack of new antibiotics entering the pipeline contributes to the demand for development of novel antimicrobial therapeutics that can tackle the challenges of treating resilient biofilm infections. Therefore, there is an urgent need for new treatment regimens to address multi-species biofilm infections.

Recent advances in nanomaterial-based therapeutics provide a promising opportunity to effectively address difficult-to-treat bacterial and biofilm infections. Polymeric nanoparticles provide versatile additions to the antimicrobial arsenal. Antimicrobial polymers mimicking host-defense peptides are an emerging class of therapeutics that exhibit broad-spectrum activity against bacteria. Their tunable properties including hydrophobicity, charge and size impart therapeutic advantages such as efficient biofilm penetration. Previously, our group synthesized a library of antimicrobial polymers based on a poly(oxanoborneneimide)(PONI) backbone, with varying alkyl chain lengths between the polymeric backbone and cationic headgroups on the sidechains. The PONI polymer with a C11 alkyl chain (PONI-C11-TMA) formed polymeric nanoparticles (PNPs) (Figure B1) that demonstrated high antimicrobial activity against single-species biofilms with minimum cytotoxicity to mammalian cells. We report here the activity of PONI-C11-TMA against clinically-relevant dual-species biofilms.
(Figure 3.1). These biofilms were formed by clinical isolates of *Escherichia coli*, *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* (MRSA), and dual-species combinations. Species were chosen based on their clinical relevance.\textsuperscript{26,27,28} PNPs were effective in eradicating dual-species biofilms as evaluated by Alamar Blue and crystal violet assays. Confocal laser scanning microscopy imaging revealed that PNPs penetrated and disrupted the dual-species biofilm matrix within 1 hour. Notably, *in vitro* co-culture of fibroblasts and dual-species biofilms of *E. coli* and *P. aeruginosa* demonstrated antimicrobial activity of PNPs against biofilms while maintaining low cytotoxicity towards mammalian cells. Taken together, PNPs present potent broad-spectrum antibiofilm activity against multi-species biofilms, offering a promising strategy for treatment of biofilm infections.

![Figure 3.1](image)

**Figure 3.1** Preparation and activity of PONI-C11-TMA PNPs. The resulting PNPs significantly reduced established dual-species biofilms while maintaining fibroblast viability.

### 3.2. Results

#### 3.2.1 Dual-species biofilm penetration profile of PNPs

The ability of the polymeric nanoparticles to penetrate the EPS of a dual-species biofilm was visualized using confocal laser scanning microscopy (CLSM). A 4-day old dual-species biofilm of DsRed-expressing *E. coli* and GFP-expressing MRSA was treated...
for 1 h with coumarin blue-tagged PNP. Micrographs revealed that PNP penetrated the EPS matrix and disrupted the biofilm as evidenced by a significant decrease in signal coming from *E. coli* and MRSA (Figure 3.2, Figure B2). The reduction in fluorescence was more prominent for *E. coli* than MRSA. A similar trend was observed for biofilm penetration of PNPs into mono-species biofilms of *E. coli* and MRSA (Figure B3-4).

**Figure 3.2**  Dual-species biofilm penetration profile of PNPs. Representative 3D views of confocal image stacks of 4-day old dual-species biofilm of DsRed-expressing *E. coli* (red channel) and GFP-expressing MRSA (green channel), coumarin blue-tagged PNPs (blue channel), and their overlay after treating the biofilms for 1 h with 1 μM coumarin blue-tagged PNPs in M9 media.
3.2.2 Minimum biofilm bactericidal concentrations of PNPs

The minimum biofilm bactericidal concentrations (MBBC) of PNPs against single- and dual-species biofilms were evaluated using an established MBBC protocol. Biofilms were formed by clinical isolates of *E. coli*, *P. aeruginosa*, MRSA and *Staphylococcus epidermidis*, and their combinations. PNPs eradicated the dual-species biofilms (Figure 3.3). In contrast, the antibiotic control, gentamicin, exhibited decreased activity when treating dual- compared to single-species biofilms (Figure B5).

3.2.3 Quantifying biofilm biomass and bacteria viability

Crystal violet and Alamar Blue assays were performed to evaluate the effect of PNPs on biomass and bacterial viability, respectively, of the two-species biofilms. Two-day old biofilms of MRSA IDRL-6169 + *E. coli* IDRL-10366, *P. aeruginosa* IDRL-11442 + *E. coli* IDRL-10366, and their single-species counterparts were treated with PNPs for 3 h. Results indicated that the ability of the PNPs to kill bacteria and disrupt biofilms was retained in dual-species biofilms (Figure 3.4, Figure B6).

3.2.4 Cell viability in an in vitro fibroblast-dual species biofilm co-culture

An in vitro co-culture model consisting of fibroblast cells and two-species biofilms of *E. coli* DH5α and *P. aeruginosa* ATCC- 19660 was used to evaluate the safety of the PNPs towards mammalian cells while eradicating biofilms. Polymeric nanoparticles were minimally toxic towards the fibroblasts, maintaining ~90% viability at the highest concentration evaluated. At the same concentration, PNPs reduced bacterial amounts up to ~6 log_{10} colony forming units (Figure 3.5, Figure B7).
Figure 3.3  Minimum biofilm bactericidal concentration of PNPs. MBBC values of PNPs against mono-species (Gram-positive: MRSA, *S. epidermidis*; Gram-negative: *P. aeruginosa, E. coli*) and dual-species biofilms (MRSA IDRL-6169 + *S. epidermidis* IDRL-7073; MRSA IDRL-6169 + *P. aeruginosa* IDRL-11442; MRSA + *E. coli* IDRL-10366; *P. aeruginosa* IDRL-11442 + *E. coli* IDRL-10366). Bars represent average of three values and stars represent individual measurements.

3.3. Discussion

The tunable physicochemical properties of polymeric nanoparticles, such as size, surface charge and hydrophobicity, allow access to a range of antimicrobial modalities, making them candidates for treating multi-species biofilm infections. In this study,
oxanorbornene-based PNPs mimic antimicrobial peptides, but synthetic approaches enable the fabrication of small size of nanoparticles, exhibiting multivalent interactions with the bacteria, as well as traverse the biofilm EPS matrix. Nanoparticles access a variety of antibacterial mechanisms, making the barrier to resistance development higher than for small molecule antibiotics that target specific processes.\textsuperscript{15-17} The antimicrobial PONI-based polymers employed in this work are functionalized synthetic polymers which consist of cationic trimethylamine (TMA) head groups bridged to the PONI backbone by hydrophobic alkyl chains. In our previous study, we explored a library of PONI-based polymers with varying alkyl chain lengths with hydrophobicity and observed the best activity from the one with a C\textsubscript{11} alkyl bridge (PONI-C\textsubscript{11}-TMA).\textsuperscript{20} ONI-C11-TMA self-assembles into polymeric nanoparticles (PNPs), with size \textasciitilde15 nm, in aqueous solution due to amphiphilicity of the system (Figure B1). Positively charged PNPs enable electrostatic interactions with negatively charged bacterial membranes and the EPS matrix.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{Figure3.4.png}
\caption{Antibiofilm activity of PNPs. a) Biomass and b) bacteria viability of 2-day-old mono- and dual-species biofilms of MRSA IDRL-6169 + \textit{E. coli} IDRL-10366 after 3 h treatment with PNPs. The data shown are averages of triplicates with the error bars indicating standard deviations.}
\end{figure}
The hydrophobic portion of the polymer also contributes to its interactions with bacteria and EPS, making them effective against dual-species biofilms, even at low concentration range. The broad-spectrum capabilities of PNPs were established using mono- and dual-species biofilms of clinical isolates of Gram-positive (S. aureus, S. epidermidis) and Gram-negative (P. aeruginosa, E. coli) bacteria.

Figure 3.5. Activity and cytotoxicity of PNPs evaluated using fibroblasts-dual species biofilm coculture. Viability of 3T3 fibroblast cells and E. coli DH5α + P. aeruginosa ATCC-19660 dual-species biofilms in the co-culture model after 3 h treatment with PNPs. Scatters and lines represent 3T3 fibroblast cell viability. Bars represent log_{10} of colony forming units in biofilms. Limit of quantification is 2 log_{10}. Data are averages of triplicates, with error bars indicating standard deviations.

Penetration and accumulation at infectious site are therapeutic challenges due to the dense biofilm matrix.^{30,31} Dual-species biofilms of red fluorescent protein DsRed-expressing E. coli and green fluorescent protein (GFP)-expressing MRSA were treated
with the PNPs tagged with a blue fluorescent dye, coumarin-blue. As shown in Figure 3.2, coumarin blue tagged-PNPs successfully penetrated across the dual-species biofilm. Reduced fluorescent signals from both *E. coli* and MRSA after treatment with PNPs demonstrates an ability to target bacteria within the matrix of the complex biofilm. Notably, there was a higher fluorescent signal reduction for *E. coli* cells than for MRSA cells, both in the mono- and dual-species biofilm settings.

PNPs both penetrate and eradicate biofilms. The PNPs demonstrated minimum biofilm bactericidal concentrations (MBBC) ranging from 8 to 16 μM for dual-species biofilms, indicating their broad-spectrum activity (Figure 3.3). The observed penetration profiles were consistent with the therapeutic efficacy of PNPs against biofilms. The MBBC values indicate that the activity of PNPs against MRSA IDRL-6169 (MBBC = 16 μM) is less compared to the other strains tested such as *E. coli* CD-2 (MBBC = 1 μM). We hypothesized that this difference in activity could be due to the structural characteristics of PNPs; PNPs are equipped with long alkyl chains and a cationic anchor group that disrupts the LPS-rich outer membrane of Gram-negative bacteria more efficiently relative to the thick peptidoglycan layer of *S. aureus*.32

It is important to benchmark new nanotherapeutics against traditional antibiotics. For this comparison, we determined the MBBC values of gentamicin, a broad-spectrum antibiotic, against MRSA IDRL-6169 + *P. aeruginosa* IDRL-11442, and *E. coli* IDRL-10366 + *P. aeruginosa* IDRL-11442 (Figure B5). Dual-species biofilms exhibited increased gentamicin tolerance: the MBBC of MRSA + *P. aeruginosa* (2000 μM) was 4-fold higher than that of *P. aeruginosa* only (500 μM), and 125-fold higher than that of MRSA only (16 μM). For *P. aeruginosa* + *E. coli*, the MBBC (> 4200 μM) was increased
by $\geq 8$-fold relative to *P. aeruginosa* only (500 $\mu$M) and $\geq 260$-fold relative to *E. coli* only (16 $\mu$M). This increase in required bactericidal concentrations emphasizes hurdles posed by multi-species biofilms for small molecule antibiotics. In contrast, the dual-species biofilm MBBC values for PNPs did not significantly increase, with values dictated by the bacterial species with the higher MBBC value in the mono-species setting. That dual-species biofilms were not more resistant than the monocultures indicates an advantage nanotherapeutics may have over traditional antibiotics.

PNPs were highly effective against preformed, established biofilms. Two-day old dual-species biofilms of *E. coli* IDRL-10366 + MRSA IDRL-6169, and *E. coli* IDRL-10366 + *P. aeruginosa* IDRL-11442 were treated with different concentrations of PNPs for 3 hours. Subsequently, biofilm viability was determined using Alamar Blue assay, and biofilm biomass was quantified through crystal violet assay. PNPs eradicated bacteria and reduced biofilm biomass in a dose-dependent manner (Figure 3.4). Remarkably, PNPs maintained activity in dual-species settings and with comparable efficacy against mono-species biofilms (Figure B6).

Selective toxicity against bacteria relative to mammalian cells is a prerequisite for therapeutic application of antimicrobial nanomaterials. Biofilm formation at infection sites may impede the healing process regulated by fibroblast skin cells. We, therefore, assessed biocompatibility of PNPs with mammalian NIH 3T3 fibroblasts at concentrations used to eradicate preformed biofilms. In an *in vitro* co-culture study of *E. coli*, *P. aeruginosa* and fibroblasts, PNPs showed no significant fibroblast toxicity at the relevant concentration range (Figure 3.5). PNPs reduced dual-species bacteria viability up to $\sim 6$ fold $\log_{10}$ colony forming units at concentrations ranging from 3 to 15 $\mu$M while
maintaining the viability of mammalian fibroblast cells. This observation was comparable to the co-culture of fibroblasts with the mono-species biofilm equivalents (Figure B7). These results suggest safety of PNPs towards mammalian cells.

In summary, the activity of cationic antimicrobial polymeric nanoparticles against dual-species bacterial biofilms was investigated. PNPs penetrated and accumulated across the dense EPS matrix of dual-species biofilms. Our study has demonstrated broad-spectrum efficacy of PNPs against dual-species biofilms with maintained fibroblast viability whereas the antibiotic control showed increased tolerance with low efficacy. Carefully designed polymeric nanoparticles may address the current challenges of conventional antibiotics as out-of-the-box therapeutics for multi-species biofilm infections. Overall, this strategy suggests a promising alternative to combat resilient mixed-microbial biofilms with potential clinical translatability.

3.4 Materials and Methods

All chemicals and solvents for syntheses were purchased from Fisher Scientific and Sigma-Aldrich and used without further purification unless otherwise stated. The chemicals were used as received. Dichloromethane (DCM) and tetrahydrofuran (THF) were used as solvent for chemical synthesis and dried per standard procedures. All reagents/materials were purchased from Fisher Scientific and used as received.

The following bacteria strains were used for this study: E. coli (IDRL-10366, DH5α), P. aeruginosa (IDRL-11442, ATCC-19660), and MRSA (IDRL-6169, IDRL-12570). Overnight cultures of the bacteria were prepared by transferring isolated colony from the agar plate to culture tubes with sterile media broth. The bacterial cultures were then incubated overnight at 37°C with aeration and agitation (275 rpm) until desired
growth phase. Isolates with code IDRL were from the Infectious Diseases Research Laboratory in Mayo Clinic. NIH-3T3 cells (ATCC CRL-1658) were purchased from ATCC. Dulbecco's Modified Eagle's Medium (DMEM) (DMEM; ATCC 30-2002) and fetal bovine serum (Fisher Scientific, SH3007103) were used for cell culture. A Pierce LDH Cytotoxicity Assay Kit was purchased from Fisher Scientific.

3.4.1 Synthesis of PONI-C11-TMA and characterization of PNPs

To a 10ml pear-shaped air-free flask equipped with a stir bar was added 1 (1 mg, 2.51 mmol, 1.0 eq) and 4ml of DCM. In a separate 10ml pear-shaped air-free flask was added Grubbs 3rd generation catalyst1 (34.16 mg, 0.038 mmol, 0.02 eq) and 1 ml DCM. Both flasks were sealed with septa and attached to a schlenk nitrogen/vacum line. Both flasks were freeze-pump-thawed three times. After thawing, Grubbs 3rd generation catalyst was syringed out and quickly added to the flask containing 3 and allowed to react for 10min. After the allotted time, ethyl vinyl ether (200 μL) was added and allowed to stir for 15 minutes. Afterwards, the reaction was diluted to two times the volume and precipitated into a heavily stirred solution of hexane (300 ml). The precipitated polymer was filtered and directly used to for a next reaction. The polymer 2 (50 mg) was added to 20ml vials equipped with a stir bar. Next, excess of the necessary tertiary amines was added (10 ml of a 1M trimethylamine solution in THF) to the vial and purged with nitrogen. First stage of the reactions involved stirring for 30 minutes at 80 °C. The polymers precipitated during this time. Half of the THF was evaporated and replaced with methanol which re-dissolved the polymers. The reaction was allowed to proceed overnight at 50 °C. Afterwards, the solvent was completely evaporated, washed with hexane two times, and dissolved into a minimal amount of water. The polymers were added to 10,000 MWCO dialysis membranes.
and allowed to stir for 3 days, changing the water periodically. The polymers were filtered through PES syringe filters and freeze-dried to yield quaternary ammonium polymer 3 (Figure 3.6). NMR indicated conversion into the desired quaternary ammonium salts (Figure B1). The freeze-dried polymer 3 was then dissolved in Milli-Q water to afford 225 μM as stock solution for further experiments.

TEM samples of polymers were prepared by placing one drop of the desired solution (10 μM) on to a 300-mesh Cu grid-coated with carbon film. These samples were analyzed and photographed using JEOL CX-100 electron microscopy.

3.4.2 Biofilm formation and penetration studies using confocal microscopy

GFP-expressing MRSA and DsRed-expressing *E. coli* were inoculated in Luria broth (LB) medium at 37°C until stationary phase. Cultures were harvested by centrifugation and washed with 0.85% sodium chloride solution three times. Concentrations of resuspended bacterial solutions were determined by optical density measured at 600 nm. 10⁸ bacterial cells/mL of GFP-expressed MRSA, DsRed-expressing *E. coli* or their combination, supplemented with 1 mM of isopropyl β-d-1-thiogalactopyranoside (IPTG), were seeded (2 mL in TSB) in a confocal dish and were allowed to grow. After 4 days, media was replaced by M9 media containing 1 μM of
coumarin-blue tagged PNPs, then incubated for 1 hour. Biofilm samples incubated with M9 media only were used as controls. Cells were imaged before and after washing with PBS three times. Confocal microscopy images were obtained on a Zeiss LSM 510 Meta microscope by using a 40× objective. The settings of the confocal microscope were as follows: green channel: $\lambda_{ex} = 488$ nm and $\lambda_{em} = LP 540$ nm; red channel: $\lambda_{ex} = 560$ nm and $\lambda_{em} = LP 640$ nm; blue channel: $\lambda_{ex} = 403$ nm and $\lambda_{em} = LP 495$ nm. Emission filter: LP = high pass.

3.4.3 Determination of antibiofilm activity

3.4.3.1 Determination of Minimum Biofilm Bactericidal Concentration (MBBC)

The MBBCs for the PNPs and antibiotic control, gentamicin, were determined using previously established protocols. Briefly, bacterial cells from overnight cultures were diluted to 1/50th using tryptic soy broth (TSB) and incubated at 275 rpm, 37°C until they reached mid-log phase. For mono-species biofilms, 150 µL of bacteria solution was added to each well of a 96-well microtiter plate with pegged lids. For dual-species biofilms, 75 µL of each component bacterial strain was added to each well of the microplates. Biofilms were cultured by incubating the plate for 6 hours in an incubator/shaker at 37°C at 50 rpm. Then, the pegged lid was washed with 200 µL PBS for 30 seconds and transferred to a plate containing two-fold serial dilutions of PONI-C11-TMA (from 0.5 to 128 µM) and gentamicin (from 4.2 to 8500 µM) prepared in a separate 96-well plate using M9 minimal media. The plate was incubated at 37°C for 24 h. Then, biofilms on the pegged lid were washed with PBS and transferred to a new plate containing only M9 media. The plate was further incubated at 37°C to determine the MBBC. The MBBC of both
antibiofilm agents was determined by visual inspection and confirmed through spectrophotometry (OD$_{600}$).

3.4.3.2 Treatment of Established Biofilms

Bacterial seeding solutions were prepared in TSB to reach 0.1 OD. For mono-species biofilms, 100 μL seeding solutions were added to each well of a 96-well microtiter plate. For dual-species biofilms, 50 μL of both bacteria seeding solutions were added to each well of the 96-well microtiter plate. M9 medium without bacteria was used as a sterile control. Plates were covered and incubated at room temperature under static conditions. Biofilms were used after 2 days. They were washed with PBS (three times) to remove the planktonic bacteria. Next, PNPs at varied concentrations, made in M9 media, were added to each well of the microplate. The microplate was then incubated at 37°C under static conditions. After 3 hours, biofilms were washed with PBS three times, and bacterial viabilities were determined using an Alamar Blue assay, following the manufacturer’s protocol. To quantify the effect of PNPs on the biofilm biomass, 2-day old biofilms were prepared and treated as mentioned. After the 3-hour treatment, biofilms were stained with 1% crystal violet for 15 min and solubilized with 96% ethanol. The optical density at 595 nm was determined as a measure of biofilm mass.

3.4.4 Cytotoxicity evaluation of PNPs using biofilm-3T3 fibroblast cell coculture

The in vitro co-culture experiment was performed using a previously reported protocol.$^{25}$ A total of 10k NIH 3T3 (ATCC CRL-1658) cells were cultured in Dulbecco’s modified Eagle medium (DMEM; ATCC 30-2002) with 10% bovine calf serum and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO2. Cells were kept for 24 hours to reach a confluent monolayer. Bacteria (P. aeruginosa and E. coli) were
inoculated and harvested as mentioned above. Afterward, seeding solutions with \(10^8\) cells/mL of each bacteria were inoculated in buffered DMEM supplemented with glucose. Old media was removed from 3T3 cells followed by the addition of 100 μL of seeding solution (1:1 \(E.\ coli\) and \(P.\ aeruginosa\)). The co-culture was then stored in a box humidified with damp paper towels at 37 °C for 6 h without shaking. PNPs were diluted in DMEM media prior to use to obtain the desired testing concentrations (1-15 μM). Old media was removed from co-culture, replaced with freshly prepared testing solutions, and incubated for 3 hours at 37 °C. Co-cultures were then analyzed using LDH cytotoxicity assay to determine mammalian cell viability using the manufacturer’s instructions. To determine bacteria viability in biofilms, the testing solutions were removed and co-cultures washed with PBS. Fresh PBS was then added and the remaining bacteria from biofilms redispersed through sonication for 20 min and mixing with a pipet. Solutions containing redispersed bacteria were then plated onto LB agar plates, and colony forming units were counted after incubation at 37 °C overnight.

3.4.5 Statistical analysis

The data in biofilm penetration profiles using CLSM imaging are representative of at least two biological replicates. For MBBC experiments, Crystal violet assays, Alamar Blue assays, fibroblast viability and quantitative colony counting, data are presented as mean of at least three replicates with error bars representing standard deviation. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. A p-value < 0.05 is considered as statistically significant. GraphPad Prism was used to perform statistical analyses.
3.5. References


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CHAPTER 4

ANTIMICROBIAL POLYMER-LOADED HYDROGELS FOR THE TOPICAL TREATMENT OF MULTIDRUG-RESISTANT WOUND BIOFILM INFECTIONS

4.1 Introduction

Wound infections are a major global healthcare challenge, annually affecting around 300 million patients worldwide. In the USA alone, approximately 2% of the total population is burdened by chronic, non-healing wounds, with estimated treatment costs reaching up to 97 billion USD every year.\(^1\,^2\,^3\,^4\) The wound bed is prone to bacterial infection and is vulnerable to biofilm formation.\(^5\,^6\) Biofilms are micro-communities of bacteria embedded within a protective barrier of the extracellular polymeric substances (EPS) matrix that allows bacteria to evade or survive antibiotic treatments and host immune responses.\(^5\,^7\,^8\) Biofilm infections further impair wound healing due to prolonged inflammation and hindered reepithelialization, which contributes to wound persistence, morbidity, and mortality.\(^5\,^9\,^10\) Topical antiseptics and long-term antibiotic therapy are among the standard wound treatment strategies in practice to eliminate the infection. However, both strategies are often inadequate in killing bacteria in biofilms because of antibiotic resistance and poor biofilm penetration.\(^11\,^12\) Without an effective clinical strategy to address biofilm-infected wounds, treatments will continue to focus on invasive methods of biofilm removal including debridement and amputation.\(^13\,^14\) These strategies entail risk, higher treatment costs, and lower patient satisfaction.\(^15\) With this lack of effective wound biofilm therapeutic, a second but more devastating pandemic is rapidly approaching where a minor skin infection could prove lethal.\(^16\) Hence, there is an urgent need for new wound
biofilm infection therapeutics that can both eradicate multidrug-resistant (MDR) biofilms and support processes that facilitate wound healing.

Polymeric nanoparticles are versatile systems relevant in the antimicrobial arsenal that provide a promising opportunity to effectively address bacterial and biofilm infections. Polymers can be engineered to possess antimicrobial activity by tuning their morphological and physicochemical properties, including size, shape, and surface chemistry. Previously, we report an antimicrobial poly(oxanorboreneneime) (PONI)-based polymer, PONI-C11-TMA, that self-assembles into nanoparticles (PNPs) that exhibit potent activity against MDR bacteria. To be an effective topical antimicrobial therapeutic, these antimicrobial polymers should be delivered using carriers that enable them to achieve and maintain maximum therapeutic dosage at the site of infection. Wound dressings particularly hydrogels provide protection of the wound and conditions advantageous to wound healing. The high moisture content, biocompatibility, and reversibility of hydrogels make them effective wound dressing. Moreover, their tunable porosity facilitate their integration with antimicrobial nanomaterials for enhanced efficacy.

