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Antimicrobial Strategies for Topical Applications

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ANTIMICROBIAL STRATEGIES FOR TOPICAL APPLICATIONS

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I dedicate this dissertation to:

My family: Pete and LeighAnn Lopez, Aleena and Matthew Burgner.

Tin Roof Brewery. Yoga and beer were my favorite stress-release.

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ABSTRACT

Bacteria can thrive in diverse environments and are often harmless or beneficial. Bacteria in digestive tracts is one example of beneficial bacteria; however, bacteria can be harmful and when this type proliferates, it can cause infections within hosts. Bacterial infections are easily treated with antibiotics in most cases. However, bacteria are also capable of developing mutations which could cause them to become multi-drug resistant and eventually, "superbugs." Therefore, the development of novel antimicrobial agents and materials capable of combating drug-resistant bacteria is necessary. Research presented in this dissertation focuses on different strategies for minimizing and preventing topical bacterial infections using Groups of Uniform Materials Based on Organic Salts (GUMBOS) and biopolymers.

The first part of this dissertation focuses on the synthesis and antibacterial activity of GUMBOS created from antiseptics and β-lactam antibiotics to combat *Neisseria gonorrhoeae*. Firstly, using ion-exchange reactions, antimicrobial GUMBOS from an outmoded antibiotic were synthesized and characterized using proton and carbon NMR, mass spectrometry, and FT-infrared spectroscopy. Improvement or bioequivalence in *in vitro* antibacterial activity was obtained on isolates of *N. gonorrhoeae*. In the second project, GUMBOS were synthesized using a currently prescribed antibiotic. GUMBOS not only showed improvement for killing *N. gonorrhoeae*, but also demonstrated increased efficacy against isolates of *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*. Overall, these studies present an alternative to current antibiotic drug therapy by using a novel group of ionic antimicrobial materials that have improved bio availabilities, multi-modal properties, and potent antimicrobial activity as a viable alternative to combating *N. gonorrhoeae* in the oropharynx. Finally, acute cytotoxicity against cervical cellular lines in addition to an assessment of intestinal permeability and bioavailability were completed.

The next part of this dissertation focuses on developing materials from biopolymers for providing potential improvements to wound care and topical infection prevention applications. In the third project, composite materials using biopolymer blends are presented. These composites were synthesized using a simple ionic liquid and were incorporated with antimicrobial GUMBOS. Composites showed great potential for combating topical *Staphylococcus aureus* infections. Moreover, composites presented high swelling capabilities, which could be translated to exudation capacity. The fourth project focuses on polyelectrolyte complexes synthesized using metathesis reactions from a biopolymer and antiseptics. These complexes were then evaluated for use as oral and topical patches for combating bacterial infections. These materials were characterized using several analytical techniques. Further, these complexes were heat pressed into films which showed steady antiseptic release over several hours.

CHAPTER 1. INTRODUCTION

1.1.Bacteriology

Bacteria are unicellular, prokaryotic organisms first discovered by Antonie van Leeuwenhoek in 1677 who initially called them "animalcules."[1] It wasn't until the 1830s, when Christian Gottfried Ehrenberg introduced the word "bacterium" to describe his observations of "non-spore forming rod-shaped bacteria."[2] They can thrive in diverse environments, such as, soil, ocean water, and human intestinal tracts. These diverse environments could be a reason why bacteria have several morphologies.[3] The different morphologies include rods, spheres, and spirals, with sizes ranging on the scale of microns. Most bacteria are either rod-shaped (bacilli) or spherical/oval (cocci). However, slight differences in morphology can lead to different classes of bacteria that are named by their shape, such as, vibrio (comma-shaped), spirilla (spiraled), and spirochetes (coiled). Bacteria can also arrange themselves into arrays that further adds to the nomenclature. For example, staphylococci form clusters while streptococci form chains.

Bacteria are structurally comprised of several intracellular and extracellular structures. Intracellular components are surrounded by cell membranes composed of phospholipids which envelope essential materials.[4] Unlike eukaryotic cells, prokaryotes do not contain membrane-bound organelles, such as a nucleus and mitochondria.[5] Their genetic material is stored as a circular bacterial chromosome of DNA in an irregularly shaped region called a nucleoid.[6] Since they lack mitochondria, bacteria use biochemical reactions to produce concentration gradients across cellular membranes to maintain cellular metabolisms and create potentials analogous to a battery.[7] One common feature between prokaryotic and eukaryotic cells are ribosomes which synthesize proteins and enzymes for routine function.[8] A cell wall, found outside of the cell membrane, is

composed of peptidoglycan, a polymer consisting of glycosaminoglycan chains cross-linked by peptides.[9]

1.1.1. Classification of Bacteria

Bacteria can be classified based on characteristics of their cell walls, which can be visualized under microscopes using a technique developed in 1882 by Hans Christian Gram called Gram-staining. This technique uses crystal violet (water-soluble dye), decolorization agent, and safranin (counterstain) to distinguish whether a bacterium is Gram-positive or Gram-negative (Figure 1.1). Gram-positive bacteria have a thick layer of peptidoglycan which retains the crystal violet dye and stains these bacteria purple. However, Gram-negative have a thin layer of peptidoglycan which does not retain the crystal violet during the decolorizing process and instead the bacteria are stained red.[10] Bacteria can also be classified on their growth response in the presence and absence of oxygen.[8] Aerobic bacteria require the presence of oxygen to survive and grow, whereas anaerobic bacteria require its absence. The most versatile are called facultative organisms which can grow in the presence or absence of oxygen.[8]

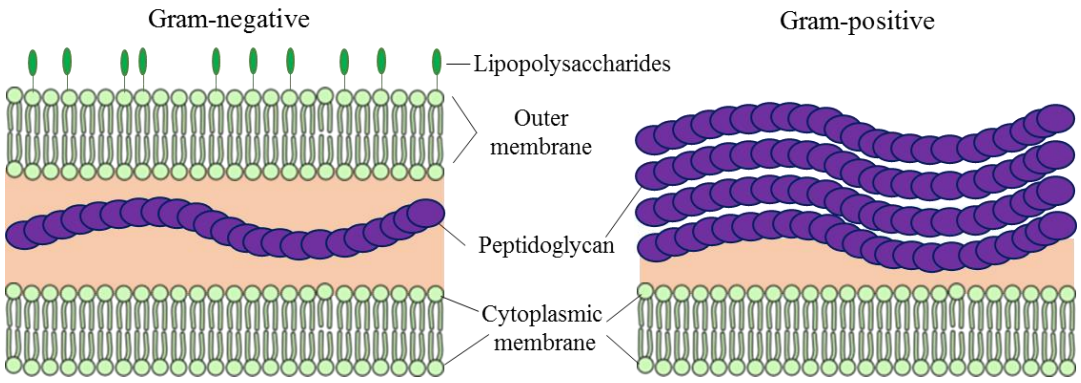

Figure 1.1. Membranes of Gram-negative vs Gram-positive bacteria

1.1.2. Pathogenic bacteria

While most bacteria are often harmless or beneficial, microorganisms that inflict disease and harm the host are known as pathogenic. The degree of pathogenicity is determined by virulence

factors and is often the result of biochemical, genetic, or structural features of the bacterium.[11] Pathogenetic bacteria inflict disease through two main methods: 1) invading tissues or cells and colonization and/or 2) toxigenesis. Pathogens can enter our bodies through the mouth, nose, eyes, or urogenital openings, or through wounds and breaks in the skin barrier.[11] These pathogens invade host cells, multiply, and produce extracellular substances that debilitate host defense mechanisms.[11] Bacteria that target these sites in the body have special adherence mechanisms which aid in overcoming specific host defenses. These mechanisms rely on two factors: a receptor and a ligand.[11] Bacterial ligands, called adhesins, are macromolecular components of bacterial cell walls that interact with host cell receptors. Receptors are typically proteins, peptides, or carbohydrates on the host cell surface.[11] Bacteria adhere to host cells through two mechanisms—nonspecific and specific adherence. Nonspecific adherence to host cells uses various interactions and forces, and Brownian motion. Specific adherence involves lock-and-key mechanisms between complementary receptor and adhesin molecules.[11]

The other method of inflicting disease is called toxigenesis, i.e., the ability to produce toxins.[11] There are two main types of bacterial toxins, exotoxins and endotoxins. Exotoxins are extracellular diffusible toxins that act on sites distant from the original point of bacterial invasion and growth, but they can also be released by cell lysis.[11] They are usually proteins that stimulate a variety of host responses by acting enzymatically or in direct contact with host cells.[11] An example of an exotoxin producing bacterium is *Staphylococcus aureus* which can cause various diseases such as skin infections, food poisoning, osteomyelitis, and endocarditis. In contrast, endotoxins are cell-associated substances, specifically lipopolysaccharide or lipooligosacharide components of the outer membrane of cell walls of Gram-negative bacteria. Although a structural component of the outer membrane, they can be released by growing bacteria or by cell lysis due to the host's

defense mechanisms or by antibiotic activity.[11] Examples of bacteria that release endotoxins are *Neisseria gonorrhoeae,* the bacterium responsible for the sexually transmitted disease, gonorrhea, and *Pseudomonas aeruginosa*, a bacterium responsible for respiratory infections. Both exotoxins and endotoxins can be transported by blood and lymph circulation and result in disease at localized and distant sites of invasion.[11] Because of the pathogenic nature of certain bacteria and their ability to cause disease, chemotherapeutics that target pathogens without harming the human host were sought after.

1.2. Introduction to Antibacterial Drugs

Antibiotics are a class of antimicrobial chemotherapeutic drugs that are widely used in the prevention and treatment of bacterial infections. It is evidenced that antibiotics have been in use since ancient times with civilizations using herbs and honey.[12] One of the more successful treatments was the topical application of moldy bread which was first documented in 1640 in *Theatrum Botanicum,* a book written by John Parkinson.[13] Even some modern antibiotics may have been available in ancient times, for example, traces of tetracycline were found in human skeletal remains discovered in Nubia.[14]

The foundation of modern antibiotics began with Paul Ehrlich, who proposed the idea of fabricating chemical compounds that could selectively target bacteria without harming the human host.[15] This idea led him in 1904 to begin a large-scale, systematic screening program to find a drug to combat syphilis, a sexually transmitted disease thought to be incurable. In 1909, Ehrlich together with bacteriologist Sahachiro Hata and chemist Alfred Bertheim synthesized Salvarsan, a drug used to treat syphilis in the early 20th century.[15] Ehrlich's systematic screening approach, which became the cornerstone of drug search strategies, led to the discovery of sulfa drugs, specifically Prontosil, synthesized by Bayer chemists Fritz Mietzsch and Josef Klarer and

evaluated by Gerhard Domagk.[15] And in 1928, Alexander Fleming discovered one antibiotic that greatly revolutionized modern medicine—penicillin.

1.2.1. Beta-Lactam Antibiotics

Beta-lactam antibiotics are a class of antibiotics containing a 4-membered cyclic amide ring with a lactone functional group, known as a beta-lactam (β-lactam) ring.[16] The first β-lactam antibiotic, penicillin, was discovered by Alexander Fleming in 1928. It was isolated from the *Penicillium* mold.[15] However, its therapeutic use did not occur until the 1940s when a protocol from an Oxford team directed by Howard Florey and Ernest Chain led to mass production and distribution of penicillin in what started the era known as the "Golden Age" of antibiotic discovery.[15, 17-18] After the discovery and commercialization of penicillin, scientists began making derivatives, naturally-occurring and semi-synthetic, which greatly expanded the spectrum of β-lactam antibiotics.[17] Today the class of β-lactam antibiotics (Figure 1.2) include penicillin derivatives (penams), cephalosporins (cephems), carbapenems, and monobactems. As a group, β-lactams are effective against many gram-positive, gram-negative, and anaerobic organisms.[19]

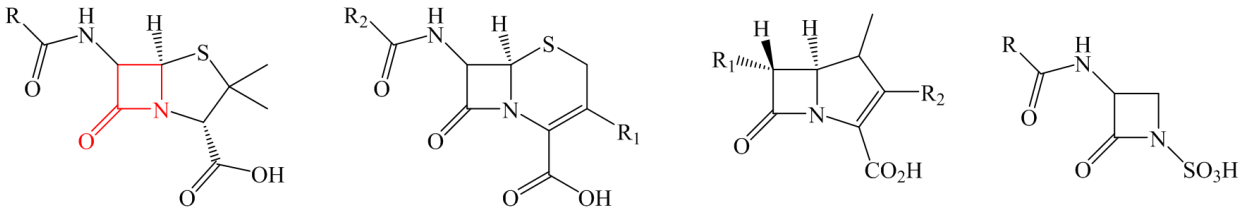

Figure 1.2. General structure of beta-lactam antibiotics. Beta-lactam ring is highlighted in red.

1.2.2. Mechanism of Antibiotic Action and Resistance

Antibiotics can be categorized as bacteriostatic or bactericidal. Bacteriostatic agents prevent the growth of bacteria, which essentially keeps them in the stationary growth phase.

Bactericidal agents, which β-lactam antibiotics are categorized as, kill bacteria.[20-21] Beta-lactam antibiotics act by inhibiting cell wall synthesis by blocking peptidoglycan crosslinking. The antibiotics irreversibly bind to penicillin-binding proteins (PBPs) preventing the catalytic cross-linking reaction. Bacteria die as a result of not being able to synthesize new cell walls during replication. In response to inhibitory effects of antibiotics, bacteria have developed "protective mechanisms", such as porins, penetration of bacterium into cytoplasm of human cells, pumps, and enzymes.[22] The most common mechanism of drug resistance to β-lactam antibiotics is the bacterial synthesis of β-lactamases, enzymes that hydrolyze the β-lactam ring rendering the antibiotic ineffective.[23] In the case of *Neisseria gonorrhoeae*, the bacterium can undergo plasmid- and chromosomally-mediated mutations which led to its resistance towards penicillin and other β-lactam antibiotics through the generation of β-lactamases .[24-25] Bacteria can also express mutated PBPs that exhibit enzymatic activity for cell wall synthesis but are not bound by β-lactam antibiotics, which is a mode of resistance for methicillin-resistant *Staphylococcus aureus* (MRSA).[26] Over time bacteria can develop resistance to many antibacterial drugs and contribute to higher incidences of infectious diseases. They become known as multi-drug resistant, or "superbugs." One group of bacteria, known as "ESKAPE" pathogens (*i.e.*, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species), have increased resistance to common antibiotics and are the leading cause of nosocomial, or hospital acquired, infections.[27] Bacteria are highly adaptable organisms which have created not only the need for antibiotics with unique mechanisms of action but also better preventable practices through the use of antiseptics and disinfectants.

1.3. Introduction to Antiseptics and Disinfectants

Antiseptics and disinfectants are chemical agents that stop or slow the growth of various microorganisms on external surfaces of the body and hard surfaces to minimize infections. They are an essential part of infection control in healthcare settings and general consumer markets.[28] Although the terms are used interchangeably, their differences can be attributed to their roles of microbial control. Antiseptics are broad-spectrum, antimicrobial agents that are applied to the body to inhibit growth of microorganisms on living tissues, while disinfectants inhibit growth on non-living objects and hard surfaces, such as countertops and handrails. Antiseptics and disinfectants also play roles in controlling microbial growth in either sterilization or preservation processes. Sterilization involves the use of antimicrobial agents to eradicate microorganisms from living and non-living surfaces.[28] For example, surgeons use surgical scrubs to sterilize their hands before surgery to minimize and prevent transmission of bacterial infections. Whereas, preservation involves the chemical nature of antimicrobial agents to inhibit growth of microorganisms in consumer products, such as pharmaceuticals, cosmetics, and food.[28] While increased antibiotic use can eventually lead to resistance, antiseptics are unlikely to induce resistance due to their nonspecific mechanism of action as entire cellular structures, rather than specific molecular targets, are affected.[29] The general mechanism of action of antiseptics can be summarized by three key functions: 1) electrostatic interaction between antiseptic and cellular surface, 2) penetration into the cell, and 3) action at the target site(s).[28] Although there are a vast number of antiseptics available with more detailed mechanisms of action, this dissertation will focus in greater detail on bisbiguanidium, bispyridinamium, and quaternary ammonium compounds, as these were the antiseptics used in the presented research.

1.3.1. Bisbiguanide

Biguanides are molecules with the formula $HN(C(NH)NH_2)_2$.[30] Compounds containing a bisbiguanide structure are an important class of compounds that have extensive medical applications, such as in oral products and surgical scrubs. An example of a bisbiguanide with potent antimicrobial efficacy is the broad-spectrum, dicationic salt, chlorhexidine (1,1'-hexamethylenebis[5-(4-chlorophenyl) biguanide]). It is bactericidal and most commonly found in oral and handwash products. The molecule is symmetrical and consists of two chlorophenyl rings and two biguanide structures connected by a central hexamethylene bridge (Figure 1.3). Chlorhexidine (CHX) is basic in nature and is available in three forms, diacetate, digluconate, and hydrochloride salts.[28] This structural modification affects physical and chemical properties such as hydrophobicity, solubility, and bioavailability; however, the antibacterial activities remain unchanged.[31] The antimicrobial activity of chlorhexidine is concentration dependent. At low concentrations, CHX affects membrane integrity whereas high concentrations cause congealing of cytoplasmic constituents.[28] Collectively, bisbiguanide compounds have activity against yeasts, protozoa, mycobacteria, sporulating and non-sporulating bacteria, and enveloped viruses.[28]

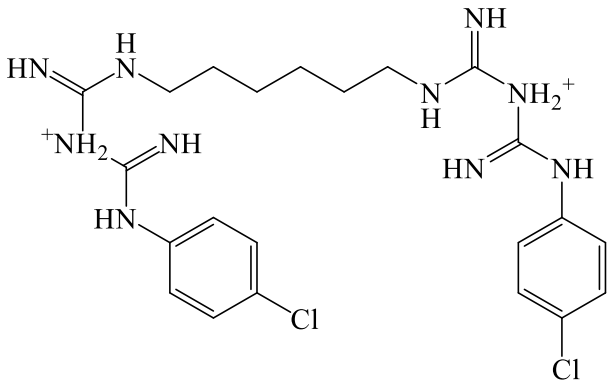

Figure 1.3. Structure of chlorhexidine base.

1.3.2. Quaternary Ammonium Compounds

Quaternary ammonium compounds (QACs) are positively charged polyatomic ions that have been shown to have antimicrobial activity. Certain QACs, primarily those with long alkyl chains, are used as antiseptics and disinfectants. Examples include, cetyltrimethylammonium, cetylpyridinium, and benzalkonium. These QACs have been used for a variety of clinical and healthcare purposes such as oral mouthwash products, application to mucus membranes, preoperative disinfection of non-broken skin, and disinfection of hard surfaces.[28] Structurally, QACs consist of a charged hydrophilic head group and an aliphatic, hydrophobic tail (Figure 1.4). The primary mechanism of action lies in the membrane activity. Quaternary ammonium compounds cause structural deformities and damage the cytoplasmic membrane within bacterial cells by penetrating the cell wall of gram-positive bacteria or outer membrane of gram-negative bacteria. This penetration perturbs the lipid bilayer causing the cellular structure to collapse and intracellular materials to leak. Further, nucleic acid and proteins become degraded, and the cell wall succumbs to autolytic enzymes.[32-33]

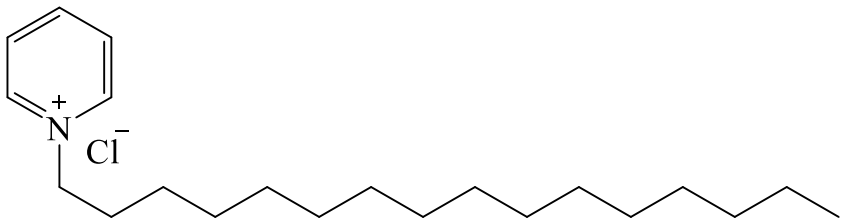

Figure 1.4. Structure of cetylpyridinium chloride as a representative quaternary ammonium compound.

1.3.3. Bispyridinium Compounds

Bispyridinamines are symmetrical molecules composed of substituted pyridinamines connected by a central alkyl chain that have shown promise as antimicrobial agents in periodontology.[34] An example of a bispydridinamine with antimicrobial properties is octenidine (N,N'-(1,10 decanediyldi-1[4H]-pyridinyl-4-ylidene) bis-(1-octanamine) (Figure 1.5). The

structure of octenidine (OCT) differs from QACs, such as benzalkonium, and CHX due to the lack of an amide and ester structure within the molecule. Octenidine is used for skin, mucus membrane, and wound antisepsis. It's a dicationic molecule and exhibits broad spectrum of antimicrobial efficacy against a wide range of microorganisms, including Gram-positive and Gram-negative bacteria, and fungi.[35-36] Octenidine binds readily to negatively charged cell wall surfaces and interacts with polysaccharides within microbial cell walls resulting in membrane destabilization, cytoplasmic leakage, and cell death.[29] Because of its strong adherence to bacterial cell wall components, OCT has shown no adverse interaction with eukaryotic epithelial and wound tissue and has demonstrated significant antimicrobial activity.[37]

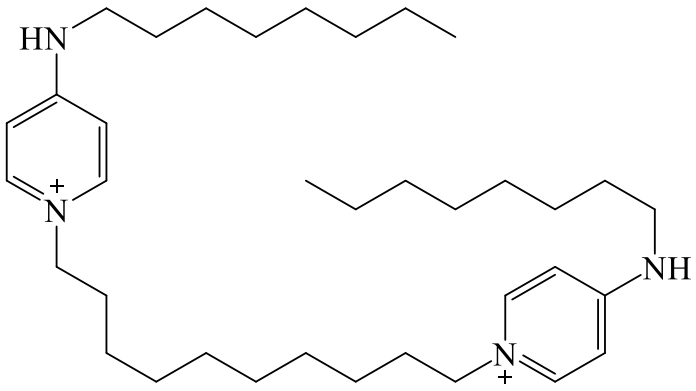

Figure 1.5. Structure of the octenidine cation.

1.4. Ionic liquids

Ionic liquids (ILs) are tunable, ionic compounds with melting points below 100 °C due to uneven packing of bulky, asymmetrical ions in the crystalline lattice structure. They are divided into two types: room temperature ILs that have melting points below 25 °C and frozen ILs that have melting points between 25-100 °C. Ionic liquids are known to have an array of physical properties such as, nonvolatility, nonflammability, negligible vapor pressure, high thermal stability, and high conductivity. Because of these unique characteristics, many ILs have been labeled as "green" as their use does not contribute to harmful environmental effects.[38] Another

sought after property of ILs is the high solvating power. Ionic liquids are able to solvate materials that traditional organic solvents cannot, such as biopolymers, including cellulose.[39-41] The high solvating power of ILs comes from the ability of the IL to act as both hydrogen bond acceptors and donors.[42]

1.4.1. GUMBOS

GUMBOS (**g**roup of **u**niform **m**aterials **b**ased on **o**rganic **s**alts) are a novel class of ionic materials developed in the Warner Research Group.[43] They consist of bulky organic or inorganic cationic and anionic moieties that can be tuned via a simple, facile metathesis reaction. In contrast to ILs which melt below 100 °C, GUMBOS are solid at room temperature and have melting points ranging from 25-250 °C. Like ILs, GUMBOS have tunable properties which can lead to changes in the chemical and physical properties.[43] These moieties typically determine the application of the compound which make them intrinsically task-specific for a variety of applications, such as sensors[44], nanotechnology[45], dye-sensitized solar cells[46], and mass spectrometry[47].

Biomedical applications of GUMBOS have been examined by exchanging the cation or anion with a therapeutic moiety. The Warner research group has reported chemotherapeutic applications by exchanging the chloride ion in rhodamine derivatives to make them more selective towards cancerous cells.[48] Similar chemotherapeutic effects were also seen with the near-infrared dye, IR-780.[49] Other applications of biomedical GUMBOS include sensors of hemoglobin, serum albumin proteins, and hydroxyl radicals.[50-52]

1.4.2. Antimicrobial Applications of GUMBOS and Ionic Liquids

Antimicrobial GUMBOS and ionic liquids can be fabricated from pharmaceutically active ingredients, like antibiotics and/or antiseptics, to increase efficacy, lower toxicity, and produce a synergistic effect. Significant improvements in the bactericidal activity of ampicillin was

demonstrated by Cole *et al.* (2011) when the ampicillin anion was combined with a QAC as the cation through ion-exchange reactions.[53] Chlorhexidine ampicillin GUMBOS demonstrated to be a viable alternative to antiseptics and antibiotics in the prevention of *Escherichia coli* O157:H7 infections.[54] GUMBOS synthesized from chlorhexidine and various outmoded β-lactam antibiotics exhibited promise as alternative combinatorial drug strategies for treating superficial wound infections caused by multi drug-resistant bacteria.[55] Lower concentrations of GUMBOS were needed to inhibit growth of multi drug-resistant bacteria better than the unreacted, stoichiometric mixtures of precursor ions. GUMBOS also extended the spectra of antibacterial activity. Moreover, GUMBOS were less toxic to common cell types, i.e. endothelial and fibroblasts, found in superficial and chronic wounds.[55]

1.4.3. Polyelectrolyte Complexes

Polyelectrolytes are macromolecules containing charged repeating units. They can be either cationic, anionic, or both.[56] Polyelectrolyte complexes (PECs) are association complexes formed between oppositely charged molecules, one of which is a macromolecule, for example, polymer-polymer, polymer-surfactant, polymer-drug, and polymer-drug-polymer.[56] PECs form due to electrostatic interactions between the ions. Polyelectrolyte complexes have a wide array of applications, such as membranes[57-58], coatings on films and fibers[59], for isolating nucleic acid[60-61], and as drug delivery agents[62-64]. In the concept of drug delivery systems, active components, i.e. drugs, can be incorporated in PECs through several ways.[65] Drug molecules can be entrapped during precipitation of the poly ion complex. Active components could be chemically bound to one of the poly ions and precipitate during complexation. Or, the active component itself could act as a poly ion and form PECs. In this last case, drug release could occur through solution equilibration, by charge interaction and decomplexation, or dissolution of the complex.[65]

1.5. Introduction to Wound Management

Wounds can occur due to various factors, including surgery, accidents or injuries, thermal damage, and chronic illnesses. Responsible and proper wound care promotes healing and recovery with minimization and prevention of bacterial infections. Keeping the area clean and clear of debris is the first step in properly caring for wounds. This allows the body to heal itself without local factors, such as contamination and/or colonization by microbes.[66] However, when breaks in the skin occur, microbes can enter causing infections. These infections can be treated with oral antibiotics and/or topical antiseptics. Antiseptics can come in many forms to help topically target microbes, such as, foams, lotions, ointments, mouthwashes, gels, etc.[67] Proper wound management also involves the use of barrier materials, an important step in defense against infection.

1.5.1. Barrier Materials for Wound Care

Caring for wounds involves the use of barrier materials, or wound dressings, to protect the wound from microbial contamination. Traditional dressings for wounds can consist of gauze, adhesive bandages, plasters, and cotton wool.[68] There are many disadvantages of these traditional materials such as failing to provide a moist environment and adhering to the wound site.[68] Traditional bandages are better suited as secondary dressings or for clean and dry wounds with low exudation.[69] Modern materials are more advanced and can facilitate the function of the wound instead of just being a covering. Modern dressings can be semi-permeable films, semi-permeable foams, hydrogels, hydrocolloids, bioactive materials, and medicated dressings.[69]

Semi-permeable films are composed of transparent and adherent polyurethane material that permits vapor permeability, conformability, and extensibility. They also promote autolytic debridement of necrotic tissue and are impermeable to bacteria.[70-71] Semi-permeable foam dressings are composed of hydrophilic and hydrophobic regions which allows gas and water vapor

exchange. These dressings are also highly absorbent and rely on exudates to facilitate healing.[72-73] Hydrogels are composed of insoluble hydrophilic polymers which have high water content allowing for soft elasticity, easy application and removal from wound site, and providing a moist environment for new tissue growth.[69] Hydrocolloid dressings consist of two layers, an inner colloidal layer and an outer water-impermeable layer, and are a combination of gel-forming agents with elastomers or adhesives.[68] These dressings are absorbent, permeable to water vapor, and moisturizing to wounds.[74] However, hydrocolloid dressings are typically used as secondary dressings.[68]

Bioactive materials are another type of modern wound dressings and are fabricated from biopolymers that play an active role in wound healing processes. These materials are known for their biocompatibility, biodegradability, and non-toxic nature and can be derived from various sources such as collagen, hyaluronic acid, alginates, chitosan, and elastin.[69] Biopolymers like chitosan, collagen, and hyaluronic acid aid in facilitating new tissue and cell growth.[75-77] Chitosan is also known to be inherently antimicrobial. Hyaluronic acid is a biopolymer produced in the extra cellular membrane with many properties such as hygroscopicity, viscoelasticity, and non-immunogenicity.[78] Bioactive dressings can also be incorporated with growth factors or antimicrobial agents to further enhance wound healing processes.[69]

1.6. Antimicrobial Testing and Preparation

1.6.1. Predictive Permeability

Membrane permeability is a highly desired trait and key property to consider during the drug design process. I*n vitro* assays assess and quantify the permeability of therapeutic agents across membranes. The Parallel Artificial Membrane Permeability Assay (Corning Gentest pre-coated PAMPA plate system; Corning Biosciences, MA) was employed in this dissertation

research. The PAMPA technique determines the passive permeability of therapeutic agents through a lipid-infused artificial membrane.[79] The configuration consists of a 96-well microtiter donor plate and an acceptor plate coated with a structured, tri-layer phospholipid membrane which is placed on top; the configuration is referred to as a "sandwich". Test solutions of known concentrations (e.g. 100-200 μM in buffer) are added to donor plates while only fresh buffer is placed in acceptor plates. Co-solvents, such as methanol, dimethyl sulfoxide (DMSO), or acetonitrile ranging in concentrations from 2-10 % can be used to aid in solubilizing test compounds in buffer. The assay is incubated for 5 hours at room temperature and the acceptor plate is measured using an ultraviolet-visible microtiter plate spectrophotometer. Permeability coefficients (Pe) are calculated based on initial concentration in donor well (C_O), concentration in donor well after 5 hours (C_D), concentration in acceptor well at 5 hours (C_A), volumes of donor (V_D) and acceptor wells (V_A), well filter area (A, 0.3 cm^2), and incubation time (t, 18000 s) using the relationship in Equation 1.1.

$$Pe\ (cm\ s^{-1}) = \frac{-\ln\left[\frac{C_A}{\frac{(C_D \times C_A) + (C_A \times V_A}{V_D + V_A}}\right]}{A \times \left(\frac{1}{V_D} + \frac{1}{V_A}\right) \times t} \quad (1.1)$$

1.6.2. Turbidity Standards for Bacterial Inoculum Preparation

For antimicrobial testing, McFarland standards are used as references to adjust the turbidity of bacterial suspensions in order to standardize the number of bacteria within a certain range. McFarland standards are prepared by mixing specified amounts of barium chloride $BaCl_2 \cdot 2H_2O$) and sulfuric acid (H_2SO_4) together producing a barium sulfate precipitate which leads to turbidity

of the solution. Preparation of a 0.5 McFarland standard matching an approximate cell density of 10^7-10^8 colony forming units (CFU)/mL as outlined by the Center for Disease Control and Prevention (CDC) consists of 0.5 mL of 1.175% w/v $BaCl_2 \cdot 2H_2O$ is added to 99.5 mL of 1% v/v H_2SO_4 under gentle stirring. The standard is dispensed into the same size vials as used for the bacterial suspensions to appropriately match turbidity. Standards should be stored in the dark at room temperature.[80]

1.6.3. Kirby-Bauer Disk Diffusion Susceptibility Test

Kirby-Bauer disk diffusion is a simple and practical qualitative, susceptibility test used to determine the sensitivity or resistance of a microorganism towards therapeutic agents.[81] Disk diffusion is performed by spreading a bacterial inoculum onto the surface of an agar plate to give a confluent lawn of growth. Agar plates can be commercially purchased, such as for *N. gonorrhoeae,* or prepared using a commercially available dehydrated base, such as for Müeller-Hinton agar. Antimicrobial solutions are pipetted onto sterile, dry 6mm paper disks. Solvent is subsequently evaporated. Paper disks impregnated with therapeutic agents are placed onto the surface of inoculated plates and incubated at 37 °C (depending on the bacterium, a 5% CO_2 atmosphere might be required) for 20-24 hours.

