Development of biocompatible multi-drug conjugated nanoparticles/smart polymer films for biomedical applications

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Development of Biocompatible Multi-Drug Conjugated Nanoparticles/Smart Polymer Films for Biomedical Applications

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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LIST OF ABBREVIATIONS AND ACRONYMS

TBSA: Total body surface area
TSS: Toxic shock syndrome
CEA: Cultured epithelial autograft
HCAS: Human cadaver allograft skin
HA: Hyaluronic acid
Ag-SD: Silver sulfadiazine
ECM: Extra cellular matrix
HDP: Host defense peptides
LOS: Length of stay
MRSA: Methicillin-resistant Staphylococcus aureus
MSSA: Methicillin-sensitive Staphylococcus aureus
Cipro: Ciprofloxicin
PenG: Penicillin G
TLC: Thin layer chromatography
TEA: Triethylamine
TMS: Trimethylsillyl
CAN: Ceric ammonium nitrate
TBAB: Tetrabutyl ammonium bromide
MIC: Minimum inhibitory concentration
ZOI: Zone of inhibition
ATCC: American type culture collection
TSA: Trypticase soy agar
TSB: Trypticase soy broth
TEM: Transmission electron microscopy
SEM: Scanning electron microscopy
AFM: Atomic force microscopy
DMSO: Dimethylsulfoxide
NMR: Nuclear magnetic resonance
RAFT: Reversible addition–fragmentation chain transfer
FTIR: Fourier transformed infrared spectroscopy
NP: Nanoparticle
PNCE: Polymeric nanoparticle-containing emulsion
MDNP: Multi-drug containing nanoparticle
CNP: Control nanoparticle
BA: Butyl acrylate
Sty: Styrene
MMA: Methyl methacrylate
EA: Ethyl acrylate
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly(lactic acid)</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(lactic-glycolic acid)</td>
</tr>
<tr>
<td>PDLLA</td>
<td>Poly(D,L-lactide acid)</td>
</tr>
<tr>
<td>PEG</td>
<td>Poly(ethylene glycol)</td>
</tr>
<tr>
<td>PLLA</td>
<td>Poly(L-lactic acid)</td>
</tr>
<tr>
<td>PGA</td>
<td>Poly(glycolic acid)</td>
</tr>
<tr>
<td>MTT</td>
<td>Tetrazolium salt</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>CZE</td>
<td>Capillary zone electrophoresis</td>
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DEVELOPMENT OF BIOCOMPATIBLE MULTI-DRUG CONJUGATED NANOPARTICLES/SMART POLYMER FILMS FOR BIOMEDICINAL APPLICATIONS

Kerriann R. Greenhalgh

ABSTRACT

It has been reported by the American Burn Association that 4,000 people die every year due to burn injury. After survival of the initial trauma, the next major obstacle that must be overcome is combating bacterial infection, the primary cause of mortality for burn victims (Chapter 1). The polyacrylate nanoparticle drug delivery system was created to provide a water-based solution for delivery of highly lipophilic antimicrobials; such as N-thiolated β-lactams, however, with the success of this system for these antimicrobials, it was extended towards other, commercially-available water-soluble antimicrobials through acrylation of the drug monomers, including those with observed bacterial resistance (Chapter 2).

Various antibiotics were incorporated into this polyacrylate nanoparticle delivery system by either encapsulation or covalent attachment, and the antibacterial activity was determined in vitro (Chapter 3). Since current treatment of burn wound infections calls for numerous antimicrobials in order to combat the vast array of microbes that may be present in the wound, a multi-drug conjugated nanoparticle system was constructed and analyzed for antibacterial activity against many pathogens commonly found in burn wounds (Chapter 4). In vitro antibacterial assays suggest that the nanoparticle delivery system rejuvenated the activity of penicillin-based antibiotics against formerly resistant microbes, such as methicillin-resistant *Staphylococcus aureus*.

The multi-drug conjugated nanoparticle emulsion had the added benefit of forming a drug-conjugated polyacrylate polymer film through air-drying and polymer coalescence. Upon topical application to a skin abrasion in a mouse model, a protective barrier was created over the wound. This film exhibits mechanical properties similar to elastin, a pliant biological material, giving it the elasticity and flexibility required to move and interact with the wound in the same fashion as intact skin (Chapter 5). This film also
permits diffusion of essential nutrients and small molecules (such as oxygen and water) required for wound healing. The emulsion was able to be combined with other biological materials, such as collage, to form a biocomposite material expressing the most optimal properties from each constituent (Chapter 6). In vitro cytotoxicity analysis (Chapter 7) and in vivo toxicity studies (Chapter 8) produced positive results indicating that the multi-drug conjugated nanoparticle emulsion is a promising new treatment for the burn wound and other topical skin and soft tissue infections.
CHAPTER 1

BURN WOUND TREATMENTS AND COMPLICATIONS

1.1 Introduction

It has been reported by the American Burn Association that 500,000 Americans seek medical attention, 40,000 are hospitalized, and 4,000 die every year due to burn injury.\(^1\) Children, who are at a higher risk of mortality from burn wounds due to weaker immune systems and who have lower body surface area, often receive wounds covering a larger area of the body, thus making treatment even more difficult, and have mortality rates even higher than seen for adults, up to 84\%.\(^2\) Mortality rates due to burn wounds are staggering when the total body surface area (TBSA) is greater than 50\%, and even greater when the majority of the wounds are third degree, extending through the dermal layer and into the subcutaneous region. This is because the skin is an essential organ involved in regulating body temperature, preventing fluid loss, and providing the body’s main defense against infection, and when damaged, leaves the entire body vulnerable to numerous pathological problems.\(^1\) When patients are able to survive the initial trauma and shock of a burn wound, the next major obstacle the patient must overcome is combating bacterial infections, which is the primary cause of mortality for these patients.\(^3\)

Burn wounds must go through several stages before the wound can be fully healed, and at each stage there are many complications, issues, and treatment options that must be addressed in order for the wound to heal properly and have a low risk of hypertrophic and keloidal scarring. Initially, burn wounds require cooling in order to terminate the spreading of internal tissue damage.\(^4\) Cooling the wound upon hospitalization has also shown to improve wound healing and has been known to decrease local oedema at the wound site,\(^4\) which is why moist occlusive dressings, and more recently hydrogel sheets, are used upon admittance to a hospital. Once cooled, the first stage a burn wound goes through is often referred to as the infectious period,\(^5\) where the wound is at its most vulnerable state to microbial invasion. It is in this stage of healing that surgical options are minimal, and the patient must rely upon topical antimicrobial treatments and prophylaxis for wound protection. At this stage of wound healing is when the patient is at the highest risk of mortality. Following this stage is when necrosis and agglutination occur,\(^5\) and the wound bed can undergo debridement procedures followed by surgical grafting procedures to provide a more substantial wound coverage and treatment plan. The final stage of wound healing only occurs once all infections have been cleared from the wound and all necrotic tissue has been excised. At this stage re-epithelialization occurs,\(^5\) as cells are able to proliferate and wound contraction is observed. Proper cell function and optimal healing conditions at this stage are crucial for minimizing scar formation.
While there are numerous treatments available for burn wounds and wound infection prevention, the best course of treatment ultimately depends on the depth and severity of the burn wound.\cite{6,7} First degree burn wounds, which only affect the epidermal layer of skin, usually heal naturally within a few days and do not require any antimicrobial treatments. These wounds are commonly inflicted by excessive sun exposure. Second degree burn wounds that extend through the epidermal layer and affect the dermal region of skin often require antimicrobial treatment and will produce blistering. These wounds often take 2-3 weeks to heal, and if deep enough, can require debridement and grafting and result in hypertrophic scarring if unattended. Third degree burns are the most severe burn wounds that penetrate through the entire cutaneous region and into the subcutaneous region. These burns produce scarring even with surgical intervention and treatment. Some of the most commonly employed treatments for third degree or full thickness burn wounds are surgical-based, including autografts, allografts from cadaveric skin, synthetic wound dressings, and xenografts from porcine skin.\cite{5,6-28}