In this present work, we describe the antibiofilm activities of PNPs and their effects on the processes involved in wound healing. Moreover, we demonstrate here that loading PNPs into hydrogels generates a clinically relevant topical treatment for chronic wound biofilm infections (Figure 4.1). We studied the efficacy of PNPs against bacterial biofilms of clinical isolates, with a focus on methicillin-resistant Staphylococcus aureus (MRSA) given its clinical relevance on chronic wound infections. We found that these PNPs feature multiple important aspects as wound biofilm therapeutics including 1) the ability to penetrate and disrupt the EPS matrix; 2) the effective eradication of bacteria embedded
in the EPS matrix including resistant and persister cells; 3) high barrier for resistance
development; 4) safety towards mammalian cells; and 5) positive effects on inflammation
and fibroblast migration. Confocal laser scanning microscopy (CLSM) revealed that PNPs
interact with both the EPS and the embedded bacteria, destroying the biofilm matrix within
one hour. This activity is supplemented by their broad-spectrum efficacy, with minimum
biofilm bactericidal concentration values ranging from 1-8 μM. Exposure of MRSA to 15
passages of sub-inhibitory doses of PNPs did not elicit resistance development, as opposed
to antibiotic control vancomycin. Using an in vitro wound infection model consisting of
MRSA biofilm, macrophages, and fibroblasts, we observed their ability to reduce
inflammation and support fibroblast migration because of their potent antimicrobial action.

Upon establishing their effects in vitro, we then formulated these nanoparticles into
poloxamer 407 hydrogels for efficient administration and enhanced duration of action in vivo. Poloxamer hydrogels are widely used wound dressings and are generally recognized
as safe (GRAS). Incorporation of nanotherapeutics into hydrogels form hybrid materials
that improve both the hydrogel and nanotherapeutic properties. The hydrogel can provide
localized and prolonged release of the PNPs while the PNPs can impart antimicrobial
effects to the hydrogel.

The resulting PNP-loaded P407 hydrogels were tested against a murine model of
chronic wound MRSA biofilm infection to evaluate their efficacy as topical therapeutics.
A luminescent MRSA strain, USA300 NRS384 (SAP-231), was used to initiate infection.
The bioluminescent signal was used to ensure that a consistent degree of infection is
established on the mice prior to treatment. A mature MRSA biofilm was allowed to develop
on the wound to mimic the severity of chronic wound infections. PNPs administered in
their solution form did not demonstrate any antimicrobial effects while PNP-loaded P407 hydrogels cleared up to ~99% (~2 log₁₀ units) of bacterial load. This maintained efficacy due to the hydrogel formulation highlights the importance of using carriers that can increase the local residence time of the nanotherapeutics at the infection site. The observed antimicrobial efficacy also translated into controlled inflammation which significantly enhanced wound healing. Overall, the unique antimicrobial properties of PNPs as well as their integration into poloxamer hydrogels generated an effective non-antibiotic topical treatment for severe wound biofilm infections caused by MDR bacteria.

Figure 4.1. Schematic representation of PNP-loaded P407 hydrogels as topical therapy to treat severe wound biofilm infection in vivo. PONI-C11-TMA self-assembles into PNPs that can penetrate, disrupt, and eradicate bacteria in biofilms while supporting processes that facilitate skin repair. Integration of PNPs into P407 hydrogels localized the antimicrobial therapeutic into the infected area which maximized contact and dosage delivery, efficiently clearing infection, and contributing to wound healing.
4.2. Materials and Methods

4.2.1. Biofilm penetration profile probed using confocal laser scanning microscopy

GFP-expressing methicillin-resistant *S. aureus* (MRSA, IDRL-12570) was inoculated in tryptic soy broth (TSB) at 37°C until the stationary phase. Bacteria cells were collected by centrifugation and washed with 0.85% sodium chloride solution thrice. Concentrations of resuspended bacterial solutions were determined by optical density (OD\(_{600}\)). In a sterile 35 mm confocal dish, \(10^8\) CFU/mL of GFP-expressing MRSA were seeded in 2 mL TSB, supplemented with 1 mM of isopropyl β-d-1-thiogalactopyranoside (IPTG) and 1 μM Alexa Fluor-647 to stain the extracellular polymeric substances (EPS) matrix. Biofilm was allowed to grow for 4 days, replacing the media with fresh supplemented TSB daily. At day 4, media was replaced by minimal M9 media containing 1 μM of coumarin blue-tagged PNPs, then incubated for 1 hour. Biofilm samples incubated with M9 media only were used as controls. CLSM images were obtained on a Zeiss LSM 510 Meta microscope using a 40x objective. The settings of the confocal microscope were as follows: green channel: \(\lambda_{ex} = 488\) nm and \(\lambda_{em} = \text{LP 540 nm}\); red channel: \(\lambda_{ex} = 560\) nm and \(\lambda_{em} = \text{LP 640 nm}\); blue channel: \(\lambda_{ex} = 403\) nm and \(\lambda_{em} = \text{LP 495 nm}\). Emission filter: LP = high pass.

4.2.2. *In vitro* wound biofilm model using MRSA biofilm-fibroblast-macrophage co-culture

We developed a co-culture model consisting of macrophages, fibroblasts, and MRSA biofilm to simulate wound biofilms *in vitro*. In brief, a total of 80k NIH-3T3 fibroblasts were seeded in a 24-well plate while 30k RAW 264.7 cells were seeded in a transwell membrane insert using DMEM with 10% FBS and 1% penicillin-streptomycin.
at 37°C in a humidified atmosphere of 5% CO₂. The transwell insert allows both mammalian cells to exist in a single well while having the means to separate them for cell viability studies. The cells were kept for 24 hours to reach a confluent monolayer. Then, the media from the wells containing fibroblasts were removed and replaced with a solution of 10⁸ CFU/mL MRSA prepared in DMEM with 10% FBS. The transwell membranes containing the macrophage monolayer were then inserted in the wells with fibroblasts and bacteria. The plate was incubated at 37°C in a humidified atmosphere of 5% CO₂ for 5 more hours to allow MRSA biofilm formation. Then, the wells were washed with PBS and the media was replenished with DMEM containing PNPs. After 3 hours, mammalian cell viabilities were determined using LDH assay while remaining viable bacteria were quantified using agar plate colony counting.

4.2.3. Preparation of PNP-loaded poloxamer 407 (P407) hydrogels

P407 hydrogels were prepared using the “cold method”. The desired concentration of PNPs was prepared in cold PBS. Then, poloxamer 407 (20% w/v) was slowly added into the PNPs solution with an adjusted temperature of 4°C while stirring, then kept at 4°C overnight with stirring. The resulting clear solution was kept refrigerated until ready for use.

4.2.4. In vivo mice experiments

4.2.4.1. Ethics statement

C57BL/6 mice were supplied by Jackson Laboratory. Mice were housed in sterile cages with a 12-hour light/ 12-hour dark cycle. Mice were allowed to acclimatize for at least a week before any of the procedures were performed. All animal experiments were
performed following the authorized protocol (IACUC Protocol ID 2648) and the policies issued by the Institutional Animal Care and Use Committee at the University of Massachusetts Amherst.

4.2.4.2. Generation and treatment of biofilm-infected murine skin wound defects

Mice were anesthetized using isoflurane and meloxicam was subcutaneously administered for pain management. The skin on the dorsum of the mouse was shaved and disinfected with alternating povidone-iodine and alcohol swabs, thrice. Subsequently, a sterile 5-mm circular full-thickness skin wound was created using a skin puncture biopsy tool (Acuderm Inc., Fort Lauderdale, FL). Using a micropipette, $10^8$ CFU of bioluminescent MRSA USA300 NRS384 strain (SAP 231)\textsuperscript{27} in saline (10 μL) was inoculated onto the wound bed. To prevent secondary bacterial contamination and allow visualization of the wound bed, semi-occlusive transparent Tegaderm® (3M, St. Paul, MN) was affixed over the wound using Vetbond®. Biofilm was allowed to form and mature for 4 days to simulate mature wound biofilm conditions. Infection was tracked through IVIS imaging of the luminescence signal from the bacteria. Then, mice were separated into four groups of six to receive one of the following: a) PBS, b) P407 hydrogel only, c) PNPs in PBS, and d) PNPs in P407 hydrogel. The treatment was administered topically, once a day for four days. On the last day of treatment, the mice were sacrificed through CO\textsubscript{2} asphyxiation. A 3-mm circular full-thickness skin sample from the inner infection area was collected using a skin biopsy punch and then homogenized for quantitative bacterial colony counting and measurement of pro-inflammatory cytokine levels. Photographs and IVIS images were taken daily, and weight, wound size, and purulence scores were noted every day.
4.2.4.3. Imaging experiments

Mice were infected with SAP-231 as above. Subsequently, Cy5.5-tagged PONI-C11-TMA was used to fabricate PNPs prepared both in PBS and loaded in P407 hydrogel. The resulting solution or hydrogel was administered to the infected wound area. Then, mice were imaged using IVIS to track the degree of colocalization of the luminescence from the infecting MRSA and the fluorescence from the Cy5.5-tagged PNPs. Mice were monitored and imaged until 24 hours then sacrificed via CO\textsubscript{2} asphyxiation.

4.3 Results and Discussion

The prevalence of antibiotic-resistant bacterial biofilms threatens the possibility of simple wound infections becoming life-threatening conditions.\textsuperscript{6} Antibiotic-free strategies, including polymeric nanoparticles, present a promising approach to addressing wound biofilm infections. Polymers can be intently designed to impart multi-modal antimicrobial properties to combat antibiotic-resistant bacteria and their biofilms.\textsuperscript{17,28} Herein, we developed clinically translatable wound biofilm therapeutics based on the integration of polymeric antimicrobial nanotherapeutics, PNPs, into a hydrogel platform as an effective strategy to treat refractory wound biofilm infections \textit{in vivo}.

4.3.1. PNPs penetrate the EPS matrix, access embedded bacteria, and destroy MRSA biofilm

The EPS matrix of the biofilms creates a physical barrier that limits the entry of therapeutics and supports microbial growth.\textsuperscript{5} We used confocal laser scanning microscopy to probe the biofilm penetration profile of PNPs (\textbf{Figure 4.2A-C}). The MRSA biofilm (red+green channel) was visualized by using a GFP-expressing MRSA strain (IDRL-
12570) to grow the bacterial biofilm (green channel) and using Alexa Fluor647 to stain the EPS (red channel). Coumarin blue-tagged PNPs (blue channel) were used to monitor the penetration profile of the polymer. The blue signal colocalizes to both the red and green channels (Figure 4.2B-C). PNPs were able to traverse the EPS matrix and interact with the embedded MRSA, indicating complete penetration. The amphiphilic nature of PNPs is posited to facilitate the transport of PNPs across the highly negative and hydrophobic dense EPS matrix. The biofilm penetrating ability of PNPs resulted in a concentration-dependent destruction of the biofilm biomass as quantified by Crystal Violet assay (Figure 4.2D). We also examined whether the PNPs would affect the components of the EPS matrix, such as extracellular DNA (eDNA), and found that PNPs disrupt the integrity of the biofilm matrix as evidenced by the decreased levels of eDNA (Figure C1).

4.3.2. PNPs are active against resistant cells and persisters without resistance selection

Biofilms comprise bacteria with heterogeneous phenotypic states that contribute to their survival. Upon overcoming the physical barrier presented by the biofilm matrix, an effective biofilm therapeutic should be active against difficult-to-kill bacteria cells: the resistant strains and persisters. PNPs exhibited a broad-spectrum eradication of antibiotic-resistant bacterial biofilms of clinical isolates, with minimum biofilm bactericidal concentration (MBBC) values ranging from 1-8 μM (Figure 4.3A). Using a two-day-old MRSA biofilm, we observed a concentration-dependent killing by PNPs after 3 h treatment, highlighting the rapid onset of their antimicrobial effects (Figure 4.3B). At 2.5 and 5 μM polymer concentrations, 6 log_{10} bacteria reduction and complete eradication of
MRSA biofilms were achieved, respectively. PNPs demonstrated excellent antibacterial activity against pre-formed biofilms.

Figure 4.2. Effects of PNPs on methicillin-resistant *S. aureus* (MRSA) biofilm. (A) Representative 3D views of confocal image stacks of 4-day-old biofilms (47.5 μm thickness) after 1 h treatment with coumarin blue-tagged PNPs (blue channel). GFP-expressing MRSA IDRL-12570 (green channel) was used to form the biofilm, staining with Alexa Fluor 647 to visualize the EPS matrix (red channel). (B) Two-channel merged images. Red+green channels display the entire biofilm image showing the EPS layer and the MRSA cells embedded within. Blue+red channels demonstrate that PNPs co-localize with the EPS layer. Blue+green channels show that PNPs also co-localize with the MRSA cells within the biofilm. (C) Merged image from the three channels. Blue+red+green channels illustrate that PNPs completely penetrate the entire biofilm, both interacting with the EPS matrix and MRSA cells. (D) Biofilm biomass of 2-day-old MRSA IDRL-6169 after 3 h treatment with PNPs. Data are presented as the average of triplicates with standard deviation expressed as error bars.

Persister cells, a subpopulation of metabolically inactive bacteria in biofilms, exhibit a dramatic reduction in susceptibility towards antimicrobials and can resume
growth after antimicrobial pressure is relieved.\textsuperscript{30} These biofilm-derived persisters repopulate biofilms, causing recurring infections.\textsuperscript{9} Persister MRSA cells were generated by exposing MRSA biofilms to 100x minimum inhibitory concentration (MIC) of gentamicin for 24 h. The surviving cells were harvested and then treated with PNPs and vancomycin. The derived persister cells were completely eradicated by PNPs while vancomycin only resulted in $\sim 2 \log_{10}$ bacteria reduction (Figure 4.3C). This efficacy of PNPs towards antibiotic-resistant cells and biofilm-derived persisters is attributed to their mechanism of action through membrane damage, which does not require cells to be in their active growth state.

Resistance selection is a critical challenge for antibacterial therapeutics.\textsuperscript{31} Exposure of bacteria to sub-lethal doses of antimicrobials and their prolonged use can generate resistance mechanisms producing MDR bacterial strains.\textsuperscript{32} We exposed MRSA to sub-therapeutic doses of PNPs (1/3x MIC) for 15 serial passages to study the resistance development of PNPs. PNPs demonstrated no observable resistance over these passages, retaining their original effective dosage (Figure 4.3D). In contrast, vancomycin elicited selection of resistance in the MRSA strain used after the 5th passage. By the 15th passage, 500x of the original MIC of vancomycin is required to kill MRSA. The high barrier to resistance development exhibited by PNPs is attributed to the multiple antimicrobial modalities they can access, particularly membrane damage due to the electrostatic and hydrophobic interactions with the cell membrane.\textsuperscript{21} These interactions create pores leading to cytosolic leakage, eventually causing cell death.
Figure 4.3. Effects of PNPs on resident bacteria cells (resistant and persister cells) in biofilms. (A) Minimum biofilm bactericidal concentrations (MBBCs) of PNPs against different bacteria strains of clinical isolates: methicillin-resistant *S. aureus* IDRL-6169, MDR *S. aureus* CD-35, *S. epidermidis* IDRL-7073, *E. coli* CD-549, *E. coli* CD-2, MDR *E. coli* IDRL-10366, *K. pneumoniae* CD-343, *A. baumannii* CD-575, MDR *P. aeruginosa* CD-40, *P. aeruginosa* CD-1006, MDR *P. aeruginosa* IDRL-11442, E. cloacae complex CD-1412. (B) Viability of MRSA IDRL-6169 after 3 h treatment of the biofilm with increasing concentrations of PNPs. (C) Viability of bacterial persisters derived from MRSA IDRL-6169 biofilm after 3 h treatment with PNPs or antibiotic control vancomycin. (D) Monitoring resistance generation of MRSA IDRL-6169 during sub-therapeutic serial exposure of PNPs or vancomycin. The fold increase in MIC compared to the original MIC value is reflected in the y-axis. Data are presented as the average of triplicates with standard deviation expressed as error bars.

4.3.3. PNPs contribute to processes that facilitate wound healing in vitro

Selective toxicity towards bacteria over mammalian cells is a prerequisite for the practical application of antimicrobial wound therapeutics. An *in vitro* wound biofilm co-
culture model was developed consisting of MRSA IDRL-6169 biofilm (infection), RAW 264.7 macrophages (inflammation phase), and NIH/3T3 fibroblasts (skin repair) (Figure 4.4A). Treatment of the three-component co-culture with PNPs revealed that at concentrations where MRSA is being killed, macrophages and fibroblasts maintained significant viability (Figure 4.4B). This selectivity is attributed to the careful design of the polymer; the charge difference between the near neutral mammalian cell membrane and highly negatively charged bacteria envelope is the basis of the selectivity of cationic PNPs toward bacteria.\(^{28}\) Moreover, the amphiphilic balance and careful placement of the hydrophobic groups on the polymer minimized their toxicity to mammalian cells.\(^{21}\)

After establishing potent antibiofilm activity and safety in mammalian cells, we then evaluated the consequential effects of PNPs on the wound healing process. Wound healing is a dynamic process that requires an orderly transition from the inflammatory stage, progressing to tissue regeneration and, finally, tissue reorganization.\(^1\) Biofilms in the wound bed prompt an excessive and prolonged influx of pro-inflammatory cytokines that slow down the repair process.\(^{12}\) Tumor necrosis factor alpha (TNF-\(\alpha\)) is one of the primary cytokines released in response to infection and indicates a pro-inflammatory state.\(^{34}\) We assessed TNF-\(\alpha\) secretion levels of RAW 264.7 macrophages in the absence and presence of MRSA biofilms, without or with PNPs (Figure 4.4C). Uninfected macrophages with PNPs alone did not prompt significant TNF-\(\alpha\) production, comparable to macrophage alone control. However, the presence of biofilms showed a considerable increase in TNF-\(\alpha\) levels at 2 h. At 24 h and 48 h after treatment with PNPs, the TNF-\(\alpha\) level is significantly reduced as opposed to untreated, infected macrophages that remained to secrete high concentrations
of TNF-α. The reduced state of inflammation is presumed to be a consequence of the effective eradication of MRSA biofilms by the PNPs.

Next, we evaluated whether PNPs can support fibroblast regeneration using wound scratch assay. The wound was simulated by making a scratch on the layer of the fibroblasts and the infection by the biofilm-conditioned media. We monitored fibroblast regeneration via imaging. We saw that after 24 hours, infected samples treated with the PNPs achieve around 90% wound closure while the infected cells are only at 30% (Figure 4.4D). Overall, PNPs not only eliminated the biofilms but also demonstrated positive effects on wound healing in vitro.

4.3.4. Developing PNPs as topical therapeutics by integration with P407 hydrogels

Given the promising in vitro antibiofilm and wound healing activities of PNPs, we then set out to develop the antimicrobial polymers for their practical utility in treating chronic wound infections. Achieving an adequate therapeutic concentration of PNPs across the entire wound area is essential to obtain optimal wound biofilm treatment. Formulations with low viscosity, such as solutions, are prone to uneven spreading both within and beyond the wound area, which can result in a loss of therapeutic efficacy. In contrast, high-viscosity formulations adhere uniformly to the entire wound bed, leading to efficient therapeutic delivery and sustained duration of action.
Figure 4.4. Effects of PNP\text嫔s on macrophages and fibroblasts, mammalian cells relevant in wound healing. (A) Schematic representation of the in vitro wound co-culture model. Fibroblasts (ATCC CRL-1658) were cultured at the bottom of the well then MRSA IDRL-6169 biofilms were grown on top. Macrophages (ATCC TIB-71) cultured on a transwell membrane insert were then placed above. (B) Viability of MRSA, NIH/3T3 fibroblasts, and RAW 264.7 macrophages in the co-culture model after 3 h treatment with PNP\text嫔s. (C) Macrophage TNF-\(\alpha\) production in macrophage-MRSA biofilm co-culture at 2 h, 24 h and 48 h after treatment with PNP\text嫔s. Macrophages with and without treatment of PNP\text嫔s were used for comparison. (D) The extent of wound closure after exposure to MRSA IDRL-6169 biofilm culture media (BCM) was assessed via percent scratch area closed in the in vitro wound scratch assay. Fibroblasts were exposed to MRSA BCM, without (infected) and with PNP\text嫔s (infected + PNP\text嫔s). Fibroblasts only (uninfected) served as control. Data are presented as the average of triplicates with standard deviation expressed as error bars.

Hydrogels are widely used wound dressings that protect the wound area while providing environments conducive for wound healing\textsuperscript{22}. Furthermore, the pores of hydrogels can be loaded with antimicrobial nanotherapeutics that can enhance the utility of both systems. Poloxamer-based hydrogels are particularly promising due to their excellent biocompatibility and stability, as well as their capacity to encapsulate a wide range of hydrophilic and hydrophobic therapeutics\textsuperscript{38}. Poloxamer 407 (P407) hydrogel is a
polyoxypolypropylene–polyoxyethylene non-ionic block copolymer composed of approximately 70% of ethylene oxide and 30% of propylene oxide \cite{39,40}. We formulated the antimicrobial polymers in 20% (w/v) P407 hydrogel to achieve the topical application of PNPs. We chose P407 hydrogel due to its commercial availability, FDA approval, and ease of administration \cite{39,40}. P407 hydrogel forms a sol at <25°C transitioning to a gel form at higher temperatures (Figure 4.5A). This thermoresponsive feature made the administration of the PNP-loaded P407 hydrogel onto wound beds straightforward. Significantly, the P407 hydrogel matrix is uncharged, minimizing potential electrostatic interaction with cationic antimicrobials, such as PNPs, that would otherwise impede their release from the hydrogel\cite{25,39,40}. These hydrogels can provide intimate and sustained contact with wounds, providing controlled and optimal delivery of PNPs.

We studied the release profile of PNPs from the hydrogel scaffold at 37°C (Figure 4.5B). We formed fluorescent PNPs using TRITC-tagged PONI-C11-TMA and incorporated these PNPs into P407 hydrogels. We incubated the hydrogel at 37°C and collected samples at different time points to determine fluorescent signals (ex/em\text{TRITC}: 541/572 nm). PNPs were steadily released, achieving 100% release after 2 h (Fig. 4B). Afterward, we ensured that the P407 hydrogel did not interfere with the antimicrobial efficacy of PNPs. MBBC studies showed that the PNPs in P407 hydrogel displayed similar antimicrobial efficacy as the solution-based PNPs (Figure 4.5C).
Figure 4.5. Integration of antimicrobial PNP s into P407 hydrogel. (A) PNP-loaded P407 hydrogel in its sol (T<25°C) and gel (T>25°C) form. (B) Release profile of PNP s from P407 hydrogel at 37°C. Data are presented as the average of triplicates with standard deviation expressed as error bars. (C) MBBC values of PNP s in solution and PNP-loaded P407 hydrogel against MRSA IDRL-6169, S. epidermidis IDRL-7073, MDR E. coli IDRL-10366, MDR P. aeruginosa IDRL-11442.

4.3.5. PNP-loaded P407 hydrogel is an effective topical wound biofilm therapeutic in vivo

We then set out to test the in vivo efficacy of PNP-loaded P407 hydrogel as topical wound biofilm therapeutics using a robust and reproducible murine model of severe wound biofilm infection\textsuperscript{41,42} (Figure 4.6A). Bioluminescent MRSA USA300 NRS384 strain (SAP-231) was chosen as the infecting bacteria to simultaneously establish infection and
track its progression. The wound was created by making a 5-mm defect at the dorsum of the mice, infected with $10^6$ CFU MRSA/animal then incubated for 4 days to develop a mature biofilm.

We first evaluated whether the hydrogel strategy is effective in localizing the PNPs at the infection site. Four-day-old biofilm-infected wounds were treated with solution-based PNPs and PNP-loaded P407 hydrogel, prepared using NIR fluorescent Cy5.5-tagged PONI-C11-TMA. Using IVIS imaging, we tracked the colocalization of the infection (bioluminescence) and the therapeutic PNPs (NIR fluorescence). As shown in Figure 4.6B, solution-based PNPs are spread outside the wound area, reducing the effective concentration at the infection site. In contrast, PNP-loaded P407 hydrogel contained the PNPs at the infected wound area, providing a means for PNPs to be in maximum contact with the biofilm and allowing efficient dosage for antimicrobial activity.

The efficacy of the PNPs in treating wound biofilms was then assessed to determine whether the localization of the therapeutic translates to efficacy. Wounds with mature biofilm infections were generated as above, then mice were randomly grouped into four to receive one of the following: 1) phosphate-buffered saline (PBS) only, 2) P407 hydrogel only, 3) PNPs in PBS or 4) PNP-loaded P407 hydrogel. Treatments were topically administered once daily for four days. On the last day of treatment, mice were sacrificed via CO$_2$ asphyxiation, and 3 mm skin samples in the inner wound area were collected for quantitative bacterial load determination. Bioluminescence was monitored to qualitatively assess the activity of the treatment groups. As shown in Figure 4.6C, mice treated with PNP-loaded hydrogel showed no bioluminescent signal on the last day of treatment while mice treated with PBS, P407 hydrogel, or PNPs in PBS resulted in little to no change in
bioluminescence comparing the images at the first and last day of treatment. Quantification of the remaining bacterial load at the infection site through agar plate colony counting revealed that PNP-loaded P407 hydrogel treatment resulted in up to 99% (2 log_{10} CFU reduction) clearance of bacterial infection in the severe conditions of wound biofilm area (Figure 4.6D). The other treatment groups exhibited lack of antibacterial activity. PNPs in PBS, despite their promising antibiofilm effects in vitro, demonstrated poor antimicrobial efficacy. This result can be attributed to the reduced amount of the antimicrobial polymer that remained at the wound site. The low viscosity of the solution-based therapeutic resulted in the significant loss of PNPs, as seen in Figure 4.5B, drastically decreasing their efficacy against established mature biofilms. This highlights the importance of using inert carriers including P407 hydrogels in developing wound biofilm therapeutics to ensure maximum contact and efficient topical delivery of PNPs to retain efficacy.
Figure 4.6. PNP-loaded P407 hydrogel as topical therapeutics for severe wound biofilm infections in vivo. (A) Schematic representation of the murine model of severe wound biofilm infection. (B) IVIS images to compare the degree of colocalization of the infected wound area and PNP s using PNP s prepared in PBS or PNP-loaded P407 hydrogel. MRSA USA300 NRS384 strain (SAP-231), a bioluminescent strain, was used as the infecting pathogen to be able to visualize the infection area. PNP s were prepared using Cy5.5-tagged-PONI-C11-TMA, NIR fluorescent polymer, to track the therapeutic. (C) Bioluminescence signals from the wound area of representative mice at the first (day 4) and last (day 7) day of treatment. (D) Bacterial load from the infected wounds treated with PBS, P407 hydrogel, PNP s in PBS or PNP s in P407 hydrogel was determined using quantitative colony counting. *** indicates P value < 0.001.