Therapeutic agents on the disks diffuse into the agar causing different size "halos" to form where bacterial growth is inhibited called zones of inhibition (Figure 1.6). The efficacy of therapeutic agents as susceptible, intermediate, or resistant is categorized based on zone diameter limits determined by the Clinical and Laboratory Standards Institute (CLSI), formerly the National Committee for Clinical and Laboratory Standards (NCCLS).[82-83] The concentration of therapeutic agent is largest closest to the disk and logarithmically decreases as distance increases because the rate of diffusion through agar is less rapid than the rate of extraction of drug out of the disk.[84-85]

The rate of diffusion is governed by many different factors such as, molecular weight, aqueous solubility, relative hydrophobicity, and intrinsic resistance of the microorganism.[86] Smaller molecules will diffuse through agar more rapidly than larger molecules and hydrophobic molecules diffuse more slowly than hydrophilic molecules.[85] Due to these factors, hydrophobic drugs can often be misidentified as poor antimicrobial agents. One major disadvantage of the disk diffusion method is that minimum inhibitory concentrations cannot be determined from the zone diameters.

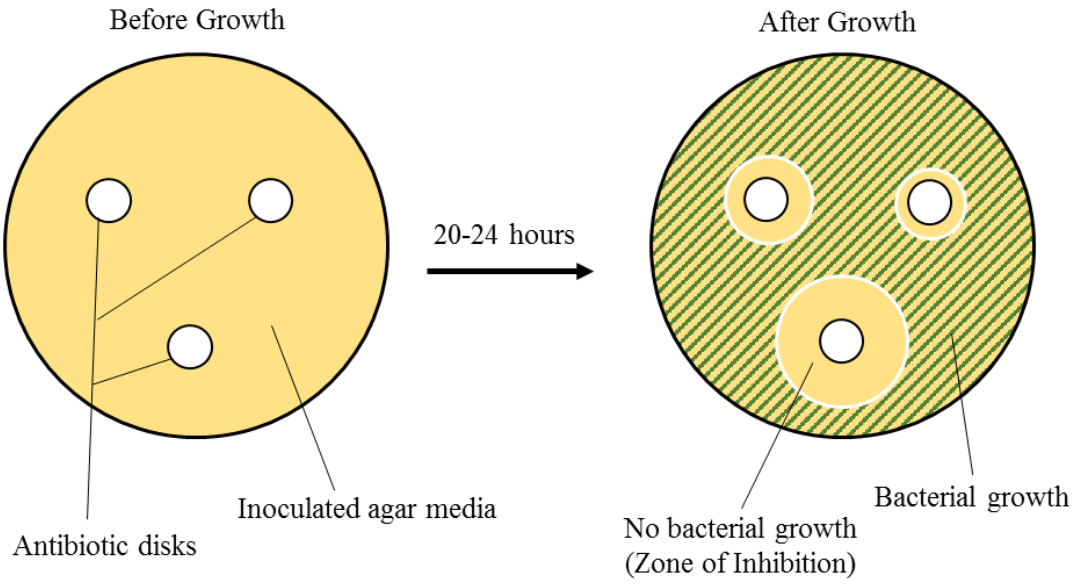

Figure 1.6. Kirby-Bauer disk diffusion susceptibility test showing zones of inhibition.

1.6.4. Liquid media for *Neisseria gonorrhoeae*

Neisseria gonorrhoeae is a fastidious bacterium that requires a complex nutrient regimen in order to be grown successfully and is often difficult to cultivate in liquid media. Commercial liquid media, defined or undefined, is not available for purchase. A fully defined, clear, protein-free liquid media was made according to methods reported by Wade and Graver (2007).[87] Various growth nutrients in defined quantities were added to a base of normal strength M199 cell culture

medium. This liquid medium allowed dense growth of *N. gonorrhoeae* for use in broth dilution tests.

1.6.5. Broth Dilution Susceptibility Test

Broth dilutions tests are quantitative measures of susceptibility of microorganisms towards therapeutic agents. In this method, depicted in Figure 1.7, two-fold serial dilutions of therapeutic agents prepared in liquid growth medium are dispensed in 96-well microtiter plates.[88] Therapeutic agents are initially prepared in deionized water or culture medium and up to 2% of methanol, DMSO, or acetonitrile can be added to aid in solvation. This concentration of organic solvent does not inhibit growth in liquid media.[89] Therapeutic agent-containing wells are inoculated with equal volume of bacterium matching a 0.5 McFarland standard. Microtiter plates are incubated for 20-24 hours at 37 °C and examined for visible signs of bacterial growth which is evidenced by turbidity. The lowest concentration of therapeutic agent that prevents visible growth of bacteria is known as the minimum inhibitory concentration (MIC). This is a high through-put method that can test up to 12 compounds over a range of 8 concentrations.

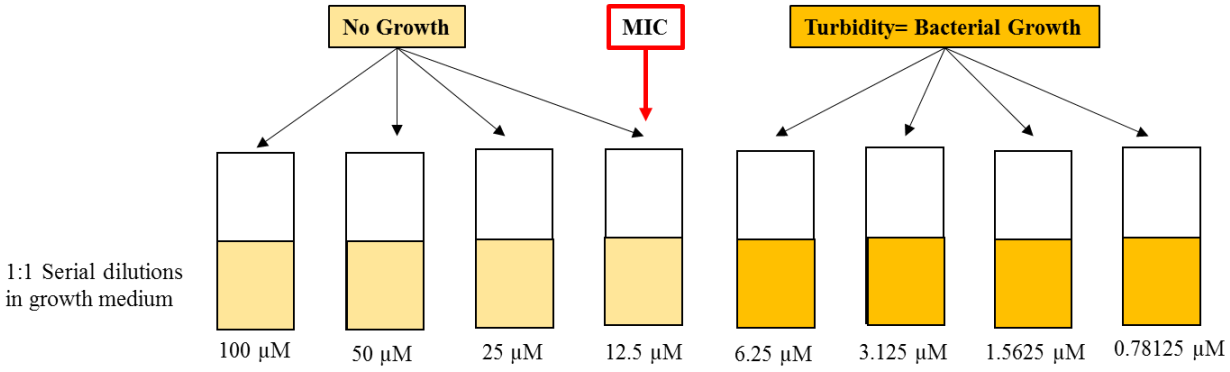

Figure 1.7. Example of broth dilution susceptibility test showing determination of minimum inhibitory concentration.

1.7. Ultraviolet-Visible Spectroscopy

Ultraviolet-visible (UV-Vis) absorption spectroscopy is an analytical technique that quantifies the molecular absorption of an analyte as light passes through a sample. Absorbance-based techniques can be used for a wide variety of applications, such as examining the photochemistry of a molecule to biomedical applications for evaluating drug release and viability of microorganisms. More specifically, this technique measures the fraction of the intensity of incident light (I_0) at a given wavelength that strikes the sample and the subsequent intensity of light after passing through the sample (I), known as transmittance (T). Equations 1.2 and 1.3 show the relationship between transmittance and absorbance (A). Absorbance is also directly proportional to concentration (C) and pathlength of light through the width of the cuvette (b). The proportionality is converted into a constant (ε) known as the molar extinction coefficient which is a measure of how strongly a chemical species absorbs light at a wavelength. This is known as Beer-Lambert's Law.[90]

$$T = \frac{I}{I_0} \tag{1.2}$$

$$A = -\log\frac{I}{I_0} = \varepsilon bC \tag{1.3}$$

The instrumental configuration and working principle of a conventional spectrophotometer is displayed in Figure 1.8.[91] A beam of light is released from a source which can be either continuum or line based. Continuum sources of light consist of deuterium, hydrogen, tungsten, or xenon lamps. Line-based light sources can consist of hollow cathode tubes or lasers. A 75-watt tungsten-halogen continuum lamp was used in the UV-Vis spectrophotometer employed in this dissertation research. Light passes through to a monochromator that mechanically selects specific wavelength ranges for the study. Light from the monochromator irradiates the sample. Samples

are placed in optically transparent sample holders called a cuvette. Cuvettes are made of either glass, quartz, or plastic. Transmitted light that passes through the sample is then recorded by the detector, which can be either a photomultiplier tube or photodiode array.

In this dissertation, absorbance measurements were used to determine *in vitro* drug release, predictive permeability, and cell viability. A 96-well microtiter plate reader was used for absorbance measurements of cell viability in place of a conventional spectrophotometer as these studies were carried out in 96-well microtiter plates. These two instruments operate on the same principle; however, a microplate reader has a vertical light beam instead of the conventional horizontal beam (Figure 1.9). Regarding Beer-Lambert's Law, the sample volume of the microplates replaces the pathlength of the cuvette.

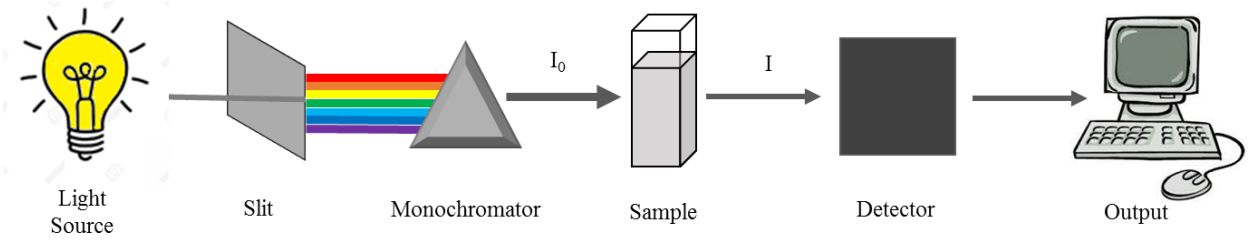

Figure 1.8. Instrumental configuration of conventional absorbance spectrophotometer.

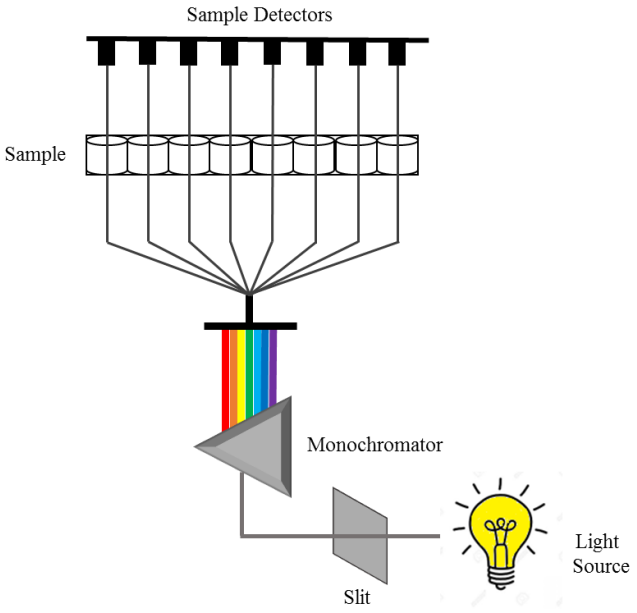

Figure 1.9. Instrumental configuration of a microtiter plate reader.

1.7.1. Mammalian Cell Cytotoxicity

In vitro cytotoxicity cellular studies are essential to determine the effect of antimicrobial drugs on the function of various cellular processes. These assays determine therapeutic efficacy and biocompatibility of the developed compounds. Several colorimetric assays with varying detection methods have been developed; however, detection of formazan dye is the most common.[92] In this method, a tetrazolium salt is reduced by dehydrogenases and reductase enzymes that are only found in living cells. Formazan dyes are the reduced product and come in a variety of colors depending on the original salt substrate. The measurement of formazan dyes is dependent on several factors, such as, cell type, number of cells, incubation time, and type of assay used.[93] In this dissertation, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) toxicity assay was used to assess the cytotoxicity of developed GUMBOS. In the presence of living cells, the yellow MTT dye is reduced by mitochondrial reductase enzymes to insoluble purple formazan crystals. This reaction is presented in Figure 1.10. Subsequently, a sodium dodecyl sulfate dimethylformamide solution is used to dissolve the purple formazan crystals and create a homogenous solution. Absorbance is measured at 570 nm and is proportional to the number of living cells, as only the living cells will have the reductase enzymes that cause the purple color.[94]

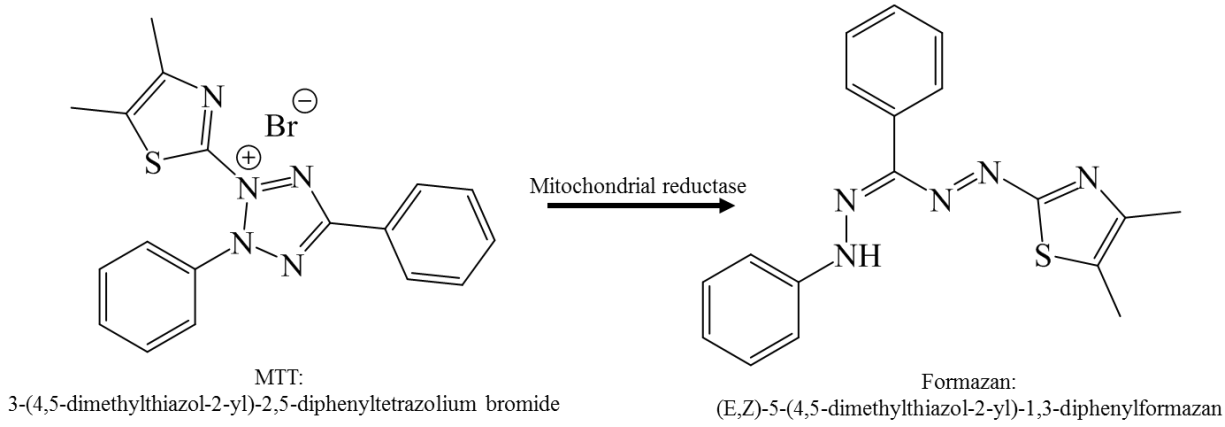

Figure 1.10. Enzymatic reaction of the cleavage of MTT to formazan in the presence of mitochondrial reductase.

1.8. Scope of Dissertation

The objective of this dissertation was to develop topical applications using GUMBOS and polymers for preventing and minimizing bacterial infections. The second and third chapter of this dissertation focused on fabricating antimicrobial GUMBOS from antibiotics and antiseptics for the prevention of oropharynx *Neisseria gonorrhoeae*. Antimicrobial efficacy, such as zones of inhibition and minimum inhibitory concentrations, was determined. Chapter two focused on recycling an outmoded antibiotic whereas Chapter three focused on synthesizing GUMBOS from ceftriaxone, the currently prescribed antibiotic for gonorrhea infections.

The fourth chapter is a description of composite materials fabricated from two polymers, cellulose and hyaluronic acid, for the purpose of wound care. Composites of differing polymer ratios were constructed and evaluated for properties such as exudation capacity. Furthermore, these composites were drug loaded and investigated as medicated patches.

The fifth chapter centers around polyelectrolyte, or ionic, complexes synthesized from polyanionic hyaluronic acid and cationic antiseptics. These ionic complexes were characterized and investigated as possible applications for skin and oral disinfection. The sixth chapter concludes this dissertation by summarizing findings and discusses future research aims for the work presented.

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CHAPTER 2. OCTENIDINE-CARBENICILLIN GUMBOS AS POTENTIAL TREATMENT FOR OROPHARYNGEAL GONORRHEA

2.1. Introduction

Neisseria gonorrhoeae is a Gram-negative diplococcus that infects human mucosal membranes. The areas of the body most commonly infected are the urethra, cervix, rectum, oropharynx, and conjunctiva.[1] This microorganism causes an inflammatory disease known as, gonorrhea, which infected more than 500,000 Americans in 2018.[2] It is the second most common notifiable infection in the United States.[3] However, the Centers for Disease Control and Prevention (CDC) estimate that approximately 1.14M infections occur annually in the United States and has named *N. gonorrhoeae* an urgent antibacterial-resistant threat.[4-5] In 2019, the CDC estimated that 550,000 infections would be antibiotic resistant to at least one prescribed antibiotic.[5] The World Health Organization (WHO) estimates that 78 million new cases of gonorrhea occur annually.[6] Asymptomatic infections are very common in both males and females,[7-8] and if left untreated, complications of the disease include pelvic inflammatory disease, life-threatening ectopic pregnancy and infertility, arthritis, and an increased susceptibility to HIV/AIDS.[9-12] Gonorrhea can be treated with antibiotics, such as, ceftriaxone and azithromycin. However, the organism has a long history of readily developing resistance to therapeutic regimens.[2]

Drug resistant *N. gonorrhoeae* isolates from the oropharynx have emerged, most likely because there is poor drug penetration into pharyngeal tissue.[13] While current studies may have limitations, it has been recently demonstrated that gonococci could possibly be transmitted person to person strictly from "deep kissing."[14-15] *N. gonorrhoeae* can also persist in the oropharynx as most cases are asymptomatic or are misdiagnosed as other kinds of pharyngitis.[16-17] The oropharynx is thought to act as a "silent reservoir" whereby gonococcus acquires its resistance determinants from commensal *Neisseria* species and other bacterial species of asymptomatic

individuals which allows for horizontal gene transfer. For this reason, decolonization of these individuals might prevent the emergence of multi-drug resistant gonorrhea.[18-20]

Currently, the CDC recommends a dual regimen of 250 mg ceftriaxone administered intramuscularly and 1 g azithromycin orally.[21] While these antibiotics remain effective generally, resistance rates are rising globally. In 2010, a Swedish heterosexual man, who presented with oropharyngeal gonorrhea required several rounds of ceftriaxone with increasing dosage.[22] In 2017, a heterosexual man from the United Kingdom acquired drug-resistant oropharyngeal gonorrhea in Southeast Asia. The infection did not respond to a high dosage of ceftriaxone (1 g) and spectinomycin, which indicates high resistance. The infection was cleared only after intravenous administration of ertapenem, an antibiotic belonging to the potent β-lactam class of carbapenems that are often agents of "last resort".[23-24]

A possible solution for oropharyngeal gonorrhea could be use of antiseptics as they are unlikely to induce resistance. Antiseptics have been explored as treatments for acute gonorrhea in the past[25] and antiseptic mouthwashes have been shown to have an antimicrobial effect on oropharyngeal gonorrhea.[26] One such antiseptic, octenidine dihydrochloride, exhibits a broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria.[27] This antiseptic has shown significant efficacy in periodontology[28] and is even sold as a commercially available mouthwash under the name Octenisept®.[29] It has also been approved for use on skin, mucosal membranes, and wound antisepsis.[30] In this study, we aim to target and minimize gonorrhea infections by synthesizing an antiseptic-based compound that can be used as alternative therapy for gonorrhea, specifically oropharyngeal gonorrhea.

Bringing new antibiotics to market requires not only years of research and development but can also cost billions of dollars.[31] In response to this global crisis, the literature supports using

GUMBOS (Group of Uniform Materials Based on Organic Salts) as antimicrobial agents. GUMBOS are a novel group of solid phase organic salts, typically using ionic liquid counter-ions; however, the melting point range of GUMBOS have been extended beyond that of ionic liquids.[32] The melting point of GUMBOS fall in the range of 25-250 °C, whereas for ionic liquids the melting point lies below 100 °C. These compounds have uniquely tunable properties that can be incorporated into the salt via careful selection of counterions.[32] GUMBOS are stable, relatively non-toxic as compared to their constituent compounds, and are as effective as or better than conventional antibiotic therapy against multi-antibiotic resistant bacteria.[33-34] Antimicrobial GUMBOS are simple and inexpensive to synthesize from existing, well known compounds such as antiseptics and antibiotics in their salt forms.

In this study, we strategically design GUMBOS synthesized from octenidine and carbenicillin, a β-lactam antibiotic with high efficacy against various Gram-negative bacteria and increased thermal and pH stability in solution.[35-37] However, carbenicillin is no longer administered due to toxicity issues at high concentrations[38-39]. By ion-exchanging the sodium ions on carbenicillin with octenidine, it is hypothesized that the toxicity will be improved.[33] The *in vitro* antibacterial efficacy of GUMBOS against *N. gonorrhoeae* ATCC 49226 and clinical isolates of *N. gonorrhoeae* was evaluated using disk diffusion susceptibility tests. Comparative analyses of octenidine-carbenicillin GUMBOS, their constituent parts, unreacted stoichiometric mixtures, and current treatments for gonorrhea confirm the potential of this approach as an alternative therapy against the threat of antibiotic resistance. The toxicities of these GUMBOS were also evaluated.

2.2. Materials

Disodium carbenicillin, methanol (MeOH), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Milwaukee, WI). Octenidine dihydrochloride was purchased from

TCI Chemicals (Japan). Cell viability MTT (3-[4,5- Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay was purchased from Promega Corporation (Madison, WI). Prepared agar plates (BD BBL Prepared Plate Media: GC II agar with IsoVitalex™ Enrichment) and Oxoid™ antimicrobial susceptibility discs (ceftriaxone, azithromycin, doxycycline, and blank disks; 6 mm) were purchased from Fisher Scientific (Pittsburg, PA). Parallel Artificial Membrane Permeability Assay (Gentest™ pre-coated PAMPA plate system) was purchased from Corning Incorporated (Tewksbury, MA).

2.3. Experimental Section

2.3.1. Synthesis and Characterization of β-lactam-based GUMBOS

Synthesis and characterization of octenidine-carbenicillin ([OCT][CAR]) GUMBOS were performed using similar methods previously reported by Cole *et al.* (2015)[33] with slight modification. In this work, octenidine-carbenicillin was synthesized using ion-exchange procedures that involved stirring stoichiometric amounts of octenidine dihydrochloride (OCT 2HCl) and disodium carbenicillin (Na_2 CAR) for one hour at room temperature in deionized water (Figure 2.1). The resulting precipitate was washed several times with cold, deionized water and removed by lyophilization overnight. The structure of [OCT][CAR] was characterized using ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR), and Fourier transform infrared spectroscopy (FT-IR). Spectra is provided in Appendix A Figures A1, A2, and A3, respectively. High resolution mass spectrometry *m/z* is not reported as no useful spectrum was obtained which can occur with carbenicillin.[40]

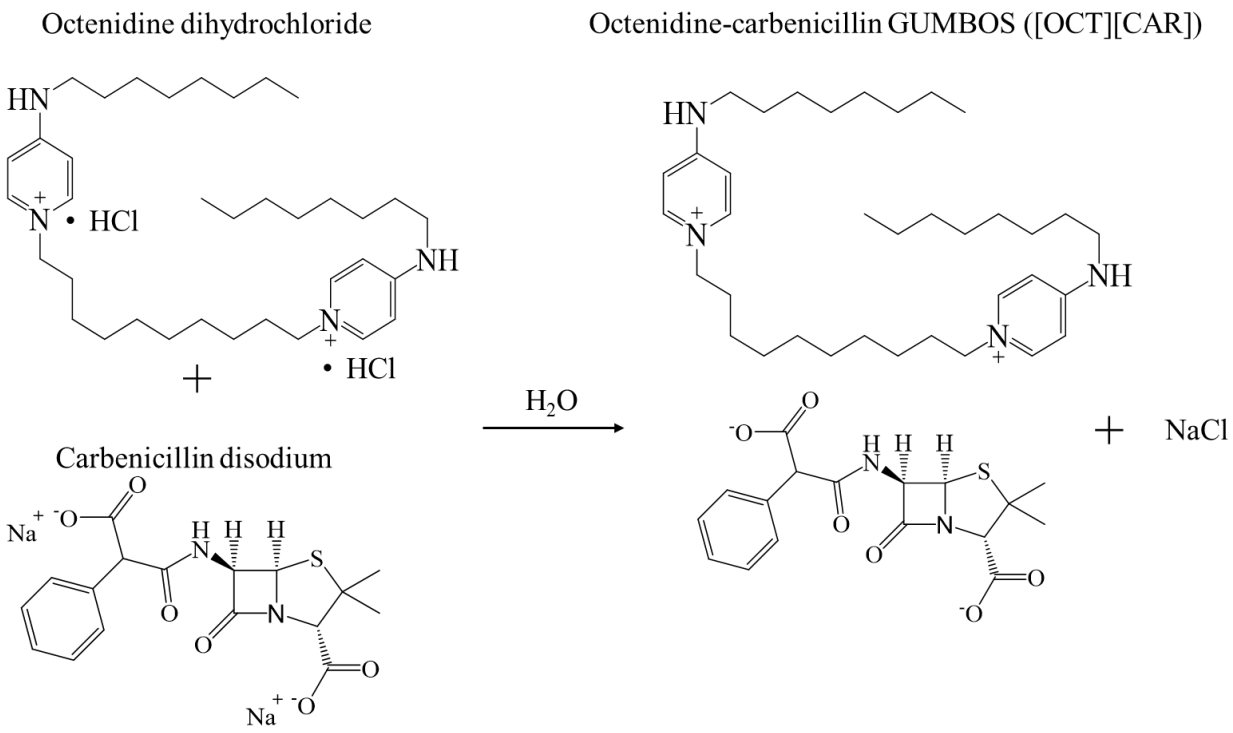

Figure 2.1. Synthesis and structures of precursor ions and octenidine-carbenicillin GUMBOS.

2.3.2. Predictive Intestinal Permeability

Parallel Artificial Membrane Permeability Assay (PAMPA) was employed as an *in vitro* model of passive, transcellular permeation. In this technique, a 96-well microtiter plate is used as a donor plate and a membrane/acceptor compartment coated with structured tri-layers of phospholipids is placed on top; this configuration is referred to as a "sandwich". Known concentrations of [OCT][CAR] (100 μM in 1X PBS, 0.25% DMSO) were added to the donor plate while only buffer was placed in the acceptor plate. The assay was incubated for five hours at room temperature and the acceptor plate was measured using a UV-Vis spectroscopy plate reader (Eppendorf PlateReader AF2200). Permeability coefficients (Pe) were calculated based on initial concentration in donor well (Co), concentration in donor well at five hours (CD), concentration in acceptor well at five hours (CA), volumes of donor (VD) and acceptor wells (VA),

well filter area (A, 0.3 cm^2), and incubation time (t, 18000 s) as calculated using the relationship in Equation 2.1.

$$\mathbf{Pe\ (cm\ s^{-1})} = \frac{-\ln\left[\frac{C_A}{\frac{(C_D \times C_A) + (C_A \times V_A)}{V_D + V_A}}\right]}{A \times \left(\frac{1}{V_D} + \frac{1}{V_A}\right) \times t} \quad (2.1)$$

2.3.3. Antimicrobial Susceptibility Testing

Kirby-Bauer disk diffusion susceptibility tests were used to determine zones of inhibition (ZOI) for *N. gonorrhoeae* (ATCC 49226) and three clinical isolates obtained from Louisiana State University Health Sciences Center New Orleans HIV Outpatient Clinic. Kirby-Bauer disk diffusion is one of the CDC's preferred methods of testing susceptibility of *N. gonorrhoeae*.[41] Testing was performed according to Clinical and Laboratory Standards Institute (CLSI) recommended procedures.[41] In this susceptibility test, six millimeter diameter paper disks were impregnated with known amounts of antimicrobial drug and solvent was evaporated. Dry, impregnated disks were placed onto prepared nutrient agar plates that were inoculated with *N. gonorrhoeae* to give a confluent lawn of growth. Suspensions of the strains were prepared in accordance with a 1.0 MacFarland standard. Inoculated agar plates were incubated for 20-24 hours at 37°C in 5% CO_2 atmosphere.

2.3.4. Cytotoxicity Assay

In order to determine cell viability, a colorimetric MTT dye assay (Promega Corp., Madison, WI) employing manufacturer's instruction was used as an indicator of cytotoxicity of GUMBOS towards healthy (HeLa) cells. HeLa cells (ATCC CCL-2) grown in Dulbecco's modified Eagle's medium-reduced serum supplemented with 10% fetal bovine serum were plated

at a density of $1x10^5$ cells/mL (10k cells/well) in 96-well plates. Concentrations of therapeutic agents up to 500 µM (1% DMSO or MeOH) were doubly diluted in cell culture media and transferred to seeded cells. Cells were incubated for 15 minutes and 60 minutes at 37 °C in 5% CO_2 atmosphere. Cells treated with only medium served as a negative control. At the end of incubation period, 15 µL of MTT was added to each well and continued incubation for an additional hour. Absorbance was measured at 570 nm in a microplate spectrophotometer. All experiments were performed in quadruplicate. Cell viability as a percentage was determined as the ratio between treated cells and untreated (control) cells taken as 100%. Reported values are the lethal concentrations able to kill 50% of the population of viable cells (IC_{50}).