![Figure 1.1. Anatomy of human skin and subdermal layers. Image obtained from the National Library of Medicine.\cite{29}](image)

These aggressive surgical procedures are being continuously improved to enhance patient care, and have been known to decrease pain, shorten hospital stays, assist infection prevention, and decrease the number of operative procedures.\cite{2,8} Early wound debridement has become one of the most common burn wound treatments since the early 1970’s, regardless of the degree of injury, and is essential for any surgical treatments to be successful. Since its introduction to the medical field, debridement procedures have been a major factor in decreasing mortality rates over the past three decades.\cite{7,10} Mechanical/surgical debridement is the most frequently performed technique and uses a scraping tool for removal of the necrosized tissue, which is one of the most painful procedures performed on burn victims and is not a selective means of necrotic tissue
removal. Recently, biotechnical advances have been made that utilize the degradatory powers of enzymes to breakdown the damaged collagen, elastin, and fibrin molecules in the wound bed, and companies now manufacture numerous enzymatic kits for such purposes. While these surgical techniques, including skin grafting and debridement procedures, assist in preventing loss of fluids, promote wound healing, and provide some degree of protection from infection, many factors, such as time, patient stability, and available donor skin, influence whether these procedures can be performed. [2, 10] Inevitably, this influences whether the patient will be at a greater risk of developing infection and sepsis.

When a burn occurs that is third degree (full thickness), the epidermis is completely compromised, along with some of the dermis as well, leaving the underlying necrotic burned eschar exposed, which is comprised of collagen, fibrinogen, fibronectin, denatured proteins, mediators, and toxins.[10, 11] Many bacteria contain receptor proteins that recognize and bind these cells, including *Staphylococcus aureus* strains that contain receptors which interact exclusively with the exposed human cellular matrix components. 8 This allows *S. aureus* to inhabit and become one of the most common pathogens found in burn wounds. 8 Infections caused by these and other microbes can slow healing, damage the remaining healthy tissue surrounding the wound, and lead to infection in the blood stream, sepsis and toxic shock syndrome (TSS). [12] In order to lower the morbidity and mortality rates in burn patients, an effective treatment is needed that will prevent the spread of infection from the wound beds thereby preventing these dire situations from developing.

While surgical graft procedures continue to rise in popularity, they require a high degree of patient stability since anesthesia and major fluid loss is associated with such procedures.[8] Patients often are not eligible for these surgical procedures until 6 to 10 days after the initial burn, during which time various wound dressings and antibiotic treatments are commonly employed in an attempt to combat infection in the wounds. [13] Research and development has moved further away from making improvements to topical antibiotic therapies and has focused more on better surgical procedures, which are only successful and can only be initiated after the patient has already recovered sufficiently and exudation of the wound has lessened,[8] during which time the patient is at a high risk of infection. A once booming area of research has thus been abandoned, leaving behind a dire need for new and innovative antibiotic development. The occurrence of nosocomial-based infections has continued to grow since this standstill, and the predominance of multi-drug resistant bacteria infiltrating burn wounds has continued to rise, while few new therapies have been explored in the past decade or more.[8, 14] Thus new and improved analgesics, antibiotics, and immunity-promoting therapies are urgently required for severe burn wound treatment. [8, 14]
1.2 Wound Dressings for Infection Treatment and Prevention

1.2.1 Various Silver-Doped Dressings

Research to improve antimicrobial wound dressings have continued to be explored by a select number of pharmaceutical companies and researchers, however, products have not been significantly improved in the past 20 years. The mainstay of burn wound care has revolved around silver-doped antimicrobial bandages as temporary wound dressings, which has been the major topic in wound dressing research for the past 20 years. These bandages contain antibacterial properties through the silver constituent, commonly presented in the form of silver salt solutions, where silver sulfadiazine is the most commonly used and is considered the benchmark for silver-containing dressings.[30] The use of such metal-doped bandages is to reduce bacterial infection at the site of the wound and also to aid in sepsis prevention.[30] Since the emergence of drug resistant microbes has limited the use of antimicrobials in wound dressings, many companies have begun manufacturing wound dressing products containing various forms of silver salts as the sole antimicrobial constituent. Some of these products include silver nylon cloth/activated charcoal (Actisorb®),[31] silver sulfadiazine with cerium nitrate (Flammacerium®),[30] silver absorbent wound dressing (SilvaSorb®),[31] silver-impregnated polyurethane (PolyMem Silver®),[31] and silver sulfadiazine-impregnated lipidocolloid wound dressing (Urgotul SSD®).[30, 31] While these dressings display antibacterial activity, they must be continuously changed due to a one time release of the silver constituent into the wound bed. The process of changing dressings can be extremely painful, potentially damaging, and highly risky.[30] The recently developed nanocrystalline silver-coated dressings Acticoat™ and Silverlon® released onto the market in 1999 have shown controlled, prolonged release of the silver constituent into the wound area, thereby decreasing the frequency of dressing changes.[30, 32, 33] Unfortunately, due to the controlled release of the silver, the levels of silver that is delivered to the wound is below the bactericidal levels necessary to prevent resistance formation.[30] Even though the need for frequent dressing changes has been reduced, daily application of dressings is still required for this type of product; therefore, the heightened risk of nosocomial-based infections and tissue damage still remains with this form of treatment.