Inflammation is an essential phase of the wound healing process, which clears the pathogens from the infection site. However, the presence and persistence of biofilms on the wound prolong this inflammatory state, slowing down or inhibiting the other processes involved in wound healing. Pus formation is indicative of infection and inflammation.
We monitored the degree of purulence of the wounds using a reported scoring system\textsuperscript{41,44} (Figure 4.7A). On the last treatment day, we observed that mice treated with PBS only and P407 hydrogel had wounds that were yellow and had high amounts of exudate, accruing scores ranging from 3-5 (Figure 4.7A-B). Groups treated with PNPs in solution and PNP-loaded hydrogels obtained lower purulence scores, with PNP-loaded hydrogels having scores ranging from 0-2 and wounds that were cleaner and had considerably less exudate than PNPs in PBS (Figure 4.7A-B). Moreover, we evaluated the wound samples for pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α). TNF-α mRNA levels were evaluated using RT-PCR. As shown in Figure 4.7C, PNPs in PBS and PNP-loaded P407 hydrogels resulted in reduced levels of TNF-α, with PNP-loaded P407 hydrogel showing significantly better inflammation control compared to PNPs in PBS. The efficacy of the PNP-loaded P407 hydrogels was also mirrored by their enhanced wound healing compared to other treatment groups (Figure 4.7D). Mice treated with PNP-loaded P407 hydrogels exhibited a greater reduction in wound size (~50%) as compared to those treated with PBS, P407 hydrogel only or PNPs in PBS. As a consequence of the maintained antimicrobial efficacy of the PNP-loaded hydrogels, inflammation was controlled and wound healing was enhanced. Overall, these findings highlight the potential of PNP-loaded P407 hydrogels as effective topical wound biofilm therapeutics for severe wound infections. Integration of antimicrobial nanotherapeutics and hydrogel materials proves to be an effective strategy to maintain and maximize antimicrobial efficacy that can consequentially aid in the wound healing process.
**Figure 4.7.** PNP-loaded P407 hydrogel enhanced wound healing in vivo. (A) Purulence scores at the day of sacrifice. (B) Representative images of mice treated with PBS, P407 hydrogel, PNPs in PBS or PNPs in P407 hydrogel at the day of sacrifice. (C) Relative TNF-α mRNA levels. (D) Degree of wound size reduction at day of sacrifice relative to day first day of treatment. *, **, *** indicates P value < 0.05, 0.01 or 0.001, respectively.

### 4.4 Conclusion

In summary, the efficacy and utility of cationic antimicrobial polymeric nanoparticles (PNPs) as topical therapeutics for severe wound biofilm infections were investigated. PNPs, mainly due to their structural design, penetrated across the dense and protective EPS matrix of mature MRSA biofilms, eradicating embedded resistant and
persistor cells. PNPs demonstrated broad-spectrum antibiofilm efficacy without resistance selection even after continuous and prolonged exposure to sub-therapeutic doses. Notably, PNPs exhibited selective toxicity towards biofilms while maintaining safety towards macrophages and fibroblasts, cells that play important roles in wound healing. We then developed PNPs into a topical therapeutic for their practical use in wound treatment. Hydrogels offer controlled and modular wound dressings for antimicrobial polymers. Incorporating PNPs into poloxamer 407 (P407) hydrogels generated a platform that provided a localized and steady release of the therapeutic without compromising its activity. The maintained efficacy of the PNPs through formulation with P407 hydrogels was confirmed in an in vivo murine model of chronic wound MRSA biofilm infection. Significant antimicrobial activity was achieved through treatment with PNP-loaded hydrogels while solution-based PNPs did not result in bacterial reduction. By effectively killing bacterial biofilms, PNP-loaded P407 hydrogels demonstrated enhanced wound healing in terms of degree of purulence, inflammation, and wound size. Taken together, integrating potent cationic antimicrobial polymers into neutral hydrogels is a promising strategy to afford effective topical therapeutics with potential clinical translatability for the treatment of resilient wound biofilm infections.

4.5. References


CHAPTER 5

ENGINEERED ANTIMICROBIAL POLYMER-SIRNA POLYPLEXES AS A DUAL-TARGETED PLATFORM FOR THE TREATMENT OF BIOFILM-INFECTED WOUNDS

5.1. Introduction

Chronic non-healing wounds are a huge clinical burden, affecting 40 million patients worldwide with total spending estimates close to USD 100 billion.\(^1\) Most chronic wounds are attributed to biofilm infections which impede the repair of 60% of non-healing wounds.\(^2\) Wound healing is a dynamic process that requires an orderly transition from the inflammatory stage, progressing to tissue regeneration and, finally, tissue reorganization.\(^3\) Biofilms that persist on wounds create a barrier that prevents antibiotics and other treatments from reaching the infected area. Long-term and high-dosage treatment with topical and/or systemic antibiotics, and frequent wound debridement remain to be the standard wound biofilm treatment in practice.\(^4\) Moreover, wound biofilms prompt prolonged pro-inflammatory responses which alters the progression of skin repair without efficiently clearing the infection.\(^5\) The prolonged presence of immune cells results in the persistent release of inflammatory factors and proteinases.

Simultaneous killing of bacteria and modulation of inflammation provides a promising strategy for treating bacterial biofilm wound infections.\(^6\) The biofilm matrix however presents a bio-barrier that protects the bacteria from antimicrobials. This dense biofilm matrix also presents an added barrier that hinders the access of therapeutic agents to target macrophages.\(^\text{Error! Bookmark not defined.}\) Previously, antimicrobial polymers with poly(oxanorbornenimide) backbones with long alkyl chains demonstrated remarkable
antimicrobial activity through selective bacterial membrane disruption. A key for this antimicrobial polymer is amphiphilicity derived from the structure of polymers consisting of positively charged anchor groups from quaternary ammonium attached to C11 alkyl chain (PONI-C11-TMA). Cationic polymers also are capable of complexing anionic small interfering RNA (siRNA) to provide polyplex systems for therapeutic delivery. Therefore, we hypothesized that the positive charge of PONI-C11-TMA could be used to generate polyplexes with siRNA through electrostatic interaction. The resulting polyplex would have the potential for dual functionality, clearing the bacterial infection and delivering siRNA targeting inflammation\textsuperscript{7,8} across the biofilm matrix into macrophages.

Matrix metalloproteinases (MMPs) are emerging therapeutic targets for the management of chronic wounds\textsuperscript{9,10}. Unregulated levels of MMP-9 excreted by macrophages and monocytes lead to excessive degradation of extracellular matrices, inhibition of growth factors and delayed migration of reparative cells, including fibroblasts\textsuperscript{11}. We report here the fabrication of the multimodal wound biofilm nanotherapeutic PONI-C11-TMA/siRNA polyplexes that eradicate bacterial biofilms while reducing excessive inflammation through MMP-9 knockdown-mediated macrophage immunomodulation (\textbf{Figure 5.1}).
5.2. Results and Discussion

In this study, PONI-C11-TMA was synthesized using ring-opening metathesis polymerization (ROMP) with a third-generation Grubbs catalyst. Briefly, the molecular weight and polydispersity index (PDI) of the polymers were measured using gel permeation chromatography (GPC, PMMA calibrated) in tetrahydrofuran. This ROMP afforded polymers with controlled molecular weight (24,000 g/mol) and a low
polydisperity (PDI: 1.03). The synthesized polymer was characterized and used for experiments, forming polyplexes through simple co-incubation with siRNA. Stable complexation between PONI-C11-TMA polymer and siRNA at varied TMA/phosphate (N/P) ratios was confirmed by gel mobility shift assay with complete complexation of siRNA observed with polymers. Dynamic light scattering (DLS) measurements showed that the polyplexes exhibited discrete particle sizes as an average diameter of 120 nm with low PDI (less than 0.1) (Figure 5.2a). Zeta potential analysis demonstrated that average charge increased with N/P ratio because of increased charge contribution by cationic PONI-C11-TMA polymers (Figure 5.2b). Transmission electron microscopy (TEM) images further confirmed particle sizes and showed that PONI-C11-TMA/siRNA polyplexes have spherical morphology, further confirming successful polyplex formation (Figure 5.2c). The encapsulation efficiency of siRNA was verified by RiboGreen assay (Figure 5.2d). All siRNA encapsulation efficiencies in polyplexes showed more than 96%, suggesting that more than 96% of the packaged siRNA is present inside the polymers. Additionally, polyplexes were prepared with serum media and incubated with RNase A to evaluate the stability of siRNA when exposed to physiological conditions. Polyplexes efficiently protected siRNA from enzymatic degradation by RNase A, while free siRNA was fully degraded. This suggests that the employed strategy improved siRNA stability through protection from enzyme degradation.
We then screened the antimicrobial activity and macrophage biocompatibility of the different polyplexes fabricated with varying N/P ratios (Figure 5.3a). Formation of the polyplexes did not compromise the antimicrobial efficacy of the PONI-C11-TMA as evidenced by the comparable activities of the PONI-C11-TMA only and their corresponding polyplex, resulting to up to ~4 log reduction of MRSA colony forming units (CFU). Furthermore, all the formulations had minimal cytotoxicity towards macrophages. Given the observed efficacy and safety of the small library of polyplexes, we decided to move forward with the formulation that resulted in the highest antimicrobial effects for the subsequent studies. We next validated if the polyplexes are successful at delivering siRNA to knockdown MMP9 in macrophages and fibroblasts, cell types relevant in the wound
healing process. As shown in Figure 5.3b, the polyplexes resulted in up to 80% MMP-9 knockdown. We also evaluate the hemolytic activity of the polyplexes and found minimal levels of hemolysis (Figure 5.3C), an important aspect for nanotherapeutics.

Figure 5.3. a) Screening antimicrobial activity and macrophage biocompatibility of the formulated polyplexes with different N/P ratios. b) Evaluating siRNA activity through MMP-9 knockdown in RAW 264.7 macrophages and 3T3/NIH fibroblasts. c) Evaluating hemolytic activity of the polyplex, and its individual components siRNA only and polymer only, compared with lysing agent Triton-X.
Having optimized delivery conditions of polyplexes, we next investigated the dual targeted platform for the treatment of polyplexes in a co-culture model of MRSA-macrophages. Dual platform of antimicrobial activity and siRNA delivery ability was tested against MRSA-infected macrophage co-culture model. RAW 264.7 cells expressing eGFP were first infected by MRSA expressing RFP at multiplicity of infection (MOI) of 1:100 for 6 h for biofilm formation. Then, PONI-C11-TMA/si_eGFP polyplexes were added and incubated for 24 h. To confirm polyplex activity, the eGFP and RFP expression profile were monitored by confocal microscopy (Figure 5.4). Analysis by confocal microscopy revealed that incubation with scrambled siRNA had no effect on eGFP expression, however, antimicrobial activity was observed through the decrease in RFP expression.

**Figure 5.4.** Evaluating simultaneous antimicrobial activity and siRNA knockdown of the polyplexes using a biofilm-macrophage co-culture model. RFP-expressing MRSA was used to grown and form the biofilm on top of a monolayer of GFP-expressing macrophages.
expression, indicating biofilm eradication. Notably, analysis confirmed that PONI-C11-TMA/si_eGFP polyplexes showed effective knockdown (~90%) of eGFP expression as well as RFP expression reduction suggesting MRSA biofilm eradication.

Having confirmed the ability of PONI-C11-TMA polymer to both eradicate biofilms and deliver siRNA for efficient GFP knockdown with macrophage-MRSA co-culture model, we next investigated whether our PONI-C11-TMA/siRNA polyplexes could enhance the retention of siRNA on the chronic inflammation condition and promote wound healing in vitro. MMP-9 is a widely studied matrix metalloproteinase (MMP) that plays a crucial role in tissue remodeling and other biological processes. Previous studies have shown that MMP-9 is overexpressed at higher levels and with strong activity in the exudates of chronic wounds compared to normal wounds. Considering chronic wounds have been found to have a significant increase in MMP-9 expression, resulting in the degradation of the extracellular matrix (ECM) and impaired healing process, inhibiting MMP-9 expression could potentially enhance remodeling of ECM to improve skin repair\(^\text{12}\).

To assess the effect of PONI-C11-TMA/si_MMP-9 on chronic wounds, we first performed a wound healing assay in vitro using 3T3 fibroblast cells. To mimic wounds in chronic infection condition, we first treated the fibroblasts with biofilm conditioned media (BCM) 24 h prior to delivery of polyplexes. Compared to the control groups, BCM significantly hinders wound closure, but compared with the control groups, a significant reduction in wound area was observed upon treatment with the polyplexes at 24 h (Figure 5.4a-b). Taken together, these results indicated that polyplexes treatment improved wound closures in chronic inflammation condition via MMP-9 knockdown.
Figure 5.5. The effect of MMP-9 downregulation on fibroblast migration in vitro under biofilm conditioned media (BCM). a) Representative images and b) summarized data showing wound healing characterized by cell migration in 3T3/NIH fibroblast cells treated with BCM, si_MMP, PONI-C11-TMA/si_scramble, or PONI-C11-TMA/si_MMP9 polyplexes at 24 h.

After confirming the dual modality of the polyplexes for the treatment of wound biofilms in vitro, we then set out to test their in vivo efficacy as topical wound biofilm therapeutics. We used a murine model of wound biofilm infection with bioluminescent MRSA USA300 NRS384 strain (SAP-231) as the infecting pathogen (Figure 5.6a). The wound was created by making a 5-mm defect at the dorsum of the mice, infected with 10^7 CFU MRSA/animal then incubated for 24 h to develop the MRSA biofilm (Figure 5.6b).

The mice were then randomly grouped into four to receive one of the following: 1) phosphate-buffered saline (PBS) only, 2) polymer only 3) PONI-C11-TMA_siMMP9 polyplex or 4) vancomycin only. Treatments were topically administered once daily for four days. 24 hours after the last day of treatment, mice were sacrificed via CO2 asphyxiation, and 3 mm skin samples in the inner wound area were collected for quantitative bacterial load determination. Quantitative colony counting revealed that the polyplexes retained their potent antibiofilm activity, showing ~2 log units of bacterial reduction, comparable to polymer only (Figure 5.6c). Bioluminescence was also monitored to qualitatively assess the activity of the treatment groups. As shown in Figure
5.6d. mice treated with polymer and polyplex showed no bioluminescent signal on the last day of treatment while mice treated with PBS or vancomycin showed minimal to no change in bioluminescence comparing the images at the first and last day of treatment.

Figure 5.6. a) Schematic representation of the murine model of wound biofilm infection. b) SEM image confirming biofilm formation on the wound site. c) Extent of bacterial reduction relative to negative control, PBS only. * indicates P value < 0.05. d) Bioluminescence signals from the wound area of representative mice at the different days of treatment.
Wound at high infection and inflammation conditions are usually indicated by pus formation. To assess effects of the treatment groups on the inflammatory phase of the wound healing process of the infected mice, we monitored the degree of purulence of the wounds using a reported scoring system\textsuperscript{13}. Mice treated with polyplexes obtained the lowest number of scores, ranging 1-2 that indicates low infection/inflammation state. Mice treated with polymer only have scores averaging at 3 (Figure 5.7a). Quantifying the MMP9 mRNA levels at the collected tissues samples revealed that treatment with polyplexes resulted in 80% knockdown of MMP-9, while polymer alone have levels comparable to negative control, PBS group (Figure 5.7b). This suggests that the polyplexes also efficiently delivered siRNA \textit{in vivo}, knocking down MMP9 levels. The successful MMP-9 knockdown further promoted wound healing, with mice treated with the polyplexes having up to 50% wound closure, significantly better than PNPs and vancomycin alone (~25% closure) (Figure 5.7c).

\textbf{Figure 5.7.} a) Purulence scores of the mice treated with PBS, polymer, polyplex or vancomycin at day of sacrifice. b) Relative MMP9 mRNA levels. c) Degree of wound size reduction at day of sacrifice relative to day first day of treatment. *, **, *** indicates P value < 0.05, 0.01 or 0.001, respectively.
5.3. Summary and Conclusion

siRNA therapy for wound biofilm treatment is a potential strategy to control excessive infection-related inflammatory response particularly the unregulated expression of matrix metalloproteinase 9 or MMP9 that delay the wound repair process. However, delivery of siRNA to macrophages is already challenging, and is further complicated by the biofilm EPS matrix. We posit that the positive charge of PONI-C11-TMA could be used to generate polyplexes with siRNA through electrostatic interaction. The resulting polyplex would have the potential for dual functionality, clearing the bacterial infection and delivering siRNA targeting inflammation, across the biofilm matrix into macrophages. Complexation with PONI-C11-TMA generated stable polyplexes with 100 nm diameter. These polyplexes effectively delivered siRNA into macrophages in vitro, providing >90% knockdown of MMP-9 in RAW 264.7 macrophages and 3T3 fibroblasts, with low toxicity. The polyplexes likewise retained the antimicrobial activity of the parent PONI-C11-TMA, killing up to 4 log reduction of bacteria in the biofilm with and without the siRNA payload. This system was further assessed using a co-culture model of GFP-expressing macrophages and RFP-expressing methicillin-resistant S. aureus biofilm. The PONI-C11-TMA/siRNA polyplexes delivered siRNA to silence GFP (> 80%) in macrophages while killing > 90% of the MRSA cells in the biofilm. Moreover, treatment of the coculture of 3T3 fibroblast cells and MRSA biofilm with the polyplexes resulted in 5 log units of bacteria reduction and maintained fibroblast viability. Results demonstrated antimicrobial potency as well as fibroblast biocompatibility of the polyplexes. The in vivo efficacy of the PONI-TMA-siRNA polyplexes was then evaluated using a murine model of wound biofilm infection. Notably, the polyplexes retained their potent antibiofilm activity, showing about 2 log
reduction of MRSA. This antimicrobial activity provided enhanced wound healing (~50%) relative to vancomycin positive controls (~15%). The polyplexes also efficiently delivered siRNA, as demonstrated by 80% MMP-9 mRNA knockdown at the wound site (RT-PCR). MMP-9 knockdown further promoted wound healing, with mice treated with the polyplexes having 50% wound closure, significantly better than PONI-C11-TMA alone (25%). Overall, these studies demonstrate a dual-action wound healing platform. Potent antibiofilm activity and efficient delivery of siRNA therapy afforded a dual-mode wound biofilm therapeutic that significantly enhances wound healing.

5.4. References


CHAPTER 6

WOUND REPAIR PROMOTER-LOADED NANOPARTICLES FOR THE MULTIMODAL TREATMENT OF WOUND BIOFILM INFECTIONS

6.1. Introduction

Biofilm infection is detrimental to the wound healing process.\(^1\) Normally, the wound repair process undergoes three stages: 1.) inflammation; 2.) new tissue formation; and 3.) remodeling.\(^2\) However, in the presence of biofilms, the wound remains in a prolonged inflammatory state while impeding progression of the proliferative healing phase.\(^3\) Persistent inflammation results in continued presence of reactive oxygen species (ROS) and proteases, further damaging the host tissue and degrading key proteins important in the wound healing cascade.\(^4\) Furthermore, biofilms were shown to suppress relevant growth factors which then impair proliferation of fibroblasts and keratinocytes.\(^5\)

Current standard wound care involves debridement of infected tissue to remove biofilms from the infection site.\(^6\) However, it has been reported that biofilms reform rapidly since the remaining tissue can still act as a nidus for continuous infection.\(^7\) On the other hand, topical antibiotics and antiseptics are inadequate in killing bacterial cells within an established biofilm.\(^8\) Beside the challenge of clearing biofilms, processes involved in the wound repair cascade should also be promoted for successful wound healing. Hence, there is an urgent need for wound therapeutics that can both eliminate biofilm infections and support the skin repair process.

Astaxanthin is a hydrophobic small molecule therapeutic that is gaining traction for wound treatment given its potent anti-inflammatory effects as well as its ability to promote wound healing by expression of growth factors and enhancing cell migration for skin
However, hydrophobic small molecules get stuck in the EPS matrix, debilitating its potential use in wound repair. Polymeric systems are suitable for multiple applications owing to their tunable physico-chemical properties, such as hydrophobicity, size and charge. Polymers can be used as nanocarriers to deliver therapeutics or as inherent therapeutic agents. Previously, our lab reported a poly(oxanorborneneimide)-based (PONI-C11-TMA) that demonstrated remarkable antibiofilm activity with minimum mammalian cell toxicity. PONI-C11-TMA forms into a cationic nanoparticle (PNP) with hydrophobic pockets. Besides being a potent antibiotic agent, PNPs can also be loaded with other hydrophobic wound repair promoters, such as astaxanthin, to achieve a multimodal wound biofilm therapeutic that actively eradicates biofilms while delivering cargos addressing other aspects of wound healing such as inflammation and reparative cell migration.

6.2. Results and Discussion

Amphiphilic polymers including PONI-C11-TMA can self-assemble in water forming micelle-like structures. The hydrophobic portion forms a densely packed core that can trap hydrophobic molecules while the hydrophilic block of the polymer allows the nanoparticle to be dissolved in water. The hydrophobic core of PONI-C11-TMA can be loaded with hydrophobic molecule astaxanthin to fabricate astaxanthin -loaded polymeric nanoparticles (PNP-Ax ), a therapeutic agent that will both eradicate biofilms in the wound site and accelerate the wound repair cascade (Figure 6.1). Astaxanthin (log P= 8.60), a carotenoid, has been shown to be relevant in wound healing through a variety of activities- collagenase inhibition, suppression of ROS and inflammatory mediators, and increased expression of fibroblast growth factor (bFGF). bFGF is significant in the
proliferative and remodeling phases of wound healing. Moreover, astaxanthin also promotes fibroblast and keratinocyte migration.\(^9\)

![Scheme of preparation of astaxanthin-loaded polymeric nanoparticles (PNP-Ax) using flash nanoprecipitation.](image)

**Figure 6.1.** Scheme of preparation of astaxanthin-loaded polymeric nanoparticles (PNP-Ax) using flash nanoprecipitation.

Astaxanthin was loaded into PNPs using a technique called flash nanoprecipitation. Flash nanoprecipitation (FNP) is a versatile nanoparticle production tool that uses rapid micromixing to create high supersaturation conditions leading to high solute encapsulation efficiency in polymer-based delivery vehicles.\(^16\) In this technique, two streams of liquid, the hydrophobic cargo and aqueous polymer solution, with high flow rate generated from a microfluidic device, creates supersaturation conditions that generates stable nanoparticles with high loading capacity\(^17\) (**Figure 6.1**). The fabricated astaxanthin-loaded polymeric
nanoparticles (PNP-Ax) have an overall positive charge (+44 mV) and diameter averaging at ~20 nm as determined by dynamic light scattering (DLS) studies (Figure 6.2), and are stable up to 30 days.

After successfully loading the cargo into the antimicrobial polymer, we checked whether the formed nanoparticle retained the activities of the components (Figure 6.3). We first evaluated the minimum biofilm bactericidal concentration (MBBC) of PNP-Ax, PNP and astaxanthin (Ax) to assess activity against methicillin-resistant S. aureus (MRSA, IDRL-6169). PNP and PNP-Ax have similar MBBC values (8 μM) while Ax did not display any antibiofilm activity at the maximum concentration evaluated. Moreover, PNP-Ax completely eradicated planktonic bacteria at 1500 nM (Figure 6.3a). Astaxanthin is a potent anti-inflammatory agent; we next assessed whether PNP-Ax retained this ability. RAW 264.7 macrophages were cultured with biofilm conditioned media to simulate infection condition, then 24 h after, cells were treated with PNP-Ax, PNP, Ax. Tumor necrosis factor alpha (TNF-α) is one of the primary cytokines released in response to infection and indicates a pro-inflammatory state; we evaluated TNF-α mRNA expression
24 h after treatment. Results showed that PNP-Ax and Ax have comparable extent of suppressing TNF-α expression, indicating that Ax remains active even after encapsulation within PNPs. Biocompatibility of PNP-Ax with 3T3 fibroblasts was also evaluated using Alamar Blue assay; PNP-Ax have minimal impact on fibroblast viability (Figure 6.3c).