2.4. Results and Discussions

2.4.1. Characterization of Octenidine-Carbenicillin GUMBOS

Off-white solid, yield 90%. ^{1}H-NMR (400Hz, DMSO-*d6*) δ 9.03 (br. s, 1 H), 8.64 (d, J=8Hz, 1 H), 8.26 (dd, J=8Hz, 2H), 8.09 (dd, J=8Hz, 2H), 7.30-7.19 (m, 6H), 6.95 (dd, J=8Hz, 2 H), 6.89 (dd, J=8Hz, 2H), 5.29-5.25 (m, 2H), 4.08 (t, J=8Hz, 4H), 3.81 (s, 1H), 3.53 (q, 2H), 3.24 (t, J=4Hz, 8Hz, 4H), 1.72 (q, 4H), 1.55-1.51 (m, 8H), 1.43 (s, 3H), 1.32-1.22 (m, 33H), 0.85 (t, J=8Hz, 6H). ^{13}C-NMR (125 Hz, DMSO-*d6*) 170.46, 170.12, 169.23, 156.20, 142.89, 140.34, 135.58, 128.82, 128.72, 128.53, 127.62, 127.39, 125.71, 110.23, 104.33, 77.50, 70.77, 57.45, 56.05, 55.12, 42.43, 41.72, 30.72, 29.69, 28.72, 28.22, 28.19, 28.13, 28.11, 27.84, 27.40, 25.85, 24.82, 21.56, 13.41

2.4.2. Predictive Permeability

Octenidine 2HCl is a dicationic molecule with two chloride anions, and this structure does not permeate through skin, mucus membranes, wounds, or the placental barrier.[42] With an anion exchange metathesis from chloride ions to carbenicillin (a dianion), permeability increased

significantly, which inevitably also increases bioavailability. Mean effective permeability coefficient for [OCT][CAR] was 3.78 x 10^{-6} (±0.85) which falls in the range of high permeability. Permeability coefficients that are greater than 1.5x10^{-6} cm/s exhibit high permeability, whereas coefficients less than 1.5x10^{-6} exhibit low permeability.[43] This suggests that [OCT][CAR] and octenidine dihydrochloride behave chemically different and thus, GUMBOS may behave as a new ion pair when used therapeutically.

2.4.3. Antibacterial Activity of Octenidine-Carbenicillin GUMBOS Using Disk Diffusion

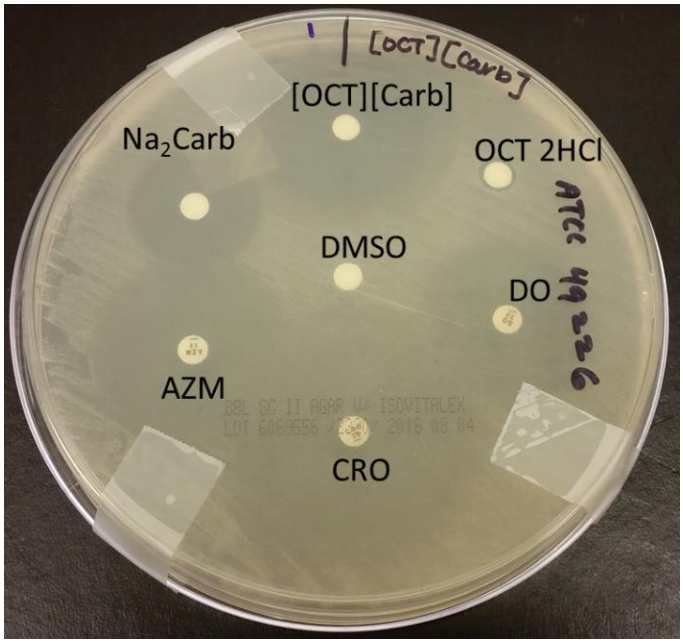

Figure 2.2. Kirby-Bauer disk diffusion susceptibility test on *Neisseria gonorrhoeae* grown on GC II agar with IsoVitalex™ Enrichment with disks of [OCT][CAR] GUMBOS, Na_2CAR, OCT 2HCl, DMSO (negative control), azithromycin, ceftriaxone, and doxycycline.

Antibacterial activity of [OCT][CAR] was compared to individual parent compounds, unreacted stoichiometric mixtures, and current therapeutic agents, ceftriaxone, azithromycin, and doxycycline using Kirby-Bauer disk diffusion (Table 2.1). Blank disks were loaded with 50 nanomoles of GUMBOS, respective constituent compounds, and equivalent stoichiometric unreacted mixtures of octenidine and carbenicillin which is equimolar to the purchased ceftriaxone disks (Oxoid™, Thermo Fisher). After 20-24-hour incubation, diameters of ZOIs were measured

using a ruler. Ceftriaxone (30 µg, 50 nanomoles), azithromycin (15 µg, 20 nanomoles), and doxycycline (30 µg, 70 nanomoles) had zone sizes within the susceptibility range set by the CLSI for *N. gonorrhoeae* (ATCC 49226) and clinical isolates tested. Antibacterial activity was improved for [OCT][CAR] when chloride ions were exchanged for the antibiotic. Through synthesis of octenidine-carbenicillin GUMBOS, antibacterial activity exhibited an additive effect for *N. gonorrhoeae* (ATCC 49226) and clinical isolates. This effect was not seen, however, for the unreacted mixture of the two drugs for either *N. gonorrhoeae* (ATCC 49226) or clinical isolates. The ZOIs of [OCT][CAR] were also larger than or equal to azithromycin and doxycycline ZOIs for *N. gonorrhoeae* (ATCC 49226). When comparing ZOIs of [OCT][CAR] to azithromycin and doxycycline for the clinical isolate population, [OCT][CAR] exhibited equal efficacy to azithromycin while exhibiting superior activity to doxycycline, an antibiotic prescribed in the case of gonorrhea and chlamydia co-infection.[44-45] As a result of these efficacy values, antibacterial activity of [OCT][CAR] is shown to be bioequivalent to azithromycin. This is significant as resistance rates for azithromycin have increased globally. Among 57 countries reporting on azithromycin susceptibility, 28 (49%) reported >5% resistance.[46] In the United States, where clinical isolates were obtained, resistance increased largely from 2014-2019. The percentage of isolates with elevated resistance rates to azithromycin increased from 2.5% to 4.6%.[3] Ceftriaxone produced large ZOIs as the clinical population still remains susceptible to it in the United States (only 0.2% of isolates had elevated resistance to ceftriaxone in 2017).[3] Figure 2.2 shows an agar plate with ZOIs from disks of [OCT][CAR] GUMBOS, Na_2CAR, OCT 2HCl, DMSO (negative control), azithromycin, ceftriaxone, and doxycycline.

Table 2.1. Zones of inhibition for *N. gonorrhoeae* ATCC 49226 and clinical isolates.

Test Materials [a]	Quantity of Material (Nanomoles)	ZOI (mm)	
		ATCC 49226	Clinical Isolates
CRO	50	51 ± 1	49 ± 4
AZM	20	40 ± 1	36 ± 4
DO	70	35 ± 0.6	28 ± 7
[OCT][CAR]	50	40 ± 0.6	37 ± 4
1 OCT:1 CAR	50	30 ± 1	34 ± 3
OCT 2HCl	50	9 ± 1	9 ± 0.3
Na_2 CAR	50	27 ± 3	28 ± 3

[a] CRO: ceftriaxone; AZM: azithromycin; DO: doxycycline

2.4.4. Cytotoxicity of Octenidine and Carbenicillin in Combination and as GUMBOS

Following characterization and antimicrobial efficacy, GUMBOS were employed in vitro to assess their cytotoxicity versus the stoichiometric, unreacted mixture and octenidine dihydrochloride. When tested in vitro, β-lactam antibiotics are known to be highly nontoxic.[47] Octenidine dihydrochloride has also shown low cytotoxic potential when tested against human primary gingival fibroblasts and human primary nasal epithelial cells.[48] For a general approximation of system toxicity and possible application beyond mouthwash, such as a vaginal douche, cervical cells were used. Cytotoxicity was assayed at 15 and 60 minutes because oral rinses are most beneficial if an individual does not drink liquids at least 15 to 60 minutes after use. If also used to eradicate infections of the cervix, this time frame allows ample time for the drug to remain in contact with cervical tissue before being removed from the body. The IC_{50} values for OCT 2HCl, unreacted mixture, and [OCT][CAR] are reported in Table 2.2.

After 15 minutes of incubation, [OCT][CAR] showed slightly lower toxicity than the stoichiometric, unreacted mixture, while OCT 2HCl was less toxic to cervical cells. After 60 minutes of incubation, toxicity was equivalent for [OCT][CAR], unreacted mixture of OCT 2HCl and Na_2 CAR, and OCT 2HCl. While the cytotoxicity potential varied only slightly between GUMBOS and its mixture, the unreacted mixture of the two antimicrobial compounds still

contains the two sodium ions, which may aggravate hypertension or congestive heart failure.[49-50] GUMBOS would, therefore, be inherently safer since the sodium ions are removed entirely and replaced with octenidine, which has much lower toxicity.

Table 2.2. Acute cytotoxicity (IC_{50}, μM) of octenidine-carbenicillin GUMBOS for 15 and 60 minutes against HeLa cells.

Test Materials	IC_{50} (μM)	
	15 min	**60 min**
OCT 2HCl	55 ± 2	26 ± 1
1 OCT: 1 CAR	48 ± 4	25 ± 1
[OCT][CAR]	50 ± 2	25 ± 0.3

2.4.5. Octenidine-Carbenicillin GUMBOS to Reduce Transmission of Gonorrhea

In 2019, a new paradigm for the transmission of extra-genital *N. gonorrhoeae* emerged. Oropharyngeal infections were noted in the absence of urogenital infections[51] and it was hypothesized that kissing or saliva exchange could be an unrecognized means of transmission.[14, 52] It was proposed that antiseptic mouthwashes might offer a condom-free control strategy.[15] In this regard, Chow *et al*. reported that use of Listerine® mouthwash as a gargle could significantly reduce the amount of *N. gonorrhoeae* in the oropharynx of men who have sex with men (MSM).[26] With this study, we propose that octenidine-carbenicillin GUMBOS could be incorporated into a mouthwash for this purpose.

The constituent components of our GUMBOS, octenidine and carbenicillin, have established safety profiles. Octenidine is currently formulated as a mouthwash and carbenicillin, like most beta-lactam antibiotics, is relatively non-toxic to mammalian cells as shown in Table 2.2. We also believe that our GUMBOS, formulated as suppositories, could be used to reduce colonization in other anatomical sites such as the vagina and rectum.

2.5. Conclusion

As the threat of antibiotic resistance in *N. gonorrhoeae* increases and oropharyngeal cases become more difficult to treat, alternative therapies are very much in need. This study suggests that octenidine-carbenicillin GUMBOS may be a viable alternative therapy for prevention and minimization of *Neisseria gonorrhoeae*. [OCT][CAR] was found to be bioequivalent to azithromycin and doxycycline as determined by Kirby-Bauer disk diffusion assays. Moreover, [OCT][CAR] exhibited higher efficacy than the constituent parent compounds and unreacted mixtures. Cytotoxicity results showed that [OCT][CAR] was also nontoxic towards cervical cells. This approach of fashioning antimicrobial agents into GUMBOS may offer an alternative approach to current drug therapies for gonorrhea and have further implications for topical, prevention strategies.

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CHAPTER 3. EFFICACY OF CEFTRIAXONE-BASED GUMBOS AGAINST *NEISSERIA GONORRHOEAE* AND CARBAPENEM-RESISTANT *ENTEROBACTERIACEAE*

3.1. Introduction

The sexually transmitted infection, gonorrhea, is a global public health threat with the Centers for Disease Control and Prevention (CDC) and World Health Organization (WHO) estimating that more than 820,000 Americans and 78 million people worldwide are infected annually.[1-2] Gonorrhea affects human superficial mucosal surfaces and is a major cause of pelvic inflammatory disease in the United States.[3-5] Gonorrhea is easily treated with antibiotics, however; a cause for great concern is that *Neisseria gonorrhoeae,* the Gram-negative etiological agent of gonorrhea, has shown a limitless capacity for increased resistance to antibiotic treatments. It has become resistant to all antibiotics, except third generation cephalosporins, elevating its status to "superbug".[6-8] Since the 1980s, doctors have had to abandon one therapy after another—penicillin, tetracycline, ciprofloxacin, and most recently cefixime.[9] This resistance is due to the ability of N. gonorrhoeae to mutate through two means: chromosomal and plasmid-mediated mutation.[6, 9] One such mutation has led to the production of beta (β)-lactamases, enzymes that provide resistance to antibiotics by breaking the antibiotics' structure rendering it non-effective, which was one of the causes for penicillin resistance.[10-13] The current treatment for gonorrhea infections, as recommended by the CDC, is now dual antibiotic therapy of ceftriaxone (250 mg intramuscularly) and azithromycin (1 g orally).[14-15] However, the current antibiotic regimen is being threatened as globally emerging and increasingly resistant strains are developing. In the United States, only 0.2% of isolates had elevated ceftriaxone minimum inhibitory concentrations (MICs) whereas azithromycin had a much larger increase of isolates with elevated MICs.[16] From 2013-2014, isolates with elevated azithromycin MICs had a sharp increase from 0.6% to 2.5% and during

2014-2017, the percentage increased from 2.5% to 4.4%.[16] While few isolates in the United States have exhibited decreased susceptibility to ceftriaxone, WHO has reported an increase in resistant *N. gonorrhoeae* cases in more than 50 countries.[17] Decreased susceptibility of N. gonorrhoeae to cephalosporins is expected to spread and the threat of returning to the pre-antibiotic era of untreatable infectious disease is very real.[18] This would significantly complicate the ability of providers to treat this bacterium as few antibiotic options are available or in development.

In response to this alarming crisis, a strategy was developed for producing antimicrobial agents as GUMBOS (**g**roup of **u**niform **m**aterials **b**ased on **o**rganic salts) through metathesis reactions.[19] This strategy aims to target *N. gonorrhoeae* using ceftriaxone-based GUMBOS. GUMBOS are tunable, solid phase organic salts that traditionally use ionic liquid counter-ions but have a defined melting point range from 25-250 °C whereas ionic liquids have melting points below 100 °C.[19] By coupling functional cations and functional anions, this approach could lead to many applications, including antimicrobial chemotherapeutics. As most antiseptics and antibiotics are in salt forms, antimicrobial GUMBOS can be easily prepared via ion-exchange reactions. GUMBOS fabricated from such antiseptics and antibiotics have been shown to have lower toxicity and higher efficacy than their constituent parts.[20-21] This study focuses on fabrication and characterization of GUMBOS from common antiseptics, chlorhexidine diacetate and octenidine dihydrochloride, and ceftriaxone disodium salt and their efficacy in killing *N. gonorrhoeae*. The efficacy of these GUMBOS against members of carbapenem-resistant *Enterobacteriaceae* (CRE), a family of multi-drug resistant Gram-negative bacteria that pose a global threat to human health, is also demonstrated.

3.2. Materials

Ceftriaxone disodium salt, octenidine hydrochloride, and azithromycin were purchased from TCI. Chlorhexidine diacetate was purchased from Acros Organics. A cell viability MTT (3-[4,5- Dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay was purchased from Promega Corporation (Madison, WI). Prepared agar plates (BD BBL Prepared Plate Media: GC II agar with IsoVitalex™ Enrichment) and Oxoid™ antimicrobial susceptibility discs were purchased from Fisher Scientific (Pittsburg, PA). Cation-adjusted Muller Hinton Brother and reagents for preparing a fully defined, clear, protein-free liquid media as described by Wade and Graver (2007)[22] were purchased from Fisher Scientific (Pittsburgh, PA).

3.3. Experimental

3.3.1. Synthesis of Ceftriaxone-based GUMBOS

Ceftriaxone-based GUMBOS were synthesized by use of ion-exchange reactions in deionized water. This reaction scheme is illustrated in Figure 3.1. Briefly, stoichiometrically equivalent amounts of disodium ceftriaxone and antiseptic, chlorhexidine diacetate (CHX 2Ac) and octenidine dihydrochloride (OCT 2HCl), were mixed for two hours at room temperature. The resulting products were washed several times with cold, deionized water and lyophilized overnight. The resulting ceftriaxone-based GUMBOS, chlorhexidine-ceftriaxone ([CHX][CRO]) and octenidine-ceftriaxone ([OCT][CRO]), were characterized using ^{1}H- and ^{13}C- nuclear magnetic resonance.

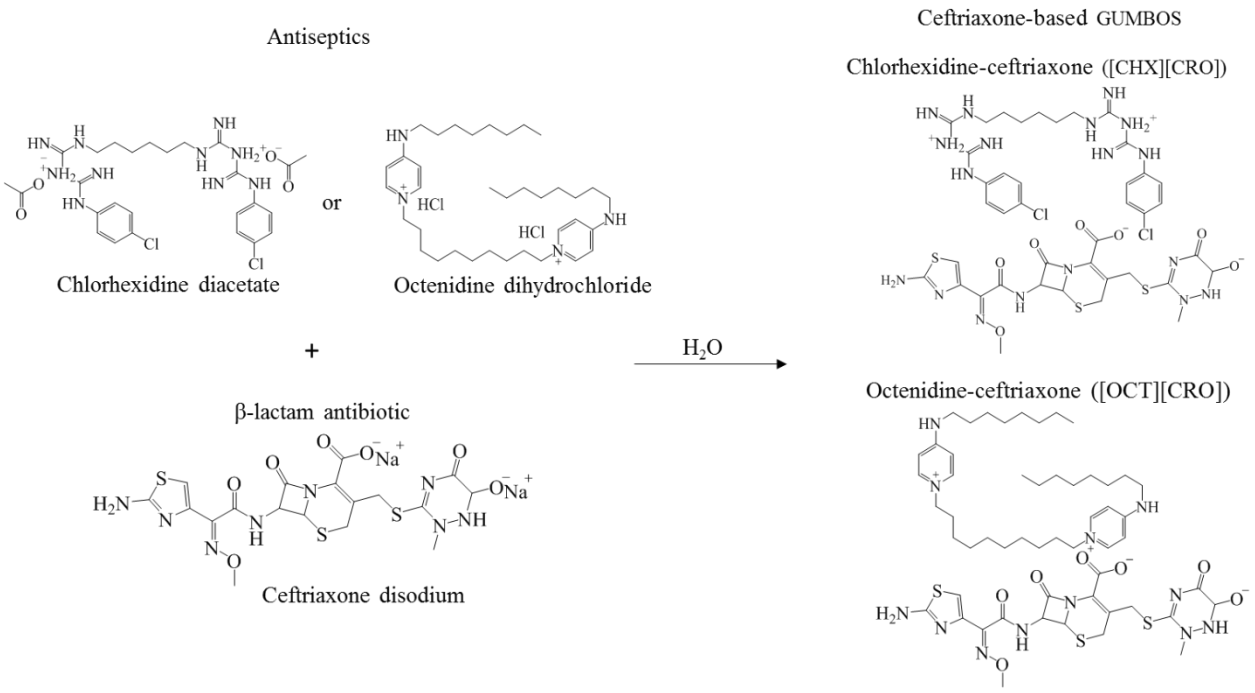

Figure 3.1. Reaction scheme for synthesis of ceftriaxone-based GUMBOS.

3.3.2. Octanol-Water Partition Coefficient

To determine the relative lipophilic strength of GUMBOS, the logarithmic octanol-water partition coefficient (Log P) was measured. It is a ratiometric parameter based on the concentration of therapeutic agent in either phase of the two-phase system (octanol and water) when at equilibrium (Equation 3.1). Specifically, a shake-flask method was used. To prevent minimal solvent miscibility, an equal amount of water and 1-octanol were mixed together for 24 hours and subsequently allowed to separate. The octanol layer was used to generate a calibration curve from the absorbance of the compound at various concentrations. An equal volume of saturated water was added to a flask of known concentration (C_i) chosen from the calibration curve. This mixture was stirred for a minimum of two hours and left undisturbed to allow the two solvent layers to separate. The absorbance of the therapeutic salt in the octanol layer was determined (C_o). Thus, the concentration of therapeutic agent in water was determined using $C_i - C_o = C_w$.

$$\textbf{Log P} = \frac{[\textbf{Octanol}]}{[\textbf{Water}]} \quad (3.1)$$

3.3.3. Minimum Inhibitory Concentrations of GUMBOS against *N. gonorrhoeae*

Clinical isolates of *N. gonorrhoeae* and *N. gonorrhoeae* ATCC 49226 were obtained from Louisiana State University Health Sciences Center New Orleans HIV Outpatient Clinic. Minimum inhibitory concentrations (MICs) of GUMBOS, constituent parent drugs, azithromycin, and unreacted mixtures were determined using micro-broth dilution in 96-well plates. Stock solutions of 1 mM were prepared in deionized water (1% DMSO). Stock solutions were diluted to a known working concentration and 100 µL were added to the first row of the plate. For susceptibility testing of *N. gonorrhoeae*, media was prepared according to methods reported by Wade and Graver (2007).[22] This liquid medium allowed dense growth of *N. gonorrhoeae* for use in broth dilution tests. Protein-free liquid media (100 µL) were added to each well and two-fold dilutions were performed. The antibiotic containing wells were inoculated with an equal volume of bacterial inoculum matching a 0.5 McFarland standard. Microtiter plates were incubated for 20-24 hours at 37 °C with 5% CO_2 and examined for visible signs of bacterial growth as evidenced by turbidity. The lowest concentration of antibiotic that prevents growth is the MIC.

3.3.4. Kirby-Bauer Disk Diffusion of GUMBOS Against *N. gonorrhoeae*

Kirby-Bauer disk diffusion assays were performed to qualitatively determine the efficacy of ceftriaxone-based GUMBOS towards *N. gonorrhoeae* (ATCC 49226) and two clinical isolates. Testing was performed according to Clinical and Laboratory Standards Institute (CLSI) recommended procedures.[23] Prepared plates containing GC II agar with IsoVitalex enrichment were used for the assays. Inoculum of *N. gonorrhoeae* matching a 1.0 MacFarland standard was spread onto the surface of the agar to allow for a confluent lawn of growth. Blank paper disks were

impregnated with GUMBOS at an amount equimolar to the commercially prepared ceftriaxone (30 µg, 50 nanomole) disk. The constituent parent compounds were prepared as equivalent stoichiometric molar amounts of GUMBOS. These disks were placed onto the agar surface and incubated at 37 °C in a 5% CO_2 environment for 20-24 hours.

3.3.5. Minimum Inhibitory Concentrations of GUMBOS against Carbapenem-resistant *Enterobacteriaceae* (CRE), *Pseudomonas aeruginosa,* and *Burkholderia cepacia*

GUMBOS, ceftriaxone, and azithromycin were tested against extended spectrum beta-lactamase (ESBL)-producing gram negative bacteria. Carbapenem-resistant clinical isolates of *Escherichia coli*, *Enterobacter cloacae*, and *Klebsiella pneumoniae* were obtained from Louisiana State University Health Sciences Center New Orleans (LSUHSC-NO) HIV Outpatient Clinic. Clinical isolates of *Pseudomonas aeruginosa* and *Burkholderia cepacia* were also obtained from LSUHSC-NO HIV Outpatient Clinic. Minimum inhibitory concentrations were determined as described above using micro-broth dilutions with two minor adjustments, cation-adjusted Mueller Hinton (MH) broth was used instead and stock solutions of test compounds were prepared directly in MH broth.

3.3.6. Cytotoxicity Assay of GUMBOS

Cytotoxicity of GUMBOS, unreacted mixtures, and antiseptics towards healthy cells were evaluated using the MTT assay, a colorimetric dye assay, according to manufacturer's instructions. HeLa (ATCC CL-2) cells were grown in Dulbecco's modified Eagle's medium-reduced serum (DMEM) supplemented with 10% fetal bovine serum and plated at a density of $1x10^5$ cells/mL (10k cells/well) in 96-well plates. Stock solutions of antimicrobial agents in cell medium (1% DMSO) were diluted to a known working concentration, doubly diluted, and transferred to seeded cells. Cells were incubated for 15 and 60 minutes at 37 °C in 5% CO_2 atmosphere. Cells treated with only DMEM were used as a negative control. After incubation, 15 µL of MTT were added to

each well and cells were further incubated for an hour. Sodium dodecyl sulfate dimethylformamide solution was added after this additional incubation time in order to dissolve the purple formazan crystals. Cell viability was quantified at 570 nm using a microplate spectrophotometer (Eppendorf PlateReader AF2200, Hauppauge, NY). Cell viability as a percentage was determined as the ratio between treated cells and untreated (control) cells taken as 100%.

3.4. Results and Discussion

3.4.1. Characterization of Ceftriaxone-based GUMBOS

[CHX][CRO]. ^{1}H-NMR (400HZ, DMSO-*d6*) δ 10.61 (s, 2 H), 9.53 (d, J=8 Hz, 1 H), 8.38 (br. s, 1 H), 7.95 (s, 1 H), 7.64 (m, 10 H), 7.29 (d, J=8.8 Hz, 4 H), 7.21 (s, 2 H), 6.74 (s, 1 H), 5.61 (dd, J= 8.2 Hz, 1 H), 5.06 (d, J=0.08 Hz, 1 H), 4.36 (d, J=12.4 Hz, 1 H), 4.14 (d, J=12 Hz, 1 H), 3.82 (m, 3 H), 3.06 (s, 4 H), 2.89 (s, 1 H), 2.73 (s, 1 H), 1.89 (s, 1 H), 1.43 (s, 4 H), 1.23 (s, 4H) ^{13}C-NMR (125 MHz, DMSO) δ 168.35, 165.78, 163.05, 162.05, 153.92, 149.07, 142.61, 132.96, 128.24, 122.06, 115.05, 108.95, 61.84, 58.23, 57.49, 34.27, 26.45. HRMS (ESI) *m/z* calc. for $C_{18}H_{18}N_8O_7S_3$, [M+H], 555.0544; found 555.0531. $C_{22}H_{30}Cl_2N_{10}$, [M+H], 505.2105; found 505.2119.

[OCT][CRO]. ^{1}H-NMR (400Hz, DMSO-*d6*) δ 9.47 (d, J=8 HZ, 1 H), 9.26 (t, J=5.6 Hz, 2H), 8.27 (d, J=7.6 Hz, 2H), 8.12 (dd, J=7.2 Hz, 2H), 7.18 (s, 2 H), 7.00 (dd, J=7.2 Hz, 2H), 6.88 (dd, J=7.2 Hz, 2H), 6.73 (s, 1H). 5.51 (dd, J=8 Hz, 1H), 4.96 (d, J=4.8 Hz, 1H), 4.31 (d, J=12.4 Hz, 1H), 4.13 (s, 1H), 4.08 (t, J=7.2 Hz, 4H), 3.82 (s, 2H), 3.51 (s, 1H), 3.46 (s, 1 H), 3.38 (s, 3H), 3.25 (dd, J=12.8 Hz, 6H), 1.72 (q, J=7.2 Hz, 4H), 1.55 (m, 4H), 1.27 (m, 33H), 0.85 (t, J= 6.8 Hz, 6H) ^{13}C-NMR (125 MHz, DMSO) δ 168.36, 163.66, 163.06, 161.67, 156.65, 155.50, 149.09, 143.48, 142.63, 141.10, 110.64, 108.99, 104.97, 61.81, 58.12, 57.39, 56.69, 31.22, 28.68, 28.65,

27.92, 26.32, 22.08, 13.94. HRMS (ESI) *m/z* calc. for $C_{18}H_{18}N_8O_7S_3$ [M+H], 555.0544; found, 555.0527. $C_{36}H_{62}N_4$ [M+H], 551.5047; found, 551.50696.

3.4.2. Predictive Lipophilic Behavior

GUMBOS were synthesized in water and are more hydrophobic than their constituent compounds. However, octanol-water partition coefficients were measured for GUMBOS using the shake flask method to further explore the lipophilic properties of these compounds. The computed Log P values for [CHX][CRO] and [OCT][CRO] were 0.2 (± 0.1) and 0.3 (±0.2), respectively. The positive Log P values for GUMBOS indicate a greater affinity for the lipid phase. By exchanging the anions on chlorhexidine with ceftriaxone, [CHX][CRO] exhibited slightly higher lipophilicity than chlorhexidine diacetate. According to Hansch *et al.* (1995), Log P for chlorhexidine is 0.08.[24] Octenidine dihydrochloride does not permeate through skin, mucosal membranes, or the placental barrier.[25-26] Thus, by anion exchanging the chloride ions for ceftriaxone, the lipophilicity was increased.

3.4.3. Antimicrobial Activities of Ceftriaxone-based GUMBOS

The antimicrobial activities of [CHX][CRO] and [OCT][CRO] were assessed against the individual precursor components, stoichiometrically equivalent mixture, and azithromycin. This is illustrated in Table 3.1. Studies were conducted with *N. gonorrhoeae* ATCC 49226 and four clinical isolate strains obtained through the LSU Health Sciences Center New Orleans HIV Outpatient Clinic. Evaluation of initial antimicrobial studies with *N. gonorrhoeae* ATCC 49226 suggested that GUMBOS were more effective than their precursor antiseptics, stoichiometric equivalent mixtures, and azithromycin. GUMBOS required concentrations of 10x less than azithromycin, and concentrations of about four time less than the stoichiometric equivalent mixtures. Ceftriaxone had a half-fold decrease in concentration as compared to GUMBOS. As

expected, ceftriaxone was highly effective against isolate populations since clinical isolates obtained in Louisiana, USA are ceftriaxone susceptible. Azithromycin required concentrations of six to nine times greater than GUMBOS for growth inhibition. Stoichiometric mixtures of antiseptic and ceftriaxone were just as effective as GUMBOS which can be attributed to the ceftriaxone component as chlorhexidine diacetate and octenidine dihydrochloride required concentrations of 50-80x times greater than GUMBOS to inhibit growth of *N. gonorrhoeae*.