1.2.1.2 Issues with Silver Dressings

Bacterial drug resistance towards the silver-based dressings has been shown to form in clinical applications despite no observation of resistance in vitro. This development is believed to form through one of two biochemical routes: bacteria can bind the silver once inside the cell forming an intracellular complex, or the silver can be excreted from the cells prior to invoking its antibacterial activity through the cell’s efflux systems.[30] Another issue with the use of silver-containing dressings is the amount of silver available in the wound and the inverse relationship with the concentration of silver required for antibacterial activity. In all products described above, if the silver is delivered to the wound in a burst effect, as is seen with silver salts, then the concentration in the wound bed is high enough for bacteriostatic and bactericidal properties; however, this activity
does not last long and the silver must be replenished often.\textsuperscript{[30]} The other form of silver delivery is through nanocrystalline silver delivered through a controlled release mechanism, yet this does not provide the wound bed with a high enough concentration of silver at a single time point to invoke a bacteriocidal effect, and thus allows the bacteria to form resistance to the silver.\textsuperscript{[30]} Recent studies have established that the use of Acticoat\textsuperscript{TM} with cultured keratinocytes and other cultured skin substitutes can be cytotoxic within one day and can inhibit re-epithelialization,\textsuperscript{[30]} while Silvazine® has been shown to have the highest degree of cytotoxicity among the silver-containing wound dressings.\textsuperscript{[30]} One benefit of the silver substances for wound treatment, especially the nanocrystalline silver incorporated in the Acticoat\textsuperscript{TM} and Silverlon® wound dressings, have been shown to promote wound healing through decreasing matrix metalloproteinases (MMP), which are biomolecules known to cause tissue destruction and cellular apoptosis.\textsuperscript{[30]} However, not all silver-based complexes have these additional beneficial properties and can even be detrimental to the wound healing process itself.

\textbf{1.2.2 New Matrix Materials Promote Healing and Antimicrobial Delivery}

Research has recently been conducted on different matrix materials for the delivery of the silver complexes in order to increase antibacterial efficiencies and promote wound healing in the wound dressing products. Absorbable collagen fiber matrices, synthetic polymer-based materials, and chitosan-based biomaterials have all been incorporated into wound dressings, each having its own advantages and disadvantages.\textsuperscript{[5, 30]} The two main ideas behind each of these new materials for wound dressings is to create a biodegradable matrix to which the silver particles can adhere and be released, and provide a controlled release of the metal or metal complex into the wound bed, thus extending the lifetime of the dressing. These materials have also been chosen in order to increase the absorbability of the dressing, which allows exudates from the wound bed to be removed without invasive procedures, essentially cleaning out the wound bed.\textsuperscript{[5]} Both of these properties contribute to promotion of wound healing and reduced time before additional reconstructive surgical procedures can be performed. However, with this absorption property comes the risk of exudates sticking to both the bandage and the wound bed, thus removing some healthy cells and tissue that have infiltrated the wound bed during dressing changes. This can cause new trauma which reverses wound healing and causes the area to have to start the wound healing process all over again.

\textbf{1.2.3 Wound Healing-Promoting Dressings}

Apart from antimicrobial dressings, research has also developed dressings that promote wound healing by including extra cellular matrix (ECM) proteins and growth promoting factors. Hybrid peptide synthesis has led to wound dressings that mimic the peptide makeup of elastin and laminin, two major ECM proteins known for promotion of cellular adhesion and proliferation. These peptides provide a facet for cellular attachments and induction of otherwise inhibited wound healing.\textsuperscript{[34]} When placed in conjunction with an
alginate fibrous dressings, the hybrid-peptide dressing was able to promote granulation tissue regeneration and epithelialization in the wound bed,[34] thus providing a new approach to wound healing that does not involve artificial skin substitutes and is optimal for chronic wounds where healing is highly impaired. However, this form of wound dressing does not provide the wound with antimicrobial treatment, and thus must be used with a topical antimicrobial agent that can impede peptide access to the wound bed and limit the effectiveness of the dressing towards wound healing. Some types of wound dressings provide the wound bed with a moist environment, which not only cools the wound but also provides the moisture it needs for healing.[4] Most dressings applied during this initial phase of treatment but often contain analgesics to help manage pain but do not contain antimicrobials, which is why by the second or third day of hospitalization the wound bed has succumbed to total microbial invasion. It is here that improvements must be made to provide the wound not only with adequate moisture, but also with antimicrobial therapy for bacterial eradication and wound coverage for protection from translocation of opportunistic pathogens.

1.3 Current Antimicrobial Treatments

1.3.1 Silver-Based Creams

Silver-containing creams and solutions have been used for infection control since the 1880’s, where silver nitrate was the first silver salt used for such an application.[31] While this silver salt was a successful bacteriocidal compound, it displayed caustic effects on human tissue,[31] and thus other forms of Ag+ and other ways of delivering Ag+ to the infection site have been an important research topic in infection treatment. The use of silver as an antimicrobial died out at the onset of antibiotic discovery, but has recently gained strength again due to the emergence of multi-drug resistant bacteria. Today, silver sulfadiazine creams (Silvazine® and Flamazine™) are common topical ointments that use silver metal substrates as the antimicrobial for infection control, where Silvazine® also has chlorhexidine digluconate as a drying agent and additional cleanser.[30, 31, 35] While both silver sulfadiazine creams and silver nitrate solutions are used clinically, silver nitrate solutions must be applied up to 12 times a day, while silver sulfadiazine creams can be applied only twice a day.[30] Silver sulfadiazine has also been coupled with cerium nitrate to provide even more efficient antibacterial activity, and in this formulation, has shown to limit wound contamination even in cases where debridement cannot be performed until over five days after burn injury.[36] Conventionally, these silver-containing ointments are used with paraffin gauze dressing, although studies have shown that this form of wound dressing with such topical antimicrobials is not optimal.[37] One of the major drawbacks of using silver-containing topical ointments is the occurrence of the light-sensitive formation of black colloidal Ag⁰ on the skin surface.[31] When this happens, the skin becomes a dark bluish black color, which is irreversible and a permanent cosmetic issue.[31] Companies producing these silver-containing creams and solutions have attempted to overcome this issue with the addition of sulfadiazine to help prevent this metal reduction by allowing the Ag⁺ to stay in the solution.[31] The efficacy
of any silver compound is greatly diminished by organic matter, i.e. burn wound exudates, interacting with chloride ions present in the wound.[30]

Another drawback of these topical silver creams is delayed wound healing and eschar separation, which prevents sloughing off of necrotic tissue from the wound bed.[30] These creams also can produce toxicity when applied frequently over a long period of time over a large surface area and can lead to hypertrophic scarring upon healing.[30] Silver nitrate solutions and silver sulfadiazine creams have shown high levels of toxicity in vitro against fibroblasts and keratinocytes and increased inflammation in clinical trials, where inflammation is more than likely due to the cream base in silver sulfadiazine.[30] In vivo and clinical studies have shown that silver can be taken up by epidermal cells and passed on to the peripheral circulation where renal toxicity can then occur due to high concentrations of silver in the blood and urine.[30] This observed toxicity is mainly due to the fact that silver, when in simple salt form, cannot differentiate mammalian cells from bacterial cells, and thus will accumulate in either; therefore, extended periods of topical silver cream application over a large wound area can cause extensive damage to the wound itself and to other systems in the body. The silver sulfadiazine creams, along with commonly used mafenide acetate and gentamicin sulphate creams all have shown bacterial resistance in clinical settings;[38] therefore, a new approach to antimicrobial treatment in burn wounds needs to be brought to light.