**Figure 6.3.** *In vitro* activity of PNP-Ax. a) Bacterial viability after treatment with varying concentrations of PNP-Ax. b) Quantification of TNF-α mRNA expression. c) Fibroblast viability after PNP-Ax treatment.
We next conducted a preliminary *in vivo* study using our established murine model of wound biofilm infection to assess PNP-Ax efficacy. Wound at the dorsum of the mice were created by making 5-mm defects followed by infection with bioluminescent MRSA SAP 231 ($10^7$ CFU/animal). After 24 hours (1-day old biofilm), mice were randomly grouped into four to receive one of the following: 1) PNP-Ax, 2) PNP, 3) Ax or 4) PBS. Treatment was then administered topically and was done once a day for 5 days. Bioluminescence was monitored via IVIS imaging to qualitatively assess degree of bacterial infection remaining on the wound site. At last day of treatment, groups treated with PNPs and PNP-Ax have bioluminescent signals almost gone, while groups treated with Ax and PBS still have high signals, almost similar to signals at day 1 of treatment. This reflects the antibiofilm activity observed *in vitro*: PNP-Ax have maintained the ability to eradicate biofilms, similar to the antimicrobial PNP alone. Skin samples from the wound site were also collected to evaluate levels of inflammatory markers including TNF-α and inducible nitric oxide synthase (iNOS). Samples are currently still being processed.

![Bioluminescent signal of the infected wound area of representative mice at first and last day of treatment.](image)

**Figure 6.4.** Bioluminescent signal of the infected wound area of representative mice at first and last day of treatment.
6.3. Summary and Outlook

The overall goal of this study is to fabricate a multimodal wound biofilm therapeutic by taking advantage of the inherent antibacterial and antibiofilm activity of PNP\textsuperscript{s} to eradicate biofilms at the infection site as well as its ability to serve as a delivery vehicle for astaxanthin, a hydrophobic small molecules that promotes wound repair. The resulting nanoparticles, astaxanthin-loaded polymeric nanoparticles (PNP-Ax), have the potential to fulfill the multi-faceted requirements for the succesful healing of biofilm-infected wounds.

Preliminary results showed that nanoparticle preparation using flash nanoprecipitation afforded stable cationic PNP-Ax with size \( \sim 20 \) nm. The formulation was able to retain both the antimicrobial effects of the encapsulating polymer, as well as the anti-inflammatory effects of the cargo, astaxanthin. Preliminary \textit{in vivo} studies also indicate that PNP-Ax have retained ability to clear biofilms, comparable to the activity of PNP alone. Experiments are still on-going to determine the effects of the fabricated nanoparticle on the processes involved in the skin repair of the infected wound area.

6.4. References


CHAPTER 7

NANOTHERAPEUTICS USING ALL-NATURAL MATERIALS. EFFECTIVE TREATMENT OF WOUND BIOFILM INFECTIONS USING CROSSLINKED NANOEMULSIONS


7.1 Introduction

Antibiotics are the current treatment of choice for bacterial infections.\(^1\) Unfortunately, bacteria are rapidly acquiring resistance against these agents through different mechanisms they have developed over billions of years to remove and/or deactivate toxins or evade their activities.\(^2,3,4\) The ability of pathogenic bacteria, including Pseudomonas aeruginosa and Staphylococcus aureus, to form biofilms results in particularly problematic infections in wounds and on implanted devices.\(^5,6\) The extracellular polymeric substance (EPS) matrix of biofilms has evolved as a barrier to some antibiotics and host immune responses. The slow growth and presence of persister cells in biofilms further foster resistance against traditional antibiotics.\(^7\) Biofilms also impede the wound healing process, resulting in chronic wounds associated with increased morbidity, mortality, and decreased quality of life.\(^8,9\) The lack of effective antibiofilm agents has led to an annual multibillion-dollar (US) burden to healthcare systems worldwide.\(^10\)

Plant-derived essential oils provide a potential resource to combat bacterial biofilm-associated infections.\(^11\) Essential oils are produced by plants as protection against infections by bacteria.\(^12\) These oils have been extensively used in traditional medicine as
antioxidant, anti-inflammatory and antibacterial agents, and their efficacy has been scientifically validated.\textsuperscript{13,14} The additional benefits of using essential oils, including aroma,\textsuperscript{15} safety,\textsuperscript{16} and sustainability, have contributed to their increasing use in therapeutics. The low aqueous solubility of essential oils, however, limits their use in combating planktonic bacterial infections.\textsuperscript{17} Moreover, these hydrophobic oils are not able to efficiently penetrate into the highly charged EPS of biofilms,\textsuperscript{18} making them of limited use against biofilm-associated infections.

Synthetic nanoparticles and polymers provide a strategy for enhancing the activity of essential oil-based antimicrobials.\textsuperscript{19,20} We set out to generate an all-natural platform that delivers active antimicrobials derived from essential oils for treatment of wound biofilms, with the potential for increased safety and community acceptance. In this platform, riboflavin (vitamin B2) is used to cross-link\textsuperscript{21,22} gelatin that stabilizes nanodroplets of carvacrol, a key antimicrobial component of oregano oil. Our approach combines nature-derived ingredients to generate well-defined nanostructured-materials with size and stability required for potent antimicrobial and antibiofilm applications.\textsuperscript{23,24} These nanoemulsions eradicated both Gram-positive and -negative bacterial biofilms in vitro. Significantly, the nanoemulsion system was highly active in vivo, reducing bacterial loads in the wound site and enhancing the rate of wound healing in a murine wound biofilm model. Taken together, the described nanoemulsions used only bio-derived GRAS (generally regarded as safe) components to provide a safe, sustainable and effective treatment for wound biofilms.
Figure 7.1. Fabrication and characterization of gelatin nanoemulsions. a) Riboflavin (UV-cross-linking initiator) was dissolved in carvacrol. The oil mixture was then emulsified in an aqueous gelatin solution and cross-linked using long wavelength UV-A light (365 nm) to fabricate gelatin nanoemulsions. b) Chemical structures of carvacrol, riboflavin, and the functional groups of gelatin participating in the cross-linking reaction. c) Proposed cross-linked structure of gelatin nanoemulsions. d) Dynamic light scattering histogram of nanoemulsions in phosphate buffered saline (150 mM). e) Transmission electron microscopy images of nanoemulsions. Scale bar is 100 nm. f) Confocal laser scanning microscopy images of green fluorescent protein-expressing Klebsiella pneumoniae (IDRL-11999) biofilm after 40 minutes treatment with Nile red-loaded nanoemulsions (XY top view, XZ side view). Scale bars are 50 μm and the thickness of this biofilm is ~37 μm. g) Spatial and time distribution of red fluorescence within the biofilm after the addition of Nile red-loaded nanoemulsions, indicating complete penetration after 35 min.

7.2 Results and discussion

The choice of oil for fabricating the nanoemulsions plays a critical role in therapeutic effects. Oregano oil is among the most potent antimicrobial essential oils.
Carvacrol is the primary active component of oregano oil, and was chosen to provide the GRAS benefits of oregano oil without the batch-to-batch variability observed with unpurified oils. Moreover, commercial gelatin was chosen as the scaffold for the oil-in-water nanoemulsion engineered to overcome the poor water solubility of carvacrol. Gelatin is naturally-derived, inherently biocompatible, biodegradable, non-cytotoxic, and has low antigenicity. Structurally, gelatin is hydrophilic with hydrophobic domains, allowing it to encapsulate and stabilize hydrophobic oils, such as carvacrol, in aqueous condition. The gelatin matrix was stabilized through a cross-linking technique used for therapeutic corneal collagen cross-linking. This strategy employs riboflavin (vitamin B2) as a photo-initiator for cross-linking gelatin fibers. Nanoemulsions were fabricated by emulsifying a suspension of riboflavin in carvacrol into an aqueous suspension of gelatin, generating a transiently stable emulsion. This emulsion was then irradiated (365 nm) to activate the cross-linking process, resulting in stable nanoemulsions.

Dynamic light scattering (DLS), transmission electron microscopy (TEM) and attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) were used to characterize the nanoemulsions. DLS analysis showed that the hydrodynamic diameter of nanoemulsions is ~310 nm with a narrow size distribution (polydispersity index = 0.017). These nanoemulsions are stable at room temperature for at least 30 days and are degraded in the presence of collagenase. TEM photographs revealed a spherical morphology of the nanoemulsions. The size observed in TEM (~100 nm) is smaller than observed by DLS, presumably due to partial collapse upon removal of carvacrol under vacuum during TEM. Finally, the chemical nature of the cross-linking was demonstrated by the emergence of a
band at 1033 cm\(^{-1}\) arising from aliphatic-aromatic ether formation, an additional aromatic ether signature at 1242 cm\(^{-1}\), and appearance of sp\(^3\) C-H stretches at 2957 cm\(^{-1}\) (Figure 7.1c and Figure D3).

Carvacrol and other hydrophobic materials fail to penetrate biofilms.\(^\text{18}\) We hypothesized that the cationic charge of gelatin at the low pH\(^\text{29}\) found in biofilms\(^\text{30}\) would facilitate transport of nanoemulsions into biofilms. Confocal laser scanning microscopy (CLSM) was used to demonstrate effective and complete penetration of gelatin nanoemulsions into biofilms. Transport was tracked by loading the vehicle with the hydrophobic dye Nile red. Nanoemulsions were incubated with 4-day old biofilms of green fluorescent protein (GFP)-expressing \(K.\ pneumoniae\) IDRL-11999. As shown in Figure 7.1f, gelatin nanoemulsions penetrated the biofilm matrix completely, as indicated by visualization of Nile red throughout the thickness of the biofilm, with colocalization of red and green signals. Time-dependent z-stack scanning demonstrated that complete penetration of nanoemulsions occurred within 40 minutes (Figure 7.1g).

We next probed the mechanism of action of gelatin nanoemulsions. We hypothesized that gelatin nanoemulsions kill bacteria the same manner as carvacrol, namely through disrupting the cell membrane. We used propidium iodide (PI) staining to monitor the membrane permeability of bacteria.\(^\text{31}\) Planktonic bacteria (\(P.\ aeruginosa\), ATCC-27853) were treated with gelatin nanoemulsions or antibiotic Ceftazidime (from 0.125X to 4X of their respective minimum inhibitory concentrations (MICs)) in the presence of PI. Gelatin nanoemulsion treatment immediately generated fluorescence inside bacteria, indicating that PI penetrated through the compromised cell membrane and bound to DNA (Figure D4). In contrast, bacteria treated with Ceftazidime did not generate
fluorescence, as Ceftazidime does not directly act on the membrane. Significantly, studies have shown that therapeutics causing membrane disruption are unlikely to induce resistance,\textsuperscript{32} as demonstrated in a recent effective treatment of burn infection models.\textsuperscript{33}

After validating the biofilm penetration profile and the mechanism of action, we used Alamar blue assay to assess antimicrobial activity of nanoemulsions against biofilms of four clinical bacterial isolates (\textit{P. aeruginosa} CD-1006, methicillin-resistant \textit{S. aureus} [MRSA] CD-489, \textit{Escherichia coli} CD-2, and \textit{Enterobacter cloacae} complex CD-1412). Treatment of these biofilms with nanoemulsions for 3 hours eliminated bacteria within the biofilms at 5\% v/v (1.95 mM of carvacrol) (Figure 7.2), with individual components of the nanoemulsions having minimal effect. Notably, treatment of bacteria with only gelatin (nutrient\textsuperscript{34}) or riboflavin (nutrient/potential quorum sensing signal\textsuperscript{35}) can enhance biofilm viability. We further quantified the minimum biofilm inhibitory concentrations (MBICs) and minimum biofilm eradication concentrations (MBECs) towards several clinical isolates, including \textit{P. aeruginosa} (CD-1006, IDRL-11442, ATCC 27583) and MRSA (IDRL-6169) to represent bacterial species that are common constituents of wound biofilm infections.\textsuperscript{36} All biofilms were suppressed or eradicated by nanoemulsions at concentrations ranging from 4\% v/v (1.56 mM) to 8\% v/v (3.12 mM). This activity was mirrored in more challenging dual-species biofilm models (MRSA/\textit{P. aeruginosa} and MRSA/\textit{E. coli}).\textsuperscript{37}
Figure 7.2. Gelatin nanoemulsions kill the bacteria within biofilms. Viability of a) *Escherichia coli* CD-2, b) methicillin-resistant *Staphylococcus aureus* CD-489, c) *Pseudomonas aeruginosa* CD-1006, and d) *Enterobacter cloacae* complex CD-1412 biofilms after 3 hours of treatment with riboflavin, gelatin, carvacrol, or nanoemulsions. e) Viability of 3T3 fibroblast cells and *P. aeruginosa* biofilms in the co-culture model after 3 h treatment with nanoemulsions. Scatters and lines represent 3T3 fibroblast cell viability. Bars represent log_{10} of colony forming units of bacteria in biofilms. f) Gelatin nanoemulsions kill the biofilm bacteria in simulated wound conditions. Log colony forming units (CFU) of methicillin-resistant *Staphylococcus aureus* IDRL-6169 biofilms after 3 hours of treatment with gelatin nanoemulsions in simulated wound fluid. Data are presented as mean ± standard deviation and represent three independent experiments.

We next used an *in vitro* bacterial biofilm-mammalian cell coculture model to evaluate antimicrobial activity and biocompatibility of gelatin nanoemulsions. *P. aeruginosa* (ATCC-27853) was seeded on the monolayer of NIH 3T3-fibroblast cells for 6 hours to mimic biofilm infections on mammalian cells. Subsequently, the cocultures were treated with gelatin nanoemulsion for three hours. The viabilities of mammalian cells and bacteria were determined using LDH assay and colony counting, respectively. As shown
in Figure 7.2e, gelatin nanoemulsions effectively reduce bacterial colonies up to 5 log10 colony forming units (CFUs), while the toxicity toward 3T3 fibroblast cells remained negligible. Moreover, the activity of the nanoemulsions was determined using a simulated wound fluid (SWF) model, mimicking the interference/deactivation of antimicrobial activity by wound fluids. In this experiment, 4-day old MRSA (IDRL-6169) biofilms were grown using tryptic soy broth (TSB)/SWF (1:1) solution. Gelatin nanoemulsions were serially diluted with SWF solution and added to the biofilms; three hours after, antimicrobial efficacy was determined using quantitative colony counting. Nanoemulsions were active against the biofilms in SWF, with 12% v/v (4.68 mM) and 16% v/v (6.24 mM) of nanoemulsions resulting in ~2 and ~5 log10 CFU reduction, respectively (Figure 7.2f).

Encouraged by the in vitro efficacy of the nanoemulsions, their in vivo activity was evaluated using a murine wound biofilm model (Figure 7.3a) designed to assess efficacy against established biofilms found in chronic wound infections. In this model, MRSA IDRL-6169 biofilms were established in a wound created using a 5-mm skin puncture, and allowed to mature for 4 days. Mice were then separated into three groups: 100% v/v nanoemulsions (39 mM carvacrol), vancomycin (110 mg/kg), and saline solution only. Treatments were administered every other day (topical application of nanoemulsions and PBS, and intraperitoneal injection of vancomycin) until the day of sacrifice. Photographs were taken daily, and wound sizes and weights of the mice were monitored every day. The degree of purulence was also evaluated daily using a purulence reaction scoring system described in Figure D5.
Figure 7.3. Gelatin nanoemulsions reduce bacterial load and expedite wound healing in a murine model in vivo. a) Schematic overview of the murine biofilm-associated wound infection model. b) Colony counts from the infected wounds treated with PBS and nanoemulsions. c) Wound size at the day of sacrifice. d) Purulence score at the day of sacrifice (*, ** = P values < 0.05 or 0.01, respectively.

Treatment with nanoemulsions significantly reduced bacterial load in the wound as compared with PBS controls, with ~1.5 log<sub>10</sub> unit reduction after administration of two treatments (Figure 7.3b), a reduced degree of killing relative to in vitro studies owing to the much greater complexity of the in vivo environment. Significantly, vancomycin only had ~0.5 log<sub>10</sub> bacterial reduction (Figure D6). The antimicrobial effect of the nanoemulsions was mirrored by their enhanced wound healing. The group treated with nanoemulsions showed a greater reduction in wound size after treatment as compared to those treated with vancomycin or PBS (Figure 7.3c), with the two control group wound beds still containing pus (Figure D7-9). The wounds were likewise better healed: mice in the nanoemulsion group had a purulence score 0, with normal-appearing healed wound beds. In contrast, vancomycin and PBS groups had purulence scores ~2, indicating the presence of pus (Figure 7.3d and Figure D10).
Histological analysis of the wound beds similarly indicated enhanced healing with the nanoemulsions. Hematoxylin and eosin staining (H&E staining) revealed regeneration of keratin and epithelial layers, and collagen matrix for the nanoemulsion group. In contrast, for the vancomycin and PBS groups, inflammatory cells were still abundantly found in the area, indicating that the wounds were in the early stages of the wound healing cascade (Figure 7.4 and Figure D11). Notably, the healed skin had a normal appearing epidermis and dermis, suggesting that nanoemulsions do not alter morphology of the skin undergoing repair. The relative lack of inflammatory cells could also suggest an anti-inflammatory role for the nanoemulsions in wound healing.

7.3 Conclusions

In summary, integration of nanostructured biomaterials with essential oil payloads provides effective treatment of challenging wound biofilms. Emulsification of oregano oil and gelatin followed by vitamin B2 cross-linking provided stable nanoemulsions comprised solely of naturally-occurring components. These described nanoemulsions effectively penetrate into biofilms, and kill embedded Gram-positive and -negative bacteria effectively in vitro. This antibacterial effect is observed in an in vivo murine model, and translates into enhancement in wound healing both in terms of wound size and degree of purulence. Crucially, this platform is highly modular, providing a general platform for the delivery of a wide variety of oils and other payloads. Overall, integration of the inherent bactericidal activity of essential oils with materials properties provided by biomaterials presents a new path of treatment for wound biofilms, with potential for treating other life-threatening bacterial infections.
Figure 7.4. Histological analysis of the tissues surrounding infected wounds show enhanced healing with nanoemulsions. a) Epidermis samples showed regeneration of keratin and the epithelial layer with nanoemulsion treatment. In contrast, inflammatory cells and proteinaceous debris were observed with PBS and vancomycin treatments. b) Substantial formation of collagen matrix at the epidermis-dermis junction was observed after nanoemulsion treatment, whereas immature epidermis and granulation were observed with PBS. Necrosis and cell debris were also detected in the vancomycin-treated sample. c) The dermis was restored with nanoemulsion treatment, while inflammatory cells were still present in the PBS and vancomycin controls.

7.4. References


CHAPTER 8

SELECTIVE TREATMENT OF INTRACELLULAR BACTERIAL INFECTIONS USING HOST CELL-TARGETED BIOORTHOGONAL NANOZYMES


8.1 Introduction

*Salmonella* is a Gram-negative intracellular pathogen that causes systemic infections such as typhoid fever and gastroenteritis. As one of the most common sources of foodborne illness, *Salmonella* pathogens remain a threat to public health worldwide,\(^1\) with ~1.3 billion cases of *Salmonella*-related illness and ~370,000 deaths reported every year.\(^2\) One of the major challenges in treating *Salmonella* infections is that this pathogen invades and resides within the host’s own cells, including dendritic cells, epithelial cells and macrophages. Macrophage invasion allows *Salmonella* to establish systemic disease in a susceptible host.\(^3,4\) The ability of these and other pathogens to hide inside of host cells protects them from both host immune defences and antimicrobial therapeutics. These intracellular infection mechanisms can result in acute life-threatening infections and long-term, recurring chronic infections that are difficult to treat.\(^4,5\)

Traditional antibiotic therapeutic strategies have limited efficacy against intracellular pathogens. Most antibiotics are not designed to penetrate mammalian cell membranes, and/or are degraded by enzymes in the cytosol.\(^6,7\) As a result, high doses of antibiotics are required to kill intracellular pathogens, which magnifies their off-target
effects on the microbiome.\textsuperscript{8} Broad spectrum antibiotics eliminate both pathogenic and
beneficial bacteria, attenuating the positive roles the microbiome plays in fighting
infections,\textsuperscript{9} while also generating resistant strains that lead to reduced drug efficacy.\textsuperscript{10}
Moreover, loss of a healthy microbiome leads to a range of gastrointestinal problems, in
particular \textit{C. difficile} infection.\textsuperscript{11,12} These challenges are all exacerbated by a lack of
progress in the development of new antibiotics, making it unlikely that a new drug effective
against intracellular infections will be available soon.\textsuperscript{13}

Localization of therapeutic activity to affected host cells is a key challenge in
treating intracellular infections.\textsuperscript{14} Bioorthogonal transition metal catalysts (TMCs)-based
provide a strategy for on-demand generation of therapeutics at the infection site. However,
their direct utilization poses significant challenges as they have limited solubility in
aqueous conditions and are prone to deactivation in biological systems.\textsuperscript{15,16} Integrating
TMCs with nanoparticles provide bioorthogonal ‘nanozymes’ (NZ) to produce drugs and
imaging agents in complex biosystems in situ,\textsuperscript{17,18} differentiating them from commonly
used nanozymes with intrinsic peroxidase-mimicking activity.\textsuperscript{19,20,21}

We report here the fabrication of a targeted nanozyme that converts pro-antibiotics
into antibiotics inside cells to effectively eradicate intracellular pathogenic bacteria. Here,
we have generated negatively charged nanozymes functionalized with mannose (Man-NZ)
that are efficiently and selectively internalized by macrophages. These nanozymes consist
of mannose-functionalized gold nanoparticle scaffolds (2 nm core, Man-AuNPs), with iron
tetraphenylporphyrin (FeTPP) serving as a bioorthogonal catalyst.\textsuperscript{22} Mannose
functionalization allows specific binding to the mannose receptor (CD206) present on the
surface of macrophages,\textsuperscript{23,24} facilitating selective uptake of Man-NZ. Once internalized,
Man-NZ efficiently converts a phenylazide-caged pro-ciprofloxacin into ciprofloxacin to specifically kill pathogenic *Salmonella* (Figure 8.1). Transwell co-culture studies using *Salmonella*-infected macrophages and non-pathogenic *Lactobacillus* species bacteria indicated that antibiotic activity was localized within the macrophages. Intracellular *Salmonella* levels were reduced significantly, and minimal toxicity to *Lactobacillus* sp. was observed. Taken together, this nanozyme strategy combines ligand-receptor targeting with bioorthogonal catalysis to create an effective, site-specific intracellular antibiotic treatment that concurrently minimizes off-target toxicity.

Figure 8.1. Macrophage uptake of mannose-targeted nanozymes (Man-NZ) followed by administration of a bioorthogonally-caged prodrug generates antibiotics inside cells.
8.2 Results and discussion

Gold nanoparticles (AuNPs) are established as safe for therapeutic delivery and feature facile synthesis and functionalization.\textsuperscript{25,26} Negatively charged gold nanoparticles (COOH-AuNPs) featuring biocompatible tetra(ethylene)glycol spacers and carboxylate headgroups were used to minimize non-specific uptake of the nanozyme.\textsuperscript{27} These particles were synthesized from pentanethiol-capped 2 nm core AuNPs using a place exchange reaction, as previously reported.\textsuperscript{17} The particles were then post-functionalized via EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide)/NHS (N-hydroxysulfosuccinimide) coupling with D-mannosamine to display mannose moieties for macrophage targeting. The density of D-mannose ligands on Man-AuNPs was quantified using an anthrone/sulfuric acid assay\textsuperscript{28,29} that has been used for quantitative analysis of carbohydrates in glyconanoparticle analysis (Figure E1).\textsuperscript{30,31,32} The surface coverage was determined to be \textasciitilde40\%. Zeta potential values were consistent with these measurements, shifting from -42.7 mV to -7.9 mV after mannose conjugation (Figure E2). Significantly, the Man-AuNPs retained negative a charge, enabling the minimization of non-specific uptake. Further characterization of both AuNPs and Man-AuNPs was performed through dynamic light scattering (DLS) measurement and transmission electron microscopy (TEM) (Figure E3-4).

Mannose nanozymes (Man-NZs) were fabricated by encapsulating 5,10,15,20-tetraphenyl-21H,23H-porphine (TPP)-iron (III) complexes (FeTPP) into Man-AuNP scaffolds. The FeTPP molecule catalyzes the reduction of aryl azides to corresponding amines\textsuperscript{22} and has been used as an efficient bio-orthogonal catalyst.\textsuperscript{33,34} Man-NZs were prepared by mixing an aqueous solution of Man-AuNPs with FeTPP dissolved in
tetrahydrofuran (THF) and stirring for 10 min. Evaporation of THF drives encapsulation of the catalyst into the hydrophobic pockets of Man-AuNPs. The amount of FeTPP encapsulated in Man-AuNPs was determined quantitatively using ICP-MS analysis (Figure 8.2a and E5). Afterwards, the catalytic properties of the nanozymes were demonstrated through efficient uncaging of a non-fluorescent resorufin-based profluorophore (pro-res)$^{33,34}$, where FeTPP catalytically reduces the azide, resulting in fragmentation that releases the fluorescent resorufin molecule (Figure 8.2b-c).$^{35,36}$ Man-AuNPs alone do not contribute to the catalytic activation of the Pro-Res.