Table 3.1. MICs (μM) of CDC recommended antibiotics, GUMBOS, their unreacted stoichiometric equivalent mixtures, octenidine dihydrochloride, and chlorhexidine diacetate

Antimicrobial Agents	ATCC 49226	Clinical Isolates
Ceftriaxone	0.04	0.02
Azithromycin	0.8	0.6 ± 0.2
Chlorhexidine-Ceftriaxone GUMBOS	0.08	0.06 ± 0.02
Ceftriaxone+Chlorhexidine mixture	3.1	0.12 ± 0.05
Octenidine-Ceftriaxone GUMBOS	0.08	0.09 ± 0.07
Ceftriaxone+Octenidine mixture	3.1	0.1
Octenidine dihydrochloride	3.1	4.7 ± 1.8
Chlorhexidine diacetate	3.1	4.7 ± 1.8

3.4.4. Zones of Inhibition for Ceftriaxone-based GUMBOS

Antimicrobial agents diffuse into the medium causing zones of inhibition (ZOI) to form corresponding to the susceptibility of the bacterium to the antimicrobial agent. The diameters of the ZOI allow determination of whether the bacterium is susceptible, intermediate, or resistant to the antimicrobial agent. While ceftriaxone-based GUMBOS produced lower MIC values than their unreacted, stoichiometric mixtures, GUMBOS exhibited indifferent ZOI diameters to their unreacted mixtures for *N. gonorrhoeae* ATCC 49226 and clinical isolates. Zone diameter values are reported in Table 2. This indifference could be attributed to the size of GUMBOS. GUMBOS are bulky ion-pairs with large molecular weights which cause them to diffuse more slowly through agar than their constituent parent compounds. They are also hydrophobic compounds which could also influence their diffusion rate through the medium. GUMBOS also produced significantly

larger ($p<0.05$) ZOIs than the antiseptics. When compared to ceftriaxone for both *N. gonorrhoeae* ATCC 49226 and clinical isolates, GUMBOS produced ZOIs that were not statistically different ($P>0.05$). However, GUMBOS did produce statistically larger ZOIs when compared to azithromycin ($P<0.05$).

Table 3.2. ZOIs (mm) of CDC recommended antibiotics, GUMBOS, their unreacted stoichiometric equivalent mixtures, chlorhexidine diacetate, and octenidine dihydrochloride.

Antimicrobial Agents	Quantity (nanomoles)	ATCC 49226	Clinical Isolates
Ceftriaxone	50	51 ± 0.6	52 ± 1
Azithromycin	20	40 ± 1	37 ± 1
Chlorhexidine-Ceftriaxone GUMBOS	50	49 ± 3	50 ± 3
Ceftriaxone+Chlorhexidine mixture	50	51 ± 1	49 ± 4
Octenidine-Ceftriaxone GUMBOS	50	46	51 ± 4
Ceftriaxone+Octenidine mixture	50	46 ± 0.6	52 ± 5
Octenidine dihydrochloride	50	11 ± 1	9 ± 1
Chlorhexidine diacetate	50	29 ± 3	25 ± 2

3.4.5. Antimicrobial Activities of Ceftriaxone-based GUMBOS against CRE, *Pseudomonas aeruginosa*, and *Burkholderia cepacia*

Table 3.3. MICs (μM) of ceftriaxone (CRO), azithromycin (AZM), and GUMBOS against CRE.

Antimicrobial Agents	*E. coli* (Isolate 1)	*E. Coli* (Isolate 2)	*K. pneumoniae*	*E. cloacae*
CRO	> 500	> 500	> 500	> 500
AZM	> 500	250	250	250
[CHX][CRO]	25	12.5	50	50
[OCT][CRO]	50	25	25	25

The unique chemistry of GUMBOS is thought to make them refractive to existing methods of antibiotic resistance such as β-lactamases. Carbapenemases are a group of enzymes capable of hydrolyzing third generation cephalosporins, such as ceftriaxone[27] and antibiotics belonging to the carbapenem class of antibiotics.[28] Carbapenems are members of the β-lactam class of antibiotics and are deemed "antibiotics of last resort".[29] GUMBOS, ceftriaxone, and azithromycin were tested against various strains of CRE (*Escherichia coli, Enterobacter cloacae, Klebsiella pneumoniae*)

to determine if GUMBOS would provide an advantage over current treatment regimes. The MICs of ceftriaxone, azithromycin, and GUMBOS tested against CRE are reported in Table 3.3. Note that high concentrations of ceftriaxone and azithromycin were required to inhibit CRE growth whereas much lower concentrations of GUMBOS were needed to inhibit growth. Chlorhexidine ceftriaxone needed concentrations of 5 to 40x less than ceftriaxone and azithromycin in order to inhibit CRE growth, whereas octenidine ceftriaxone needed concentrations of 5 to 20x less than ceftriaxone and azithromycin. This antimicrobial activity could be attributed to the bulkiness of the chlorhexidine and octenidine cations. The bulkiness could sterically hinder attachment of β-lactamases allowing ceftriaxone to remain active. This may impart a new treatment approach for resistant infections when enzymes are used in antibiotic deactivation mechanisms.

Table 3.4. MICs (μM) of ceftriaxone (CRO), azithromycin (AZM), and GUMBOS against *P. aeruginosa* and *B. cepacia*.

Antimicrobial Agents	*P. aeruginosa*	*B. cepacia*
CRO	>100	>100
AZM	>100	>100
[CHX][CRO]	100	>100
[OCT][CRO]	100	100

GUMBOS were also tested against *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Like CRE, these bacteria are gram-negative and rod-shaped; however, they're aerobic and non-lactose fermenters. Antimicrobial activities are illustrated in Table 3.4. GUMBOS were less susceptible to these two bacteria than CRE. There was also no discernable difference in antimicrobial efficacy between GUMBOS, ceftriaxone, and azithromycin. This could be due to the structure of the cell wall of *P. aeruginosa* and *B. cepacia*. The outer most layer of the outer membrane of gram-negative bacteria contains lipopolysaccharides (LPS), large molecules consisting of lipid and polysaccharide structures.[3] LPS aids in maintaining structural integrity and protecting the membrane from antibiotic drugs.[3] In the case of *P. aeruginosa* and *B. cepacia*,

divalent cations (Mg^{2+} and Ca^{2+}) attach to LPS, preserving and stabilizing the integrity of the membrane and preventing charged molecules from crossing the membrane.[30-32] CRE lack these divalent cations and rely on other defense modes against antibiotics.[33]

3.4.6. Cytotoxicity Assessment

Table 3.5. Acute cytotoxicity (IC_{50}, μM) of GUMBOS, unreacted mixtures, and antiseptics for HeLa cells.

Antimicrobial Agents	IC_{50} (μM)	
	15 min	60 min
Chlorhexidine diacetate	81 ± 4	25 ± 1
Octenidine dihydrochloride	55 ± 1	26 ± 1
[CHX][CRO]	84 ± 5	45 ± 1
1 CHX: 1 CRO	60 ± 6	50 ± 1
[OCT][CRO]	67 ± 8	23 ± 1
1 OCT: 1 CRO	68 ± 4	22 ± 1

Cytotoxicity of GUMBOS, antiseptics, and unreacted, stoichiometric mixtures were assessed in order to determine in vitro safety. The antiseptics, chlorhexidine diacetate and octenidine dihydrochloride, have been shown to be non-toxic to various cell lines.[21, 34] Cervical cells were used in these studies to determine an overall, systemic toxicity and to evaluate probable use in eradicating gonorrheal infections of the cervix (Table 3.5). After 15 minutes, cell toxicity caused by GUMBOS was less for [CHX][CRO] than [OCT][CRO]. Chlorhexidine-ceftriaxone was significantly less toxic than its unreacted, stoichiometric mixture ($p<0.05$); however, octenidine-ceftriaxone showed indifferent toxicity to its unreacted, stoichiometric mixture of parent compounds. In comparison to chlorhexidine diacetate, [CHX][CRO] was only slightly less toxic. However, [OCT][CRO] was significantly less toxic than octenidine dihydrochloride. After 60 minutes, cell toxicity caused by [OCT][CRO] GUMBOS still showed indifferent toxicity to its stoichiometric mixture and octenidine dihydrochloride. [CHX][CRO] GUMBOS were slightly

more toxic than its mixture after 60 minutes, however; this was not statistically significant ($p>0.05$).

3.5. Conclusion

As a result of isolates with elevated ceftriaxone and azithromycin MICs on the rise, it is of utmost importance that alternative treatments for gonorrhea be explored. Such urgency is paramount since *Neisseria gonorrhea* has steadily become resistant to all previous treatments. Thus, it is only a matter of time before susceptibility to ceftriaxone disappears. As expected, ceftriaxone remained susceptible against the clinical isolates obtained for these studies. GUMBOS did not prove to be more effective than ceftriaxone, however; ceftriaxone-based GUMBOS proved to be more effective than azithromycin according to disk diffusion and micro-broth dilution studies. Azithromycin resistance is increasing more rapidly than ceftriaxone resistance and alternative therapies need to be explored. By fashioning ceftriaxone into GUMBOS using antiseptics, the unique structure of these ion-pairs is also shown to be much less affected by beta-lactamases than ceftriaxone disodium salt, thereby extending its spectrum of antibacterial activity against *N. gonorrhoeae*. While stoichiometric equivalent mixtures of antiseptic and ceftriaxone proved to be as effective as GUMBOS, GUMBOS should still be considered as an alternative therapy since they are less toxic and much less affected by beta-lactamases than ceftriaxone. It has also been proven by previous studies that by forming ion-pairs from antiseptics and β-lactam antibiotics, the reduced aqueous solubility leads to greater lipophilicity and increased intramolecular interactions.[21] Reduced solubility also relates to potentially greater bioavailablity.[21] Overall, this approach may offer an alternative strategy to tackling the impending resistance of *N. gonorrhoeae* towards ceftriaxone and azithromycin.

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CHAPTER 4. HYALURONIC ACID-CELLULOSE COMPOSITES AS PATCHES FOR MINIMIZING BACTERIAL INFECTIONS

4.1. Introduction

Hyaluronic acid (HA) is a glycosaminoglycan that is found throughout connective, endothelial, and neural tissues.[1] It has unique properties, such as, high hygroscopicity, viscoelasticity, biocompatibility, non-immunogenicity, and does not generate toxic products upon degradation.[2] These unique properties are thought to be beneficial in wound care bandage materials as HA has been investigated for a number of clinical applications, including lubrication and mechanical support of arthritic joints[3-4], as a surgical aid in ophthalmological surgery[5-6], drug delivery agent[7-9], and to facilitate surgical wound healing.[10-12] Hyaluronic acid has also recently been used in skin-care products, such as facial moisturizers, as a result of its aqueous viscous consistency and non-allergenic tissue-friendliness.[13] However, one disadvantage of HA is its lack of adequate mechanical properties.[14] To overcome this disadvantage, our laboratory focuses on fabrication of composites from HA and cellulose in this manuscript.

Cellulose (CEL) is a polysaccharide and is the primary constituent of plant cell walls. It is biocompatible and biodegradable. Cellulose was chosen for this study because it lends chemical stability and mechanical strength to composite materials.[15] Different forms of cellulose have recently been used as additives in composite biopolymer fabrication. For example, Huang *et al* (2016) fabricated berberine-enriched carboxymethylcellulose-hyaluronic acid hydrogels with

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excellent viscosity to provide anti-inflammatory and antibacterial functions for minimizing post-surgical complications. However, these composites required polyvinyl alcohol as a base to assist membrane formation.[16] Jia *et al* (2015) has shown that bacterial cellulose (BC) and hyaluronic acid can be combined into composites with enhanced tensile strength and Young's Modulus.[17] While BC has been widely used for various applications, cellulose derived from cotton (as used in this manuscript) is thermodynamically more stable than BC.[18] In addition, fabrication of these composites required cross-linking reactions or soaking BC for several days in HA.[17] In another example, Domingues *et al* (2015) determined that cellulose nanocrystals and hyaluronic acid can be combined into hydrogel materials for injectable purposes. However, these materials also required the covalent modification of HA and CEL using additional cross-linkers.[19]

Tran *et al*. (2012) used ionic liquids to combine chitosan and cellulose into composite materials with enhanced tensile strength as well as antimicrobial properties. In addition, materials reported by Tran *et al* exhibited an increased antimicrobial effect on a wider range of bacteria than other chitosan-based materials.[15, 20-21] By fabrication of these biopolymers into composite materials, we rationalize that the combined properties of mechanical integrity, hygroscopicity, and biocompatibility could be beneficial for potential wound care devices. In order to obtain these composites, we hypothesize that use of ionic liquids would be a viable and facile alternative for dissolving biopolymers, such as HA and CEL, as compared to previously mentioned procedures.

Ionic liquids (ILs) are organic salts that are liquid at temperatures below 100°C.[22] ILs have various, unique chemical and physical properties including high thermal stability, high solubility power, low volatility, and negligible vapor pressure. Because of these properties, ILs have been labeled as "green" alternatives to traditional, organic solvents.[23] In addition, due to their high solvating ability, ILs have been used as solvents in dissolving biopolymers, including cellulose,

extensively.[15, 24-27] However, limited information was found regarding ILs to dissolve HA. For this reason, we explored ILs as potential solvents for composite fabrication. In this study, we demonstrate that a simple ionic liquid, 1-butyl-3-methylimidazolium chloride ([Bmim][Cl]), solubilizes both hyaluronic acid and cellulose, and allows composite formation without the use of chemical modifications or cross-linking reactions. One anticipated challenge of [Bmim][Cl] is the high viscosity associated with the halide anion,[28] but viscosity was not an issue with the elevated temperature needed for fabrication. Overall, this method proved simple, green, and completely recyclable.

We also note that composites impregnated with antimicrobial agents may be used as topical medicated devices. Previous studies support the use of antimicrobial GUMBOS (group of uniform materials based on organic salts) as a chemical approach for combating various gram-negative and gram-positive bacteria.[29-30] GUMBOS are a group of novel materials, similar to ILs, composed of bulky inorganic and/or organic counter-ions with melting points between 25°C and 250°C.[31] GUMBOS employed in this manuscript were formulated from chlorhexidine, an antiseptic, and beta-lactam antibiotics, oxacillin and cephalothin. These materials have been proven to be more effective than the constituent parent compounds against various strains of bacteria, most notably *Staphylococcus aureus*, which is one of the most common bacteria found on the skin and hair as well as noses and throats of humans and animals.[13] This bacterium is capable of manifesting into severe clinical infections.

Herein, we explore the development and characterization of a green and recyclable method using ionic liquids to fabricate composites from biocompatible and biodegradable biopolymers (e.g. hyaluronic acid and cellulose) and exploit their biomedical use as wound care devices for minimizing bacterial infections. The main advantages of this approach are the ease of fabrication,

cross-linking agents are not necessary, and high swelling abilities of resulting composites. Preparation of these biocomposites demonstrate promising applications as patches for use in wound care.

4.2. Materials

Cellulose (medium, fibrous from cotton linters), phosphate buffered saline tablets, oxacillin sodium salt, and cephalothin sodium salt were obtained from Sigma Aldrich. Hyaluronic acid sodium salt (1.5 to 2.2 million Dalton) and chlorhexidine diacetate were obtained from Acros Organics. 1-chlorobutane was obtained from Alfa Aesar. Methylimidazole was obtained from TCI Chemicals. *Staphylococcus aureus* (ATCC 29213) was grown in Brain Heart Infusion (BHI) broth and sub-cultured on Mannitol salt agar. Inoculates were prepared in BHI and spread on Mueller Hinton agar. All growth media was obtained from Accumedia.

4.3. Experimental

4.3.1. Synthesis and characterization of HA/Cellulose composite

Composites were fabricated in various ratios (1:1, 1:2, 2:1, 1:4, and 4:1) of hyaluronic acid to cellulose and did not exceed 5% w/w HA. However, 1:1 and 2:1 (HA:CEL) yielded the most stable composites that did not wash away upon removal of [Bmim][Cl]; thus, this manuscript focuses only on these two ratios. Structures of starting materials are illustrated in Figure C1 (Appendix C). Composites were fabricated by first dissolving sodium hyaluronan (5% w/w) in [Bmim][Cl], and were synthesized according to procedures reported in literature by Crowhurst *et al* (2003).[32] The solution was stirred in a vial at 100-110 °C in a mineral oil bath. Once all HA was dissolved, CEL (2.5% or 5% w/w) was added in 10% increments to the vial. After CEL dissolved and the solution homogenized, the mixture was transferred to a silicone mold and placed in a refrigerator overnight to allow gel formation. The ionic liquid, [Bmim][Cl], was recovered by

soaking the composite in deionized water. Water was subsequently removed from [Bmim][Cl] by lyophilization. Confirmation of [Bmim][Cl] removal was concluded using FTIR characterization and is detailed in Figures C2 and C3 (Appendix C). Silver nitrate ($AgNO_3$) ion test was also performed to confirm removal of all [Bmim][Cl] from the composite. These composites were cut to size (1.27 cm x 1.27 cm), lyophilized overnight, and stored on the bench top. No crosslinking agents or chemical modifications were used in this synthesis. These HA/CEL-based composites were then characterized by Fourier transform- infrared spectroscopy (FT-IR), thermogravimetric analysis (TGA), X-ray diffraction (XRD), and scanning electron microscopy (SEM). They were also evaluated for swelling capacity and drug release properties. An overall scheme of the fabrication and application of these composites is illustrated in Figure 4.1.

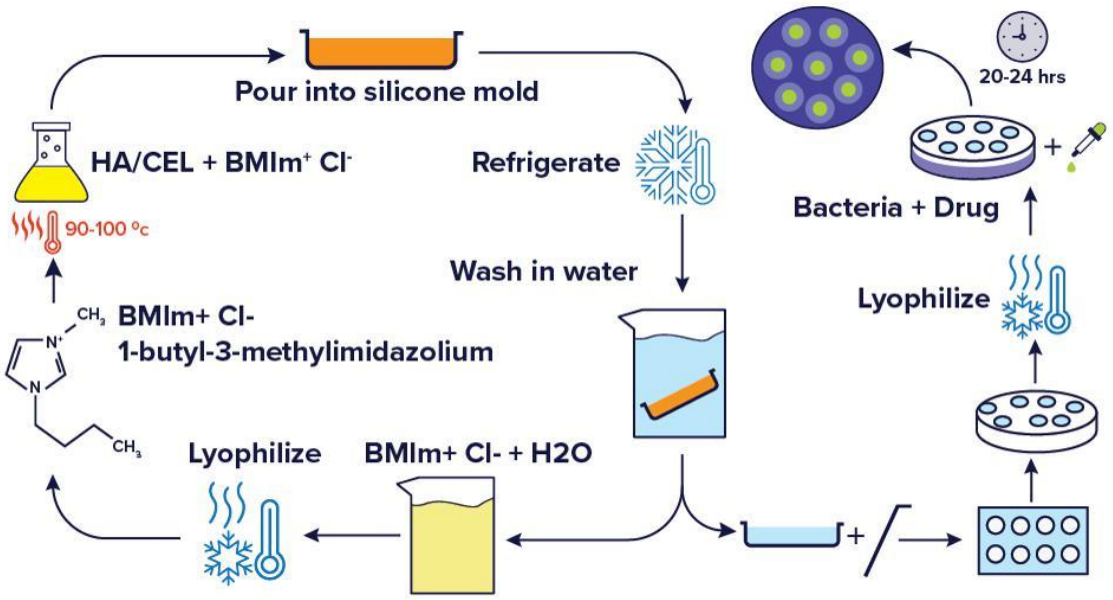

Figure 4.1. Fabrication and application of hyaluronic acid/cellulose composites and recycling of 1-butyl-3-methylimidazolium.

4.3.2. Synthesis and Characterization of Chlorhexidine-based GUMBOS

Chlorhexidine-di cephalothin ([CHX][Ceph]) and chlorhexidine- di oxacillin ([CHX][Oxa]) GUMBOS were synthesized using ion-exchange procedures similar to those reported in literature.[30] Antibacterial susceptibility to various multi-drug resistant Gram-negative

and Gram-positive bacteria, as well as cell viability has been previously reported by Cole *et a*l (2015). These GUMBOS have also been shown to have increased pharmacokinetic properties as well as increased intestinal bioavailability.[30] Structures of starting materials are shown in Figure C4 (Appendix C).

4.3.3. Swelling Capacity

Dried composites were weighed and soaked in various media, such as 0.9% saline solution, distilled water, and 1X phosphate buffered saline (PBS; pH 7.4), at room temperature up to 3 hours. Weights of the wetted composites were measured at defined intervals up to 3 hours. Prior to weighing, filter paper was wiped across the surface of the composite to remove excess water. The swelling percent (S%) was determined using the following equation.

$$\mathbf{S}\% = \frac{\mathbf{W_s - W_d}}{\mathbf{W_d}} \times \mathbf{100}\% \qquad (4.1)$$

In Equation 4.1, W_d is the weight of the dried composite and W_s is the weight of the respective swollen composite. The swelling percent is defined as the fractional increase in the composite weight due to absorption. All swelling studies were performed in triplicate.

4.3.4. Drug Loading

The drugs, [CHX][Ceph] and [CHX][Oxa], were dissolved in methanol and diluted to a desired concentration in 500 µL of methanol. Fifty microliters of solution were drop casted repeatedly onto the composite with evaporation of methanol between each loading. It is presumed that drug loading is 100%. The composite was then dried overnight using evaporation to ensure complete removal of methanol. Fluorescence microscopy was also performed to determine

consistent drop casting of drugs. Fluorescein sodium salt (2 μM) was drop casted onto composites using the method reported above and is illustrated in Figure C5 (Appendix C).

4.3.5. Drug Release

Composites were placed in vials with 5 mL of 0.9% saline solution and were constantly shaken using a VWR S-500 orbital shaker. At fixed time intervals, 4 mL of 0.9% saline solution was removed and replaced with equal volume of fresh saline to maintain a constant volume. The amount of drug released was assayed using a Shimadzu UV-3101PC UV-VIS-NR spectrophotometer (Shimadzu Europe) at λ= 231 nm. All drug release studies were performed in triplicate.

4.3.6. Kirby-Bauer Disk Diffusion

Testing was performed according to Clinical and Laboratory Standards Institute (CLSI) recommended procedures.[33] Kirby-Bauer disk diffusion assays[34-35] were performed on *Staphylococcus aureus* (ATCC 29213) to qualitatively demonstrate that therapeutic agents can be released from these dry composites and thus, could minimize potential bacterial infections. Before lyophilization, composites were molded with a 7 mm plastic straw. These rounds were freeze-dried overnight and subsequently impregnated with therapeutic agents. Controls containing the same concentration of therapeutic agents were prepared using standard 6 mm paper disks to effectively compare bacterial control relative to the composite. *S. aureus* was grown in brain heart infusion (BHI) medium for 24 hours and sub-cultured onto mannitol salt agar. Inoculum matching a 1.0 McFarland standard was prepared in BHI medium and spread evenly over the surface of a Mueller Hinton agar plate to allow a confluent lawn of growth. Composite rounds and disks were placed on the agar plate and incubated upside down for 20-24 hours at 37 °C. After incubation,

zones of inhibition had formed around the composites and standard disks. Zone diameters were measured using a ruler. All experiments were performed in triplicate.

4.4. Results and Discussions

4.4.1. FT-IR Analysis

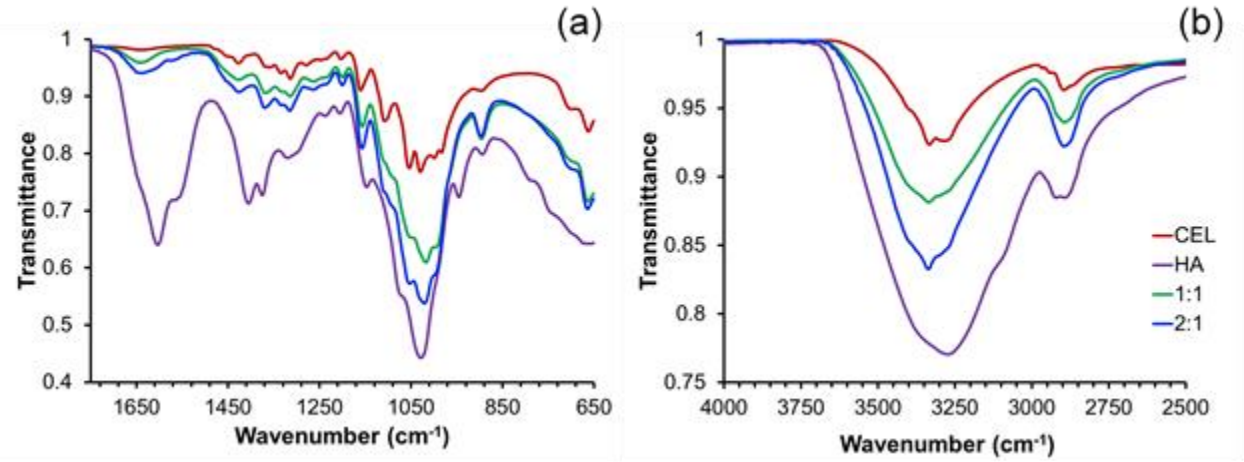

Figure 4.2. FTIR spectra of CEL, HA, 1:1, and 2:1 composites (a); O-H Stretching region (b).

FT-IR spectroscopy was employed to determine the chemical structure of the composite materials. Spectra were measured using a Bruker Tensor 27 (Billerica, MA) under transmission wavenumber ranging from 650 to 4000 cm $^{-1}$ (Figures 4.2a and b). The HA spectrum had absorption bands at 1604 cm^{-1} and 1405 cm^{-1}. These are indicative bands of the carboxylate asymmetric stretching vibration and carboxylate symmetric stretching, respectively.[36] Bands at 1145 cm^{-1} and 1027 cm^{-1} are C-O-C stretching vibration of the HA skeleton.[37] Two signals at 1560 cm^{-1} and 1322 cm^{-1} are indicative of amide bands.[36]. In the region of 1150-650 cm^{-1}, there is little difference between the spectra of the composites and HA. This verifies the presence of HA in these composite materials. All spectra have a strong absorption band between 3600-3000 cm^{-1} which is indicative of the O-H stretching region (Figure 4.2b). While these peaks are not as broad in composite materials, they are broader than the cellulose O-H peak. This narrow peak for cellulose is presumably due to hydrogen bonding within the glucose monomer network. This change in peak

shape within the composites suggests hydrogen bonding interactions between the HA and cellulose in the composite materials[38].

4.4.2. Thermal Stability of Composites

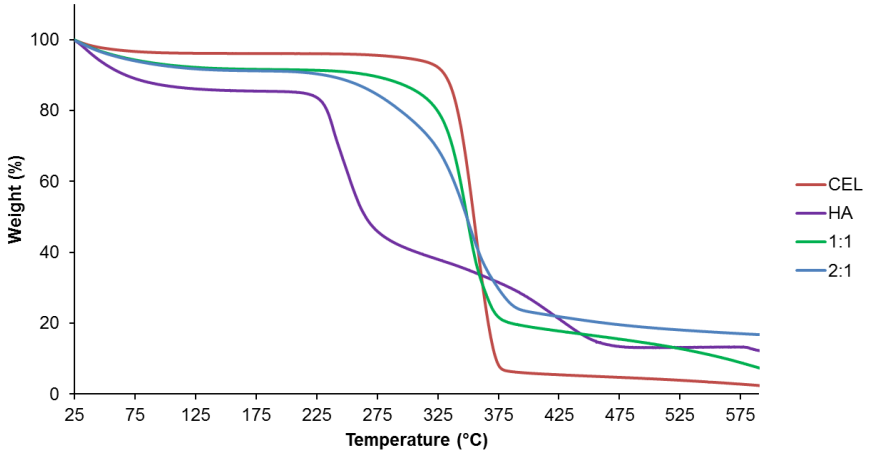

Figure 4.3. TGA spectra of cellulose, HA, and composites.

Table 4.1. Onset temperatures of the composites and parent biopolymers.

Compound	Onset Temperature (°C)
Hyaluronic acid	228.1
Cellulose	332.7
1:1 composite	324.3
2:1 composite	305.4

Figure 4.3 is a display of TGA spectra for the parent biopolymers and composite materials. TGA was performed using a Hi Res Modulated TGA 2950 Thermogravimetric Analyzer (TA Instruments, New Castle, DE). Small pieces weighing about 20 mg were placed in a platinum pan and heated from room temperature to 600°C at a rate of 10°C/min. Onset temperatures for cellulose and hyaluronic acid were 332.70°C and 228.12°C, respectively (Table 4.1). Onset temperatures of the composites decreased by 9 and 30 °C, as compared to cellulose for 1:1 and 2:1, respectively. The 1:1 composite had an onset temperature of 324.27°C while the 2:1 composite had an onset temperature of 305.44°C. The 2:1 composite most likely had a lower degradation temperature due to the higher concentration of HA, which is known to be a thermally sensitive material.

4.4.3. X-Ray Diffraction (XRD)

X-ray diffraction was employed to determine how the varying amount of HA affects the crystallinity of cellulose. XRD was performed using an Empyrean X-ray diffractometer (Malvern Panalytical, United Kingdom). Evaluation of data presented in Figure 4.4 confirms that HA is completely amorphous. The supramolecular structure of fibrous cellulose exists as cellulose I and its crystallinity is a result of a ratio between two forms (I_β and I_α). As shown in Figure 4.4, fibrous cellulose has five characteristic peaks at 14.9°, 16.4°, 20.6°, 22.7°, and 34.5° which corresponds to the 101, 10$\bar{I}$, 021, 002, and 040 planes, respectively.[39-40] The crystallinity pattern of fibrous cellulose is more characteristic of a higher concentration of cellulose I_β than I_α, a ratio more prevalent in woody plants and cotton, which is the origin of cellulose used in this manuscript.[18] In general, the composites maintain the characteristic peaks of cellulose. However, there is a slight rise in amorphous scattering between 14.9° and 22.7° diffraction angles in both 1:1 and 2:1 composites.