### 1.3.2 Peptide-Containing Dressings

Recently, peptide research has looked into forming synthetic and naturally occurring antimicrobial peptides for their antibacterial properties. Synthetic peptides such as Novispirin G10[39] and natural host defense peptides (HDP)[38] have been explored for such purposes. HDPs have been looked at because of their role in the innate immune response to foreign pathogens and are transcribed in response to a pro-inflammatory response initiated by pathogens.[38] After burn injury, local expression of HDP is greatly reduced, which allows microbes to colonize the wound bed;[38] therefore, an obvious method of treatment would be to re-introduce this peptide into the wound bed in an attempt to make up for its lack of cellular production after burn trauma. A common mode of action for antimicrobial peptides is to alter the permeability of the microbe’s membrane, however, they also have great wound healing benefits and display a broad spectrum antimicrobial activity.[39] While both naturally occurring and synthetic peptides have their own benefits and flaws, naturally occurring peptides have shown cytotoxic effects and have high costs associated with manufacturing processes,[39] therefore, synthetic means of peptide manufacturing for antimicrobial applications may be the most optimal form of antimicrobial peptides.
1.3.3 Drawbacks of Current Topical Antimicrobials

The use of topical chemotherapy has helped to improve the survival of patients with major burns and to minimize the incidence of burn wound sepsis, a leading cause of morbidity and mortality in these patients.\[^{30}\] Unfortunately, with the use of topical ointments or creams for treatment of skin wound infections, frequent application and scraping off of the wound region is required, often leading to increased pain and trauma for the patient.\[^{5, 31}\] This limits the use of topical creams, ointments, and gels for wound infection management, especially in wounds that cover a large region of the body where spreading on such treatments would cause more trauma and damage than is acceptable. Added to this issue is the fact that many creams can form an overlying slough on the wound bed that renders it difficult to assess the wound for its degree of healing.\[^{37}\] It should also be mentioned that in many hospitals, doctors will take the added precaution of administering prophylaxis treatments of water soluble antibacterials while a patient is in the infectious stage as a precautionary measure in case bacteria should migrate into the blood stream where sepsis is initiated. This helps to decrease the occurrence of sepsis and TSS in burn patients during this vulnerable time in the wound recovery process. However, prophylaxis treatment is often ineffective once the bacterial load in a wound has reached the blood stream, therefore, improvements must be made in topical antimicrobials to make them more efficient at reaching bacteria deep in the burned tissue as well as at the surface of the wound bed without having detrimental side effects to wound healing.

Table 1.1. Ideal properties for biological-based temporary wound dressings.\[^{23}\]

<table>
<thead>
<tr>
<th>Optimal Properties for a Burn Dressing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biocompatibile</td>
</tr>
<tr>
<td>Water Permeability and Heat Retention</td>
</tr>
<tr>
<td>Protective Barrier against Pathogens and Trauma</td>
</tr>
<tr>
<td>Reduces Pain</td>
</tr>
<tr>
<td>Adherent, yet Flexible and Conforming</td>
</tr>
<tr>
<td>Single Application</td>
</tr>
<tr>
<td>Moves with Patient</td>
</tr>
<tr>
<td>Low Cost</td>
</tr>
<tr>
<td>Long Shelf Life/ Simple Storage</td>
</tr>
</tbody>
</table>
1.4 Skin Grafts as Prominent Treatment

1.4.1 Human Autografts as Treatment Mainstay

Immediate removal of necrotic tissue through wound debridement followed by effective wound coverage is essential for adequate wound treatment and rapid wound healing.\[9\] One of the best forms of permanent wound coverage and rapid wound repair currently available to burn patients is human skin autografts,\[13\] where small patches of split thickness skin from unburned areas of the patient are excised and permanently grafted onto the wound bed.\[2, 9, 13\] These patches are often referred to as postage-stamp autografts due to their postage-stamp-like size and shape.\[13\] This gives the skin cells a matrix onto which new skin can grow. This technique is used to conserve the amount of donor skin needed, and is preferably harvested from the scalp when available.\[13\] Since the grafted skin is from the host itself, no rejection or immune response is observed with this procedure, and thus the body immediately accepts the implanted skin and begins to reconnect to it. This procedure minimizes the area of skin that must be rebuilt for the burn to heal. Cellular migration, which normally must cover the entire wound, now only stretches to the autografts, thereby decreasing the time required for wound healing. Also, since the grafted skin is a living system and its cells are viable and unaffected by the burn trauma, its cells can migrate outward into the wound bed and assist in the repair of the wound and skin replacement. The expansion ratio of autografted tissue can range from 1:4 to 1:9 in the wound bed,\[13\] and thus a small skin sample can cover a large area after a short period of time.

![Expansion Ratio 1:4](image1.png)  
**Expansion Ratio 1:4**

![Expansion Ratio 1:9](image2.png)  
**Expansion Ratio 1:9**

**Figure 1.2.** Depiction of postage-stamp autograft tissue expansion within wound bed. Patchwork pieces of 1cm\(^2\) placed 1cm apart from one another produces an expansion ratio of 1:4, whereas patchwork pieces placed 2cm from one another yields a 1:9 expansion ratio.\[13\]
Autografts are generally only a few cell layers thick, which makes them fragile and difficult to handle. This also limits the durability of the graft and can cause it not to fully adhere to the wound bed. These grafts, while being able to fully heal the wound with time by expanding the skin sample into the wound area, do not cover the entire wound initially and therefore must be used in conjunction with some form of allograft or mesh to obtain maximal coverage of the wound. These allografts are used as a temporary protection only and can be used individually as well as in conjunction with the autografts for wound protection, since autografts alone are extremely susceptible to bacterial infestation.

Figure 1.3. Patchwork appearance of skin on the back of the legs upon postage-stamp autografting procedure (left) and one year after procedure (right). Reprinted from Burns, Vol. 24, Pgs. 264-269, by Chang et. al. Copyright 1998 with permission from Elsevier.

Some of the major drawbacks for this type of wound coverage treatment is the occurrence of additional scarring and/or morbidity at the split thickness skin donor site. Yet this treatment remains the best option for wound coverage due to its permanent nature and the ability of both dermal and epidermal skin layers to be grafted onto the wound bed. While this form of autografting can achieve significant expansion once grafted to the wound bed, the grafting procedure itself is very difficult due to the fragility and slightness of the postage-stamp sized skin samples and the grafted skin is easily dislodged from the wound bed. Due to the non-continuous coverage of postage-stamp autografts, a patchwork design appears on the skin after wound healing due to hyperpigmentation of areas receiving the grafts and pink-to-red discolorations in between each skin graft area.