**Figure 8.2.** Characterization of Man-NZ. a) ICP-MS quantification of FeTPP catalyst loading in Man-NZ based on results from Figure S5; b) Activation of pro-resorufin to red fluorescent resorufin by Man-NZ; glutathione (GSH) was used as the cofactor; c) Percent conversion of 20 μM pro-resorufin into resorufin over time by 500 nM Man-NZ or Man-AuNPs, with 5 mM GSH as cofactor. Pro-res only served as control. Error bars represent standard deviation (n=3).
After generating the nanozyme, the selective uptake of Man-NZ by macrophages was quantified using ICP-MS (Figure 8.3, E6-7). As expected, Man-NZ was internalized by RAW 264.7 macrophages in a dose-dependent fashion and was retained even after 72 hours (Figure E7). In contrast, Man-NZ was not internalized by HepG2 hepatocytes, even at the highest dose, due to its negative charge. Significantly higher levels of macrophage uptake were observed for the targeted Man-NZ relative to the untargeted COOH-NZ (Figure 8.3a). This selective uptake by macrophages over HepG2 also indicates expected safety of the platform towards healthy liver cells.\textsuperscript{57,58} Moreover, M2 phenotype macrophages (i.e., stimulated by IL-4) displayed higher uptake compared to those of M0 and M1 phenotype (Figure 8.3b, E7b), further confirming the mannose receptor-based targeting by our nanozyme system (M2-polarized macrophages possess increased CD206). For M2 phenotype cells, Man-NZ has substantially greater uptake than COOH-NZ (Figure 8.3c). Man-NZ was non-toxic to the macrophages even at high concentrations, as demonstrated by Alamar Blue assay (Figure E8).

The intracellular activity of Man-NZ was demonstrated by activation of non-fluorescent pro-res. Non-polarized macrophage cells were incubated with Man-NZ overnight, washed, and then the pro fluorophore was added and incubated for an additional 2 h followed by washing (Figure 8.4). Confocal microscopy imaging revealed that cells treated with both Man-NZ and pro-res had noticeably more intense red fluorescence than cells treated with pro-res or Man-NZ alone. Significantly, cellular fluorescence was observed to be distributed throughout the cytosol, a prerequisite for addressing intracellular infections.
After confirming the activity of the Man-NZ inside macrophages, we next assessed the therapeutic efficacy of the nanozyme strategy using pro-antibiotics. We synthesized an azidophenyl-caged ciprofloxacin (pro-ciprofloxacin; pro-cip) that is converted to the active antibiotic, ciprofloxacin, by Man-NZ (Figure 8.1, Figure E9). This system was tested against the causative pathogen for typhoid, *Salmonella enterica* subsp. *enterica* serovar Typhimurium (Figure 8.5a). Treatment of planktonic bacteria with both Man-NZ and pro-cip resulted in an approximately 4 log reduction of *Salmonella* colony forming units (CFUs), similar to the activity of ciprofloxacin. Groups treated with pro-cip only or Man-NZ only resulted in no significant *Salmonella* reduction compared to the untreated
[phosphate buffered saline (PBS) only] control group, demonstrating that activity arises from effective bioorthogonal catalysis by Man-NZ.

Figure 8.4. Confirming intracellular catalytic activity of Man-NZ using confocal laser scanning microscopy. Confocal images of non-polarized RAW 264.7 macrophages incubated with Man-NZ overnight and then with pro-res for 2h (left). Macrophages treated with pro-res only (middle) and Man-NZ only (right) served as controls.

Following confirmation of antibiotic activation through killing of planktonic bacteria, we determined the efficacy of the Man-NZ+pro-cip system in an intracellular macrophage Salmonella infection model. In brief, RAW 264.7 macrophages were seeded in a 96-well plate, then treated with Man-NZ overnight to facilitate uptake. Following, macrophages were infected by incubating the cells with Salmonella (1:100 multiplicity of infection) for an hour. The macrophages were then washed and incubated with gentamicin for 30 minutes to remove extracellular bacteria. Afterwards, pro-ciprofloxacin was added. The macrophages were then lysed and the surviving bacteria were collected and quantified through CFU counting. Treatment with Man-NZ+pro-cip reduced intracellular bacterial CFUs to a similar extent as ciprofloxacin did (Figure 8.5b). Importantly, concentrations of pro-cip, ciprofloxacin, and Man-NZ+pro-cip used in this study were confirmed to be non-toxic to the macrophages by Alamar Blue assay (Figures E10-11). Treatment with Man-NZ only or pro-ciprofloxacin only did not significantly reduce Salmonella CFUs, demonstrating the ability of the Man-NZ to generate active antibiotics inside macrophages for killing of intracellular pathogenic bacteria. This activity was further validated in an
additional murine macrophage type, J744 (Figure E12a). We also tested our strategy using methicillin-resistant Staphylococcus aureus (MRSA)-infected RAW 264.7 macrophages; MRSA is another prevalent intracellular pathogen. As before, treatment with Man-NZ+pro-cip resulted in reduction of intracellular bacteria viability, comparable to the activity of the active drug (Figure E12b).

Figure 8.5. Antimicrobial activity of Man-NZ+pro-cip against Salmonella. a) Viability of Salmonella after 24 hr treatment with pro-ciprofloxacin and Man-NZ, compared to controls, as determined by quantitative colony counting. b) Viability of Salmonella residing inside macrophages after 24 hr treatment with pro-cip and Man-NZ or controls, as determined by quantitative colony counting in a Salmonella-macrophage infection model. Data are presented as mean ± standard deviation, n=3 (*, **, *** = P values < 0.05, 0.01, 0.001, respectively).
Traditional antibiotic therapies for intestinal infections generally result in a substantial reduction of beneficial gut bacteria, leading to an array of indirect effects. We hypothesized that generation of antibiotics inside of macrophages would result in higher local concentrations of therapeutics. As a result, the in situ generation of antibiotics would allow lower overall dosing, minimizing effects on the surrounding microbiome. To test this possibility, we developed a transwell membrane model in which *Lactobacillus* species, a dominant organism in the human gut, was co-cultured with *Salmonella*-infected RAW 264.7 macrophages (Figure 8.6a). Briefly, Man-NZ loaded macrophages were plated at the bottom of a well and infected with *Salmonella*, while *Lactobacillus* sp. (BioK+) were added to the transwell membrane (0.4 μm pore size). Pro-ciprofloxacin or ciprofloxacin was then added to the top of the well. Quantitative colony counting after 24 h of treatment revealed that pro-ciprofloxacin treatment of Man-NZ loaded macrophages resulted in significant *Salmonella* colony reduction (>3 log units) while *Lactobacillus* CFUs were minimally affected (<1 log unit; Figure 8.6b, E13). In contrast, traditional ciprofloxacin treatment killed both *Salmonella* and *Lactobacillus* to significant extents, as expected due to the broad-spectrum activity of the antibiotic. As in the previous experiments, the pro-ciprofloxacin alone control did not affect CFU counts of either bacteria type. Taken together, this study demonstrates the strong potential of nanozyme-based systems to treat intracellular infections while maintaining overall gut microbiome diversity.
Figure 8.6. Evaluating activity of Man-NZ+pro-cip using *Salmonella*-infected macrophages and *Lactobacillus* sp. coculture model. a) Schematic of transwell co-culture assay with *Salmonella*-infected, Man-NZ-loaded macrophages and *Lactobacillus*. b) Viability of *Lactobacillus* sp. and intracellular *Salmonella* after 24 h treatment with pro-ciprofloxacin and Man-NZ, and controls in a transwell co-culture model as determined by quantitative colony counting. Data are presented as mean ± standard deviation, n=3. *, **, *** = P values < 0.05, 0.01, 0.001, respectively.

8.3. Conclusions

In summary, we have generated macrophage-targeted bioorthogonal nanozymes to produce antibiotics at the sites of intracellular bacterial infections. Negatively charged nanozymes post-functionalized with a mannose terminal headgroup demonstrated high selectivity for uptake by macrophages. Following internalization, the nanozyme retained its catalytic activity as demonstrated by the conversion of non-fluorescent pro-resorufin
into the fluorescent resorufin. Activation of pro-ciprofloxacin by Man-NZ inside of Salmonella-infected macrophages resulted in effective killing of intracellular Salmonella. The ability to generate effective concentrations of antibiotic inside of the macrophages provides high selectivity for intracellular infections and an important new strategy for treatment of these infections without harming the surrounding microbiome. This bioorthogonal approach has the potential to effectively treat intracellular infections ranging from typhoid to tuberculosis -- diseases that affect millions globally every year.

8.4. References


CHAPTER 9

ANTIMICROBIAL-LOADED BIODEGRADABLE NANOEMULSIONS FOR EFFICIENT CLEARANCE OF INTRACELLULAR PATHOGENS IN BACTERIAL PERITONITIS

9.1 Introduction

Multidrug-resistant (MDR) bacterial infections represent a global health threat associated with ~5 million deaths in 2019.\textsuperscript{1,2,3} As adaptable pathogens, bacteria have developed multiple mechanisms to evade the host immune system and antimicrobial agents. These processes include drug resistance development,\textsuperscript{4} biofilm formation\textsuperscript{5} and intracellular infections.\textsuperscript{6} Bacteria (e.g. \textit{Staphylococcus aureus}) can survive and replicate inside mammalian cells, leading to chronic and recurrent illnesses.\textsuperscript{6,7} Immune cells such as macrophages are the first line of defense during infections through phagolysosomal killing,\textsuperscript{8,9} however, bacteria can evade this mechanism and instead use the macrophage as a protective niche against antibiotics and host immune responses.\textsuperscript{6,7,10}

Intracellular infections are central to many therapeutically challenging bacterial illnesses, with the resulting pathogen reservoir associated with recurring and persistent systemic infections, including peritonitis. Peritonitis is a severe and often fatal infection in patients with cirrhosis, and is a major contributing cause of death in patients on peritoneal dialysis.\textsuperscript{11,12} At present, the standard treatment strategy involves antibiotic therapy,\textsuperscript{13} however, the efficacy of antibiotics is compromised by drug resistance\textsuperscript{4,14} and the inability of antibiotics to access the intracellular bacteria and maintain therapeutic concentrations within host mammalian cells.\textsuperscript{13} Even antibiotics of last resort, including vancomycin, can be ineffective in eradicating intracellular bacteria.\textsuperscript{15,16} Hence, there is an urgent need for
antimicrobials that can efficiently penetrate cell membranes, accumulate inside host cells, and eliminate intracellular MDR bacteria.

Phytochemicals are potent antimicrobials that are emerging alternatives to traditional antibiotics for combatting MDR bacteria.\textsuperscript{17} Poor stability, low aqueous solubility, and challenging in vivo partitioning of the hydrophobic species, however, limit their practical therapeutic potential.\textsuperscript{18,19} Nanomaterial vehicles provide a strategy for the delivery of phytochemicals, increasing their efficiency, stability, solubility and biocompatibility.\textsuperscript{20,21,22} As an example, biodegradable polymeric nanoemulsions (BNEs) using positively-charged guanidinium-functionalized poly(oxanorborneneimide) (PONI-GMT) polymers generate stable nanoemulsions with phytochemicals that effectively penetrate biofilms and kill bacteria.\textsuperscript{23,24}

Polymeric nanoparticles can be engineered to bind to mammalian cells and deliver cargo.\textsuperscript{13,25} Polymeric scaffolds decorated with positively-charged guanidinium moieties provide versatile platforms for the delivery of a wide range of biologics into mammalian cells.\textsuperscript{25,26} We hypothesized that the guanidinium groups of PONI-GMT could interact with the negatively-charged cell surface of macrophages, facilitating intracellular delivery of antimicrobial phytochemicals, targeting and killing resident pathogens. This hypothesis was tested by screening four phytochemical-loaded BNEs (carvacrol, eugenol, linalool and methyl eugenol) for their activity in vitro against intracellular pathogens including methicillin-resistant S. aureus (MRSA) (Figure 9.1). The eugenol-loaded BNE (E-BNE) was selected for the subsequent studies due to its exhibited high antimicrobial activity, high macrophage accumulation, and low cytotoxicity. E-BNE delivered eugenol into macrophages via cholesterol-dependent membrane fusion as shown by confocal laser
scanning microscopy and flow cytometry. Significantly, E-BNE was able to reverse the immunosuppressive strategies of intracellular MRSA, restoring normal TNF-α production and caspase-3/7 levels. Unlike conventional antibiotics, MRSA did not develop resistance towards E-BNEs even after multiple exposures to sub-therapeutic doses.

E-BNEs were next tested against a murine model of MRSA-induced peritonitis to establish the translatability of the observed in vitro efficacy (Figure 9.1). A luminescent MRSA USA300 NRS384 strain (SAP-231) was injected intraperitoneally into mice to initiate bacterial invasion, enabling imaging of the infection. Notably, E-BNEs cleared 99% of bacterial load while vancomycin, a standard treatment for peritonitis caused by S. aureus, showed no efficacy. Overall, this study demonstrates the potential of the BNE platform as a non-antibiotic alternative for the eradication of intracellular bacteria. The therapeutic efficacy of this system provides a promising strategy against a wide range of refractory intracellular infections that currently have few effective treatments in the clinic.
Figure 9.1. Fabrication of antimicrobial phytochemical-loaded biodegradable nanoemulsions (BNEs) for the treatment of intracellular infections including peritonitis. Phytochemicals (carvacrol, eugenol, linalool, methyl eugenol), loaded with DTDS crosslinker, were emulsified with aqueous solution of PONI-GMT forming the BNEs. Eugenol-loaded BNEs (E-BNEs) were found to have optimum properties as intracellular infection therapeutic and were used to treat MRSA-induced peritonitis using a murine model.
9.2 Methods

9.2.1. Fabrication of phytochemical-loaded BNEs

BNEs were prepared through the emulsification of DTDS-loaded essential oils (carvacrol, eugenol, linalool and methyl eugenol) into an aqueous solution of PONI-GMT. 3 μL of the essential oil with solubilized DTDS (3%) was added to 497 μL PONI-GMT solution (6 μM final concentration). Using an amalgamator, the resulting mixture was then emulsified for 50 s. Emulsions were allowed to rest overnight before use.

9.2.2. Intracellular antimicrobial activity of E-BNE

For intracellular killing assays, a modified version of a previously published protocol was performed. \(^{29}\) RAW 264.7 cells were seeded at a concentration of 10,000 cells/well and cultured in Dulbecco’s modified Eagle medium with 1% antibiotics and 10% bovine calf serum in a humidified atmosphere of 5% CO\(_2\) at 37°C overnight. A solution of MRSA in culture media without antibiotics was then added to the wells to achieve 100:1 multiplicity of infection (MOI) and incubated for 1 h. The wells were washed with gentamicin three times, then incubated in culture media containing 50 μg/mL gentamicin for 30 min to remove extracellular bacteria. The cells were washed with PBS once and treated with different concentrations of E-BNEs for 2 h. Then, cells were washed once and macrophage cells were lysed using 0.1% Triton X. The lysate was collected for quantitative colony counting. Three biological replicates were generated and the experiment was repeated on two different days.
9.2.3. Confirming delivery of essential oils inside macrophages using confocal laser scanning microscopy

RAW 264.7 cells were seeded at a concentration of 200,000 cells/dish in a 35 mm confocal dish 24 h before the experiment. Red fluorescent dye, Nile red, was incorporated into the BNEs by dissolving the dye in the essential oils (1 mg Nile red/mL oil). The cells were washed with PBS and then incubated with media containing dye-loaded BNEs for 2 h. Media was then aspirated and the cells were washed with PBS to remove excess material. Cells were stained with Hoechst 33342 solution (Thermo) about 20 minutes before imaging. Confocal microscopy images were obtained on a Zeiss LSM 510 Meta microscope by using a 60X objective. The settings of the confocal microscope were as follows: red channel: \( \lambda_{\text{ex}} = 560 \) nm and \( \lambda_{\text{em}} = \text{LP} 640 \) nm; blue channel: \( \lambda_{\text{ex}} = 403 \) nm and \( \lambda_{\text{em}} = \text{LP} 495 \) nm. Emission filter: LP = high pass.

9.2.4. Ethics statement

BALB/c mice were supplied by Jackson Laboratory. Mice were housed in sterile cages with a 12-hour light/12-hour dark cycle and were allowed to acclimatize for at least a week before any of the procedures were performed. All animal experiments were carried out following the authorized protocol (IACUC Protocol ID 3023) and the policies issued by the Institutional Animal Care and Use Committee at the University of Massachusetts Amherst.

9.2.5. Dosing and biodistribution studies

A dosing study was done to determine the concentration of E-BNEs to be used to evaluate the efficacy of the nanoemulsions in treating murine peritonitis. Mice were
randomized into 4 groups of 3 to receive one of the following treatments: 1) PBS only; 2) 100 μL 100% E-BNE (0.6 mg eugenol/mouse); 3) 250 μL 100% E-BNE (1.6 mg eugenol/mouse); 4) 500 μL 100% E-BNE (3.2 mg eugenol/mouse). The abdomen of the mice was disinfected with alcohol pads then the test materials were administered via IP injection. The mice were then closely monitored for any signs of distress. Survival rates were noted 24 hours post-injection.

To evaluate the biodistribution profile of E-BNEs upon intraperitoneal administration, E-BNEs loaded with NIR750 dye (5 μM) were prepared. After disinfection, mice were IP injected with 500 μL dye-loaded E-BNE. IVIS imaging (IVIS SpectrumCT) was done 1- and 24 h post-injection. Then, mice were sacrificed through CO₂ asphyxiation, harvesting peritoneal fluid, blood, liver and spleen for ex vivo imaging.

9.2.6. In vivo murine peritonitis model

The abdominal area of the mice was disinfected using alcohol pads. Using a sterile 28-gauge syringe, 150 μL of bioluminescent MRSA (MRSA USA300 NRS384) in PBS was intraperitoneally injected into the mice (10⁶ CFU/mouse) and infection was allowed to ensue for 24 hours. Mice were then imaged using IVIS SpectrumCT to confirm infection. Mice that did not show a bioluminescent signal 24 hours post-infection were excluded from the study. Infected mice were then randomly separated into three groups to receive one of the following treatments: 1) PBS, 2) vancomycin control and 3) E-BNE. A single dose of the test solutions was administered intraperitoneally. After 24 hours, mice were again imaged, then euthanized via CO₂ asphyxiation.

A previously reported protocol was adapted and modified to evaluate antibacterial efficacy.²⁹,³⁰ Mice were injected with 3 mL sterile HBSS intraperitoneally. Peritoneal fluid
was then collected to quantify total CFU count, as well as extracellular and intracellular count. For the total CFU count, the collected fluid was immediately used for quantitative colony counting. The remaining fluid was then equally separated into 2 fractions: 1) extracellular quantification and 2) intracellular quantification. For fraction 1, the sample was centrifuged at 300xg for 10 min at 4°C and the supernatant was collected for quantitative colony counting. For fraction 2, the sample was centrifuged at 300xg for 10 min at 4°C and the pellet was resuspended in HBSS with gentamicin (50 μg/mL) and incubated for 30 min. Gentamicin was then removed by washing the cells thrice with PBS. Then, pellets were resuspended in HBSS with 0.1% Triton-X. After 10 min, the solution was used for quantitative colony counting. All colony counting was done using tryptic soy agar plates.

9.3 Results

9.3.1. Fabrication and screening of phytochemical-loaded BNEs

Cross-linked poly(oxanorborneneimide) polymers (PONI) can be used to stabilize and improve the antimicrobial properties of essential oils. In the current study, PONI polymers were functionalized with guanidinium, maleimide and tetraethylene glycol monomethyl ether moieties to produce a cationic amphiphilic polymer, PONI-GMT. The tetraethylene glycol monomethyl ether units impart amphiphilicity to the polymer which enables PONI-GMT to self-assemble around the hydrophobic essential oil, forming stable biodegradable nanoemulsions (BNEs). The maleimide moieties then allow further emulsion stabilization by forming a cross-linked structure via maleimide-Michael addition reactions with the biodegradable cross-linker, disulfanediylid(ethane-2,1-diyl) bis(11-
sulfanylundecanoate) (DTDS). Guanidinium groups were used to confer an overall positive charge on the nanoemulsions. These positively charged guanidinium groups interact with negatively charged macrophage cell surfaces to facilitate nanoemulsion entry and payload delivery. Guanidinium ligands interact with membrane lipids through electrostatic interactions and hydrogen bonding, inducing non-disruptive and non-cytotoxic membrane permeabilization.31,32

We screened a small library of BNEs formulated with carvacrol (C-BNE), eugenol (E-BNE), linalool (L-BNE), and methyl eugenol (ME-BNE), as these phytochemicals demonstrate good antimicrobial activity.33, 34, 35, 36 The oils were first loaded with DTDS crosslinker and then subsequently emulsified with an aqueous solution of PONI-GMT to form stable nanoemulsions (Figure 9.1). Analysis with transmission electron microscopy (TEM) and dynamic light scattering (DLS) showed BNEs with spherical morphology and sizes ranging from 160 to 350 nm (Figure 9.2A-E). As expected, the afforded BNEs had positive zeta potential values (Figure 9.2F) due to the cationic guanidinium functionalities of PONI-GMT.
Figure 9.2. Characterization of phytochemical-loaded BNEs. Transmission electron microscopy images of (A) C-BNE; (B) E-BNE; (C) L-BNE; (D) ME-BNE. Scale bar is 200 nm. (E) Dynamic light scattering histogram of BNEs in phosphate-buffered saline (150 mM). (F) Zeta potential of BNEs.

We then screened the BNEs in terms of three parameters to identify the optimum BNE to proceed to further studies: 1) antimicrobial efficacy; 2) macrophage biocompatibility and 3) macrophage accumulation. Antimicrobial potency of the BNEs was evaluated by their corresponding minimum inhibitory concentrations (MICs) against planktonic MDR S. aureus (CD-35) and methicillin-resistant S. aureus (IDRL-6169), as well as other pathogens commonly involved in intracellular infections, MDR K. pneumoniae (CD-343) and Salmonella (ATCC 29630) (Figure 9.3A and Figure F1). C-BNE (MIC: 0.12 mg/mL) and E-BNE (0.24 – 0.48 mg/mL) demonstrated the highest efficacy, followed by ME-BNE (0.48 – 3.84 mg/mL) then L-BNE (0.96 – 3.84 mg/mL). The potent antimicrobial effects of carvacrol and eugenol are attributed to the phenolic
hydroxyl group in their chemical structure. The phenolic hydroxyl group interacts with the bacterial cell membrane which results in cellular component leakage that eventually leads to bacterial death. The hydroxyl group is also reported to inactivate microbial enzymes.

![Figure 9.3](image)

**Figure 9.3.** Screening of phytochemical-loaded BNEs. (A) Minimum inhibitory concentrations (MIC) of the BNEs against planktonic MDR S. aureus CD-35 and MRSA IDRL-6169. (B) Macrophage viability after treatment with increasing concentrations of the BNEs. (C) Selectivity indices (SI= CC50/MICMRSA) of the phytochemical-loaded BNEs. Data are presented as mean ± standard deviations and represent at least three independent experiments.

Therapeutic agents for intracellular macrophage infections require low host cell toxicity. We evaluated the viability of the BNEs towards RAW 264.7 macrophage cells
using Alamar Blue assay (**Figure 9.3B**). C-BNE demonstrated a substantial decrease in cell viability after treatment. Exposing macrophages to 0.48 mg/mL of C-BNE resulted in ~40% decrease in cell viability. In contrast, E-BNE, L-BNE and ME-BNE demonstrated minimal levels of cytotoxicity (80% viable at the highest concentration tested). The selectivity index (SI) of a therapeutic signifies the safety range of dosage that achieves maximum efficacy and minimum cytotoxicity. Using the half-maximal cytotoxic concentrations of the BNEs (CC$_{50}$) and their corresponding MIC against MRSA, we calculated selectivity indices (SI = CC$_{50}$/MIC$_{MRSA}$) to identify safety margin of the therapeutics (**Figure 9.3C**). C-BNE, although showing the most potent antibacterial activity, displayed high macrophage cytotoxicity which decreased its SI value. E-BNEs displayed the highest selectivity index, indicating the largest concentration range that is toxic to bacteria while safe for therapeutic use.

The potency of the BNEs against intracellular bacteria strongly relies on their ability to be internalized by the host cell; accumulation inside macrophages is an important criterion for intracellular infection therapeutics. Confocal laser scanning microscopy (CLSM) was used to establish the delivery of antimicrobial essential oils by the BNEs and their subsequent macrophage accumulation. Different BNEs were loaded with a hydrophobic red fluorescent dye (Nile Red), and were prepared as above using a solution of the dye dissolved in the oils (1 mg/mL). The resulting Nile Red-loaded BNEs were then incubated with macrophages stained with Hoechst nuclear dye. Quantification of red fluorescence signals from the resulting images was used to assess the extent of macrophage uptake of the BNEs (**Figure F2**). Results showed that BNEs were effective in delivering
the antimicrobial essential oils, with C-BNE and E-BNE having the highest amounts of Nile Red delivered, followed by ME-BNE and then L-BNE.

Taken together, E-BNEs display potent antimicrobial activity and significant cell accumulation while maintaining minimal toxicity towards macrophages. E-BNEs exhibited optimum properties for an intracellular infection therapeutic among the four fabricated BNEs and were used for the subsequent experiments.