Crystallinity indices (CI) of these materials were determined using the Segal method (Eq. 4.2), whereby I_{002} is the intensity of the 002-lattice diffraction and I_{am} is the intensity of diffraction at $2\Theta = 18°$.[40]

$$\mathbf{CI}\% = \frac{\mathbf{I_{002} - I_{am}}}{\mathbf{I_{002}}} \times \mathbf{100}\% \qquad (4.2)$$

The crystallinity indices are presented in Table 4.2. The CI of cellulose is 85.9%. As shown in Table 4.2, CIs of both composites were lower than that of cellulose with the 2:1 composite having the lowest CI. This can be attributed to the higher content of amorphous HA in relation to cellulose within the composite network. The amorphous material disrupts the crystalline lattice of the cellulose, thus lowering its CI.

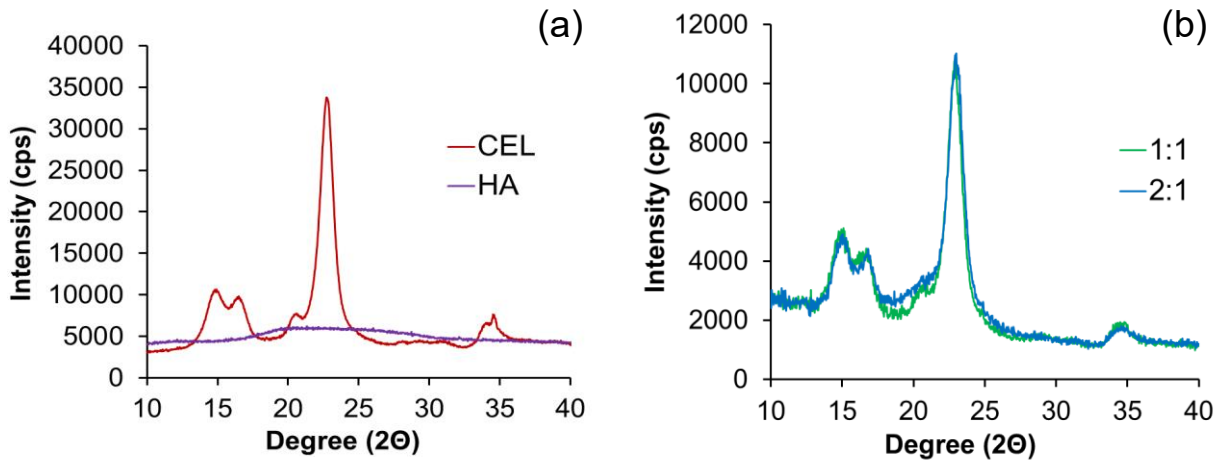

Figure 4.4. XRD spectra of CEL and HA (a), 1:1 and 2:1 composites (b).

Drug-loading of the composites were also evaluated using XRD (Figure C6). Moreover, powder XRD was employed to elucidate the crystalline nature of [CHX][Oxa] and amorphous nature of [CHX][Ceph] (SI Figure C7). There is no obvious difference between XRD spectra of plain composites versus drug-loaded. However, there are slight changes in the crystallinity indices, which were calculated for drug-loaded composites (Appendix C Table C1). [CHX][Oxa] had a larger effect on CI; for example, in 1:1 composites, [CHX][Oxa]-loading lowered the CI from 78.1% to 75.3%. This could possibly be due to adsorption of the drug onto the surface of the materials. In contrast, with 2:1 composites, [CHX][Oxa]-loading increased the CI from 76.6% to 77.9% as a result of greater interactions of [CHX][Oxa] within the network of these composites.

Table 4.2. Crystallinity indices (%) of the composites and parent biopolymers.

Compound	Crystallinity Index (%)
Hyaluronic acid	N/A
Cellulose	85.9
1:1 composite	78.1
2:1 composite	76.6

4.4.4. Scanning Electron Microscopy (SEM)

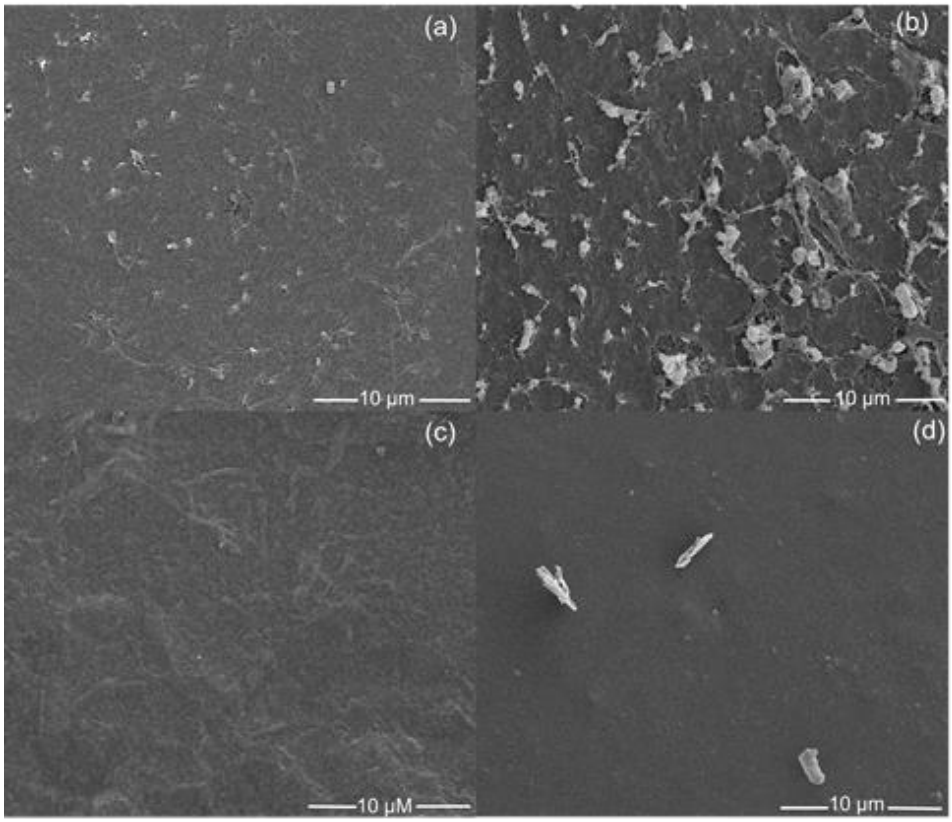

Figure 4.5. The morphology of 1:1 composite (a), 2:1 composite (b), [CHX][Ceph] loaded 1:1 composite (c), and [CHX][Ceph] loaded 2:1 composite (d).

Composites were cut into small pieces and the surfaces (before and after drug loading) were spin-coated with a thin layer of conductive platinum. Surface morphologies were evaluated by scanning electron microscopy (JSM -6610 LV SEM, JEOL USA) at 5 kV. All images (Figure 4.5) are at 5,000X. Higher magnification images can be found in Appendix C (Figures C8 and C9). The composites exhibited a textured, porous surface; however, for a 2:1 composite, the pores were larger with more irregularity in shape. The fibrous network of the CEL and HA are clearly delineated within the pores. A 1:1 composite exhibited a smoother, planar texture. Drug loaded composites also exhibited a smoother-looking surface indicating loading of the drug on the surface and in the pores. The fibrous networks were no longer observable after drug loading in both 1:1

and 2:1 materials. It is presumed that loading of [CHX][Oxa] would also exhibit the same smooth-looking surface.

Cross-sectional areas (before and after drug loading) were also evaluated by SEM at 5kV. These images can be found in Figures C10 and C11 (Appendix C). Cross-sectional areas of composite materials (1:1 and 2:1) show no discernable difference between the drug loaded and non-drug loaded composite. However, 1:1 composites exhibited a network of higher density within the matrix as compared to 2:1 composites. Similar to the surface, 2:1 composites exhibited a much more porous network within the composite matrix.

4.4.5. Swelling Studies

Swelling studies were performed in order to demonstrate the exudation capacity of these potential wound care composites, as well as investigate the effect of varying polymer ratios on capacity. Swelling percentages are reported in Figure 4.6. In all media, the 2:1 (HA:CEL) composite had much higher swelling than the 1:1 (HA:CEL) material. These results indicate that the swelling ratio increased as the pore size and amorphous nature of the composites increased as amorphous regions are able to absorb more water than crystalline regions.[41] The higher ratio of hygroscopic HA could also play a role in the 2:1 composite absorbing more media. A larger degree of swelling could also be attributed to the higher concentration of sodium ions on HA that may have aided in balancing the internal osmosis.[42] Interestingly, all composites maintained structural integrity which can be attributed to the mechanical strength of cellulose. Images of dry and swollen composites are found in Appendix C (Figures C12 and C13). In comparison to hydrogels fabricated by Domingues *et al.* (2015), swelling of composites synthesized in this manuscript was significantly higher.[19] This could be due to no cross-linking agents used in the fabrication process. Instead of creating physical crosslinks, this process is more akin to solution blending, thereby

maintaining the hygroscopic functional groups of HA. Fibrous cellulose also has relatively higher absorbent properties which could make it a better choice for topical applications such as wound management.[43]

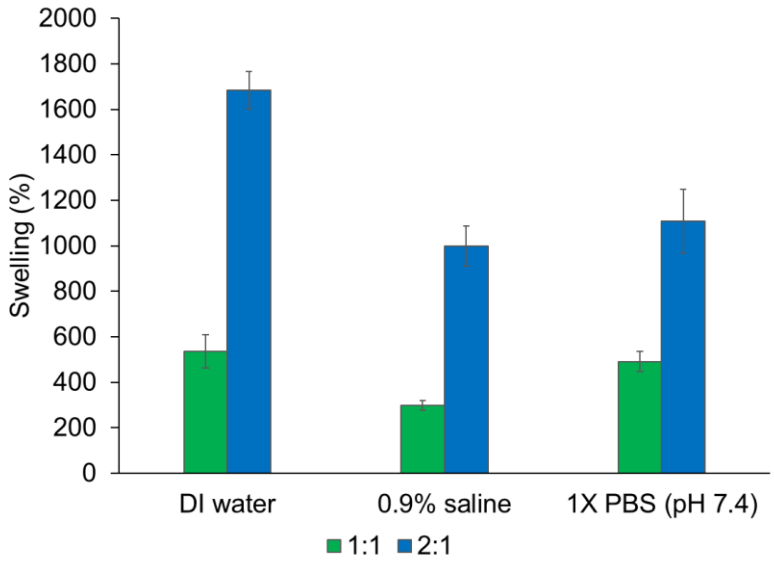

Figure 4.6. Swelling of composite materials in deionized water, 0.9% saline, and 1X PBS (pH=7.4).

4.4.6. Release Properties

The release of two antimicrobial GUMBOS was determined spectrophotometrically in 0.9% saline solution at λ=231 nm. The cumulative amount of drug released, and percentage of drug released as a function of time for [CHX][Ceph] and [CHX][Oxa] is shown in Figures 4.7 and 4.8, respectively. Upon placement of composites into the saline medium, an initial large amount of drug is released in what is known as "burst release".[44] After initial burst, the rate at which drugs are released decreases and all drug has been released between 4-6 h. This is evident from the stable plateau between the 4-6 h time point. Burst release could be beneficial for wound treatment as quick release could ensure rapid reduction of bacteria from wound sites.[44-46] Overall, the 1:1 composite showed a much higher release of drugs than the 2:1 ratio. In both drug release studies, the 1:1 composite exhibited quantitative drug release as shown in Figures 4.7b and 4.8b. In

contrast, 2:1 material did not achieve quantitative release over the same time period. This could be attributed to the morphology of the surface and inner network of the composites. The 1:1 composite, as seen in Figure 4.5a, exhibits less pores than the 2:1 composite and a denser inner network. Presumably, drug molecules are not physically able to penetrate the 1:1 network as deeply and concentrates more on the surface. In effect, these drug molecules are then released from the surface in a "burst" manner when placed into saline. In contrast, the 2:1 composite exhibited a much more porous surface (Figure 4.5b) and less dense inner network which could allow trapping of the drug further within the fibrous network and impede the rate and quantity of release of organic molecules. Both composites (1:1 and 2:1) released more [CHX][Ceph] than [CHX][Oxa] which we hypothesize could be a result of [CHX][Oxa] having a stronger interaction with the composite material than [CHX][Ceph].

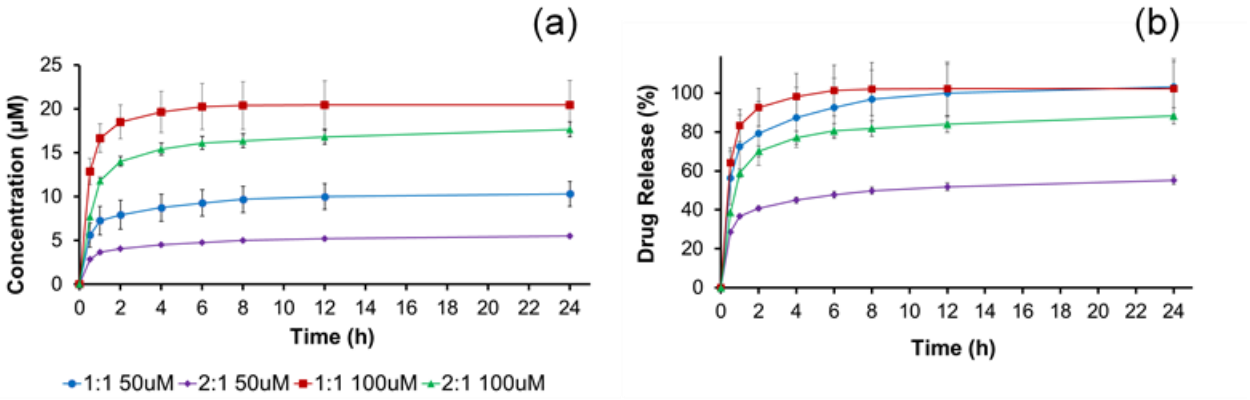

Figure 4.7. Cumulative drug release (a) and percent drug release (b) for [CHX][Ceph].

Drug-loaded and drug-released composites were also evaluated using electron-dispersive X-ray spectroscopy to confirm drug release (EDS; Quanta™ 3D DualBeam™ FEG FIB-SEM with an EDAX Pegasus EDS/EBSD detector). GUMBOS structures contain sulfur atoms (Figure C4). EDS spectra of plain composites can be found in Appendix C (Figures C14 and C15). After evaluation drug-loaded composites using EDS, it was confirmed that the sulfur-containing drugs

were present in the loaded samples. We also observed that the sulfur signal decreases in drug-released samples (Figure C18).

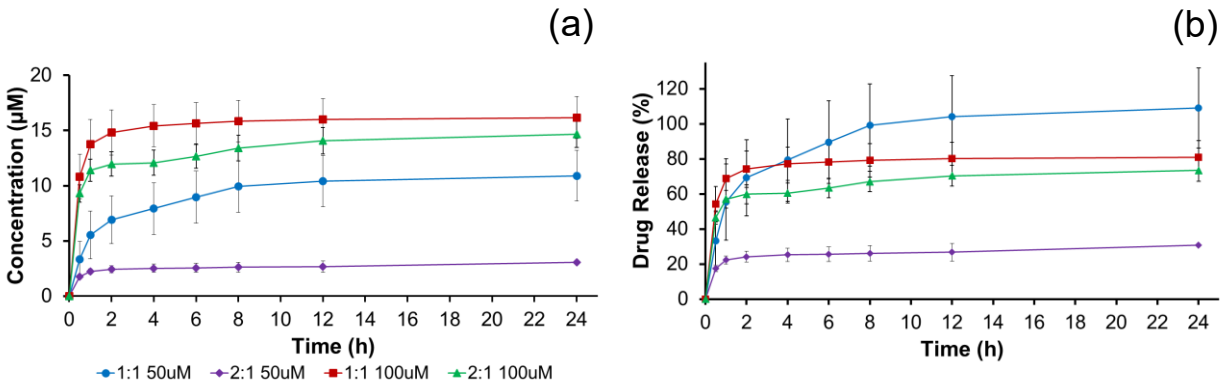

Figure 4.8. Cumulative drug release (a) and percent drug release (b) for [CHX][Oxa].

4.4.7. Disk Diffusion

Kirby Bauer disk diffusion is a qualitative susceptibility test used to determine the sensitivity or resistance of a microorganism towards an antimicrobial agent.[34] In this study, disk diffusion was performed to demonstrate drug release from composites onto an agar surface. Three concentrations were loaded onto the disks and composite rounds: 10, 50, and 250 μM. During incubation, drug diffuses from composites and disks into the agar. After 20-24-hour incubation, zones of inhibition (ZOI) formed around the disks and composites, and diameters were measured using a ruler. The zone of inhibition is a circular area around the disk (or composite) in which no bacteria has grown due to susceptibility of the bacterium to the drug. The rate of drug diffusion through agar can be governed by many different factors such as, relative hydrophobicity, aqueous solubility, molecular weight, and intrinsic resistance of the microorganism.[35] These ZOI values confirm that drug release occurred from the composites into the agar. The 1:1 composite loaded with [CHX][Ceph] had larger zone diameters than the 2:1 composite of the same drug (Table 4.3). However, an opposing trend was seen for zone diameters of [CHX][Oxa] (Table 4.4). Overall, [CHX][Oxa] produced larger ZOIs versus [CHX][Ceph]. This could be due to higher susceptibility

of *S. aureus* to [CHX][Oxa] as the standard disks impregnated with [CHX][Oxa] produced larger zone diameters than [CHX][Ceph]. Moreover, it has been proven in previous studies that the water solubility of [CHX][Oxa] is higher than [CHX][Ceph], which allowed for better diffusion of this drug through agar.[47] Evaluation of data shows that the porous nature of these biopolymer composites allows for drug release, and this method of drug delivery has potential for minimizing staph infections. Representative images of incubated agar plates with drug loaded composites and standard disks can be found in Appendix C (Figure C19).

Table 4.3. ZOIs (mm) of composites and standard disks for [CHX][Ceph].

Concentration (μM)	1:1 Composite	2:1 Composite	Standard Disk
10	15.7 ± 1.2	14.7 ± 0.6	17.6 ± 1.1
50	27.3 ± 1.5	25.7 ± 0.6	28.2 ± 1.0
250	36.3 ± 2.5	34.7 ± 1.5	37.3 ± 1.0

Table 4.4. ZOIs (mm) of composites and standard disks for [CHX][Oxa].

Concentration (μM)	1:1 Composite	2:1 Composite	Standard Disk
10	21.3 ± 0.6	23 ± 0	19.2 ± 3.2
50	31.7 ± 0.6	36.3 ± 0.6	34.2 ± 1.8
250	38.3 ± 1.2	41.7 ± 2.9	42.2± 1.7

4.5. Conclusion

In summary, hyaluronic acid/cellulose-based composites were developed by dissolution in [Bmim][Cl], an ionic liquid, with no chemical modification. These composites were developed at two ratios (1:1 and 2:1 HA to CEL) and showed a variety of morphological and structural changes that may prove applicable for wound care devices. Since IL can be removed from the composite material through washing with water and recovered by lyophilization, this method is also recyclable. Several spectroscopic and imaging techniques, including FT-IR, TGA, XRD, and SEM, were used for characterizing composite materials and monitoring [Bmim][Cl] recovery.

These composites showed great swelling capacity with the 2:1 composite having much higher capacity. This could translate into an ability to absorb more wound exudate.

By loading with antimicrobial GUMBOS, these composites were demonstrated to be possible medicated devices for use in wound care. When both composites were drop casted with drug, there was uniform coverage on both materials. In drug release studies, burst release was seen from these composite materials with higher release of drug from 1:1 composites. Examination of results of *in vitro* disk diffusion tests showed that both composites allowed diffusion of drug into the medium for combating *Staphylococcus aureus* infections. These results in combination with inherent biocompatibility strongly suggest potential biomedical applications of these composites. We will further investigate 1) incorporation of drug materials within the composite network and 2) in vivo wound healing properties of these composites using animal models.

4.6. References

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CHAPTER 5. INTERACTION OF SODIUM HYALURONATE WITH CATIONIC ANTISEPTICS TO FORM POLYELECTROLYTE COMPLEXES

5.1. Introduction

Hyaluronic acid (HA) is a linear, anionic mucopolysaccharide made up of repeating units of glucuronic acid and N-acetylglucosamine linked via alternating β-1,4 and β-1,3 glycosidic bonds.[1] This polymer is found naturally throughout connective, endothelial, and neural tissues, and is a component of the extracellular matrix.[1] It is thought to be involved in several mechanisms of the wound healing process.[2] Due to various properties, such as hygroscopicity, non-immunogenicity, biocompatibility, and viscoelasticity, HA has a wide array of applications in surgery, cosmetics, and hygiene medicine.[1, 3] Hyaluronic acid and its derivatives have also been explored extensively as drug delivery agents for steroid drugs, anti-inflammatory drugs, polypeptides and protein drugs, and anticancer drugs[4-5] through various routes of administration, including ophthalmic, nasal, pulmonary, parenteral, and topical.[6-7] Some reports detail that HA can specifically bind to cell receptors on certain tissues, such as liver, kidney, lymphatic vessels, and most tumor tissue under various conditions.[8-9] In the field of dentistry, HA is thought to play a role in mitigating periodontal diseases due to anti-inflammatory, anti-oedematous, and antibacterial properties.[10]

Antiseptics also play roles in mitigating infections on external surfaces of the body, i.e. skin and mucus membranes.[11] While increased antibiotic use can eventually lead to resistance, antiseptics are less likely to induce resistance due to their nonspecific mechanism of action, as an entire cellular structure, rather than specific molecular targets, is affected.[12] When applied to skin, antiseptics can offer solutions for infection prevention.[13-14] Antiseptics are also used in the field of dentistry as active ingredients in mouth rinses.

Due to the anionic nature of hyaluronic acid and cationic nature of antiseptics, this work aims to report on the synthesis and characterizations of polyelectrolyte, or ionic, complexes prepared from hyaluronic acid and oral/topical antiseptics. Polyelectrolyte complexes have a wide array of applications, including biomedical applications such as drug delivery and gene therapy.[15-16] Polyelectrolyte complexes are synthesized from oppositely charged molecules, for example, polymer-polymer, polymer-surfactant, and polymer-drug.[15] In past years, ionic complexes prepared from hyaluronic acid have been studied. Tolentino et al. (2012) studied the complexes of HA and alkyltrimethylammonium surfactants bearing octadecyl, eicosyl, and docosyl groups.[17] The authors found that complexes self-organized in biphasic layered structures characteristic of comb-like amphiphilic systems. More recently, Gamarra et al. (2018) fabricated HA complexes with alkanoylcholine surfactants of varying chain lengths which also arranged in comb-like structures.[18] The authors prepared these complexes as nanoparticles and encapsulated the chemotherapeutic, doxorubicin. Researchers found that these complexes suitable for drug transport and targeted delivery in anticancer chemotherapy.[18]

Herein, the sodium ion on hyaluronic acid was exchanged with cationic antiseptic surfactants, cetylpyridinium chloride and chlorhexidine diacetate, that are commercially available as mouthwashes and skin disinfectants. In this regard, the active pharmaceutical ingredient is part of the ionic complex and further drug encapsulation is not needed. By forming antiseptic complexes, these materials could be used in preventing diseases of the skin or mouth. Researchers have shown that by combining cetylpyridinium and hyaluronic acid in a mouth rinse, these two molecules had similar efficacy as chlorhexidine mouth rinses in preventing plaque accumulation.[19] Therefore, complexes of these molecules could play a similar role in the oral cavity by preventing bacterial infections of the mouth.

5.2. Materials

The sodium salt of hyaluronic acid (sodium hyaluronate, 1.5 to 2.2 million Dalton) and chlorhexidine diacetate were purchased from Acros Organics. Cetylpyridinium chloride was purchased from MP Biochemicals. Phosphate buffered saline tablets (0.01M) and lysozyme (from chicken egg white) were purchased from Sigma-Aldrich.

5.3. Experimental

5.3.1. Ionic Complex Preparation

Complexes of cetylpyridinium and hyaluronic acid were prepared by mixing aqueous solutions of sodium hyaluronate (NaHA) and cetylpyridinium chloride (CPC) at 40°C (denoted as [CPC+HA]). Complexes of chlorhexidine and hyaluronate were prepared by mixing an aqueous solution of NaHA and methanolic solution of chlorhexidine diacetate (CHX) at room temperature (denoted as [CHX+HA]). All solutions were agitated to facilitate ion-exchange reactions. Molar ratios of 1:1 (10mM:10mM) and 10:1 (100mM:10mM) of antiseptic surfactant to HA monomeric weight (402 g/mol) were used to fabricate ionic complexes. The resulting complexes (Figure 5.1) formed by precipitation were separated by centrifugation and washed several times with deionized water. Water was removed by lyophilization overnight. Further, freeze-dried complexes were heat pressed at 250°C to produce films.

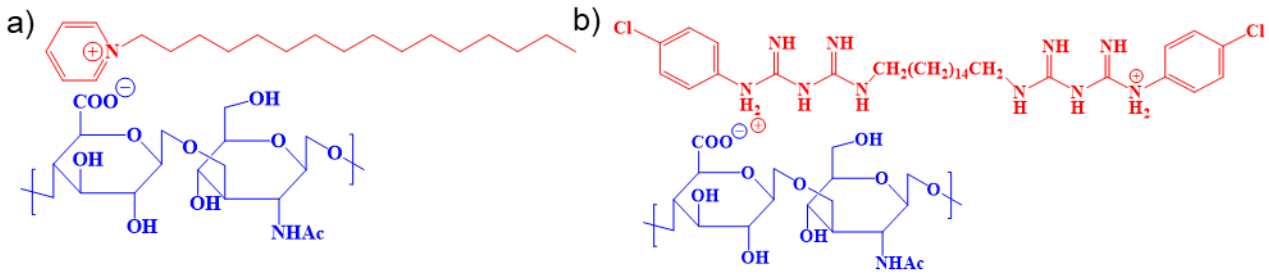

Figure 5.1. Chemical structure of the ionic complexes made of HA and antiseptics (a) cetylpyridinium and (b) chlorhexidine.

5.3.2. Structure and Thermal Characterization

Fourier transform infrared spectroscopy (FT-IR) was used to determine the chemical structure of ionic materials. The spectra were measured using a Bruker Tensor 27 (Billerica, MA) under transmission wavenumber ranging from 4000 to 650 cm^{-1}. The morphology of the complexes was analyzed using scanning electron microscopy (JSM -6610 LV SEM, JEOL USA) at 15kV. Calorimetric measurements were performed using a TA 2920 Modulated Differential Scanning Calorimeter (DSC) calibrated with indium. Sample weights of 3-5 mg were examined in a temperature range of 25 to 150°C under a nitrogen environment at a rate of 5°C/min. Thermal degradation was measured with an EXSTAR 6300 TG/DTA from 25 to 325°C under an argon environment at a rate of 10°C/min.

5.3.3. Cumulative Release of Antiseptics *in vitro*

Complexes and films of 1:1 and 10:1 antiseptic to HA weighing 10 ± 0.2 mg were placed in vials containing 5 mL of 1X phosphate buffered saline (PBS, pH=6.8) and placed in a water bath at 37°C. At predetermined time intervals, 5 mL of buffer was removed from the vial and absorbance was measured using a Shimadzu UV-3101PC UV-VIS-NR spectrophotometer (Shimadzu Europe). Equal volume of fresh PBS was added to the vial to maintain a constant volume. Absorbance was measured at λ= 259 nm and λ=254 for cetylpyridinium and chlorhexidine, respectively.

Phosphate buffered saline was prepared using tablets purchased from Sigma Aldrich. To prepare the PBS, five tablets were dissolved in one liter of deionized water to yield 0.01 M phosphate buffer (1X PBS). The pH was adjusted to 6.8 using hydrochloric acid.

5.3.4. Film Erosion *in vitro*

Pressed films were immersed in 10 mL of 1X PBS (pH=6.8) containing 5 μg/mL of lysozyme at 37°C. Samples were weighed prior to immersion in the erosion solution (W_0). After specific times, samples were removed from the solution, freeze-dried, and weighed (W_t). The weight loss percent (W_L%) was calculated using Equation 5.1. Each set of experiments was performed in triplicate.

$$\mathbf{W_L\%} = \frac{\mathbf{W_0 - W_t}}{\mathbf{W_0}} \times \mathbf{100} \tag{5.1}$$

5.4. Results and Discussion

5.4.1. Synthesis and Chemical Characterization of Complexes

Sodium hyaluronan and antiseptic surfactant complexes were synthesized by ion-exchange reactions. The complexes presented as large white precipitates as a result of ionic coupling between the polyanion and cationic, antiseptic surfactants. FT-IR spectra recorded for each complex are compared in Figures 5.2 and 5.3 for [CPC+HA] and [CHX+HA], respectively. As expected, spectra for all [antiseptic+HA] complexes show the presence of both counterparts. Hyaluronic acid has absorption bands at 1604 cm^{-1}, 1405 cm^{-1}, and 1027 cm^{-1}. These are indicative bands of the carboxylate asymmetric stretching vibration, carboxylate symmetric stretching, and C-O-C stretching vibration of the HA skeleton, respectively.[20-21] The large, broad absorption band between 3600 to 3000 cm^{-1} is indicative of the O-H and amide stretching region. In the region of 1150-650 cm^{-1} there is little difference between the spectra of the complexes and HA. This verifies the presence of HA in these poly-ionic materials.

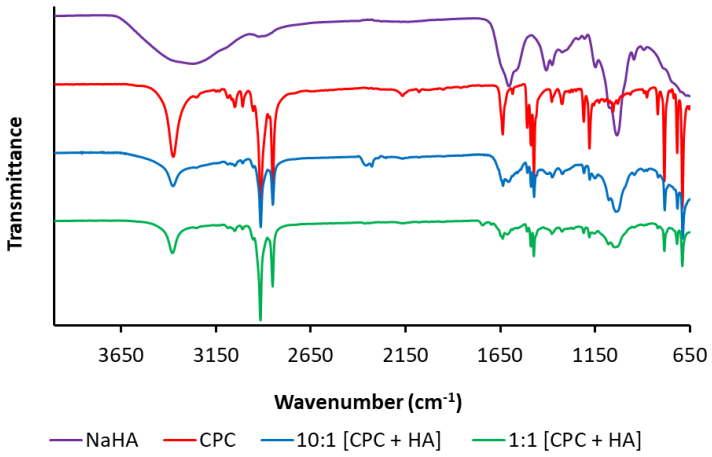

Figure 5.2. FTIR spectra of sodium hyaluronate, cetylpyridinium chloride, and [CPC + HA] complexes at 1:1 and 10:1 ratio.