1.4.1.2 Cultured Epithelial Autografts (CEAs)

While autografts remain the best form of surgical treatment for wound repair, unfortunately, when the burn TBSA is over 50%, there is little donor tissue available for
such a technique.\textsuperscript{[16, 17]} Often times, this is the case for children and especially neonates who have very little available skin for replacement, and scarring at the donor site can be substantially increased with aging.\textsuperscript{[18]} Thus an alternative to the conventional autograft treatment was approached that still incorporates the patient’s own skin sample for treatment, thereby maintaining the permanent aspect of the original wound coverage.\textsuperscript{[9, 16]}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figures/figure1_4.png}
\caption{Cultured epithelial autograft treatment progress in clinical practice.\textsuperscript{[16]} A and B are examples of a cultured epithelium on a fibrin matrix for CEA treatment. C depicts split-thickness meshed human cadaver allograft patches for temporary coverage prior to CEA treatment. D shows CEA/fibrin-treated skin 3.5 years after procedure. Images reprinted with permission from \textit{Transplantation}, Vol. 70, Pgs. 1588-1598 by Ronfard et al. Copyright 2000 by Lippincott, Williams, and Wilkins.}
\end{figure}

The cultured epithelial autograft (CEA) treatment relies on the culturing of epidermal keratinocyte cells \textit{in vitro} that are obtained from a small biopsy of the patient’s skin. The area of skin needed for this procedure is extremely small, ranging from a 2 mm\textsuperscript{2} area to a 2-3 cm\textsuperscript{2} area, depending on the extent of damage,\textsuperscript{[9, 19, 20]} and thus reduces the added pain of skin harvesting as is the case for traditional autografts.\textsuperscript{[5]} The biopsy sample is treated to remove any subcutaneous tissue then the epidermal and dermal layers are separated so that only the keratinocytes remain in the harvested skin sample.\textsuperscript{[9, 20]} These cells are then placed onto a biological matrix and allowed to expand onto the matrix to form a large
sheet of epidermis, usually 8-10 cell layers thick.\cite{8, 9, 16, 18-20} Many scientists have identified numerous matrix materials for such applications, including polyurethane membranes, silicon-collagen membranes, collagen only membranes, hyaluronic acid-based membranes, collagen sponges, and fibrin glue.\cite{8, 9, 16, 20} These materials act to not only provide a basis for the keratinocytes to replicate, but also as a pseudo-dermis when applied to the wound bed. However, the lack of a true dermal layer has limited the use of CEA in hospitals.\cite{7,8}

**Scheme 1.1.** Process for culturing CEA tissue, originally proposed by Rheinwald and Green.\cite{9, 20}

Since the discovery of this culture technique by Rheinwald and Green in 1975 and the initiation of its clinical use by O’Connor et al. in 1981, this treatment has saved many lives and improved the overall quality of life for thousands of burn victims.\cite{9} This treatment method has now become the one of choice for doctors around the world when the TBSA is greater than 65-70%, where traditional autografts are not suitable treatment options.\cite{9} This treatment has shown in hundreds of patients the ability to shorten the time required for wound healing through numerous advantageous properties, including the ability to stimulate wound re-epithelialization from both wound edges and within the wound bed itself,\cite{19} and is able to regenerate itself many years after the transplant.\cite{16} Researchers are still looking to understand the full extent of this stimulatory action exhibited by the CEA treatment.\cite{19} The appearance of redness and scarring upon complete wound contracture has been significantly reduced through this procedure as well.\cite{18} Burn areas covered with CEA, as opposed to split thickness autografts, have a normal epidermal histology, a smoother surface, and elasticity in the skin after a few
years of healing, indicating that the epidermis is able to regain its original biological processes of continual regeneration. These grafts also have a higher probability of “taking” or being accepted by the wound bed as compared to traditional allografts, by at least 30%, thus making them a much more permanent treatment for wound closure. However, recent studies have reported that the meshed autograft still remains the best option for full thickness burns in terms of length of hospital stay, procedural costs, number of readmissions for re-opening of wounds and reconstruction of scar contractures, and bacterial susceptibility. One drawback of this technology is that only the epidermis can be reconstituted, and therefore it is not an approach that can be used to treat large full thickness wounds that enter the subcutaneous region since no keratinocytes are available in this area for the CEA to interact with. Even when it is beneficial to use CEA as a means of permanent wound coverage, in order to obtain the CEA tissue, a 3-4 week period of cell culturing for a decent size epithelium sheet to grow is required, however, advances in the matrix used for epithelium construction have recently shortened this time period to 2-3 weeks. Within this time period a 3 cm² biopsy of donor tissue cultured under these conditions can grow to be 5000-10,000-fold larger, yielding a sample large enough to cover an entire adult while the patient is undergoing continuous treatment for burn shock, burn wound excision, and wound preparation including bacterial eradication for the CEA graft. There are still many questions that need to be answered before this technique can become an optimal treatment for burn wound closure, regardless of the degree of TBSA burned, including issues of cellular confluency, optimal cell type for culturing, and optimal matrix constituents, as well as gaining a better understanding of the benefits of this technology on a cellular level.

| Table 1.2. Comparison of patients treated with CEA versus traditional allograft techniques used when TBSA >90%.[7] |
|--------------------------------------------------|--------------------------------------------------|
| Patients Receiving CEA Treatments | Patients Receiving Other Treatments |
| Total Operations | 13 | 8 |
| Days of Hospitalization | 128 | 89 |
| Cost per patient ($1k) | 304 | 178 |

1.4.2 Donor Allograft Skin as an Alternative Treatment

For deep tissue burns where the dermal layer is compromised, full or split thickness allografts are often used as temporary wound closures and for protection against bacterial invasion and fluid loss. Studies have established that when this treatment is performed within six to ten days of the initial burn wound it can reduce the length of stay (LOS) at a hospital by 15.7 days. Yet, this treatment was not able to significantly reduce the
mortality rate in patients.[22] This again is due to the length of recovery time needed by the patient prior to this treatment, during which time bacterial invasion is at its highest probability. Skin and tissue banks have only recently began to harvest cadaver skin for allograft purposes, some as recently as 1998,[22] thus the popularity and low cost associated with this burn wound treatment has only recently been established in the treatment of burn wounds. Of course, there are inherent issues when using donated cadaveric skin for allografts, including discoloration of the skin post-mortem and variances in skin texture and tone among races.[22] While tissue banks are growing in size and number of donations daily, public awareness is still significantly low,[22] and large strides need to be made in order to make this treatment more affordable and available to patients in all areas of the world.

Adherence of human cadaver allograft skin (HCAS) to a wound bed has previously been thought to signal physicians that a bacterial load in a wound bed is minimal, thereby indicating when further permanent autograft treatment can be initiated.[15] However, recent studies have shown that this is not the case, and that bacterial infections are still able to manifest within the wound bed even with allograft coverage.[15] Dermal allografts from human cadaver skin are also possible and can be used in conjunction with CEAs and other epidermal-based grafts without any observed rejection.[8] There are many issues that can arise when using HCAS, such as transfer of microbes, viruses, and viral diseases from donor cadaver skin to burn patients, whose immune systems are already compromised.[2, 8] The issue of host rejection of the allografts is currently being studied and efforts are being made to suppress the host immune response to such grafts, thereby extending the length of application of the allograft to the wound.[2] A topical treatment containing cyclosporine A has been reported to give site-specific immunosupression when applied to the graft, thereby extending the time of the graft placement on the host by 50-75% over the norm.[2]

1.4.3 Porcine Xenografts as a Last Resort Treatment

As medicine has progressed so have the treatment options available for burn wound management, yet in many cases the original treatment is still the best option. Xenografts, or skin grafts originating from a different species, have been in use since the 16th century and are often still the grafting technique of choice for many hospitals around the world.[23, 24] This is especially true for third world countries and areas that cannot afford expensive alternative treatments and when donor skin is limited.[22-24] Many regions around the world do not have skin banks or places where they can obtain donor skin from humans and they must rely solely on autografts, family members for donated skin, or on xenografts for wound coverage.