9.3.2. Intracellular activity of E-BNEs

The activity of E-BNEs against intracellular MRSA was then studied. MRSA was incubated with macrophages [multiplicity of infection (MOI) 100:1] for 1 h to establish intracellular MRSA macrophage infection. Then the cells were washed and incubated with gentamicin for 30 min to remove extracellular MRSA. The infected macrophages were treated with different concentrations of E-BNEs for 2 h. The cells were washed and macrophages were lysed using Triton X to release bacteria. Surviving MRSA cells were then quantified using colony counting with tryptic soy agar plates (Figure 9.4A). E-BNEs exhibited a concentration-dependent killing, achieving 99.999% (5 log units) intracellular bacterial clearance at 0.72 mg/mL and complete eradication at 0.96 mg/mL. Notably, while bacteria were killed as a result of E-BNE treatment, macrophages exhibited maintained viability. This highlights the selectivity of E-BNEs against bacteria relative to macrophages.
Figure 9.4. Activity of E-BNEs against intracellular MRSA. (A) Efficacy of E-BNEs against intracellular MRSA IDRL-6169 infection of macrophages. (B) Selection of resistance of MRSA IDRL-6169 after serial passaging in the presence of sub-therapeutic doses of E-BNE or vancomycin. The y-axis indicates the fold increase in MIC compared to the initial values. (C) Secretion of TNF-α by untreated and treated, non-infected or MRSA-infected RAW 264.7 macrophages measured by ELISA. (D) Apoptosis of untreated and treated, non-infected or MRSA-infected RAW 264.7 macrophages measured by Caspase-Glo 3/7 assay. Data are presented as mean ± standard deviations and represent three independent experiments.

Given the serious challenge of antibiotic resistance, we also evaluated the ability of MRSA to develop resistance against E-BNEs when exposed to sub-lethal doses. The effective antibacterial concentration of E-BNEs did not change even after 15 passages of sub-therapeutic E-BNE dosage, indicating no resistance development (Figure 9.4B). In contrast, vancomycin (the standard clinical antibiotic for MRSA infections) became antibiotic resistant at the 5th passage and required 500 times more than the original...
therapeutic dose upon reaching the 13th passage. Vancomycin disrupts bacterial cell wall synthesis,\textsuperscript{41,42} and vancomycin resistance in MRSA is facilitated by \textit{van} gene clusters, replacing D-alanyl-D-alanine with D-alanyl–D-lactate or D-alanyl–D-serine to weaken the binding of vancomycin. The contrasting inability of MRSA to generate resistance against E-BNEs is attributed to the predominant bactericidal mechanism of eugenol, membrane disruption and lysis.\textsuperscript{34,38,43} Moreover, the multi-modal antibacterial mechanisms of action conferred through the formulation of phytochemicals with nanomaterials mitigated the selection of resistance.\textsuperscript{20}

Intracellular pathogens such as MRSA survive inside immune cells including macrophages by manipulating the immune response to their advantage.\textsuperscript{44} Intracellular MRSA influences macrophage biology to bypass its pro-inflammatory responses as well as apoptosis which are responsible for getting rid of pathogens.\textsuperscript{44,45} Pro-inflammatory responses and apoptosis are part of the host defense mechanism for the elimination of pathogens, depriving bacteria of their replication niche while simultaneously alerting the immune system to take action.\textsuperscript{44,45,46} Tumor necrosis factor α (TNF-α) plays an important role in initiating inflammation to clear infection.\textsuperscript{47} E-BNE alone did not significantly affect TNF-α, indicating that the nanoemulsions do not trigger pro- nor anti-inflammatory responses (\textbf{Figure 9.4C}). At 2 h post-infection, an increase in TNF-α levels in response to infection was observed. However, 24-h post-infection, untreated infected macrophages had decreased levels of TNF-α despite the high bacteria count. Significantly, E-BNE-treated infected macrophages had increased but not excessive levels of TNF-α (\textbf{Figure 9.4C}), signifying that E-BNEs reinforced immune response for bacterial elimination. Furthermore, regulated cell death through apoptosis is an innate defense mechanism that
destroys intracellular bacteria; caspase 3/7 levels are indicative of apoptosis. Invading pathogens hijack apoptosis to prolong the life of macrophages that they use as a place to hide and multiply.

E-BNEs alone did not elicit apoptotic responses; uninfected macrophages treated with E-BNEs had caspase 3/7 levels comparable to untreated uninfected macrophages (Figure 9.4D). On the other hand, E-BNE treatment of infected macrophages demonstrated more active levels of apoptosis compared to the untreated infected macrophages (Figure 9.4D). These results suggest that treatment of infected macrophages with E-BNE alleviates bacteria-induced responses to subvert apoptosis as a consequence of their antimicrobial efficacy.

9.3.3. BNEs delivered eugenol inside macrophages

After confirming the effects of E-BNEs on infected macrophages, we next probed the mechanism of delivery of eugenol by the E-BNEs using CLSM. GFP-expressing MRSA was used to infect macrophages (MOI 100:1). Nile Red-loaded E-BNEs were then incubated with the infected macrophages for 2 h. As suggested by the visualization of red fluorescence inside the macrophages, Nile Red-loaded eugenol was efficiently internalized by the macrophages, co-localizing with the infecting MRSA (Figure 9.5A). The rapid diffusion of Nile Red fluorescence throughout the cytosol is consistent with delivery of eugenol by E-BNEs through a non-endocytic route. In further mechanistic studies, macrophages were pretreated with small molecule inhibitors of different mechanisms of uptake before adding Nile Red-loaded E-BNEs. Imipramine inhibits micropinocytosis while chlorpromazine targets clathrin-mediated endocytosis. On the other hand, methyl-β-cyclodextrin (MBCD) is a membrane fusion inhibitor that depletes cell membrane
cholesterol, a key mediator of membrane fluidity and membrane fusion-like uptake processes.\textsuperscript{50} Using flow cytometry, we quantified the extent of eugenol delivery reflected by the fluorescence signal coming from the loaded Nile Red (\textbf{Figure 9.5B}). Results showed that pretreatment with MBCD resulted in the highest reduction of fluorescent signals (>70%), followed by imipramine (35% decrease) then chlorpromazine (<10% reduction). This suggests that BNEs delivered eugenol primarily through cholesterol-dependent membrane fusion-like mechanism, with potential minor contributions from micropinocytosis and caveolar uptake followed by an essentially immediate endosomal escape.

We also formulated E-BNEs using Texas Red-tagged PONI-GMT (red fluorescent) and coumarin-loaded eugenol (blue fluorescent) to visualize both PONI-GMT and eugenol after E-BNE treatment of macrophages. The CLSM images show that only the coumarin-loaded oil gets delivered inside the macrophages, indicated by the diffused blue fluorescent signals, while PONI-GMT remains outside the cells (\textbf{Figure 9.5C}). PONI-GMT allowed interaction of E-BNEs with the membrane of the macrophages, followed by the entry of hydrophobic eugenol through membrane fusion. without the polymer scaffolding being uptaken.
Figure 9.5. Delivery of eugenol using BNEs. (A) Representative confocal images of GFP-expressing MRSA-infected macrophages (stained with blue fluorescent Hoechst dye) treated with Nile Red-loaded E-BNEs. Scale bar is 50 μm (top photos) and 10 μm for the zoomed-in image. (B) Quantified delivery [mean fluorescence intensity (MFI)/cell] of Nile Red-loaded E-BNE to macrophages pretreated with inhibitors. Values are expressed as mean ± standard deviation of ≥ 3 replicates. (C) Representative confocal images of macrophages treated with E-BNEs formulated with blue fluorescent coumarin-loaded eugenol and Texas Red-tagged PONI-GMT. Scale bar is 50 μm.

9.3.4. E-BNEs are active against MRSA-induced peritonitis in vivo

The clinical relevance of E-BNEs was evaluated by testing their efficacy in treating MRSA-induced peritonitis, a life-threatening condition identified by the CDC as a “serious health threat”. Murine peritonitis models (Figure 9.6A) are established representative models for intracellular bacterial infections, providing a means to isolate peritoneal macrophages with intracellular MRSA. Before evaluating efficacy against bacterial
peritonitis, the \textit{in vivo} safety of E-BNEs was first evaluated. Mice were intraperitoneally (IP) injected with different doses of E-BNEs and observed for any signs of distress, including loss of weight, decreased mobility or death. At 24 hours post-treatment, all mice, even with the highest dose administered (3.2 mg eugenol/mouse), survived and were scored healthy. Next, the biodistribution of E-BNEs was studied using E-BNEs formulated with a near-infrared dye (NIR750 dye, 5 μM) that can be probed via IVIS imaging (Figure 9.6B). The NIR dye-loaded E-BNEs were prepared as above, and IP administered to the mice (3.2 mg eugenol/mouse). Quantification of fluorescence signals from \textit{ex vivo} samples collected 24 hours post-administration revealed that E-BNEs had high accumulation in the liver as well as the peritoneal fluid, with weak signals observed from spleen and blood (Figure 9.6C, F3A).

For the murine peritonitis treatment, the bacterial infection was initiated by injecting bioluminescent MRSA\textsuperscript{27} into the peritoneum of BALB/c mice, taking advantage of bacterial luminescence to confirm established infection. At 24 h post-infection, mice that did not exhibit bioluminescence were not used for further study (Figure F3). The remaining mice were then treated with one of the following: a) PBS, b) vancomycin or c) E-BNEs. After 24 h, mice were sacrificed, and the peritoneal fluid was collected for quantitative colony counting to determine the remaining bacterial load. As reported, vancomycin, the current standard antibiotic treatment for MRSA-induced peritonitis,\textsuperscript{28} did not show any efficacy while E-BNEs were able to clear 99\% of bacteria infection (Figure 9.6D). The lack of efficacy of vancomycin treatment can be attributed to its slow antibacterial activity and poor intracellular penetration.\textsuperscript{52} In this regard, E-BNEs prove to
be a more viable and effective treatment for bacterial peritonitis compared to antibiotics including vancomycin.

Figure 9.6. Efficacy of E-BNE in treating MRSA-induced murine peritonitis. (A) MRSA-induced peritonitis model using BALB/c mice. (B) IVIS images of mice 1 h and 24 h post-IP injection with NIR750 dye-loaded E-BNEs (C) Quantified biodistribution of E-BNEs 24 h post-administration via IP injection. (D) Remaining intracellular bacterial load 24 h after treatment with PBS, E-BNEs or vancomycin. * indicates P value < 0.05.

9.4 Discussion

Intracellular bacterial infections caused by multidrug-resistant bacteria present a clinical challenge that cannot be well-addressed by antibiotic therapy. Most antibiotics are not efficiently internalized by host cells, including macrophages, allowing the survival of residing bacteria. Intracellular bacteria then multiply and manipulate the biology of macrophages to their advantage, resulting in persistent and chronic infections. Here, we report a non-antibiotic platform to address challenges presented by intracellular bacteria.
infecting macrophage cells. Phytochemicals, plant-derived essential oils, have emerged as potent antimicrobial agents that remain active against antibiotic-resistant bacterial strains.\(^{17,23}\) Carvacrol, eugenol, linalool and methyl eugenol were of particular interest because of their broad-spectrum antibacterial properties through membrane destabilization and lysis.\(^{33,34,35,36}\) However, poor aqueous solubility and their limited ability to cross mammalian cell membranes have restricted their use as therapeutics against intracellular infections.\(^24\) The formulation of hydrophobic phytochemicals with nanomaterial-based platforms has been explored to increase their antimicrobial utility.\(^{22,23,24}\) The unique physicochemical properties of nanomaterials, including size, shape and surface functionality, can be carefully tuned to achieve a delivery system for antimicrobial phytochemicals that can penetrate inside infected macrophages.

A poly(oxanorborneneimide)-based polymeric scaffold, PONI-GMT, was selected to encapsulate hydrophobic antimicrobial phytochemicals to form stable biodegradable nanoemulsions (BNEs). The choice of the surface ligands in the design of PONI-GMT allowed the utility of phytochemical-loaded BNEs as intracellular infection antimicrobials. Tetraethylene glycol monomethyl ether moieties increased the amphiphilicity of the polymer which allowed encapsulation of the oil, achieving nanoemulsions with good solubility in aqueous media. Maleimide units provided further stability to BNEs by forming cross-linked structures. Finally, the guanidinium groups imparted an overall positive charge to the BNEs and allowed non-disruptive interactions with mammalian cell membranes for antimicrobial delivery.

All resulting BNEs, carvacrol-loaded BNE (C-BNE), eugenol-loaded BNE (E-BNE), linalool-loaded (L-BNE) and methyl eugenol-loaded BNE (ME-BNE), formed
stable cationic spherical nanoemulsions with size ranging from 165-350 nm (Figure 9.2). The efficacy of BNEs as therapeutics against intracellular infections strongly depended on their 1) antimicrobial potency, 2) macrophage biocompatibility and 3) delivery efficiency. All the BNEs were screened using these parameters to determine the optimum BNEs to combat intracellular infections (Figure 9.3, F1-2). The balance between efficacy and safety was assessed by calculating the selectivity index (SI), the ratio of the toxic concentration against host mammalian cells over the toxic concentration against infecting bacteria. Higher SI values for a therapeutic indicate a wider safety window for practical use.\cite{39} In this account, together with the efficient delivery into macrophages, E-BNE was identified to be optimal for the treatment of intracellular infection and hence, was used for further experiments.

We then proceed to evaluate the effects of E-BNEs on macrophages infected with MRSA. The persistence of MRSA inside macrophages presents a clinical challenge as they thrive in an environment protected from therapeutics and immune responses. E-BNEs displayed effective clearance of MRSA cells residing inside macrophages, which indicates that the nanocarrier strategy employed was successful in leveraging antimicrobial phytochemicals to combat MDR intracellular pathogens (Figure 9.4A). The presented strategy also afforded E-BNEs a high barrier for resistance selection (Figure 9.4B), an important consideration for developing antibacterial treatments given that existing antibiotics are rendered ineffective by resistance mechanisms.

Intracellular MRSA manipulates macrophage biology to bypass defense mechanisms responsible for getting rid of bacteria.\cite{44,45,46} Appropriate but not excessive levels of tumor necrosis factor α (TNF-α) and caspase 3/7 were observed when MRSA-
infected macrophages were treated with E-BNEs (Figure 9.4C-D). Untreated infected macrophages exhibited decreased pro-inflammatory and apoptotic response despite the propagation of infection. As a consequence of their potent antimicrobial activity, E-BNEs were able to subvert the immunosuppressive tactics of MRSA to increase survival inside macrophages.

The overall activity and efficacy that the E-BNEs demonstrated are attributed to the bactericidal mechanisms of eugenol coupled with the physicochemical properties conferred by formulation with polymeric nanoemulsions. The guanidinium functionalities of the PONI-GMT polymers facilitated the interaction of E-BNEs with macrophages, delivering antimicrobial eugenol mainly via non-endocytic membrane fusion-like uptake mechanism, with minimal contributions from endocytosis followed by a fast endosomal escape (Figure 9.5). Co-localizing with the bacterial cells inside macrophages, eugenol was able to exert its antibacterial activity resulting in the successful clearance of intracellular MRSA.

Encouraged by the in vitro efficacy of E-BNEs, we proceed to evaluate the translatability of this efficacy using a MRSA-induced peritonitis murine model (Figure 9.6). No death or loss of weight was observed after intraperitoneal administration of E-BNEs. Biodistribution studies revealed that E-BNEs accumulate in the liver and peritoneal fluid 24 h after injection. Importantly, infected mice treated with E-BNEs exhibited 99% clearance of MRSA infection, demonstrating that the nanoemulsion strategy using PONI-GMT and antimicrobial phytochemicals such as eugenol was effective in treating intracellular infections. On the contrary, vancomycin, the standard antibiotic treatment for MRSA-induced peritonitis, displayed no efficacy. This lack of antimicrobial activity from
vancomycin could be attributed to its poor intracellular penetration. The results highlight the ability of the E-BNE platform to treat bacterial infections that cannot be efficiently addressed by traditional antibiotics.

9.5 Conclusions

This work demonstrated that the integration of phytochemicals into polymer-based nanoemulsions provides an efficient non-antibiotic therapeutic platform for the treatment of MDR intracellular infections, opening new avenues in combating invasive MDR bacterial infections. The careful engineering of the cationic nanoemulsions not only allowed solubilization and stabilization of hydrophobic antimicrobial phytochemicals but also facilitated delivery and internalization by infected macrophages. This strategy features macrophage biocompatibility and broad-spectrum activity against common intracellular pathogens, especially MRSA, without the danger of resistance generation. The clinical potential of the E-BNEs was demonstrated using an in vivo MRSA-induced peritonitis model, demonstrating better antimicrobial activity compared to vancomycin. Overall, this approach demonstrates the potential of nanoemulsions as a therapeutic strategy for MDR bacterial peritonitis. The antimicrobial efficacy and low host cell toxicity makes these systems promising for a wide range of difficult-to-treat intracellular infections.

9.6. References


CHAPTER 10

SUMMARY AND OUTLOOK

Nanotherapeutics present an emerging ‘outside of the box’ approach for treatment of recalcitrant MDR planktonic bacteria and biofilm infections. The tunable properties of nanomaterials, particularly their surface functionalities, provide design spaces that can be fine-tuned to maximize therapeutic effect while minimizing host toxicity. Nanotherapeutics can access multi-modal antibacterial mechanisms that are novel, slowing or stopping the generation of drug resistance.

In this dissertation, I have demonstrated that polymer-based nanotherapeutics can do what antibiotics cannot: penetrate and destroy biofilms, and effectively eliminate intracellular bacteria both in vitro and in vivo. The first part of this thesis focuses on the different strategies explored to address the challenges in treating wound biofilm infections. Chapters 2-6 discuss how I developed antimicrobial polymer, PONI-C11-TMA, into a topical therapeutic for wound biofilms. Due to its structural design, PONI-C11-TMA possess potent antibiofilm efficacy. To further its practical use and clinical translatable, several strategies were explore including 1) synergistic combination therapy with antibiotics; 2) integration with hydrogel materials and 3) assembly formation with negatively-charged cargos such as siRNA or hydrophobic small molecules including astaxanthin. These strategies afforded a polymer-based nanotherapeutics that are active against multidrug-resistant bacteria and their biofilms, biocompatible with mammalian cells, have high barrier for resistance selection and effective in treating wound biofilm infections in vivo. In related work, natural polymers are particularly promising materials for delivery of therapeutics, as they are intrinsically biocompatible and biodegradable. In
chapter 7, we introduced another nanotherapeutic formulated using a nature-derived components, carvacrol, vitamin B2 and gelatin. These gelatin-based nanoemulsions effectively reduce bacterial burden and promote wound healing in vivo.

The latter part of this dissertation (Chapters 8 and 9) presents the strategies we explored to address intracellular infections including MRSA-induced peritonitis. Macrophage-targeted bioorthogonal nanozymes to produce antibiotics at the sites of intracellular bacterial infections was reported. The ability to generate effective concentrations of antibiotic inside of the macrophages provides high selectivity for intracellular infections and an important new strategy for treatment of these infections without harming the surrounding microbiome. On another approach, we demonstrated that the integration of phytochemicals into polymer-based nanoemulsions provided an efficient non-antibiotic therapeutic platform for the treatment of MDR intracellular infections, opening new avenues in combating invasive MDR bacterial infections. The clinical potential of this strategy was demonstrated using an in vivo MRSA-induced peritonitis model, demonstrating better antimicrobial activity compared to vancomycin.

Although I have presented evidences showing that different nanotherapeutics aid in combating difficult-to-treat bacterial infections, all the reported strategies still need further studies prior to their use in clinical trials. Biosafety of these nanomaterials is still a matter of concern, even though we have demonstrated potential safety through various in vitro and in vivo model. Future work in pursuit of further understanding the long-term effects of the nanotherapeutics as well as alternative antimicrobial strategies demonstrated in this dissertation should address these concerns.
APPENDIX A

SUPPLEMENTARY INFORMATION: FUNCTIONALIZED POLYMERS ENHANCE PERMEABILITY OF ANTIBIOTICS IN GRAM-NEGATIVE MDR BACTERIA AND BIOFILMS FOR SYNERGISTIC ANTIMICROBIAL THERAPY


Figure A.1. TEM image of a) P8 and b) P9 Polymer nanoparticles.
**Figure A.2.** Checkerboard titration between colistin and P8 polymer against *P. aeruginosa* (CD-1006). Dark cells represent higher cell density.

**Figure A.3.** Checkerboard titration between colistin and P7 polymer against a) *Bacillus subtilis* b) *S. epidermidis* and c) methicillin-resistant *S. aureus* (CD-489). Dark cells represent higher cell density. The combinations did not show any significant increase in the efficacy of the antibiotics.
Figure A.4. Change in bacteria membrane permeability assayed by zeta potential in presence of PNP, colistin and PNP-colistin combination. The figure shows different combination of PNP-colistin exhibiting increased ability to permeate bacterial membrane.

Figure A.5. Checkerboard broth microdilution assays between colistin and P8 PNPs against uropathogenic biofilm *E. Coli* (CD-2). The combination shows upto 16-fold increase in the efficacy of colistin at sub-MBEC dosage of P8 PNPs.
Figure A.6. Sample of checkerboard titration plate with varied colistin and polymer concentrations. Concentration of colistin is decreasing with 2-fold dilutions from top to bottom while polymer concentration is decreasing from right to left.

Table A.1. Table showing fold-increase in antibiotic efficacy obtained for the combination of PNPs and antibiotics tested against multiple strains.

<table>
<thead>
<tr>
<th>Species (Strain)</th>
<th>Polymer</th>
<th>Fold-increase in antibiotic efficacy</th>
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</thead>
<tbody>
<tr>
<td>E. coli (CD-2)</td>
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<tr>
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<td>P5</td>
<td>0</td>
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<td>8</td>
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<tr>
<td>E. coli (CD-2)</td>
<td>P9</td>
<td>8</td>
</tr>
<tr>
<td>P. aeruginosa (CD-1006)</td>
<td>P7</td>
<td>16</td>
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<tr>
<td>En. Cloacae (CD-1412)</td>
<td>P7</td>
<td>16</td>
</tr>
<tr>
<td>E. coli (CD-549)</td>
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</tr>
<tr>
<td>Acinetobacter species (CD-575)</td>
<td>P7</td>
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<td>P. aeruginosa (CD-1006)</td>
<td>P8</td>
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</tbody>
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APPENDIX B

SUPPLEMENTARY INFORMATION: POLYMERIC NANOPARTICLES ACTIVE AGAINST DUAL-SPECIES BACTERIAL BIOFILMS

Adapted with permission from “Makabenta, J.M.V. †; Park, J. †; Li, C-H.; Chattopadhyay, A.N.; Nabawy, A.; Landis, R.F.; Gupta, A.; Schmidt-Malan, S.; Patel, R.; Rotello, V.M. Molecules 2021, 26 (16), 4958.” Copyright (2021) MDPI.

Figure B.1. a) 1H NMR spectrum of PONI-C11-TMA. The polymer self-assembles into cationic polymeric nanoparticles (PNPs) characterized by b) dynamic light scanning (DLS) measurement and c) transmission electron microscopy (TEM) imaging.
Figure B.2. Representative 3D views of confocal image stacks of 4-day old dual-species biofilm of DsRed-expressing *Escherichia coli* (red channel) and GFP-expressing methicillin-resistant *Staphylococcus aureus* (green channel), coumarin blue-tagged PNPs (blue channel), and their overlay after treating the biofilms for 1 h with 1 μM coumarin blue-tagged PNPs in M9 media. The biofilms were imaged immediately without washing. Untreated biofilm, serving as the negative control, was prepared similarly without treatment with PNPs.
Figure B.3. Representative 3D views of confocal image stacks of 4-day old biofilm of DsRed-expressing *Escherichia coli* (red channel) and coumarin blue-tagged PNPs (blue channel), and their overlay after treating the biofilms for 1 h with 1 μM coumarin blue-tagged PNPs in M9 media. The biofilms were imaged immediately without washing. Untreated biofilm, serving as the negative control, was prepared similarly without treatment with PNPs.
Figure B.4. Representative 3D views of confocal image stacks of 4-day old biofilm of GFP-expressing methicillin-resistant *Staphylococcus aureus* (green channel) and coumarin blue-tagged PNPs (blue channel), and their overlay after treating the biofilms for 1 h with 1 μM coumarin blue-tagged PNPs in M9 media. The biofilms were imaged immediately without washing. Untreated biofilm, serving as the negative control, was prepared similarly without treatment with PNPs.
Figure B.5. MBBC values of gentamicin against mono-species [Gram-positive: Methicillin-resistant *Staphylococcus aureus* (MRSA) IDRL-6169; Gram-negative: multi-drug resistant (MDR) *Pseudomonas aeruginosa* IDRL-11442, MDR *Escherichia coli* IDRL-10366] and dual-species biofilms (MRSA + MDR *P. aeruginosa*; MDR *P. aeruginosa* + MDR *E. coli*). Bars represent average value while stars represent individual measurements.