For [CPC+HA] complexes (Figure 5.2), both ratios contain peaks in the 3100-3000 cm^{-1} and 3000-2800 cm^{-1} region which is indicative of alkene and alkane stretching for cetylpyridinium, respectively.[22] There is also much overlap in the fingerprint region for both ratios, indicating a strong presence of cetylpyridinium. Both ratios also maintain the strong N-H stretching band in the 3400-3350 cm^{-1} region. For [CHX+HA] complexes (Figure 5.3), both ratios contain peaks in the 3000-2800 cm^{-1} region which is indicative of the alkane stretching region of chlorhexidine.[23] Chlorhexidine-HA-based complexes also contain peaks in the 1500-1400 cm^{-1} region which overlap with chlorhexidine. The peak at 1486 cm^{-1} corresponds with C=C stretching on the aromatic rings.[23] Also evidenced from FT-IR spectra, these complexes are hygroscopic. In aqueous environments, hydrogen bonding occurs between adjacent carboxyl and N-acetyl groups of hyaluronic acid. This bonding allows HA to retain water.[24] FT-IR spectroscopy was measured of complexes after being exposed to atmosphere for 60 minutes. Spectra can be found in Appendix D (Figure D1 and D2). Noise in the 4000 to 3400 cm^{-1} region and 2000 to 1300 cm^{-1} is indicative of water and carbon dioxide absorption.[25] The peak in the 3400-3350 region for 1:1 [CPC+HA] complexes (Figure D1) broadened significantly which could indicate that 1:1 complexes are more

hygroscopic than 10:1 [CPC+HA] complexes. Those complexes only exhibited noise in the 2000 to 1300 cm^{-1} region. For [CHX+HA] complexes, noise in the 2000 to 1300 cm^{-1} region is present on both spectra (Figure D2); however, it is not determinable which complex is more hygroscopic.

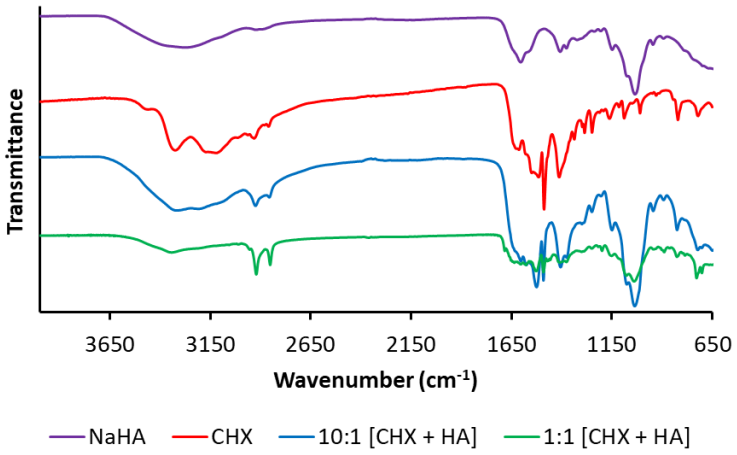

Figure 5.3. FTIR spectra of sodium hyaluronate, chlorhexidine diacetate, and [CHX + HA] complexes at 1:1 and 10:1 ratio.

5.4.2. Scanning Electron Microscopy (SEM)

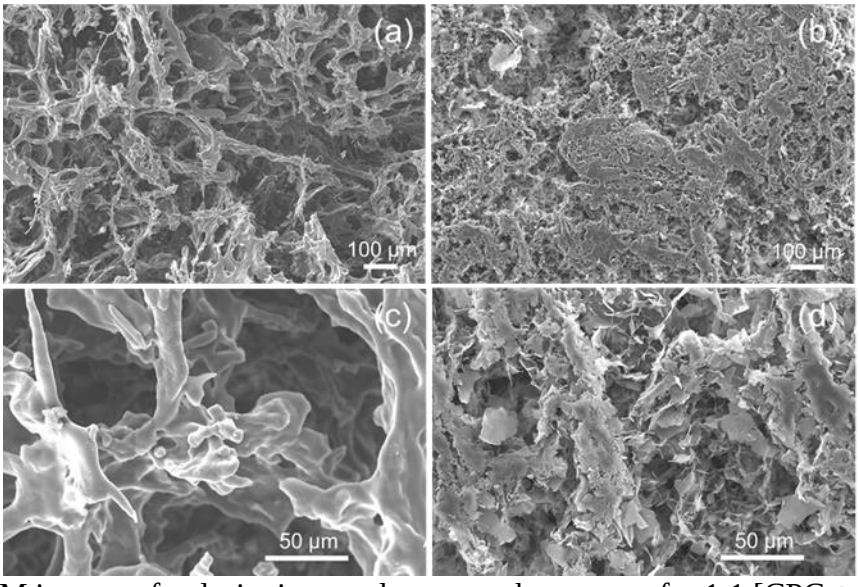

Figure 5.4. SEM images of poly-ionic complex network structure for 1:1 [CPC + HA] (a) and 10:1 [CPC + HA] (b) at 100X, and 1:1 [CPC + HA] (c) and 10:1 [CPC + HA] (d) at 500X.

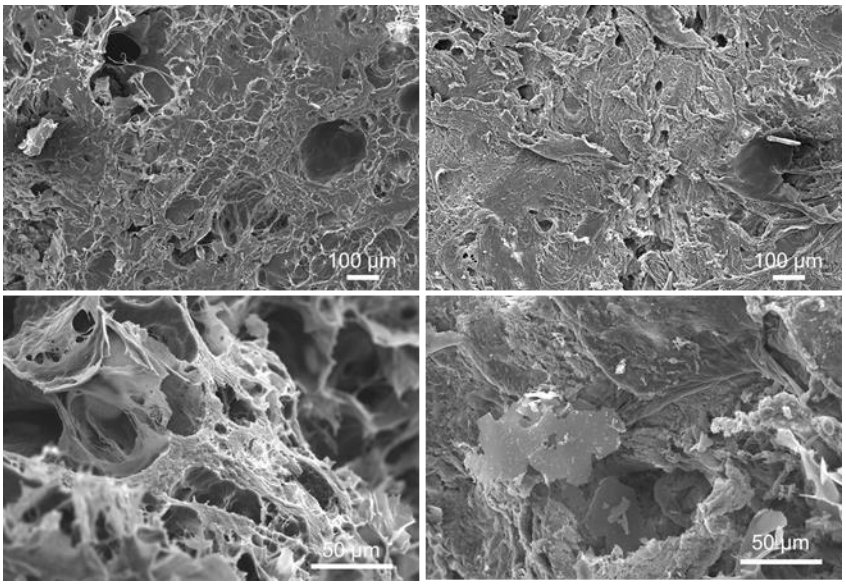

Figure 5.5. SEM images of poly-ionic complex network structure for 1:1 [CHX+HA] (a) and 10:1 [CHX+HA] (b) at 100X, and 1:1 [CHX+HA] (c) and 10:1 [CHX+HA] (d) at 500X.

The surface and inner network morphologies of the bulk poly-ionic complexes were examined using scanning electron microscopy (SEM). Figure 5.4a and b shows SEM images of 1:1 and 10:1 [CPC+HA] complexes, respectively. Larger pores can be observed for 1:1 [CPC + HA] whereas smaller pores can be observed for 10:1 [CPC+HA] indicating that the higher concentration of antiseptic leads to the formation of denser ionic complexes. Higher magnification (Figure 5.4c and d) shows the inner network of 1:1 [CPC + HA] as more fibrous than 10:1 [CPC + HA]. The inner network of 10:1 [CPC + HA] appears to have a randomly assorted, sheet-like morphology. This could be attributed to the higher concentration of cetylpyridinium used in synthesizing these ionic complexes.

SEM images of [CHX+HA] complexes are displayed in Figure 5.5. Like [CPC+HA] complexes, 1:1 [CHX+HA] complexes had less dense, more porous inner networks than 10:1 [CHX+HA] complexes. Fibers are clearly delineated within the network. The inner network of 10:1 [CHX+HA] is made up of denser, thicker fibers with sheet-like morphologies. This is

presumably from CHX. Chlorhexidine-HA-based complexes also appear to be denser than cetylpyridinium-HA-based complexes.

5.4.3. Thermal Properties

The thermal behavior of all complexes was examined using differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). Data afforded using TGA is presented in Table 5.1. Thermogravimetric traces of all antiseptic-HA-based complexes and sodium hyaluronate, as well as their first derivative curves, are illustrated in Figure 5.6. Water evaporation is evidenced on the traces before thermal decomposition was detectable around 180-224°C. By exchanging the sodium ion for antiseptics, the onset temperature of HA is lowered by approximately 20-45°C. Therefore, the thermal stability of HA is reduced after complexation.

Cetylpyridinium lowered the onset decomposition temperature of HA more so than chlorhexidine. This could be due to the long alkyl chain attached to the pyridinium ring. The first derivative of the TGA curves (Figure 5.6b) shows that 10:1 [CPC + HA] complexes decompose through a process that involves two steps: the first one at 180.1°C and the second one at 222.5°C. The first derivative of the TGA curves also shows a slight two-step process for 1:1 [CPC+HA] as well. This two-step process could be due to cetylpyridinium degrading by a Hofmann elimination to form hexadecane and pyridine.[26-27] When comparing the two ratios of each complex, the higher content of antiseptic did not appear to play a large role in affecting the onset temperature.

Table 5.1. Thermal properties of [antiseptic+HA] complexes

Material	TGA T_d [a] (°C)
Sodium Hyaluronate	223.9
1:1 [CPC+HA]	184.5
10:1 [CPC+HA]	180.1 222.5
1:1 [CHX+HA]	201.8
10:1 [CHX+HA]	199.7

[a] Onset decomposition temperature calculated by the tangent method

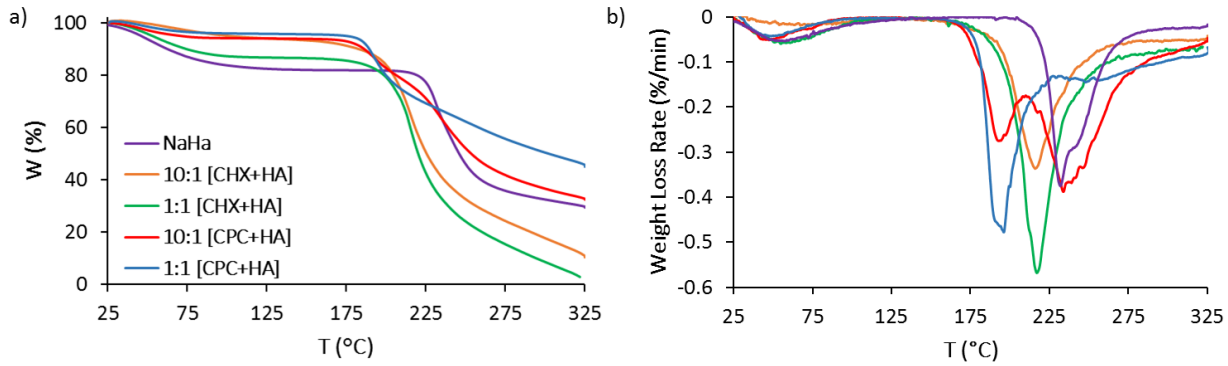

Figure 5.6. Comparison of TGA traces for all HA-based complexes and sodium HA (a) and their first derivative curves (b).

The DSC traces for complexes are compared in Figure 5.7. The DSC analysis reveals that 10:1 [CPC+HA] complexes had melting peaks at 78.9 °C. This peak can be attributed to the paraffinic phase of the long alkyl chains of CPC molecules. However, the DSC trace of 1:1 [CPC+HA] did not reveal melting peaks. This could be due to hyaluronate disrupting the molecular arrangement of these complexes. Chlorhexidine-HA-based complexes did not show any sign of melting either. The structure of chlorhexidine along with amorphous HA could be hindering any crystalline packing arrangement of these complexes.

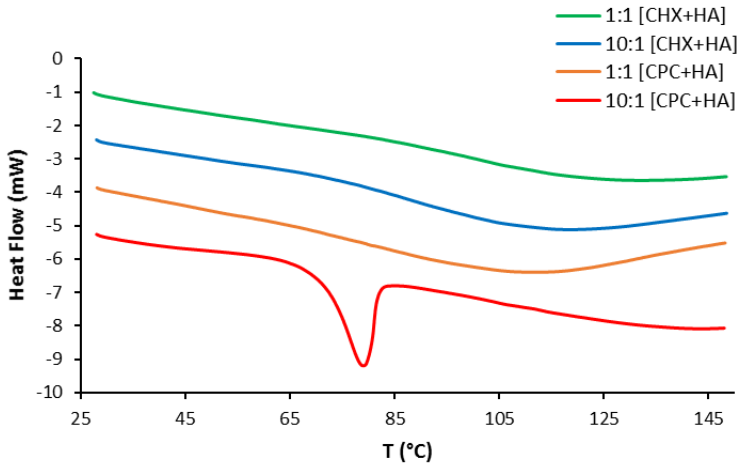

Figure 5.7. DSC traces of all antiseptic-HA-based complexes.

5.4.4. *In vitro* Antiseptic Release

The nature and speed of drug release is an important feature for evaluating new drug delivery systems. Cumulative drug release studies were performed in vitro to understand the rate of drug release. The release curves of [CPC+HA] and [CHX+HA] complexes and films in 1X PBS buffer (pH=6.8) at 37°C are illustrated in Figures 5.8 and 5.9, respectively.

Cetylpyridinium-HA-based complexes exhibited burst release between 0 minutes and 15 minutes. The correlation coefficients (R^2) of antiseptic release for cetylpyridinium-HA- and chlorhexidine-HA-based complexes are displayed in Table 5.2. Complexes of 1:1 [CPC+HA] released CPC more akin to steady release (R^2=0.94) than other cetylpyridinium-HA-based material. Interestingly, 1:1 [CPC+HA] complexes and films released statistically significant ($p<0.05$) more CPC overall as compared to 10:1 [CPC+HA] complexes and films. This could presumably be due to greater hydrophobic and electrostatic interactions when using higher concentrations of CPC. When comparing the bulk material to its respective film ratio, there is no statistically significant drug release.

Both the bulk material and films for [CHX+HA] released CHX at a steady rate over five hours as can be interpreted from correlation coefficients (Table 5.2). Bulk complexes of 1:1 [CHX+HA] released more CHX than bulk 10:1 [CHX+HA]; however, the amount was not statistically significant. Bulk complexes also exhibited an initial burst release between 0 minutes and 15 minutes. However, steady release of drug occurred for the remainder of time studied. Films of 10:1 and 1:1 [CHX+HA] released a statistically significant ($p<0.05$) more amount of CHX than their bulk counterparts. This could be due to CHX being more accessible for release in films than in bulk material. Heat pressing bulk material into films increases the surface area from which drug can be released.

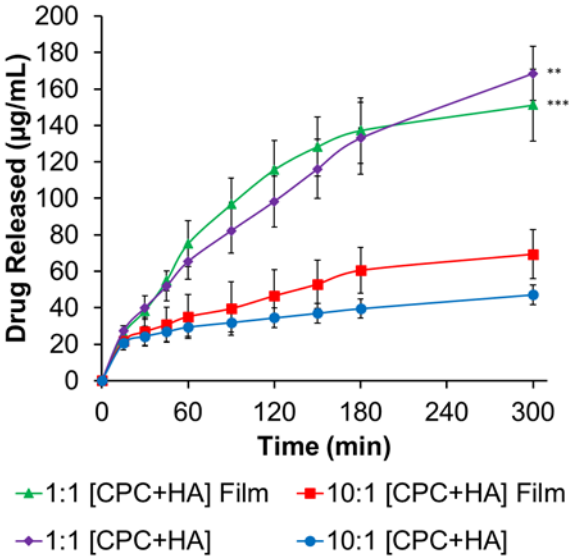

Figure 5.8. Cumulative drug release of cetylpyridinium from bulk material and films of 10:1 and 1:1 [CPC+HA]. ** and *** represent p≤0.05 when comparing bulk 1:1 [CPC+HA] to bulk 10:1[CPC+HA] and 1:1 [CPC+HA] film to 10:1 [CPC+HA] film, respectively.

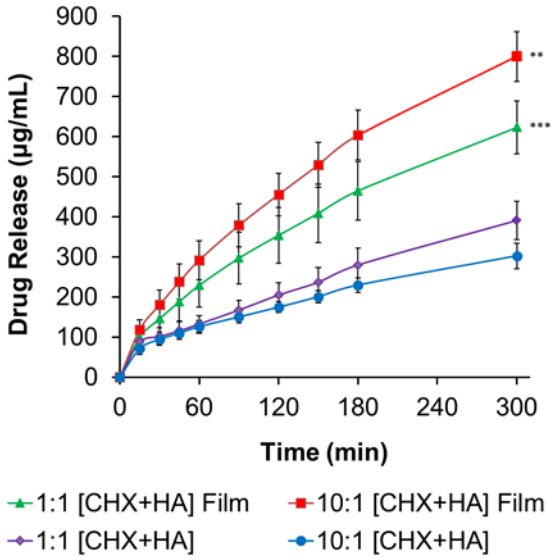

Figure 5.9. Cumulative drug release of chlorhexidine from bulk material and films of 10:1 and 1:1 [CHX+HA]. ** and *** represent p≤0.05 when comparing 10:1 [CHX+HA] film to bulk 10:1 [CHX+HA] and 1:1 [CHX+HA] film to bulk 1:1 [CHX+HA], respectively.

Overall, [CHX+HA] complexes released more drug than [CPC+HA] complexes. This could be due to weaker electrostatic interactions between CHX and HA. Also, pH of the solution could be playing a role in the high drug release. Chlorhexidine is pH dependent and is most optimal

within a range of 5.0 to 7.4.[28] At pH=6.8, CHX could have dissociated from HA more rapidly than CPC. While CPC is also optimal around physiological pH, it has been shown to increasingly bind to HA over time in PBS.[29] This could explain the lesser degree of drug release and supports the theory of greater electrostatic interactions between CPC and HA.

Table 5.2. Summary of correlation coefficient R^2 derived from entire fit of the cumulative amount of drug released.

Material	R^2	Material	R^2
1:1 [CPC+HA]	0.94	1:1 [CHX+HA]	0.96
1:1 [CPC+HA] Film	0.83	1:1 [CHX+HA] Film	0.95
10:1 [CPC+HA]	0.72	10:1 [CHX+HA]	0.93
10:1 [CPC+HA] Film	0.85	10:1 [CHX+HA] Film	0.95

5.4.5. Enzymatic Film Erosion *in vitro*

Oral drug delivery systems experience degradation and erosion due to various enzymes in the mouth.[30] To understand the rate of erosion of the proposed antiseptic-HA-based films, weight loss of films was analyzed in 1X PBS (pH=6.8) at 37 °C with the addition of lysozyme (5 µg/mL). Percent weight loss of films is displayed in Figure 5.10.

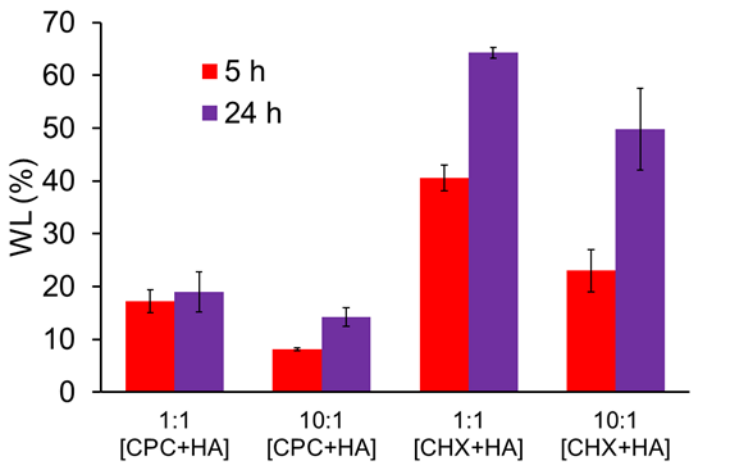

Figure 5.10. Weight loss percent during film erosion in PBS (pH= 6.8) with 5 µg/mL of lysozyme.

Chlorhexidine-HA-based films experienced a higher degree of weight loss due to enzymatic erosion as compared to cetylpyridinium-HA-based films. This could be due to weaker hydrophobic and electrostatic interactions between CHX and HA. Due to high film degradation, 1:1 [CHX+HA] films could be more suitable as skin patches as opposed to oral patches. The 10:1 [antiseptic+HA] films experiencing less erosion than their 1:1 counterpart could be explained by their high density (Figures 5.4 and 5.5) which could slow liquid and lysozyme penetration.

5.5. Conclusion

Ionic complexes of HA and antiseptic surfactants, cetylpyridinium and chlorhexidine, were readily prepared. The hygroscopic properties of HA are still maintained in these complexes which could be ideal for wound care management. Moisture prevents wounds from drying out and scabs from forming, which minimizes the likelihood of scars. By replacing the sodium atoms for antiseptic surfactants, the complex itself becomes medicated and no further drug encapsulation is needed. These materials could have potential applications in topical skin treatments or as oral patches as the antiseptics used are commercially available active ingredients in mouthwashes and skin washes. Overall, chlorhexidine-HA-based materials released more antiseptic than cetylpyridinium-HA-based complexes presumably due to weaker ionic bonding interactions. Cetylpyridinium-HA-based complexes experienced less enzymatic film erosion which could have implications for oral patches. Further investigation into cytotoxic potential and mucoadhesion tests of these HA-based films need to be investigated.

5.6. References

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CHAPTER 6. CONCLUSIONS AND FUTURE WORK

6.1. Conclusions

This dissertation discusses different antimicrobial strategies for combating bacterial species on topical surfaces using GUMBOS and biopolymers. The studies in Chapters 2 and 3 investigate the antimicrobial properties of GUMBOS synthesized from antiseptics and antibiotics towards *Neisseria gonorrhoeae* for the purpose of combating oropharyngeal gonorrhea. GUMBOS synthesized in Chapters 2 and 3 could offer an alternative approach to current drug therapies for gonorrhea and have further implications for topical, prevention strategies. Chapter 2 focused on using an outmoded antibiotic, carbenicillin, and an oral antiseptic, octenidine to generate novel GUMBOS. These studies presented in this chapter showed that octenidine-carbenicillin GUMBOS were more effective than their unreacted, stoichiometric mixtures. GUMBOS were also bioequivalent to azithromycin and were more effective than doxycycline, two antibiotics prescribed for gonorrhea. Chapter 3 focused on GUMBOS fabricated from one of the currently prescribed antibiotics for the treatment of gonorrhea, ceftriaxone, and either chlorhexidine or octenidine. These GUMBOS were ten times more effective than azithromycin in preventing growth of *N. gonorrhoeae*. Octenidine-ceftriaxone and chlorhexidine-ceftriaxone GUMBOS were also resistant towards existing methods of antibiotic resistance, i.e. β-lactamases, that might have negated the therapeutic usefulness of constituent drugs. When tested against Carbapenem-resistant *Enterobacteriaceae*, GUMBOS were 5-40X more effective than ceftriaxone and azithromycin.

Chapters 4 and 5 focused on the development of materials from polymers for wound care and infection prevention applications. Studies presented in Chapter 4 showed that a simple ionic liquid could be used to fabricate composites from hyaluronic acid and cellulose without the addition of chemical crosslinkers for the purpose of wound treatments. These composites showed

great swelling properties. Composites swelled 200-1600% their dry weight when soaked in different media (DI water, PBS, and 0.9% saline solution). These materials showed high potential for being used as drug delivery platforms for skin infections. The studies presented in Chapter 5 investigated drug delivery properties and enzymatic erosion of poly-ionic complexes fabricated from sodium hyaluronan and cationic antiseptics (cetylpyridinium chloride and chlorhexidine diacetate) that could potentially be used as either antimicrobial oral or skin patches. Poly-ionic complexes of chlorhexidine-hyaluronic acid achieved steady release of chlorhexidine and by pressing into films, release of antiseptic can be increased. However, these films exhibited a higher degree of enzymatic erosion. On the contrary, cetylpyridinium-hyaluronic acid complexes showed initial burst release before steadily releasing drug. Films of [CPC+HA] were not as affected by lysozyme as [CHX+HA] films. These films showed promise as potential oral patches.

6.2. Future Work

This dissertation primarily focuses on development of therapeutic materials and *in vitro* studies for combating bacterial infections. It would be of interest to study the behavior of these materials *in vivo* as well. *Neisseria gonorrhoeae* is evolving to become resistant to the currently recommended antibiotics, ceftriaxone and azithromycin. It would be interesting to examine the efficacy of GUMBOS synthesized from 3rd generation cephalosporins that have yet to be examined for antibacterial properties towards *N. gonorrhoeae*. Ceftriaxone resistance rates have increased over the years and there is a dearth of new drugs in the drug development pipeline to combat gonorrhea. One newly developed drug has shown promise and has advanced through trials; however, this drug only targets gonorrhea in the genital tract. Furthermore, formulation of a mouthwash for the prevention and treatment of oropharyngeal gonorrhea could be of interest for preliminary *in vivo* studies. Additionally, *in vivo* studies of hyaluronic acid-cellulose composites

on murine wound models would provide further insight into whether these materials can be classified as aiding in wound healing. Hyaluronic acid, one of the key components of composites presented in this dissertation, is a key component of the extracellular matrix and is known to be involved in several mechanisms of the wound healing process. Investigating these materials for adhesive properties to wound sites would also be beneficial; as commercial bandages adhering to wound sites can cause pain upon removal and potentially re-open the wound. In addition, mucoadhesion studies for antiseptic-hyaluronic acid-based poly-ionic complexes would provide further knowledge of possible clinical applications of these materials.

APPENDIX A. SUPPORTING INFORMATION FOR CHAPTER 2

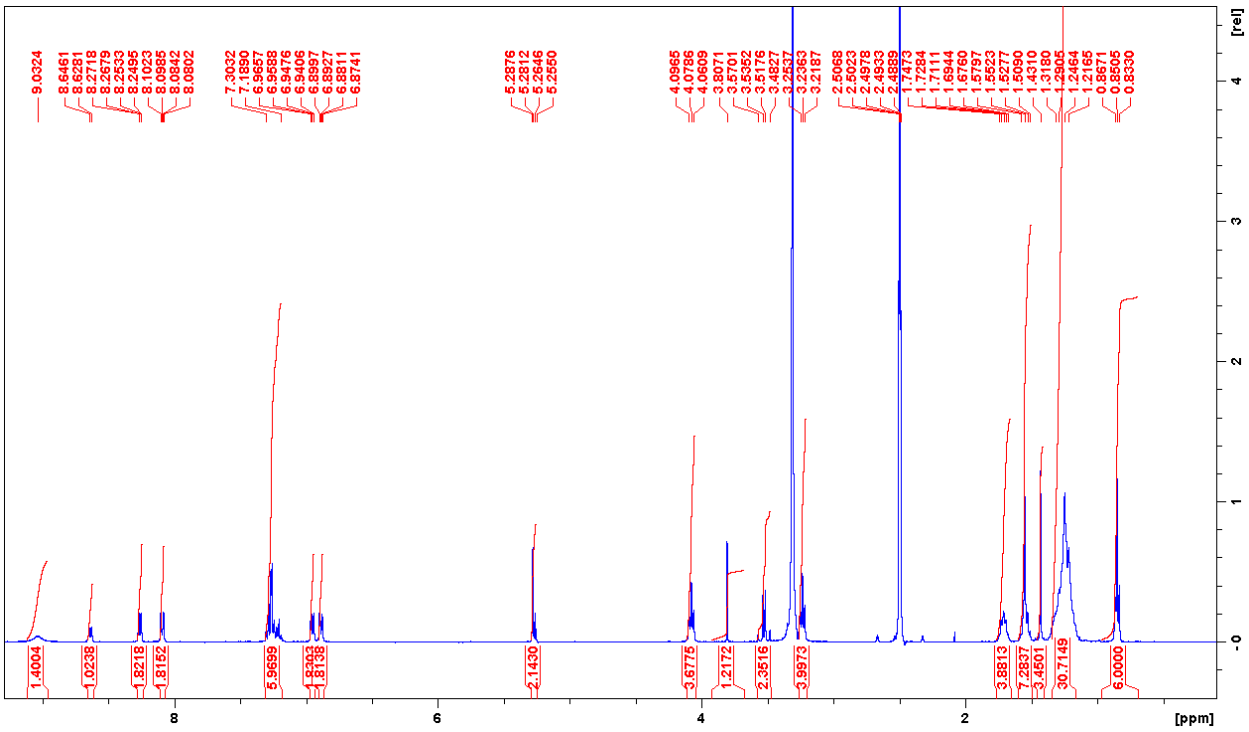


Figure A1. ^{1}H-NMR for [OCT][Carb] GUMBOS.