The most frequently used skin type for xenografts is porcine skin, which is grossly comparable to the anatomy of human skin and has been in use since the 1960’s,[23, 24] however, pigs are not the only species whose skin has been used for wound coverage. When xenografts first originated as a treatment option, skin from chickens, rats, pigeons,
frogs, lizard, cats and dogs was used, and frog skin is still in use in some countries today.\textsuperscript{[8, 23]} One major benefit of using xenografts for burn wound treatment is the large amount of tissue available for such procedures at a much lower cost than human cadaver skin.\textsuperscript{[23]} Since xenografts and allografts are not permanent additions to the body, as is the case with autografts, they must be able to cover the entire wound for complete protection from fluid loss and bacterial infection.\textsuperscript{[6]} Both of these grafts are temporary covers for burn wounds, but unlike allografts that are rejected by the body and undergo devascularization, xenografts are ejected from the wound surface, which leads to a shorter period of use and a higher number of surgical procedures.\textsuperscript{[13, 17, 23]} However, both grafting techniques are temporary and require more than one grafting procedure before the wound can be completely healed in most full thickness wounds,\textsuperscript{[6]} thus being able to harvest a large amount of skin specimen is essential. This is often what limits the use of allografts for these procedures and is why xenografts have remained in use since the 16\textsuperscript{th} century, even with the advance of newer technologies. Both HCAS and porcine skin specimens are able to be preserved by glycerol, which reduces antigenicity, virality, and bacterial loads inherent in the tissue;\textsuperscript{[8, 23]} however, this procedure is very detrimental to the HCAS due to loss of viability and vascularization.\textsuperscript{[8]} While xenografts contain a viable dermal matrix, they can never be used as a permanent treatment option due to the inherent immune response and eventual ejection of the graft from the wound bed.\textsuperscript{[8]}

![Figure 1.5. Porcine skin xenograft covering a deep full thickness burn wound.][23]

Many companies have developed manufacturing processes for porcine skin so that it is more readily available to hospitals and is treated for viral and microbial contaminants prior to use, yet many hospitals around the world prefer to perform the xenograft preparation themselves.\textsuperscript{[23]} Companies specializing in xenograft manufacturing have also looked into chemically modifying the skin specimens to reduce the antigenicity of them in an attempt to increase their half life after grafting, which unfortunately also reduced the binding efficiency of the graft to the wound bed.\textsuperscript{[23]} While this method of treatment is still of great importance in burn wound treatment, especially in Asia where xenografts are highly preferred over HCAS, there appears to be many areas where this treatment method can and needs to be improved to make this a more safe and effective method of treatment.
1.4.4 Synthetic and Biological-Based Artificial Skin Grafts are Promising New Treatment Options

Artificial wound dressings have been explored by burn specialists since the early 1970’s as an alternative to xenograft and allograft treatments.[25] They can have various functions in burn wound therapy depending on numerous factors, ranging from microbial treatment, temporary wound coverage, permanent wound coverage, and promotion of wound healing.[25] Due to recent advances in tissue engineering, artificial skin grafts are being fervently studied in hopes of creating a permanent treatment option that combines all of the advantages of each currently available graft type.[8] Many different types of artificial skin grafts are currently being explored for treatment of deep tissue wounds, including acellular grafts and biological-based full thickness bilayer skin equivalents,[8, 26-28] yet the use of these skin substitutes for burn wound treatment is still not widespread.[8]

Recently, bioengineered bilayer skin grafts have been formed from autologous keratinocytes covering a layer of allo-based (originating from another human) dermal fibroblasts in a native collagen matrix, which is set on top of a biological-based material matrix, thus forming the first multi-layered artificial skin graft.[8, 26-28] One trademark brand for this type of skin substitute is Apligraf, which has been FDA approved for use in diabetic ulcers and has been shown to promote healing, yet is not a permanent treatment.[28] Many of the autologous and allogous cell-based skin grafts have evolved towards the use of matrix materials to enhance cellular delivery into the wound bed.[8, 26-28] Optimally these materials will be completely biocompatible and will be biodegradable once applied to the wound bed, thus releasing the cultured cell(s) into the wound for active proliferation and replication, thereby promoting wound healing. One commonly used material for this matrix is hyaluronic acid (HA), which is a common extracellular matrix component.[8] Gelatin scaffolds are also being explored for such basal matrices, in combination with soluble collagen or by itself, and have shown good results in vitro and in vivo thus far.[8]

Acellular artificial skin grafts are also a promising treatment option for wound management. Many of the currently available grafts can work in conjunction with cultured epithelial autografts,[8] instead of relying upon human cadaver allografts that are limited by donor availability. The acellular skin grafts can be biological based, such as using decellularized porcine skin to yield an acellular dermal matrix,[8] or completely synthetic skin substitutes that can be formed from polymer, collagen, nylon, or silicone films, to name a few.[8, 25] While these forms of artificial skin will not “take” as is seen with living skin grafts nor will they make up for lost tissue layers by delivering tissue to the bed for cells to adhere to, they can aide in wound contraction as well as help deliver viable cells to the wound beds. Some of these forms of skin grafts, mainly the collagen-based grafts, have been shown to establish a “neodermal layer” in the wound bed when the fibers are aligned properly.[10, 27] This provides a platform for cells to build the true dermis and epidermis upon. Unfortunately, these forms of skin substitutes have not shown nearly the high degree of protection from bacterial invasion as basic split
thickness allografts \textit{in vivo},\textsuperscript{25} and thus should only serve as a temporary means of wound management.

One of the main drawbacks for using skin substitutes in any form of wound treatment is the high costs associated with such materials.\textsuperscript{26} Also, it has been shown that depending on the matrix material often allo-based dermal and epidermal skin substitutes are rejected or necrosized upon grafting to the wound bed,\textsuperscript{28} thereby limiting the use of manufactured skin substitutes. A secondary drawback for using artificial skin substitutes as grafts is the lack of hair follicles in the graft, which essentially equals a lifetime of hairlessness in that particular region of the body.\textsuperscript{28} Until a skin substitute is created that produces less scarring, is more durable, and requires no other surgical procedures for recovery, the cost-to-benefit analysis will continue to be in favor of the more traditional grafting procedures.

\subsection*{1.5 Conclusions}

There are many different options available to burn patients today for treatment of burn wounds. Surgical procedures have made many great advances recently and wound management has now become multi-dimensional employing numerous techniques for optimal treatment. With the various options available for grafting procedures, the patient can choose which treatment plan will work best given the circumstances and the degree of burn injury. However, these treatment options are only available once the patient has survived the initial infection stage of wound healing, where morbidity and mortality are at the highest occurrence rate.