Figure B.6. a) Biomass and b) bacteria viability of 2-day-old mono- and dual-species biofilms of MDR *Escherichia coli* IDRL-10366 + MDR *Pseudomonas aeruginosa* IDRL-11442 after 3 hours of treatment with PNP. The data shown are average of triplicates and the error bars indicate the standard deviation.
Figure B.7. Viability of a) 3T3 fibroblast cells and *Pseudomonas aeruginosa* ATCC-19660 biofilm and b) 3T3 fibroblast cells and *Escherichia coli* DH5α biofilm dual-species biofilms in the co-culture model after treatment with PNPs. Scatters and lines represent 3T3 fibroblast cell viability. Bars represent log_{10} of colony forming units in biofilms. The data are average of triplicates, and the error bars indicate the standard deviations.
C.1. Experimental Section

C.1.1. Materials

All solvents and chemicals used for synthesis were purchased from Fisher Scientific and Sigma-Aldrich. The chemicals were used as received, without further purification unless otherwise stated. PONI-C11-TMA was synthesized and PNPs were prepared as previously described.1

C.1.2. Bacterial and mammalian cell culture

Bacteria used in the studies are clinical isolates obtained from the Cooley Dickinson Hospital Microbiology Laboratory (strains labeled CD) or from the Infectious Diseases Research Laboratory at Mayo Clinic (strains labeled IDRL). Bioluminescent MRSA USA300 NRS384 strain (SAP-231) was provided by Dr. Roger Plaut.2

RAW 264.7 macrophages (ATCC TIB-71) and NIH-3T3 fibroblasts (ATCC CRL-1658) were purchased from the American Tissue Culture Collection (ATCC). Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS; Fisher Scientific) were used in cell culture.

C.1.3. Quantifying biofilm biomass using Crystal Violet assay

Bacterial seeding solutions (0.1 OD_{600}) were prepared in TSB. Seeding solutions (100 μL) were added to each well of a 96-well microtiter plate. Plates were kept and
incubated at room temperature under static conditions until the biofilm matures (2-day old). On day 2, biofilms were washed with PBS to remove planktonic bacteria. Then, different concentrations of PNPs (0.25-32 μM), prepared in M9 media, were added to each well of the microplate. The microplate was then incubated for 3 hours at 37°C under static conditions. After the incubation period, biofilms were washed with PBS thrice and stained with 0.1% Crystal Violet (CV) for 15 min. The wells were then washed with PBS four times to remove excess CV, then the plate was left to air-dry. The stained wells were then treated with 80:20 (v/v) ethanol: acetone to solubilize the dye. Optical density (590 nm) was determined to quantify biofilm biomass.

C.1.4. Determination of minimum biofilm bactericidal concentration (MBBC)

The MBBCs of PNPs against different bacteria strains were determined using previously established protocols. Briefly, bacterial cells grown to stationary phase were diluted (1:50) using TSB and incubated at 275 rpm, 37°C until mid-log phase. Bacteria solution (150 μL) was added to each well of a 96-well microtiter plate with pegged lids. Biofilms were allowed to grow by incubating the plate for 6 hours in an incubator/shaker at 37°C and 50 rpm. The pegged lid was then washed with 200 μL PBS for 30 seconds before transferring to a new plate containing different concentrations of PNPs (0.5-128 μM). The plate was further incubated for 24 h at 37°C. Then, biofilms on the pegged lid were washed with PBS and transferred to a new plate containing only media. The plate was again incubated for another 24 h at 37°C to determine the MBBC. The MBBC was identified by visual inspection and confirmed through spectrophotometry (OD₆₀₀).
C.1.5. Evaluating effects of PNPs on bacteria embedded in biofilms

Two-day-old biofilms were prepared as above. Varying concentrations of PNPs (1-10 μM) were used to treat the biofilms for 3 hours at 37°C. After 3 hours, wells were washed three times with PBS to remove PNPs. Sterile PBS (200 μL) was added to each well, then the plate was sonicated for 10 minutes to release the remaining bacteria from the EPS matrix. A 100 μL sample was collected from each well and was used for standard quantitative colony counting.

C.1.6. Bactericidal activity against persister cells

Persisters were derived from biofilms following a reported protocol.4 Bacterial seeding solutions (100 μL, 0.1 OD_{600}) were added to each well of a 96-well microtiter plate and then incubated for 24 h at 37°C. The resulting 2-day-old biofilms were washed with PBS, to remove planktonic bacteria, then treated with gentamicin (100x MIC) for 24 h. After the incubation time, wells were washed with PBS to remove the antibiotic and any planktonic bacteria. The remaining persister cells were dispersed from the biofilm by adding fresh PBS to the wells and then sonicating the plate for 10 min. Then, the persisters were treated with PNPs or antibiotic control for 3 hours. Surviving bacteria were then quantified through agar plate colony counting.

C.1.7. Resistant development assay via therapeutic serial passaging at sub-lethal dosages

MRSA (IDRL-6169) was inoculated in media containing sub-therapeutic concentrations (1/3x MIC) of PNPs or Vancomycin. The resulting bacterial populations
from each therapeutic were defined as the first passage. These cells were harvested, and their respective MICs were determined. Afterward, subsequent generations (or passages) were derived by exposing the preceding generation with 1/3x MIC of the corresponding therapeutic. MIC values were determined for each new generation.

C.1.8. Quantifying extent of inflammation in vitro using TNF-α levels

In a 96-well plate, 10k RAW 264.7 cells were seeded and cultured for 48 h. Media was then replaced with DMEM containing 10^8 CFU/mL of MRSA. The plate was incubated further at 37°C in a humidified atmosphere of 5% CO₂ for 5 more hours to allow MRSA biofilm formation. Then, the wells were washed with PBS and the media was replenished with DMEM, with or without PNPs. Samples were then collected at 2, 24 and 48 h after treatment for TNF-α level measurement by ELISA (R&D Systems, MN, USA). The procedure specified by the manufacturers was followed. Experiments were performed in triplicates.

C.1.9. Scratch assay to evaluate fibroblast migration in simulated wound biofilm conditions

Scratch assay to simulate wound biofilm infection was performed according to a reported protocol with modifications.5 Biofilm-conditioned media (BCM) was used to mimic biofilm infection environments and was prepared before the experiment. In brief, 3-day-old MRSA biofilms were grown in 0.2 μm transwell membrane insert by inoculating 10^8 CFU/mL MRSA in TSB. The inserts were then placed in a 6-well plate containing 1 mL TSB, then incubated at 37°C for 72 h, replacing media with fresh TSB every 24 h. Afterward, the inserts were washed with PBS and transferred to a fresh plate containing
1.5 mL DMEM and kept for further incubation. Every 24 h for 7 days, the media was collected and replenished with fresh DMEM. The collected DMEM (referred to as BCM) were pooled and filtered, to remove any bacteria, then stored at -20°C until ready to use.

200k NIH-3T3 fibroblasts were grown in a sterile 35 mm confocal dish until 80-90% confluence was reached. The cells were then scratched using a 1000 μL sterile plastic pipet tip to imitate the wound. Cells were then washed with PBS twice and then replenished with biofilm-conditioned media, with or without PNPs. The scratched cultures were then imaged to obtain the initial scratch area. The dishes were incubated in humidified 5% CO$_2$ incubator at 37°C, imaging at 12 h and 24 h after the scratch. Images were taken using a 4x objective on a Keyence BZ-X810 microscope and analyzed using ImageJ.

**Figure C.1.** Quantification of extracellular DNA, a component of the EPS matrix, after treatment of 4-day old MRSA biofilm with vancomycin or PNPs. Yellow arrow indicate bands for eDNA.
C.2. Supplementary References:


D.1. Experimental Methods

D.1.1. Materials

Riboflavin, carvacrol, and Nile red were purchased from Acros. Gelatin (Type B, 100 Bloom) and Luria-Burtani (LB) liquid medium were purchased from Fisher Chemical. Phosphate-buffered saline was purchased from HyClone. Sodium chloride and Pepton were purchased from Fisher BioReagents. M9 minimum medium was purchased from Teknova. Tryptic soy broth (TSB) was purchase from Becton Dickinson. AlamarBlue™ cell viability reagent was purchased from Invitrogen. Fetal bovine serum (FBS) was purchased from Gibco. Pegged lids were purchased from Nunc. Paraformaldehyde (PFA) was purchased from Sigma-Aldrich.

D.1.2. Preparation of gelatin nanoemulsions

Nanoemulsions were prepared through emulsification of a suspension of riboflavin in carvacrol into an aqueous gelatin solution, followed by irradiation with 365 nm UV-light. Briefly, suspension of riboflavin in carvacrol (3 μL, 0.1 wt%) was added to the gelatin aqueous solution (497 μL, 0.24 mg/ml). This solution was then emulsified for 50 seconds using an amalgamator. The emulsion was then exposed to UV lamp for 20 minutes.
The concentration of this nanosemulsion stock solution was defined as 100 v/v% (39 mM of carvacrol)

D.1.3. Characterization

The hydrodynamic diameter of nanoemulsions was measured in triplicate using DLS (Malvern Zetasizer). TEM samples were prepared on 300 square mesh nickel grids with Formvar film (Electron Microscopy Sciences). IR was performed on a Bruker Alpha FTIR spectrophotometer fitted with a platinum ATR QuickSnap sampling module.

D.1.4. Biofilm penetration study

Red fluorescent nanoemulsions were prepared as above, using a solution of Nile red in carvacrol (1 mg/1000 μl). GFP-expressing K. pneumoniae biofilms were prepared using the preparation method described below for mono-species biofilms. These biofilms were then treated with prepared red fluorescent nanoemulsions (5 v/v%) for 1 hour at room temperature. A Nikon A1 spectral detector confocal with FLIM module was used to monitor penetration profile of the nanoemulsions. A penetration profile study was performed using Nikon A1 resonant scanning confocal with TIRF module. The images were processed using NIS-Elements.

D.1.5. Evaluation of antimicrobial activity in vitro

Frozen (-80 °C) cultures of all bacteria strains used: a.) Clinical isolates E. coli (CD-2), MDR E. coli (IDRL-10366), P. aeruginosa (CD-1006), MDR P. aeruginosa (IDRL-11442), E. cloacae complex (CD-1412), MRSA (CD-489), MRSA (IDRL-6169); b.) GFP-expressing K. pneumoniae (IDRL-11999); and c.) reference strain P. aeruginosa
(ATCC 27583) were grown aerobically using Luria-Burtani agar. Overnight cultures of bacteria were prepared by transferring isolated colony from the agar plate to culture tubes with sterile LB broth. Bacterial cultures were then incubated overnight at 37 °C with agitation (275 rpm), until the stationary phase was reached. Bacteria were then collected by centrifugation (7000 rpm, 5 min) and washed thrice with sodium chloride (0.85%). Subsequently, the culture was resuspended in PBS (1 mL) to determine its OD$_{600}$ (SpectraMax M2, Molecular Devices). All clinical isolates with code CD were obtained from Cooley Dickinson Hospital, while those denoted by IDRL were from the Infectious Diseases Research Laboratory in Mayo Clinic.

For mono-species biofilms, bacteria (except S. aureus) were prepared by dilution with M9 medium to 0.1 OD$_{600}$ for biofilm formation. For S. aureus biofilms, cultures were prepared in M9/TSB (85:15) to the same concentration. Subsequently, the seeding solutions (100 μL) were added to each well of a 96-well clear flat-bottomed plate. The plate was covered and incubated under static conditions at room temperature overnight. The seeding solutions were removed, and biofilms were washed thrice with PBS. Gelatin nanoemulsion solutions ranging from 0 to 48 v/v% were then administered (100 μL) to the biofilms. The plates were incubated statically at 37 °C. After 3 h, the biofilms were washed thrice with PBS; then alamarBlue™ cell viability reagent¹ (10 v/v%) was added to each well, and incubated for 1 h. Biofilm viability was determined by measuring fluorescence intensity (excitation: 560 nm; emission: 590 nm). Readings from the wells containing alamarBlue™ cell viability reagent (10 v/v%) alone were considered as the blank ($I_{\text{blank}}$), and readings from wells with untreated biofilms were used as growth controls ($I_{\text{control}}$). Biofilm viability was calculated using the equation below:
Biofilm viability (%) = $100\% \times \frac{I_{\text{sample}} - I_{\text{blank}}}{I_{\text{control}} - I_{\text{blank}}}$

Biofilm-3T3 fibroblast cell coculture model was performed using the previously reported protocol. Briefly, NIH 3T3 (ATCC CRL-1658) cells (20k/ well) were cultured in wells of a 96 well plate overnight to form a monolayer. Afterward, the mammalian cells were washed and 100 μL of bacterial seeding solutions ($10^8$ CFU/mL) were added. The cocultures were then stored at 37 °C for six hours without shaking. Gelatin nanoemulsions and other control solutions were diluted in DMEM media prior to use to obtain the desired testing concentrations. The cocultures were washed again and the freshly prepared testing solutions were then added. After 3 h incubation at 37 °C, the cocultures were then analyzed using LDH cytotoxicity assay. To determine the bacteria viability in biofilms, the testing solutions were removed and cocultures were washed with PBS. Fresh PBS was then added, and the plate was sealed and sonicated for 20 min to disperse biofilms. The solutions containing dispersed bacteria were quantitatively determined using colony counting forming unit method.

For the SWF experiment, we followed an established SWF protocol. Briefly, bacterial cultures were prepared using the method described above. Biofilm seeding solutions were prepared in SWF/TSB (1:1) solutions. SWF was comprised of fetal bovine serum (50%) and sodium chloride (50%) in Pepton water (0.1%). This seeding solution (100 μL) was added to each well of the 96-well plate. The plate was covered and incubated under static conditions at ~23 °C for 4 days. The activity of gelatin nanoemulsions towards this model was determined using quantitative colony counting.

MBIC and MBBC assays of single- and dual-species biofilms were carried out using a Calgary biofilm device. For the single-species biofilms, bacteria were grown in
TSB (2.5 mL) at 275 rpm and 37 °C until the concentration reached 0.5 McFarland standard. These solutions (150 μL) were then transferred to each well of a 96-well plate. The plate was covered with a pegged lid and incubated for 6 h at 50 rpm and 37 °C. The pegged lid was then removed, rinsed with PBS for 30 seconds, and then transferred to a plate with antimicrobial agents (200 μL) in each well. MBIC values were determined after the plate was incubated 24 h at 37 °C statically. Subsequently, the same pegged lid was rinsed with PBS for 30 seconds again, and transferred to a plate with broth (200 μL) in each well. MBBC values were determined after the plates were statically incubated for another 24 hours at 37 °C. For dual-species biofilms, the same procedure was followed except that 75 μL of each of the component bacterial species were added into the 96-well microplate then mixed.

D.1.6. Study of killing mechanism of gelatin nanoemulsions

Bacteria solution (OD_{600} = 0.5) containing propidium iodide (PI) (10 μL/1000 μL) was prepared for the killing mechanism study. The bacteria solutions (50 μL) were added to each well of a black 96-well flat-bottomed plate. Fluorescence intensities were measured immediately after adding 50 μL of PBS containing 0.125 to 4X MIC of gelatin nanoemulsions or Ceftazidime (Excitation/Emission: 535 nm/617 nm).

D.1.7. Ethics statement

C57BL/6 mice were supplied by Jackson Laboratory. Mice were housed in sterile cages with a 12 hours light/12 hours dark cycle. They were allowed to acclimatize for at least a week before any of the procedures were carried out. All animal experiments were performed in accordance with the authorized protocol (IACUC Protocol ID 2018-0011)
and the policies issued by Institutional Animal Care and Use Committee at University of Massachusetts Amherst.

D.1.8. *In vivo* wound biofilm murine model

The biofilm model was generated using C57BL/6 mice that were anesthetized and the skin on their dorsum shaved and disinfected using a sterile alcohol pad. Afterwards, a sterile 5-mm circular full thickness skin wound was punched using a skin puncture biopsy tool. Using a micropipette, $10^7$ colony forming units (CFU) of a clinical isolate of MRSA (IDRL-6169) in saline (10 μL) was inoculated onto the wound bed. Semi-occlusive transparent Tegaderm® was placed over the wound using Mastisol® as an adhesive to prevent secondary bacterial contamination. Biofilm was then allowed to form for four days. The mice were then separated into three groups of five: one group treated with nanoemulsions (100% v/v; 39 mM), a second with vancomycin (110 mg/kg) that served as the positive control and a third with vehicle control (saline solution only). Test agents (100 μL) were administered every other day until the day of sacrifice (day 5); nanoemulsions and saline were administered topically, while vancomycin was injected intraperitoneally. Photographs were taken daily, and purulence scores, wound sizes and weights of the mice were monitored every day. On the day of the sacrifice, the mice were euthanized via CO$_2$ asphyxiation. Then, 10-mm circular full thickness skin covering the infection area was collected using a skin biopsy punch for histological analysis.

A separate set of mice was used to assess bacterial reduction midway through the wound healing process. After formation of 4-day old MRSA biofilm on the wound as above, mice were separated into two groups of five: one treated with nanoemulsions (100% v/v; 39 mM) and the other with saline solution only. Treatments were administered every
other day until the day of sacrifice (day 2). At day 2, mice were sacrificed 3 h after the test materials were administered. The mice were euthanized via CO₂ asphyxiation. Then, 3-mm circular full thickness skin in the inner portion of the infection area was collected using a skin biopsy punch for quantitative colony counting. Skin samples were homogenized in PBS, diluted and plated into mannitol salt agar to quantitatively determine remaining bacteria counts.

D.1.9. In vivo wound closure measurement and purulence score grading

All photographs were taken from a standard height at the same time over the entire treatment period. Three blinded observers determined the sizes of the wounds using the taken images with ImageJ software and graded the degree of pus formation using a standard purulence scoring system. At the same time, a blinded observer present through the duration of the study measured wound size using a digital caliper (Neiko tools) and rated the degree of purulence of the mice.

D.1.10. Preparation of skin samples for histological analysis

The skin tissue was fixed in paraformaldehyde (4%) in 4 °C overnight and transferred into PBS. After 24 hours, the tissue was dehydrated in a series of ethanol washes and stored at 4 °C. The tissue samples were cut, dividing the wound in half (Figure S8, Supporting Information). Subsequently, the tissues were cleared in xylene for 1 hour, with a xylene change after 30 minutes. After 1 hour, xylene was removed and replaced with paraffin wax, followed by fresh wax changes every 30 minutes. Half tissue sections were aligned in the wax and sectioned at 7 μm.
D.1.11. Hematoxylin and eosin (H&E) staining

The sectioned tissues were deparaffinized and rehydrated for subsequent procedures. Slides were then stained with hematoxylin for 45 seconds, placed under gently running tap water for 1 minute, submerged in Scott’s Tap Water Substitute (20 g MgSO₄ and 3.5 g NaHCO₃ in 1 L of Milli-Q® H₂O) for 1 minute, and then washed in still tap water for another minute. Slides were quickly dipped into ethanol (95%), stained with eosin for 15 seconds, and then again washed in ethanol (95%) with two 2-minute washes in ethanol (100%). Lastly, slides were washed with xylene three times for 1 minute each, and then sealed with Cytoseal™ 60. H&E stained sections were imaged with a Panoramic MIDI II slide scanner (3DHISTECH).

D.2. Supplementary Display Items

![Stability and biodegradation of gelatin nanoemulsion. a, Gelatin nanoemulsion demonstrates ≥ 30 days stability at room temperature. Cross-linked nanoemulsions were stable in storage, with only a modest change in DLS. b, Collagenase type I degrades the gelatin nanoemulsion at 37°C. The broadened DLS profile indicates the degradation of nanoemulsion.](image)

**Figure D.1.** Stability and biodegradation of gelatin nanoemulsion. a, Gelatin nanoemulsion demonstrates ≥ 30 days stability at room temperature. Cross-linked nanoemulsions were stable in storage, with only a modest change in DLS. b, Collagenase type I degrades the gelatin nanoemulsion at 37°C. The broadened DLS profile indicates the degradation of nanoemulsion.
Figure D.2. Additional transmission electron microscopy images of the gelatin nanoemulsions. Scale bar is 500 nm.

Figure D.3. IR spectra of nanoemulsion cross-linking and control experiments. As reported previously\textsuperscript{7,8}, irradiation of riboflavin with UV-A light generates singlet oxygen, oxidizing the imidazole moiety of the histidines of collagen to electrophilic imidazolones. These imidazolones then react with hydroxyl moieties of hydroxyproline, serine, or tyrosine, resulting in cross-linking. Cross-linking after irradiation was demonstrated by the emergence of a band at 1033 cm\textsuperscript{-1} arising from aliphatic-aromatic ether formation, similar to that obtained from riboflavin and gelatin without carvacrol. Compared with irradiation of gelatin and riboflavin alone, carvacrol nanoemulsions featured broadening at 1033 cm\textsuperscript{-1}, an additional aromatic ether signature at 1242 cm\textsuperscript{-1}, and appearance of sp\textsuperscript{3} C-H stretches at 2957 cm\textsuperscript{-1}, consistent with an imidazolone reaction with the hydroxyl groups of carvacrol, imparting further hydrophobic domains and additionally stabilizing the oil domains. Irradiation in the presence of the singlet oxygen inhibitor sodium azide resulted in no new bands, consistent with the proposed cross-linking mechanism. All three reactions underwent dialysis and were lyophilized to remove by-product noise (riboflavin, residual carvacrol oil, sodium azide, and water) prior to the IR measurement.
Figure D.4. Killing mechanism of gelatin nanoemulsions. *P. aeruginosa* (ATCC-27853) was treated with gelatin nanoemulsions and Ceftazidime in concentrations ranging from 0.125 to 4X of MIC. Upon addition, gelatin nanoemulsions quickly disrupted bacterial cell membrane, therefore allowing propidium iodide to bind to nucleic acids and generate fluorescence. However, no fluorescence was observed with Ceftazidime, as its mechanism of action is through inhibition of enzymes for cell-wall synthesis rather than membrane disruption.

Figure D.5. Photographs of infected wounds treated with gelatin nanoemulsions. Photographs were taken daily over the duration of the experiment. Images were used for the blinded evaluation of degrees of purulence.
Figure D.6. Photographs of infected wounds treated with PBS. Photographs were taken daily over the duration of the experiment. Images were used for the blinded evaluation of degrees of purulence.

Figure D.7. Photographs of infected wounds treated with vancomycin. Photographs were taken daily over the duration of the experiment. Images were used for blinded evaluation of degrees of purulence.
Figure D.8. Purulence scoring system. We used a 6-scale system, from 0 to 5, to evaluate the degree of pus formation in wound beds. Scores of 5 indicate that the wound is heavily infected, with pus extending beyond the wound edge. Scores of 4 indicate that the infected wound has significant pus formation but is limited to the wound bed. Scores of 3 are given to wounds completely covered with whitish exudate. Scores of 2 are assigned to wounds with a whitish exudate, and a visible wound bed. Scores of 1 are assigned to wounds with a slightly turbid exudate, while scores of 0 indicate a normal appearing wound without any sign of exudate.

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<td><img src="image2" alt="Slight turbid exudate" /></td>
<td><img src="image3" alt="Mild whitish exudate" /></td>
<td><img src="image4" alt="Whitish exudate" /></td>
<td><img src="image5" alt="Yellowish exudate" /></td>
<td><img src="image6" alt="Gross pus" /></td>
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<tr>
<td>Description</td>
<td>normal wound bed</td>
<td>slight turbid exudate</td>
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<td>whitish exudate</td>
<td>yellowish exudate</td>
<td>gross pus</td>
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Figure D.9. Purulence scores of wounds treated with nanoemulsions, PBS, and vancomycin. Photos of infected wounds were taken daily and were used to rate the extent of pus in a blinded fashion. The results showed that treatment with gelatin nanoemulsions resulted in better wound healing than treatment with vancomycin or PBS.
<table>
<thead>
<tr>
<th>excised skin after the preparation</th>
<th>histological sample</th>
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<tr>
<td><a href="image">Nanoemulsion</a></td>
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<td><a href="image">PBS</a></td>
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<td><a href="image">Vancomycin</a></td>
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**Figure D.10.** Excised skin after histological sample preparation – macroscopic and microscopic images. The red dashed line indicates where the histological sample was taken. The green dashed circles indicate the location of wound infection. Scale bar is 500 μm.
Figure D.11. Additional histological samples of skin surrounding infected wounds. a, Epidermis samples showing regeneration of keratin and epithelial layer with nanoemulsion treatment. Inflammatory cells and proteinaceous debris were observed with PBS and vancomycin treatments. b, Formation of collagen matrix in epidermis-dermis junction after nanoemulsion treatment. Immature epidermis and granulation were observed with PBS. Necrosis and cell debris were detected in the vancomycin-treated sample. c, The dermis was restored with nanoemulsion treatment, while inflammatory cells were still present in other controls.

D.3. Supplementary References


APPENDIX E

SUPPLEMENTARY INFORMATION: SELECTIVE TREATMENT OF INTRACELLULAR BACTERIAL INFECTIONS USING HOST CELL-TARGETED BIOORTHOGONAL NANOZYMES


E.1. Experimental Methods

E.1.1. Materials

All chemicals and materials for experiments were obtained from Sigma Aldrich (USA) or Fisher Scientific (USA). Further purification was not performed unless otherwise indicated. Chloroauric acid used for gold nanoparticle synthesis was purchased from Strem Chemicals Inc. (Newburyport, MA, USA).