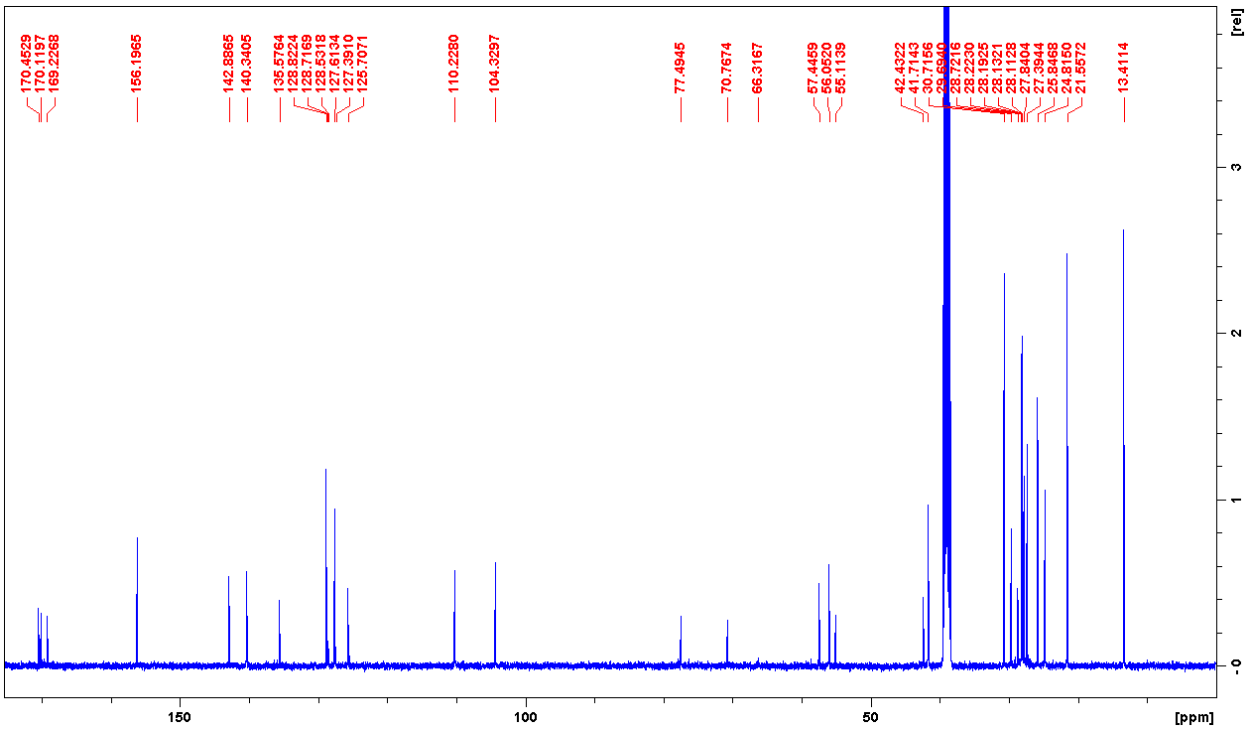


Figure A2. ^{13}C-NMR for [OCT][Carb] GUMBOS.

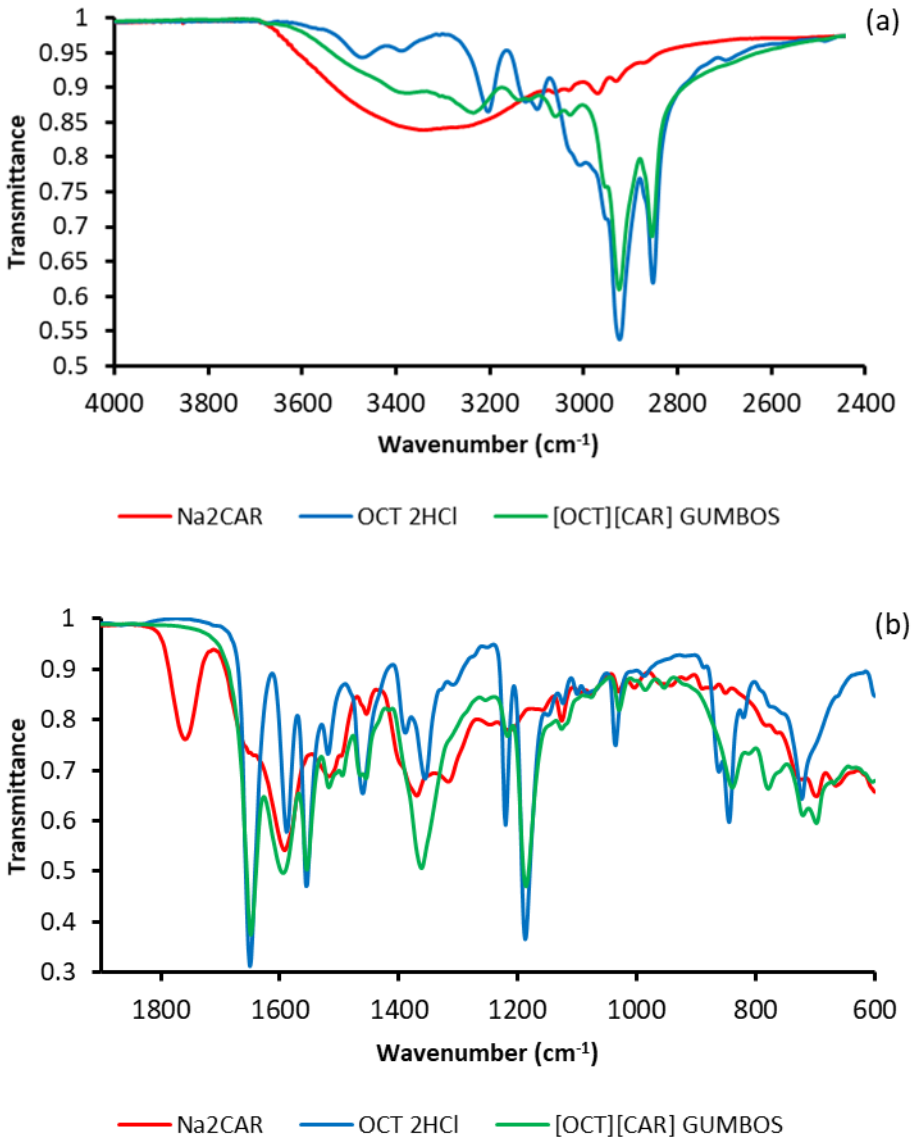


Figure A3. Fourier transform infrared (FT-IR) spectra for [OCT][CAR] and parent compounds; (a) 4000-2400 cm^{-1}, (b) 1900-600 cm^{-1}.

APPENDIX B. SUPPORTING INFORMATION FOR CHAPTER 3

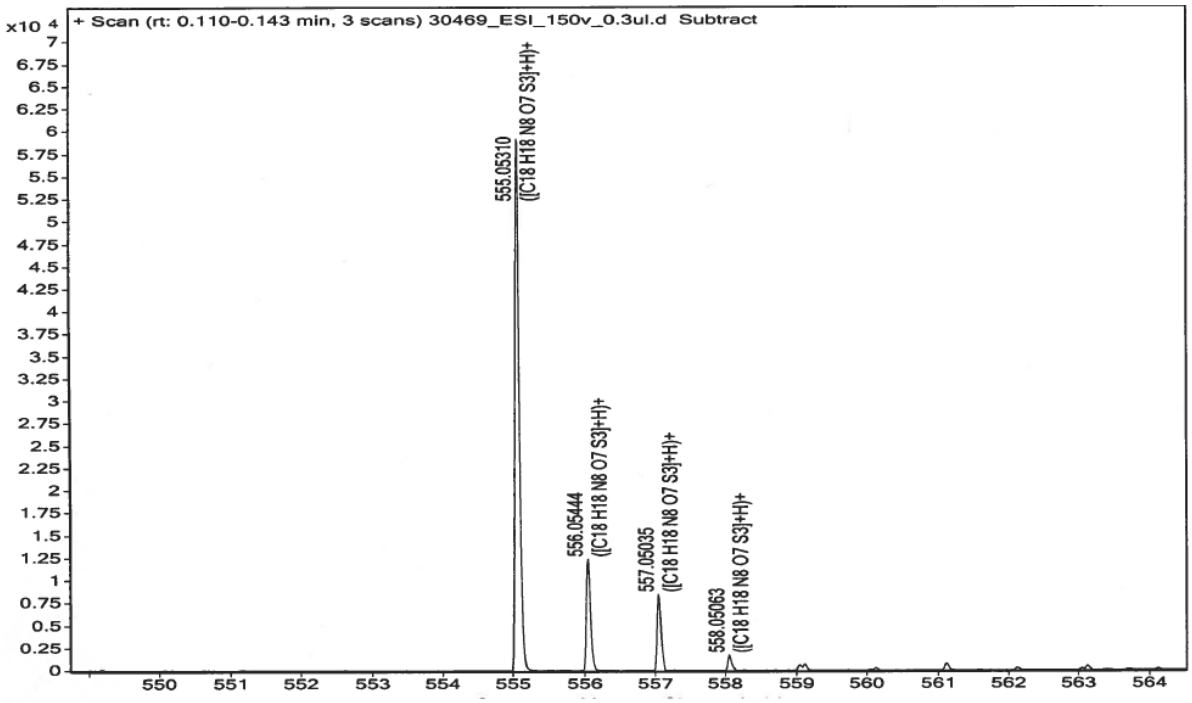


Figure B1. High res mass spectrum for ceftriaxone of [CHX][CRO] GUMBOS.

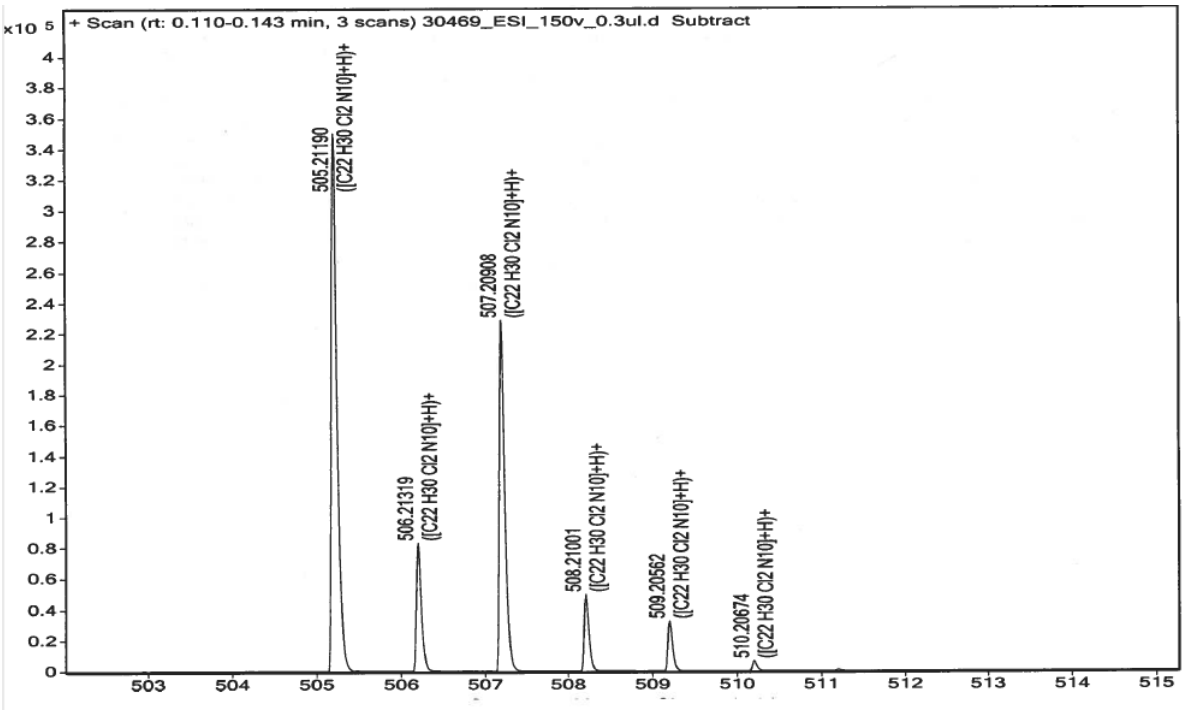


Figure B2. High res mass spectrum for chlorhexidine of [CHX][CRO] GUMBOS.

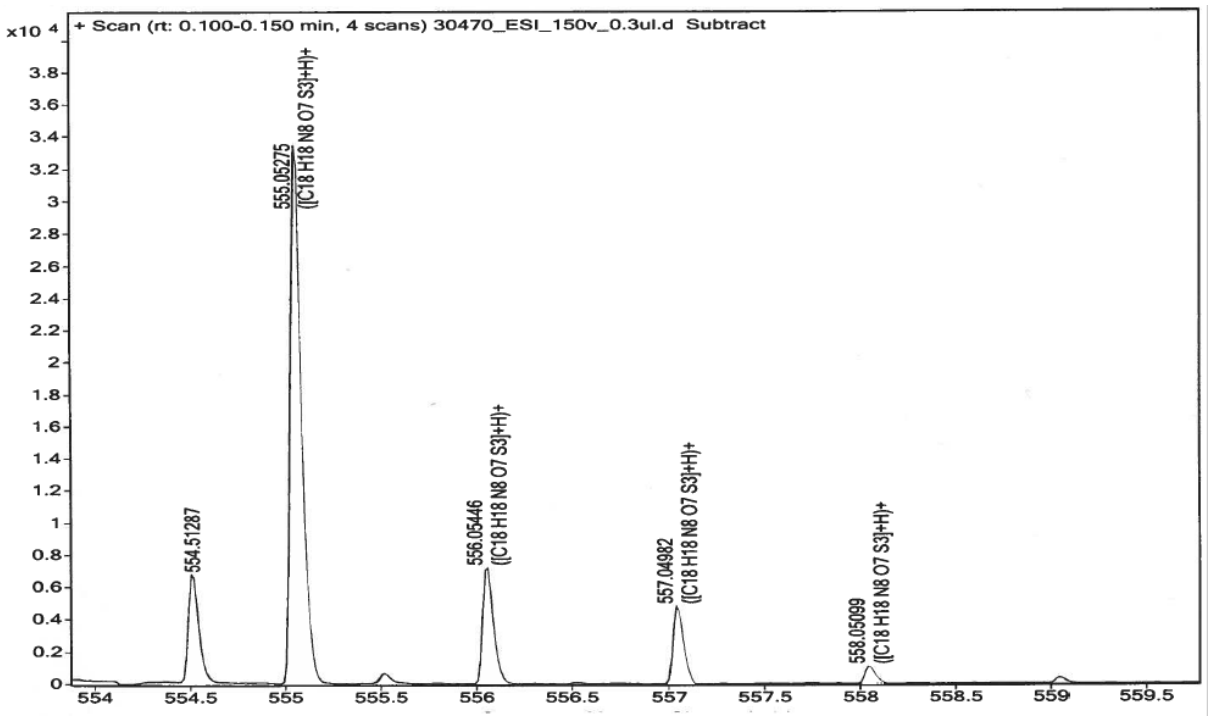


Figure B3. High resolution mass spectrum for ceftriaxone of [OCT][CRO] GUMBOS.

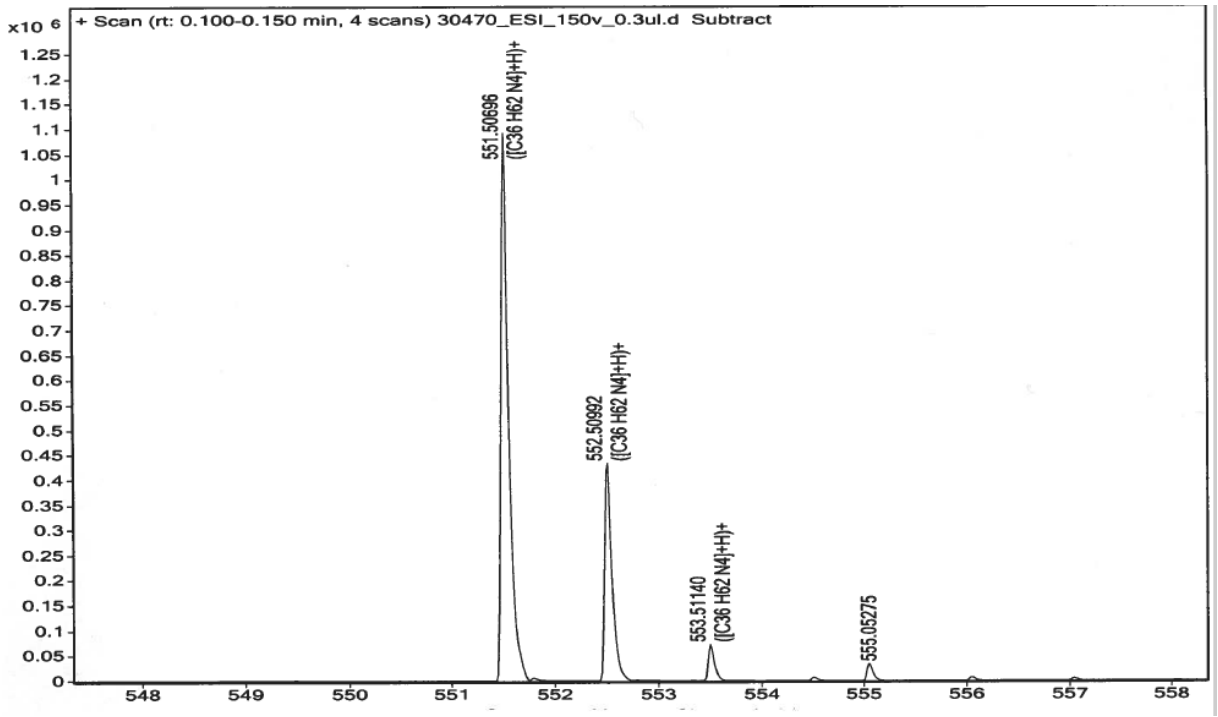


Figure B4. High resolution mass spectrum for octenidine of [OCT][CRO] GUMBOS.

APPENDIX C. SUPPORTING INFORMATION FOR CHAPTER 4

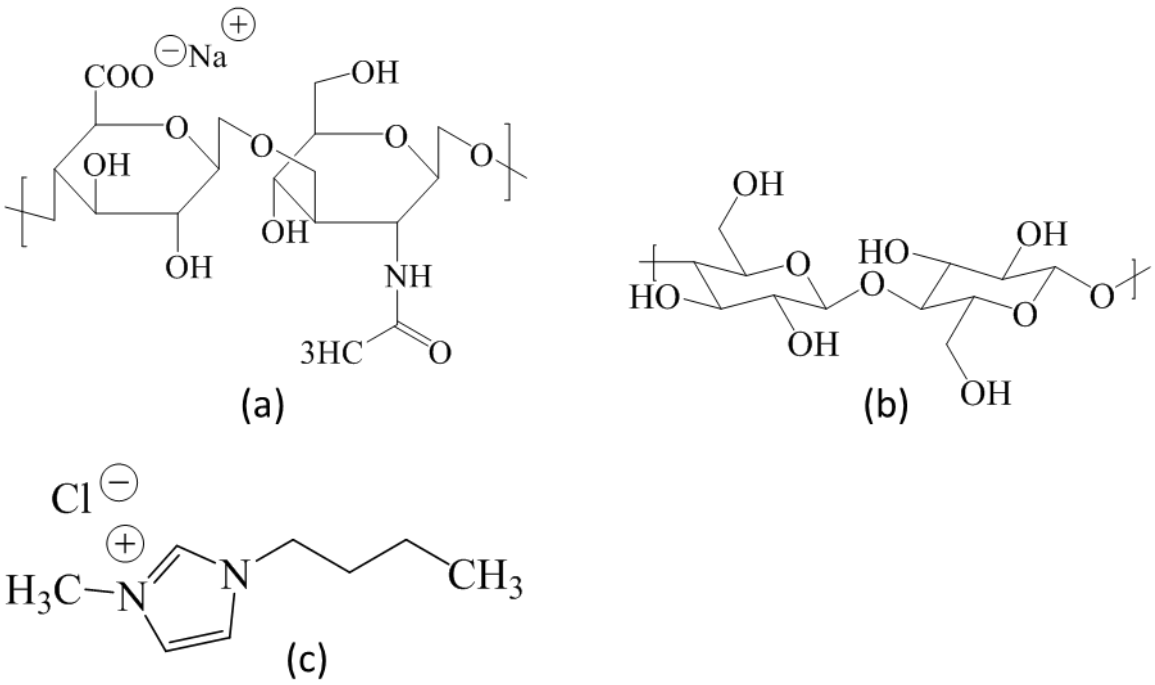


Figure C1. Structures of hyaluronic acid sodium salt (a), cellulose (b), and [Bmim][Cl] (c).

Removal and Recovery of [Bmim][Cl]

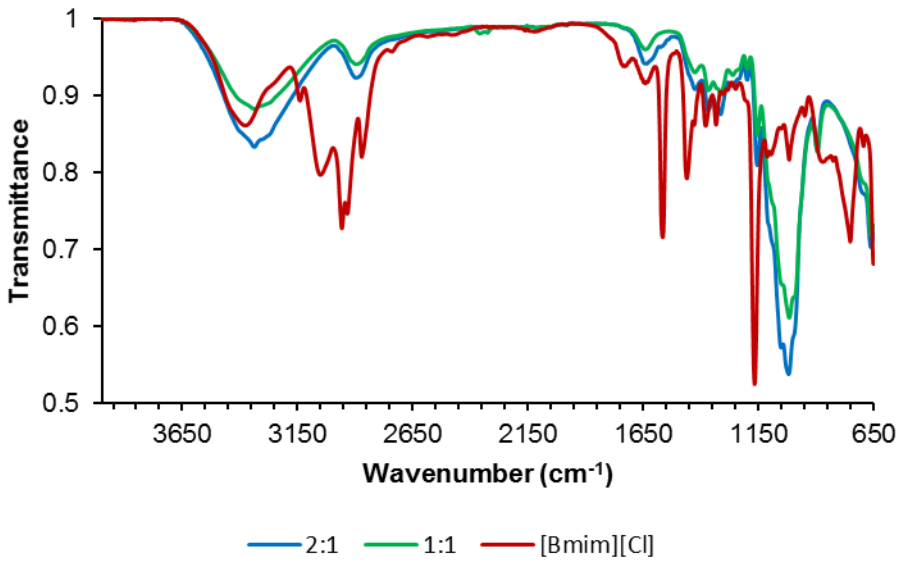


Figure C2. FT-IR spectra of 1:1 composite and 2:1 composite to ensure complete removal of [Bmim][Cl].

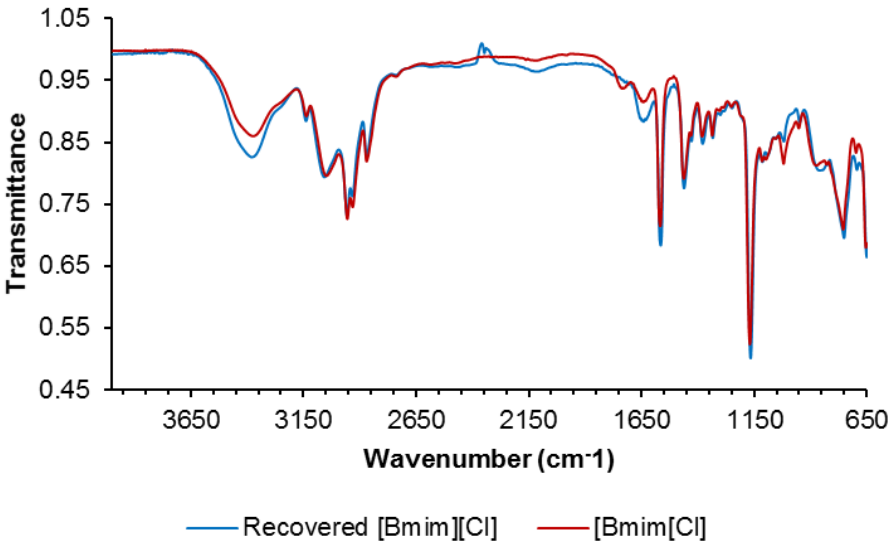


Figure C3. FTIR spectra of recovered [Bmim][Cl] versus [Bmim][Cl].

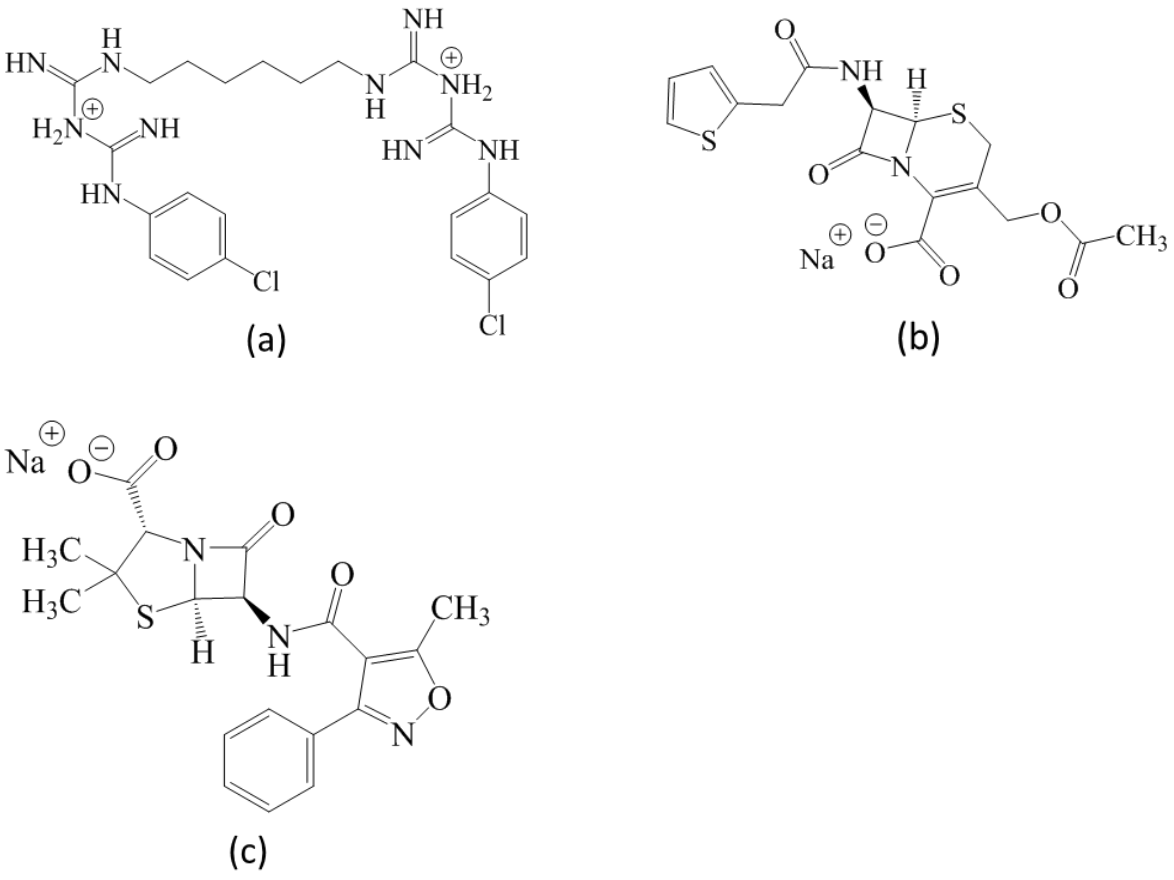


Figure C4. Structures of compounds used to synthesize GUMBOS. Chlorhexidine (a), cephalothin sodium salt (b), and oxacillin sodium salt (c).

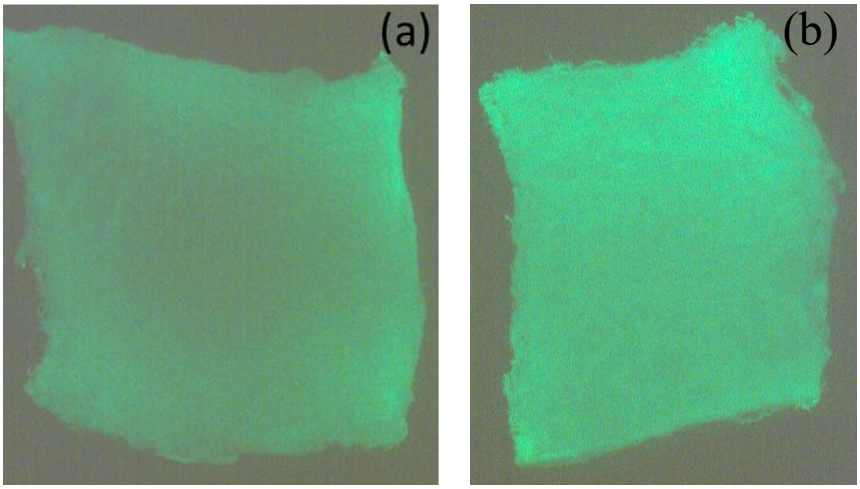


Figure C5. Fluorescence microscopy images of 1:1 composite (a) and 2:1 composite (b) drop casted with fluorescein sodium salt.

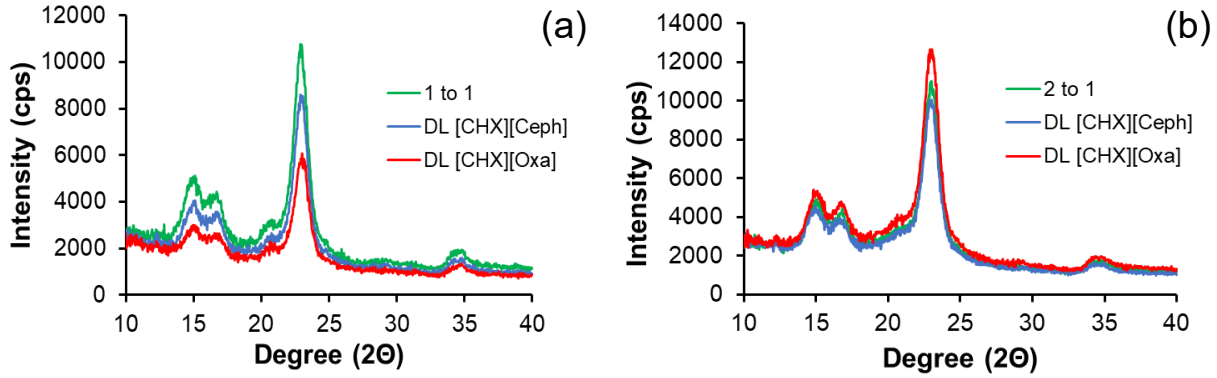


Figure C6. XRD of plain composites and drug-loaded (DL) composites; 1:1 composites (a) and 2:1 composites (b). Composites were drug-loaded with 100 µM.