A tissue that has been exposed to such heat as to cause second and third degree burns requires cooling immediately after the trauma in order to stop the damage being caused by the burn. It also calls for a moist environment initially as well as throughout the entire treatment in order for the wound bed not to dry up and begin cracking or have wound contraction begin prior to healing of the deeper tissue areas. Unfortunately, there are no current treatments that provide the wound bed with the moisture it needs and provide it with an antimicrobial to help control the onset of an infection in the wound bed. It is here that improvements must be made to provide the wound with moisture and antimicrobial therapy for bacterial eradication, and also protection from microbial translocation into the wound bed.

While undoubtedly antimicrobial treatment is a must for initial burn wound infection management, there are no currently available treatments that do not cause some additional pain and trauma for the patient. The use of topical ointments and creams are effective at controlling the infection at the wound site, however, their painful application and cleaning process, which must be repeated continuously until surgical procedures can be instilled, are very damaging to the patient and often times microbial resistance and delays in wound healing accompany such therapies. While this issue is able to be avoided through the use of antimicrobial bandages, often containing some form of silver species,
the fact that these bandages must be changed frequently still poses a risk of wound bed disruption. Use of either of these treatments yields an increased threat of nosocomial infection due to the frequent hands-on interaction of the patient with the hospital staff.

In the next few chapters of this thesis, a hands-free water-based sprayable antimicrobial system that allows for antimicrobial infiltration deep into the wound bed with targeted drug delivery and high drug efficacy will be described, which provides wound bed protection through a synthetic elastin-simulating polymer film formed on the surface of the wound bed. This system combines the advantages of the moist hydrogels, the antibacterial wound dressings, and the antimicrobial ointments without the occurrence of any of the common drawbacks associated with these treatment options, thereby providing a novel treatment for initial burn wound management that has all the benefits without any apparent downsides.

1.6 References


CHAPTER 2

ANTIMICROBIALS AND MRSA TREATMENTS

2.1 Introduction

Burn wounds provide one of the most optimal environments for bacterial cultivation. Staggering amounts of bacteria initiate infection in burn wounds by the second or third day after injury, and if left untreated, can infiltrate the blood stream and cause sepsis and eventually death. Numerous bacterial species are typically involved in burn wound infections; it has been found that up to 20 different species and strains of bacteria can inhabit a burn wound at the same time.\textsuperscript{[1]} Many of the bacteria found in burn wounds usually live harmlessly on the surface of the skin, however, when an abrasion or wound to the skin occurs, these opportunistic bacteria become pathogenic and invade the wound or abrasion causing an infection. Bacteria like \textit{Staphylococcus epidermidis}, \textit{Staphylococcus aureus}, and others that comprise the common skin flora turn deadly upon wound infiltration. Upon initial thermal injury, gram positive microbes such as \textit{S. aureus} and \textit{S. epidermidis} are prevalent, and resistant strains of these microbes quickly arise during this time. However, as the wound progresses, gram negative microbes, especially motile microbes such as \textit{Pseudomonas aeruginosa} and \textit{Escherichia coli} become predominant within the wound due to their opportunistic and incursionary nature.\textsuperscript{[1]} The majority of these microbes come from the normal flora present within the gut and intestinal lining, and migrate into the wound only a few days after injury. Therefore, the need to treat these wounds with effective antimicrobials prior to bacterial resistance formation and secondary gram negative invasion is paramount to expedited wound healing and survival.

In 1998,\textsuperscript{[2]} the Turos laboratory reported a new type of \(\beta\)-lactam antibiotic called \(N\)-thiolated \(\beta\)-lactams that possess good antimicrobial activity against select bacteria, including methicillin-resistant \textit{S. aureus} (MRSA). MRSA infections readily occur in burn wounds and are a major threat to patients if optimal treatment cannot be provided. While numerous penicillin-based analogues have been developed since the initial onset of MRSA in the 1960s, relatively few are able to resist the ring-opening action of this microbe’s penicillin defense enzyme, \(\beta\)-lactamase.
2.2 Synthesis of N-Alkylthio β-Lactams

2.2.1 Introduction

Figure 2.1. N-thiolated β-lactam structure.

Figure 2.1 shows the chemical structure of a typical N-thiolated β-lactam. The bioactivity of these N-thiolated lactams is unlike all other β-lactam compounds previously studied, which suggests a completely different mode of action than has been previously described for this class of antibacterials.\[3\] The spectrum of antimicrobial activity has been largely characterized,\[3-9\] however, no activity has been observed for gram negative bacteria or most gram-positives. It is significant and very curious that only a select number of pathogens are sensitive to the N-thiolated lactams, including staphylococci (S. aureus, MRSA, and S. epidermidis) as well as numerous Bacillus species, including B. anthracis.

The activity of this class of drug seems to be insensitive to changes at the C\(_3\) and C\(_4\) centers of the lactam ring to an extent.\[3-9\] The original alkynyl substituents present at the C\(_4\) position of the lactam ring has been substituted with E or Z alkenyl, alkyl, aryl, and heteroaryl moieties without any apparent effect on the biological activity.\[3\] Yet what is key for the activity of these compounds is the presence of a short chain alkyl group on the N-thiolated portion of the ring structure. When the thiol group is removed,\[6\] or the alkyl group is changed to a bulkier group, activity is lost. As well, the ring structure can also be expanded to a 5-membered ring, such as in 2-oxazolidinones, and activity remains with the existence of the N-thiolated portion for these as well (Figure 2.2).\[3\]

Figure 2.2. Structure of N-alkylthiolated oxazolidinones.
2.2.2 $N$-Alkylthio $\beta$-Lactams for Nanoparticle Encapsulation

The $\beta$-lactams discussed in this chapter are highly lipophilic molecules that are completely insoluble in water, and only moderately soluble in dimethylsulfoxide (DMSO), which displays cytotoxicity in mammalian fibroblasts when concentrations of solutions contain greater than 10% of this solvent. Therefore, there is an obvious problem when attempting to study the antibacterial activity of these drug monomers applied to $\textit{in vivo}$ settings. Also, this solubility issue greatly limits the clinical potential of these antibiotics. Since the $\beta$-lactams have shown potent anti-MRSA activity, and MRSA is one of the leading causes of sepsis and toxic shock syndrome (both blood borne infections) that are associated with high mortality rates, it is imperative that a delivery system be established for these drug monomers in order to make them accessible to these types of infections. Here, the synthetic design of these $N$-alkylthio $\beta$-lactams is described that will later be incorporated into a water-based nanoparticle drug delivery system (Chapter 3) that will provide the drug with systemic application capabilities as well as treatment capabilities in lipophilic regions of the body where many life-threatening infections are initiated.