RAW 264.7 cells (ATCC TIB-71) and HEPG2 cells (ATCC HB-8065) were purchased from the American Tissue Culture Collection (ATCC, Manassas, VA). Dulbecco’s modified Eagle’s medium (DMEM; for RAW 264.7 cultures), Eagle's Minimum Essential Medium (EMEM; for HEPG2 cultures), fetal bovine serum (FBS; Fisher Scientific) were used in cell culture. Salmonella enterica subsp. enterica serovar Typhimurium (ATCC 29630) was purchased from ATCC and cultured using lysogeny broth (LB; Fisher Scientific). Lactobacillus species used were obtained from probiotic BioK+ and cultured using Lactobacilli De Man, Rogosa and Sharpe (MRS) broth (Fisher Scientific).
E.1.2. Synthesis of mannose-decorated gold nanoparticles (Man-AuNPs)

AuNPs used in this study were synthesized as reported previously.\textsuperscript{1} Briefly, the Brust-Schiffrin two-phase synthesis method was used to synthesize 1-pentanethiol protected 2 nm AuNPs. A carboxylate-terminated ligand (COOH ligand) was synthesized using previously reported procedures.\textsuperscript{1,2} Murray place-exchange method was followed to attach the ligand to the AuNP core. 30 mg of pentanethiol-conjugated AuNPs were dissolved in 4 mL distilled dichloromethane (DCM) and mixed with 90 mg of COOH ligand dissolved in 4 mL of 1:1 DCM:methanol, and stirred for 3 days at room temperature under nitrogen environment, followed by removal of DCM and methanol under reduced pressure. Excess ligands, pentanethiol, acetic acid, and other salts present in the AuNP solution were removed by washing with hexane thrice and DCM twice, followed by re-dispersion in de-ionized water and dialysis (membrane molecular weight cut-off = 10,000) for 3 days. After dialysis, the particles were filtered through a syringe filter to remove impurities [Amicon (USA), Ultra 4, 10K]. The carboxylate AuNPs were then post-functionalized with mannose to give Man-AuNPs by adding 1 mM mannose amine, 3 mM N-Hydroxysuccinimide (NHS) and 3 mM 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) on an ice bath. The reaction was allowed to proceed overnight and the mixture was then collected and washed with Milli-Q water multiple times using ultracentrifugal filters (Amicon Ultra 4, MWCO=10 KDa, pore size = 0.2 μm) at 4,000 rpm to remove the excess reactants. The final AuNPs solution was filtered through a 0.2 μm polyethersulfone (PES) membrane filter and then characterized by dynamic light scattering (DLS) and transmission electron microscopy (TEM).
E.1.3. Zeta potential characterization of Man-AuNP

Zeta potential was measured in deionized (DI) water using a Malvern Zetasizer Nano ZS instrument.

E.1.4. DLS and TEM characterization of Man-AuNP

Hydrodynamic diameters of Man-AuNPs and mannose nanozymes (Man-NZs) were measured by DLS in DI water using a Malvern Zetasizer Nano ZS instrument by using a measurement angle of 173° (backscatter). Data were analyzed by the “multiple narrow modes” (high resolution) method based on non-negative-least-squares (NNLS). TEM imaging samples were prepared separately by placing a droplet of 1 µM Man-AuNP or Man-NZ solution onto a 300-mesh Cu-grid coated with carbon film. The samples were analyzed using a JEOL JEM-2000FX (located in the Institute of Applied Life Sciences) with an accelerating voltage of 200 keV electron microscope. These characterization data indicated that there was no aggregation before or after catalyst encapsulation.

E.1.5. Carbohydrate density determination

A freshly prepared anthrone solution in concentrated H2SO4 (0.5 wt %, 1 mL) was added to various concentrations of D-mannose in water (0.5 mL) in an ice bath with stirring. The solution was then heated to 100 °C and stirred for 10 min. After cooling to room temperature, the absorbances of the resulting solutions were measured at 620 nm on a Spectramax M2 Microplate Reader (Molecular Devices, San Jose, CA, USA). The data were plotted against the concentrations of D-mannose and used as the calibration curve for the calculation of ligand density on Man-AuNPs. Ligand density experiments for Man-AuNPs were carried out by dissolving freshly-prepared D-mannose-conjugated AuNPs (5
µM) in 0.5 mL Milli-Q water, and treating solutions with anthrone/H₂SO₄ following the same protocol described above. The final data shown are mean values obtained from three measurements. The non-functionalized COOH-AuNPs were treated with anthrone/H₂SO₄ in the same manner, and the absorbance at 620 nm was used as the background deducted from the total signal measured from D-mannose-conjugated AuNPs. The density of immobilized D-mannose was then determined using the calibration curve.

**E.1.6. Catalyst encapsulation in AuNP monolayer to fabricate mannose nanozyme (Man-NZ)**

2 mL of iron (III) tetraphenyl porphyrin (FeTPP) solution (2 mg/mL) in tetrahydrofuran (THF) was mixed with an equal volume of aqueous Man-AuNP solution (10 µM) and stirred for 10 min. The organic layer was slowly evaporated to induce the encapsulation of FeTPP into the NP monolayer, with excess catalyst precipitating from solution. Excess FeTPP was removed by 0.22 µm PES membrane filter. Then the dispersion was washed with ultracentrifugal filters (Amicon Ultra 4, MWCO=10 KDa, pore size = 0.2 µm), washing with Milli-Q water multiple times at 4,000 rpm until no color was observed in the flow through, to obtain the purified Man-NZ solution. The concentration of Man-NZ was determined by measuring the absorption at 506 nm, and the FeTPP amount was determined by ICP−MS by tracking $^{56}$Fe and $^{197}$Au.

**E.1.7. Quantification of FeTPP/Man-NZ using ICP-MS**

The quantification of Fe catalyst amounts per particle were performed by first adding 10 µL of Fe-NZ solution (10-30 µM) to a glass bottle containing 0.5 mL of H₂O₂/HNO₃ in a 1:1 ratio. The sample was dried overnight at 120 °C. Then, 0.5 mL of
fresh aqua regia was added to provide the Fe/Au stock solution. The resulting solution was then diluted to 10 mL with de-ionized water. Sample composition was then analyzed on a Perkin-Elmer NexION 300X ICP mass spectrometer (located in the Institute of Applied Life Sciences). $^{197}$Au and $^{56}$Fe were measured under the standard mode: nebulizer flow rate, 0.95 L/min; rf power, 1600 W; plasma Ar flow rate, 18 L/min; dwell time, 50 ms. Standard gold solutions (0, 20, 50, 100, 200, 250, 375 and 500 ppb) and iron solutions (0, 0.2, 0.5, 1, 2, 5, 10, 20, 30, 40, 45 and 50 ppb) were prepared via serial dilutions for the calibration curve.

**E.1.8. Activation of pro-resorufin by Man-NZ**

Each kinetic experiment was performed in a 96-well black plate with 100 μL of PBS solution containing the Man-NZ (500 nM), pro-resorufin (pro-res, 20 μM), and 5 mM of glutathione. Samples were then immediately inserted into the plate reader at 37°C to analyze the evolution of fluorescence (Ex. 568 nm, Em. 588 nm).

**E.1.9. Mammalian and bacterial cell culture**

All mammalian cells were grown in T75 cell culture flasks using standard growth media [DMEM supplemented with 10% FBS and 1% Penicillin-Streptomycin (Pen-Strep)] for RAW 264.7 cells and EMEM supplemented with 10% FBS and 1% Pen-Strep for HEPG2 cells) under physiological conditions (37 °C in a humidified atmosphere of 5% CO$_2$). Once the cells were at sufficient density (~80% confluent), they were washed with phosphate-buffered saline (PBS) three times and detached using 0.05% trypsin. The cells were centrifuged and resuspended in fresh culture media. 10 µL of cell solution was mixed with trypan blue in a 1:1 ratio by volume and counted by hemocytometer. Cell solutions
were diluted in the same media type to obtain the indicated concentrations for subsequent experiments.

Overnight cultures of bacteria were prepared by transferring an isolated colony from the designated agar plate to culture tubes with sterile LB broth for Salmonella and MRS broth for Lactobacillus. Bacterial cultures were then incubated overnight at 37 °C with agitation (275 rpm), until the stationary phase was reached. Bacteria were then collected by centrifugation (7000 rpm, 5 min) and washed thrice with 0.85% sodium chloride. Subsequently, the culture was resuspended in 1 mL of PBS to determine its OD$_{600}$ (SpectraMax M2, Molecular Devices, San Jose, CA).

**E.1.10. NZ cellular uptake**

RAW 264.7 and HEPG2 cells were seeded at concentrations of 50,000 cells/well in a 24-well plate overnight. The next day, the cells were treated with Man-NZ/COOH-NZ at various concentrations for 24 h. The wells were washed three times with PBS and the cells were lysed by using 500uL of 1X Cell lysis buffer. The cell lysates were analyzed for gold content by ICP-MS.

For M2 phenotype of RAW 264.7 cells, 50,000 cells/well were stimulated by IL-4 (30 ng/mL) and seeded on a 24-well plate overnight, followed by indicated treatments.

**E.1.11. Evaluation of NZ retention by macrophages**

RAW 264.7 cells were seeded at a concentration of 20,000 cells/well in three 24-well plates overnight. The next day, the cells were treated with Man-NZ at various concentrations for 24 h, 48 h and 72 h, respectively. The wells were washed three times with PBS and the cells were then lysed and further analyzed for gold content by ICP-MS.
Standard gold solutions were prepared via serial dilutions (0, 0.2, 0.5, 1, 2, 5, 10, and 20 ppb) for the calibration curve.

**E.1.12. Intracellular pro-fluorophore activation**

RAW 264.7 cells were seeded at a concentration of 75,000 cells/well in a 4-well chamber Lab-Tek II chambered coverglass system 24 h before the experiment. The cells were washed with PBS and then incubated with 500 nM Man-NZ overnight. Media was then aspirated and the cells were washed with PBS to remove excess Man-NZ. 20 μM pro-res was then added in fresh media, and the cells were incubated for 2 h. The media was then replaced with PBS. Confocal microscopy images were obtained on a Zeiss LSM 510 Meta microscope by using a 40× objective. The settings of the confocal microscope were as follows: red channel: λex = 560 nm and λem = LP 640 nm. Emission filter: LP = high pass.

**E.1.13. Cytotoxicity**

RAW 264.7 cells were seeded at a concentration of 10,000 cells/well in a 96 well plate overnight. The next day, the cells were treated with one of the following: Man-NZ, pro-cip, ciprofloxacin and Man-NZ+pro-cip at various concentrations for 24 h. After the incubation period, the cells were washed three times with PBS to remove dead cells and excess NZ. 10% Alamar Blue reagent (Invitrogen, Waltham, MA) in serum containing media was added to each well and incubated 2 h further at 37 °C and 5% CO2. Cell viability was then determined by measuring the fluorescence intensity (ex/em: 560/590 nm) using a SpectraMax M5 microplate spectrophotometer. Three biological replicates were performed for viability determination.
E.1.14. Ninhydrin assay to determine pro-ciprofloxacin activation

Ninhydrin (Sigma Aldrich) assay was performed to demonstrate the activation of pro-cip, following the protocol provided by the vendor. 100 μL ciprofloxacin solutions of varying concentrations were prepared to generate a calibration curve. Then, 10 μL of 2% ninhydrin (in ethanol) solution was added to each cip solution and allowed to incubate for 10 min with shaking. Absorbance was then measured at 350 nm. 50 μL of pro-cip solutions of varying concentrations were mixed with 50 μL of 1000 nM Man-NZ to give a final concentration of 500 nM. Following this, the ninhydrin assay was performed as above and absorbance values were obtained. Concentration of activated pro-cip to cip was determined using the calibration curve.

E.1.15. Bacteria killing assays

For the Salmonella-only infection model, a single colony of Salmonella enterica subsp. enterica serovar Typhimurium (ATCC 29630) was inoculated into LB broth and incubated at 37 °C overnight to reach stationary phase. Then 60 μL of the stationary phase solution was diluted to 3 mL using fresh LB broth and incubated at 37 °C until bacteria attained log phase growth. The resulting bacteria solution was then centrifuged, washed three times with 0.85% NaCl, and resuspended in PBS. Then, a solution containing 1x10^6 CFU/mL Salmonella was prepared using LB broth. Subsequently, the test materials, 500 nM Man-NZ, 1 and 0.5 μM pro-ciprofloxacin, and 15 nM ciprofloxacin, were prepared using LB broth. 50 μL each of the bacteria and the test material were added to a 96-well microplate and incubated at 37 °C overnight with shaking. Quantitative colony counting was then performed to determine the numbers of bacteria that survived the treatment. Wells containing bacteria solution only served as the growth control while wells containing LB
broth only served as the sterile control. Cultures were generated and analyzed in triplicates, and at least two identical, independent experiments were performed on different days.

For intracellular killing assays, a modified version of a previously published protocol was performed. RAW 264.7 cells were seeded at a concentration of 17,000 cells/well in growth media lacking antibiotics with 500 nM Man-NZ in a 96-well plate, and allowed to grow overnight. Salmonella was then added to the wells to achieve 1:100 multiplicity of infection (MOI) and incubated for 1 h. The wells were washed with gentamicin three times and either pro-ciprofloxacin or ciprofloxacin was added in fresh media, then incubated overnight. The following day, the media was removed, the mammalian cells were lysed using 0.01% Triton X, and the lysate was collected for quantitative colony counting. Three biological replicates were generated and analyzed for each group.

Transwell membrane bacterial co-culture assays. To model the selective killing of intracellular macrophage pathogens over non-pathogenic gut bacteria by Man-NZ+pro-ciprofloxacin, a transwell membrane assay was developed. The probiotic Bio-K+ (Lactobacillus sp.) was used as a model of the intestinal flora. Briefly, RAW 264.7 cells were seeded overnight in a 6-well plate in DMEM with 10% bovine calf serum and 500 nM Man-NZ at 37 °C. Intracellular Salmonella infection was performed using the same procedure described above. On the other hand, Bio-K+ was cultured in MRS broth overnight at 37 °C with shaking to reach stationary phase. Then, a solution of Bio-K+ was prepared using 1:1 MRS:DMEM media and added to a transwell membrane (0.4 μm pore size), which was inserted into the 6-well plate containing infected macrophages. Subsequently, the test materials were prepared using 1:1 MRS:DMEM media and added to
each of the wells. The plate was incubated overnight at 37 °C. The media was then collected and used for quantitative colony counting using MRS agar to determine numbers of surviving *Lactobacillus* sp. The infected macrophages were washed using PBS and lysed with 0.01% Triton X. The lysate was recovered and plated in Xylose Lysine Deoxycholate (XLD) agar to quantitatively determine surviving numbers of *Salmonella*.

**E.1.16. Statistical analysis**

For all bacteria and macrophage viability experiments, data are presented as mean ± standard deviation of at least three replicates. Statistical analysis was carried out using one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test. A P value < 0.05 is considered as statistically significant. GraphPad Prism was used to perform statistical analyses.

**E.2. Supplementary Figures**

![Calibration curve obtained from treating various concentrations of D-mannose with anthrone/sulfuric acid and measuring the absorption at 620 nm.](image)

**Figure E.1.** Calibration curve obtained from treating various concentrations of D-mannose with anthrone/sulfuric acid and measuring the absorption at 620 nm.
Figure E.2. Zeta potential of AuNPs before (left) and after (right) functionalization with mannose.

Figure E.3. Size determination of AuNPs before (top) and after (bottom) functionalization with mannose.
Figure E.4. TEM image of Man-NZ. Scale bar represents 20 nm.
Figure E.5. Standard calibration curves for Au (top) and Fe (bottom).
Figure E.6. Au calibration curve for Man-NZ uptake by macrophages.
Figure E.7. ICP-MS analysis of gold accumulation in a) RAW 264.7 macrophages following 24, 48, and 72 h incubation with Man-NZ and b) M1 and M2 polarized macrophages after 24h Man-NZ incubation. Error bars represent standard deviation (n=3).
Figure E.8. Cytotoxicity of Man-NZ to RAW 264.7 macrophages. Data are presented as mean ± standard deviation, n=3. All treatment groups have no statistically significant difference from the control group.
Figure E.9. Validation of pro-cip conversion to ciprofloxacin using Ninhydrin assay. 
a) Calibration curve for Ninhydrin assay. b) Equivalent ciprofloxacin concentration from activated pro-drug. c) Percent conversion of 50 μM pro-cip with 500 nM Man-NZ over time. Data are presented as mean ± standard deviation, n=3.
Figure E.10. Cytotoxicity of pro-ciprofloxacin and ciprofloxacin to RAW 264.7 macrophages. Data are presented as mean ± standard deviation, n=3.

Figure E.11. Cytotoxicity of Man-NZ+pro-cip in RAW 264.7 macrophages. Increasing pro-ciprofloxacin concentration was used while Man-NZ was kept constant at 500 nM. Viability was monitored by Alamar Blue assay after 24- and 48-hours of incubation. Data are presented as mean ± standard deviation, n=3.
Figure E.12. Activity of Man-NZ+pro-cip against different intracellular infection models. a) Viability of \textit{Salmonella} residing inside J774 a.1 macrophages after 24 hr treatment with Man-NZ+pro-cip. b) Viability of MRSA residing inside RAW 264.7 macrophages after 24 hr treatment with Man-NZ+pro-cip quantified by colony counting. Data are presented as mean ± standard deviation, n=3.
Figure E.13. Viability of *Lactobacillus* sp. and intracellular *Salmonella* after 6-, 12- and 48 h treatment with Man-NZ+pro-cip determined by quantitative colony counting. Data are presented as mean ± standard deviation, n=3.

E.3. Supplementary References


SUPPLEMENTARY INFORMATION: ANTIMICROBIAL-LOADED BIODEGRADABLE NANOEMULSIONS FOR EFFICIENT CLEARANCE OF INTRACELLULAR PATHOGENS IN BACTERIAL PERITONITIS

F.1. Experimental Section

F.1.1. Materials

All chemicals and materials for experiments were obtained from Sigma Aldrich (USA) or Fisher Scientific (USA). Further purification was not performed unless otherwise indicated.

F.1.2. Bacterial and mammalian cell culture

RAW 264.7 cells (ATCC TIB-71) were purchased from the American Tissue Culture Collection (ATCC). Dulbecco’s modified Eagle’s medium and fetal bovine serum (FBS; Fisher Scientific) were used in cell culture. MDR K. pneumoniae (CD-343) and MDR S. aureus (CD-35) are clinical isolates obtained from the Cooley Dickinson Hospital microbiology Laboratory (Northampton, MA). Clinical isolate of methicillin-resistant S. aureus (IDRL-6169) was from the Infectious Diseases Research Laboratory at Mayo Clinic. Bioluminescent MRSA USA300 NRS384 strain (SAP-231) was provided by Dr. Roger Plaut. Salmonella enterica subsp. enterica serovar Typhimurium (ATCC 29630) was purchased from ATCC.

F.1.3. Synthesis of PONI-GMT and DTDS

PONI-GMT and DTDS were synthesized according to a previously published protocol.¹
F.1.4. Characterization of BNEs

Hydrodynamic diameters of the BNEs were measured using DLS (Malvern Zetasizer Nano ZS) with a measurement angle of 173° (backscatter). Data were analyzed using the “multiple narrow modes” method (high resolution) based on non-negative least squares. TEM imaging samples were prepared on 300 square mesh nickel grids using JEOL JEM-2000FX with an accelerating voltage of 200 keV electron microscope.

F.1.5. Screening the antimicrobial activity of BNEs

Standard protocol for minimum inhibitory concentration (MIC) determination was employed. Briefly, a single colony of the test bacteria [MDR S. aureus (CD-35), MRSA (IDRL-6169), MDR K. pneumoniae (CD-343) and Salmonella (ATCC 29630)] was inoculated into rich media and incubated at 37 °C overnight to reach stationary phase. Then 60 μL of the stationary phase solution was diluted to 3 mL using fresh media and incubated at 37°C until bacteria attained log phase growth. The resulting bacteria solution was then centrifuged, washed three times with 0.85% NaCl, and resuspended in PBS. Then, a solution containing 1x10^6 CFU/mL bacteria was prepared. Subsequently, the essential oil-loaded BNEs were prepared and different concentrations were made by serial dilution. 50 μL each of the bacteria and the test material were added to a 96-well microplate and incubated at 37 °C overnight with shaking. Wells containing bacteria solution only served as the growth control while wells containing media only served as the sterile control. The MIC is defined as the lowest concentration of the BNEs that inhibited visible growth (turbidity) as observed with the unaided eye. Cultures were performed in triplicates, and at least two identical, independent experiments were repeated on different days.
F.1.6. Macrophage cell viability assay

RAW 264.7 cells were seeded at a concentration of 10,000 cells/well in a 96-well plate and were cultured in Dulbecco’s modified Eagle medium with 1% antibiotics and 10% bovine calf serum in a humidified atmosphere of 5% CO₂ at 37°C overnight. The next day, the cells were replaced with fresh media containing BNEs at various concentrations and incubated for 2 h. The cells were then washed three times with PBS to remove dead cells and excess BNEs. 10% alamarBlue reagent (Invitrogen, Waltham, MA) in serum-containing media was added to each well and incubated further for 2-4 h at 37°C and 5% CO₂. Cell viability was then determined by measuring the fluorescence intensity (ex/em: 560/590 nm) using a SpectraMax M5 microplate spectrophotometer. Cells-only control was considered 100% viable. Three biological replicates were performed and repeated on two different days.

F.1.7. Measurement of TNF-α levels and Caspase-Glo 3/7 assay

RAW 264.7 cells were infected with MRSA and treated with E-BNEs as above. Uninfected macrophages were also seeded and treated for comparison. At 2 h and 24 h after the addition of E-BNEs to macrophages, culture media was collected for TNF-α level measurement by ELISA (R&D Systems, MN, USA) and assess caspase activity using Caspase-Glo 3/7 assay (Promega). The procedure specified by the manufacturers was followed. Experiments were performed in triplicates.

F.1.8. Evaluation of uptake mechanism

RAW 264.7 cells were seeded at 1.5 x 10⁵ cells/well 48 h prior to the experiment and cultured in Dulbecco's modified Eagle medium (DMEM; ATCC 30-2002) with 10%
bovine calf serum and 1% antibiotics at 37°C in a humidified atmosphere of 5% CO2. Cells were washed with PBS once and then cultured in media containing a dissolved quantity of small molecule inhibitor, methyl-β-cyclodextrin (7.5 mg/mL), chlorpromazine (1.5 μg/mL), or imipramine for 1 hour at 37°C, 5% CO2. After this treatment, cells were washed with PBS twice and then treated with BNEs for 1 h. Afterward, cells were washed with PBS and trypsinized for Flow Cytometry. Flow imaging techniques utilized an Amnis ImageStream MkII Imaging Flow Cytometer (Luminex). Cells were then resuspended in a modified flow buffer. 5mM DRAQ5 fluorescent probe (Thermo Fisher) was added and allowed to incubate for ~20 minutes. Cells were then processed at a flow rate of 1,200 cells/sec and images were taken with 60X magnification. All flow cytometry quantification was performed in triplicate (3 wells per condition), with three experimental replicates collected for each sample at 1000 events per replicate. Error is reported throughout as standard deviation by population. Data were analyzed by Amnis IDEAS software. Focused cell images were isolated through a Gradient RMS histogram of the widefield (Ch01).

Subsequently, coumarin-blue loaded eugenol (1 mg dye/mL oil) and Texas Red-tagged PONI-GMT were used to prepare nanoemulsions to track both the oil and polymer upon macrophage uptake. RAW 264.7 cells were seeded at a concentration of 200,000 cells/dish in a 35 mm confocal dish 24 h before the experiment. The cells were washed with PBS and then incubated with media containing BNEs with blue dye-loaded oil and red dye-tagged polymer for 2 h. Media was then aspirated and the cells were washed with PBS to remove excess material. Confocal microscopy images were obtained on a Zeiss LSM 510 Meta microscope by using a 60X objective. The settings of the confocal
microscope were as follows: red channel: $\lambda_{ex} = 560$ nm and $\lambda_{em} = LP 640$ nm; blue channel: $\lambda_{ex} = 403$ nm and $\lambda_{em} = LP 495$ nm. Emission filter: LP = high pass.

Figure F.1. Minimum inhibitory concentrations of the BNEs against MDR $K.\ pneu,m,nia,e$ (CD-343) and $Salmonella\ enterica$ subsp. enterica serovar Typhimurium (ATCC 29630).
Figure F.2. (A) Representative confocal laser scanning microscope images of RAW 264.7 macrophages treated with different BNEs loaded with red fluorescent Nile Red (1 mg/mL oil). Scale bar is 10 μm. (B) Quantified fluorescence from the uptaken Nile Red-loaded BNEs by macrophage cells.
**Figure F.3.** (A) IVIS images of whole mice (top) and harvested samples (bottom) 24 h post-IP administration of NIR750-loaded E-BNEs. Numbers indicate the mouse and its corresponding harvested fluids: PF = peritoneal fluid; B = blood; and organs: L = liver; S = spleen. (B) IVIS images of mice at 24 h-post infection with bioluminescent MRSA administered intraperitoneally. Yellow arrows indicate mice dropped from the study due to lack of bioluminescent signal.

**F.2. Supplementary References**


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