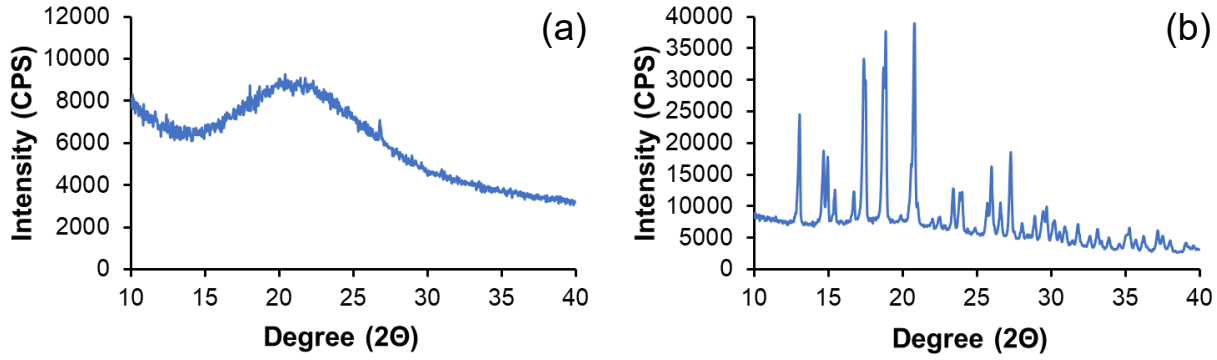


Figure C7. Powder XRD of [CHX][Ceph] (a), and [CHX][Oxa] (b).

Table C1. Crystallinity indices for drug-loaded (DL) 1:1 and 2:1 composites.

Materials	Crystallinity Index (%)
1:1 DL [CHX][Ceph]	78.1
1:1 DL [CHX][Oxa]	75.3
2:1 DL [CHX][Ceph]	75.9
2:1 DL [CHX][Oxa]	77.9

Composites were drug-loaded with 100 µM.

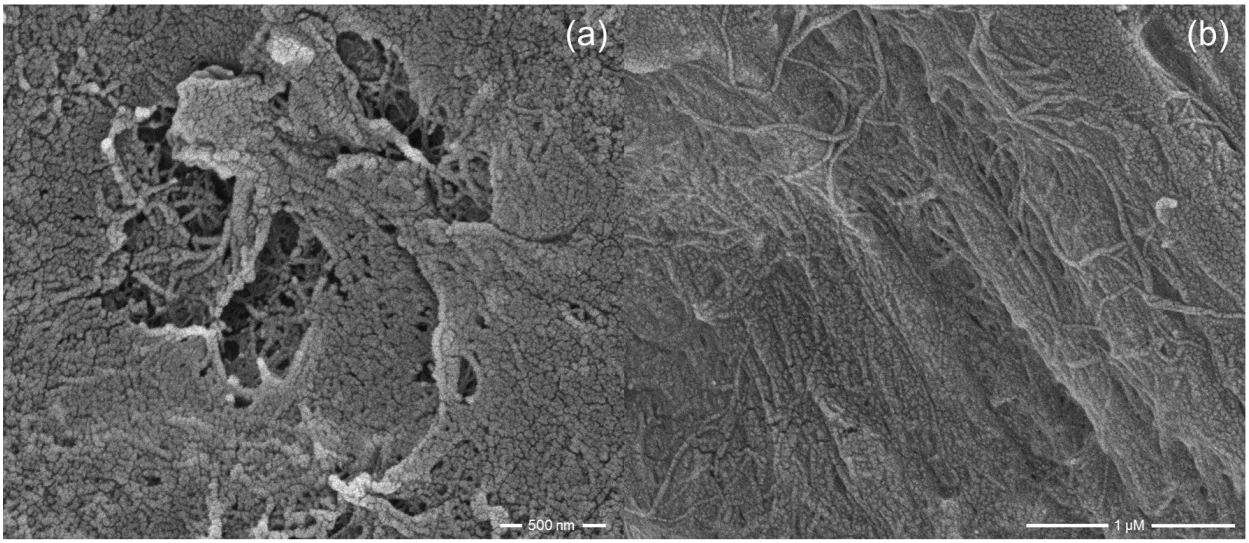


Figure C8. 50,000X SEM images of 1:1 composite (a), and drug loaded 1:1 composite (b).

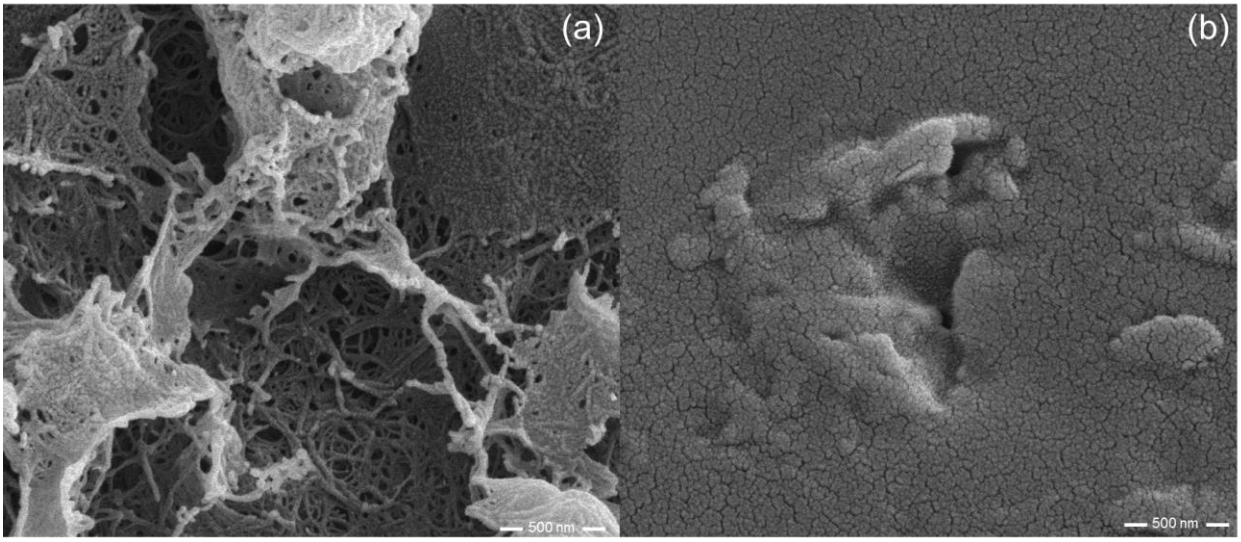


Figure C9. 50,000X SEM images of 2:1 composite (a), and drug loaded 2:1 composite (b).

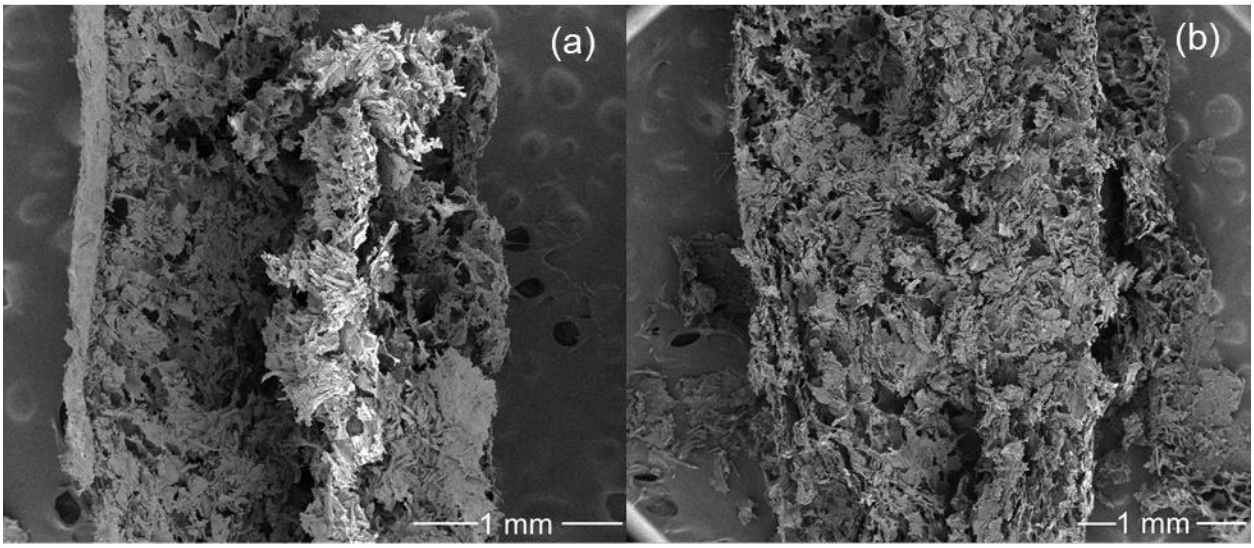


Figure C10. Cross-sectional SEM images of 1:1 composite, 50X (a), and [CHX][Ceph] loaded 1:1 composite, 40X (b).

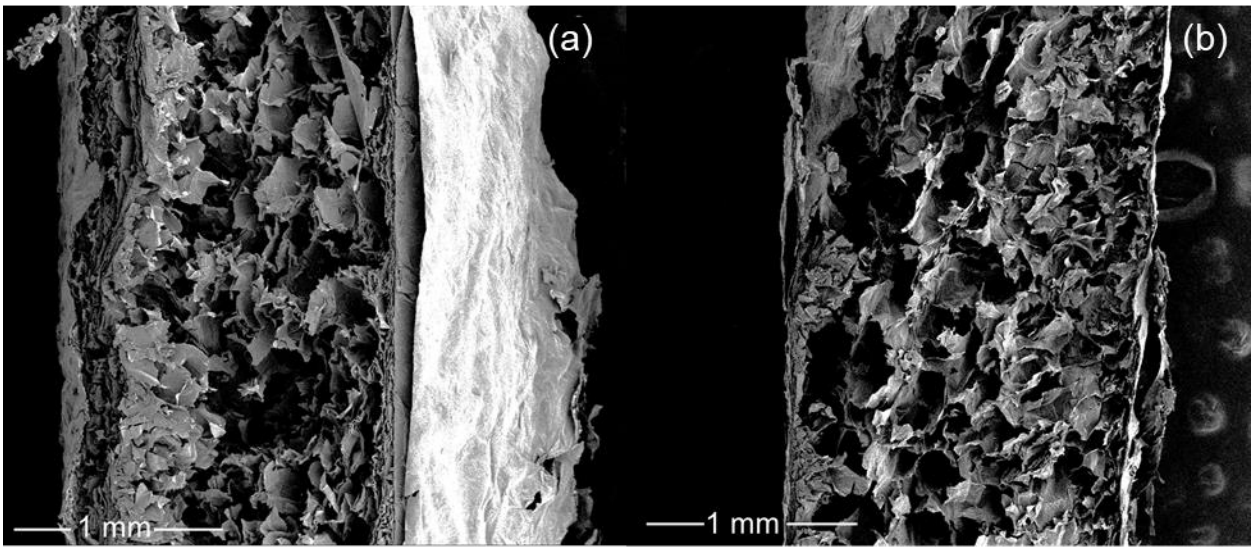


Figure C11. 50X Cross-sectional SEM images of 2:1 composite (a), and [CHX][Ceph] loaded 2:1 composite (b).

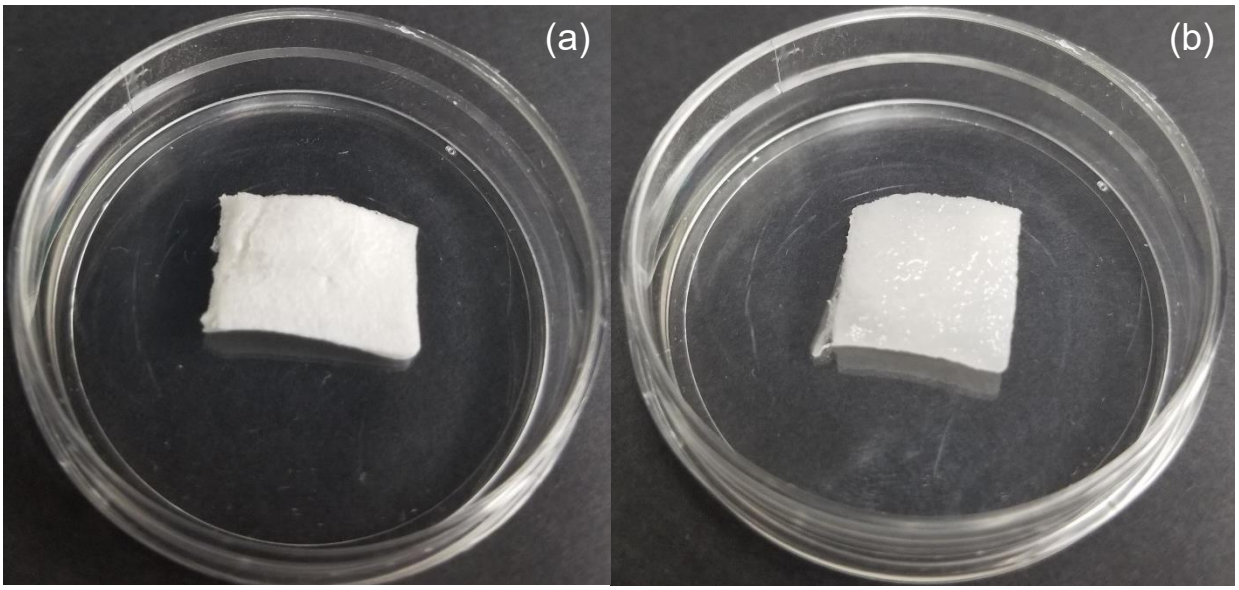


Figure C12. Dry 1:1 (HA:CEL) composite (a), and swollen 1:1 composite in 0.9% saline (b).

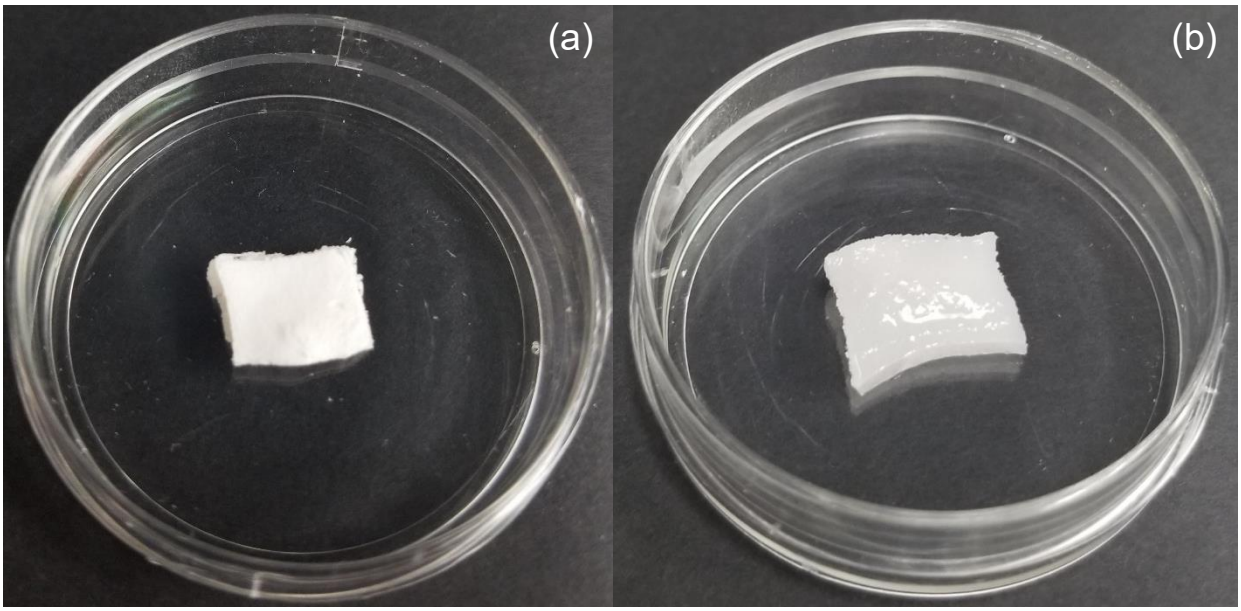
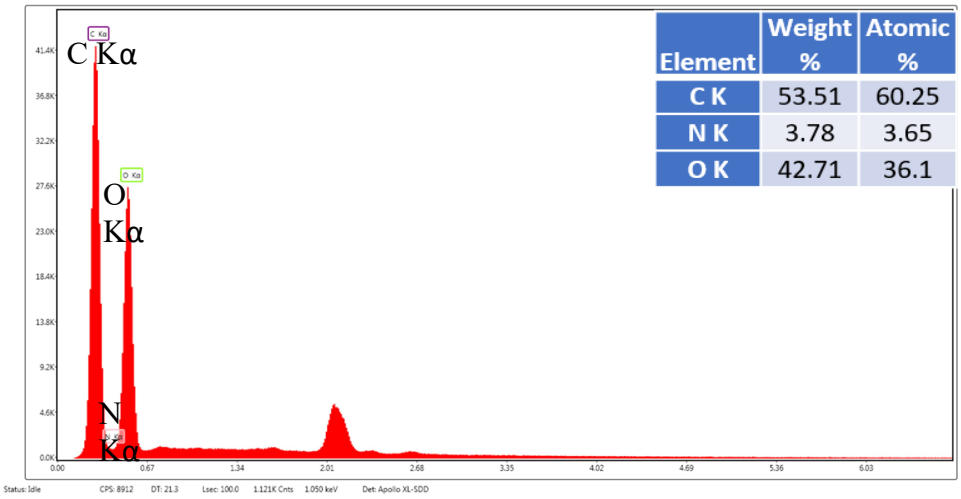
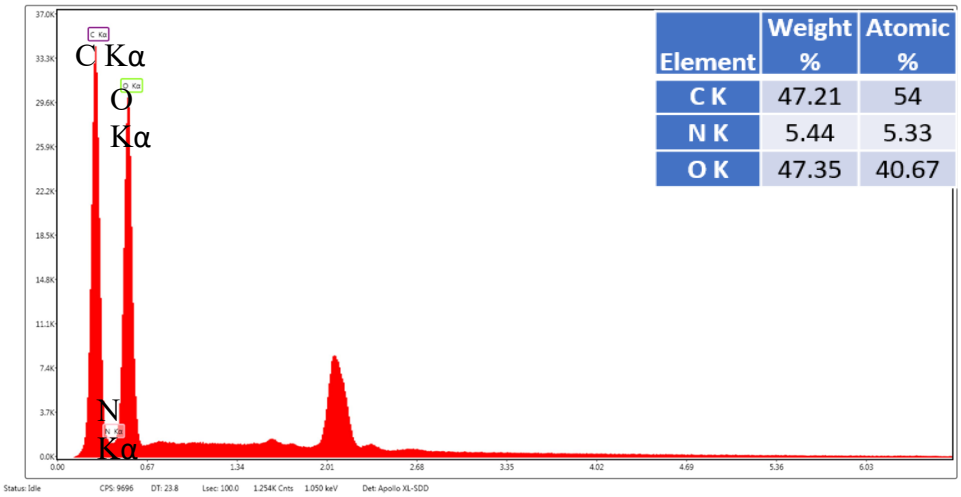


Figure C13. Dry 2:1 (HA:CEL) composite (a), and swollen 2:1 composite in 0.9% saline (b).



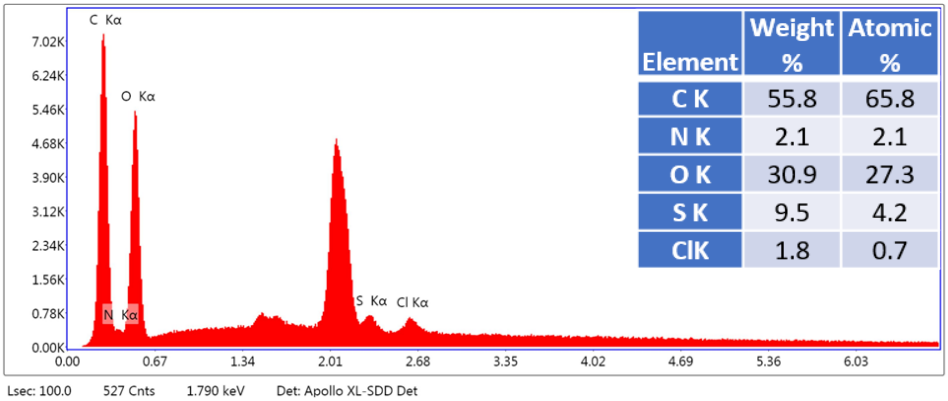
Element	Weight %	Atomic %
C K	53.51	60.25
N K	3.78	3.65
O K	42.71	36.1

Figure C14. EDS spectrum of 1:1 HA:CEL composite.



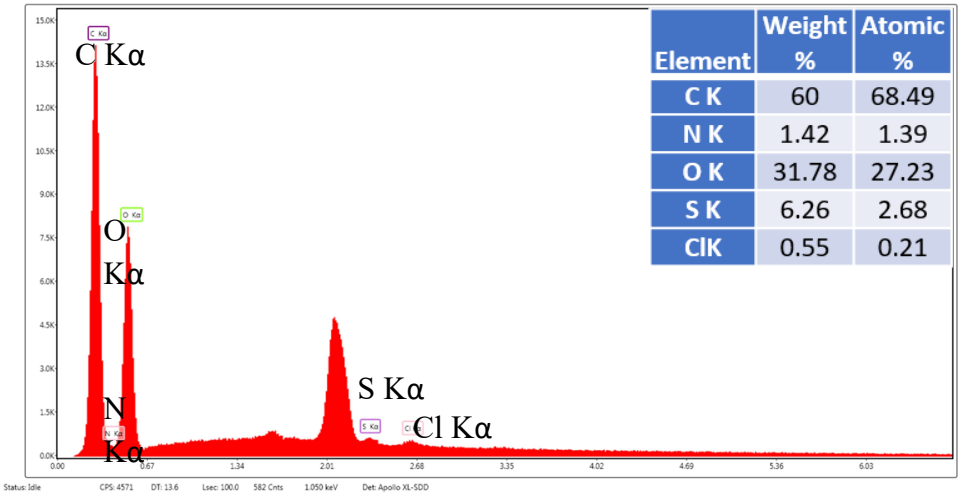
Element	Weight %	Atomic %
C K	47.21	54
N K	5.44	5.33
O K	47.35	40.67

Figure C15. EDS spectrum of 2:1 HA:CEL composite.



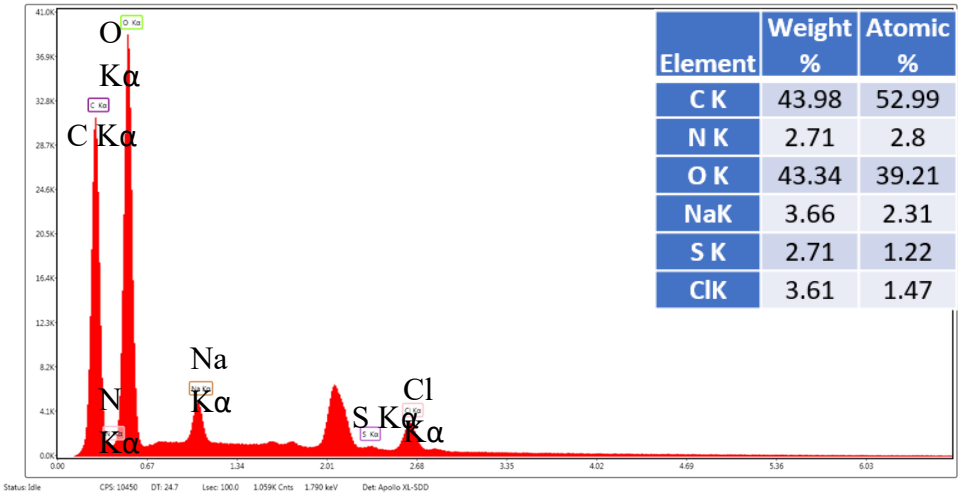
Element	Weight %	Atomic %
C K	55.8	65.8
N K	2.1	2.1
O K	30.9	27.3
S K	9.5	4.2
ClK	1.8	0.7

Figure C16. EDS spectrum of 1:1 composite drug-loaded with [CHX][Oxa] (100 μM).



Element	Weight %	Atomic %
C K	60	68.49
N K	1.42	1.39
O K	31.78	27.23
S K	6.26	2.68
ClK	0.55	0.21

Figure C17. EDS spectrum of 2:1 composite drug-loaded with [CHX][Oxa] (100 μM).



Element	Weight %	Atomic %
C K	43.98	52.99
N K	2.71	2.8
O K	43.34	39.21
NaK	3.66	2.31
S K	2.71	1.22
ClK	3.61	1.47

Figure C18. EDS spectrum of 2:1 drug-released composite (previously loaded with 100 μM [CHX][Oxa]). Presence of Na and Cl from 0.9% saline solution.

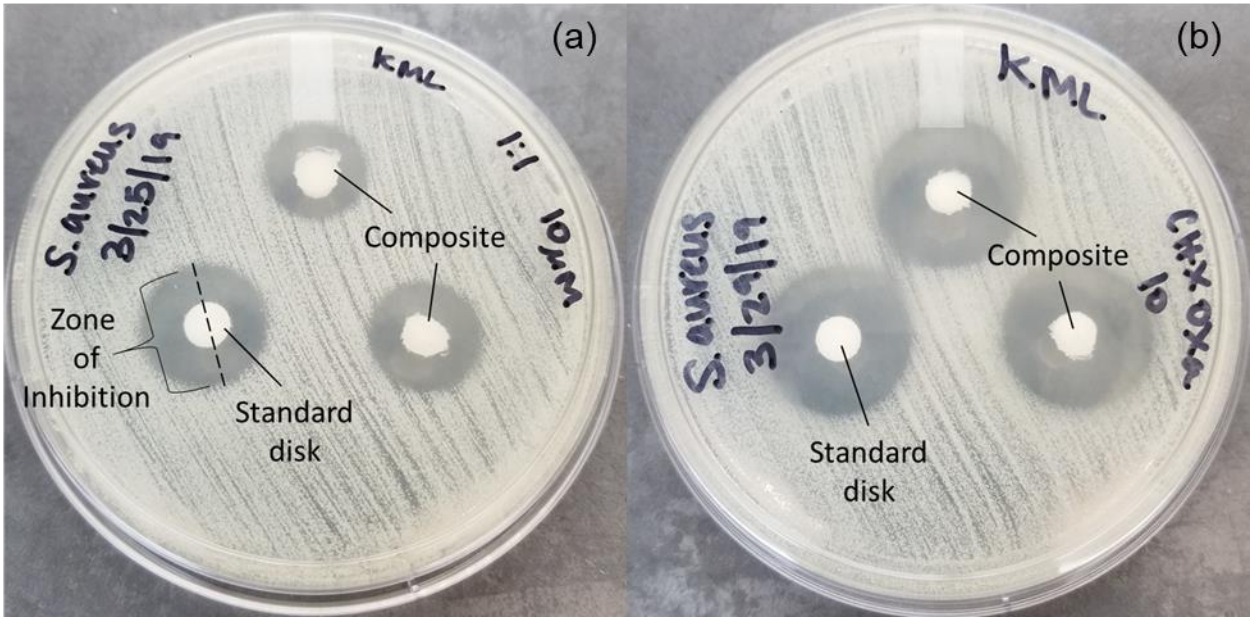


Figure C19. Representative images of composites and standard disks on agar plates inoculated with S. aureus (ATCC 29213) after 20-24-hour incubation period. The circular areas where no bacteria have grown are called zones of inhibition (ZOI). 10 μM [CHX][Ceph] loaded 1:1 composite (a), and 10 μM [CHX][Oxa] loaded 1:1 composite (b).

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APPENDIX D. SUPPORTING INFORMATION FOR CHAPTER 5

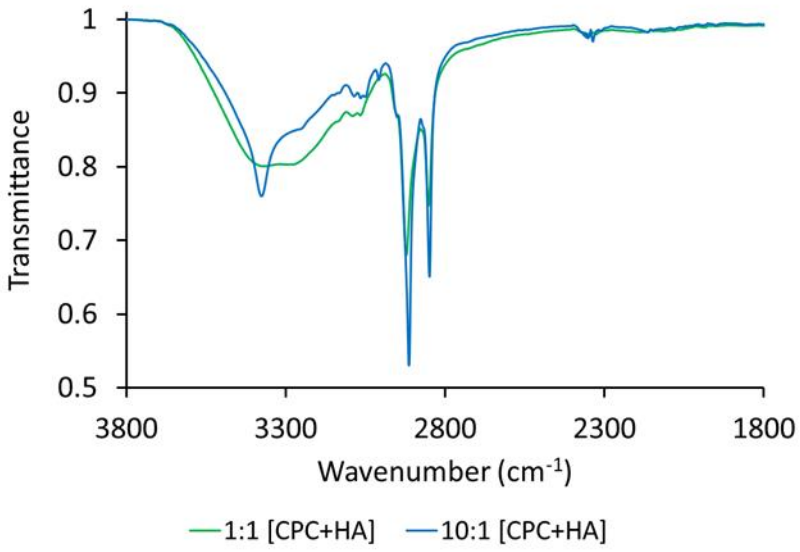

Figure D1. FT-IR spectra for [CPC+HA] complexes exposed to atmosphere for 60 minutes.

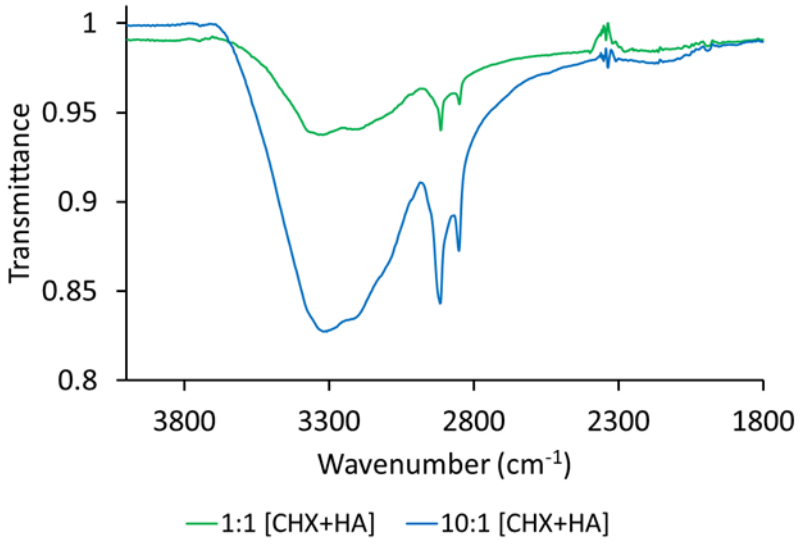

Figure D2. FT-IR spectra for [CHX+HA] complexes exposed to atmosphere for 60 minutes.

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VITA

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