**Scheme 2.1. Synthesis of lactam monomers 2, 4, 6 and 10.**

\[\text{Cl}_2\text{O} + \text{OCH}_3\quad \text{cat. CSA} \rightarrow \text{Cl} \quad \text{N} \quad \text{Cl} \quad \text{base} \rightarrow \text{N} \quad \text{OCH}_3\]

\[\text{RO} + \text{N} \quad \text{O} \quad \text{H} \quad \text{2, 4, 6, 10}\]

$N$-Alkylthio $\beta$-lactams 2, 4, 6, and 10 were prepared by the series of reactions illustrated in Scheme 2.1. The four step synthetic sequence was initiated by the synthesis of $N$-(4-methoxyphenyl)imine 103 from $o$-chlorobenzaldehyde (101) and $p$-anisidine (102). Staudinger coupling of alkoxyacetyl chloride (104) and imine 103 by a formal [2+2] cycloaddition led to the formation of the racemic \textit{cis}-(3$S$,4$R$)-substituted $\beta$-lactam 105.
Removal of the \( p \)-anisidine protecting group through an oxidative cleavage mechanism was accomplished with the use of ceric ammonium nitrate (CAN) in a strict volume:mass ratio of the water and acetonitrile solvents per mg of reactant used (0.03mL CH\(_3\)CN per mg lactam and 0.00909mL H\(_2\)O per mg CAN). The deprotected \( N \)-protiolactam 106 was then thiolated using the synthesized sulfenylating reagent 107a or 107b to provide the \( N \)-thiolated \( \beta \)-lactams 2, 4, 6, and 10. Each reaction is further detailed in the following subsections.

2.2.2.1 Synthesis of \( C \)-aryl imine 103

\( N \)-(4-Methoxyphenyl)imine 103 was prepared from the condensation of \( o \)-chlorobenzaldehyde (101) and \( p \)-anisidine (102) (Scheme 2.2). Prior to the reaction, the commercially supplied \( p \)-anisidine reagent was recrystallized using water heated to 60\( ^\circ \)C and subsequently dried \textit{in vacuo} in order to remove any impurities formed upon packaging and storage of the reagent. The \( o \)-chlorobenzaldehyde was used without further purification processes. Aldehyde 101 and \( p \)-anisidine (102) were dissolved in dry dichloromethane. A catalytic amount of camphorsulfonic acid was added to the mixture, which was then stirred at room temperature for 1-2 hours. However, in most cases the condensation was completed within 30 minutes where progression of the reaction was monitored by thin layer chromatography (TLC). The resulting imine was then recrystallized in cold methanol to remove any excess anisidine and to obtain the pure final product, \( N \)-(4-methoxyphenyl)imine 103.

![Scheme 2.2. Synthesis of \( N \)-aryl imine 103.](image)

2.2.2.2 Synthesis of \( N \)-aryl protected \( \beta \)-lactams by Staudinger coupling

Staudinger coupling was employed for synthesis of the mono-cyclic \( \beta \)-lactam ring formation, as has been previously reported.\[^{[2-9]}\] This reaction involves a formal [2+2] cycloaddition of an acetyl chloride (104a and 104b) to the prepared imine (103), to form the \( N \)-aryl protected \( \beta \)-lactams 105a and 105b (Scheme 2.3). The imine was initially deprotonated with triethylamine (TEA) at 0\( ^\circ \)C in dry dichloromethane, followed by dropwise addition of the acid chloride and subsequent removal of the ice bath. After 3-4
hours, the product was dried *in vacuo* and the lactam product was recrystallized in methanol to afford a white crystalline solid in 60-80% yield depending on the alkoxy acid chloride used.

**Scheme 2.3.** Staudinger [2+2] condensation of *N*-aryl imine 103.

![Scheme 2.3](image)

The mechanism proposed for the β-lactam formation is depicted in Figure 2.3.[8] The *N*-aryl imine synthesized in Scheme 2.2 forms mainly in the *E*-configuration, which has shown to lead mostly to the formation of the *cis* diastereomer of the racemic β-lactams. This preference for the *cis* compound is thought to form through a kinetic pathway and is often the single diastereomer observed when the acid chloride reagent is added in a dropwise fashion to the reaction mixture (Figure 2.3). Proton NMR spectral analysis was employed in order to determine the relative stereochemistry of the β-lactam, where the *cis* β-lactam displays coupling constants for the C₃ and C₄ protons between 4 and 5Hz, and the *trans* lactams display couplings constant between 2-3Hz.[8,10]
Figure 2.3. Theoretical mechanism for Staudinger-based ring closing reaction for formation of the β-lactam ring.:[5, 8]

Synthesis of the alkoxy acid chlorides (104a and 104b) is described in Scheme 2.4. Acetoxyacetic acid (108a) was prepared from glycolic acid and acetyl chloride in quantitative yields, whereas methoxyacetic acid (108b) was commercially available. Compound 108a and 108b were reacted individually with thionyl chloride to form the alkoxyacetyl chlorides (104a and 104b), yielding over 70% of each product after distillation. Preparation of the acid chloride through this scheme was found to be more cost effective than purchasing the acyl chloride and therefore was commonly employed for the preparation of the C₃-alkoxy β-lactams.

Scheme 2.4. Synthesis of alkoxyacetyl chlorides 104.
2.2.2.3. Deprotection of N-protiolactam 105

Deprotection of the N-aryl lactam was accomplished using ceric ammonium nitrate (CAN) in a ratio of water to acetonitrile (CH$_3$CN) as the solvent system. For this reaction, the lactam was dissolved in CH$_3$CN (0.03mL CH$_3$CN per mg lactam) and CAN was dissolved in distilled water (0.00909mL H$_2$O per mg CAN), then the CAN solution was slowly added to the lactam solution (over 30-40 minutes) at -15°C. This slow addition of CAN to the reaction mixture and the low temperature was critical for high product yields with minimal by-product formation, and only an additional 20-30 minutes of stirring was required after addition of the CAN. TLC analysis of the reaction mixture showed complete conversion of starting material after only one hour reaction time. The overall product yield was between 80-95%, depending on the R group.

Scheme 2.5. Removal of the N-$p$-methoxyphenyl protecting group from lactam monomer 105.

\[ 105 \xrightarrow{\text{CAN, } -15^\circ C} 106a: R = \text{Ac} \]
\[ 106b: R = \text{Me} \]

2.2.2.4 Deprotection of the C$_3$ alcohol acetoxy group on the lactam ring

In order to synthesize the N-methylthiolated lactam monomer 4, an additional step was needed prior to thiolation of the lactam ring. Hydrolysis of the C$_3$-acetoxy moiety was accomplished under mild basic conditions in order to avoid ring-opening. Addition of potassium hydroxide to the lactam acetate 106a dissolved in methanol afforded C$_3$-hydroxyl lactam 108 in 95% yield (Scheme 2.6).$^{[11]}$

Scheme 2.6. Deprotection of C$_3$-hydroxy group on lactam 106a.
2.2.2.5 Thiolation of the N-protiolactams 106 and 108

Thiolation of the deprotected β-lactam ring was accomplished through the use of an N-thiolated phthalimide reagent prepared in house (Scheme 2.8). This method was first reported by Miller et. al. and due to the good product yields and high purity after column chromatography this procedure was established as the method of choice for thio transfer reactions with the lactam ring. The β-lactam monomer (106, 108) was dissolved in dry CH₂Cl₂ followed by addition of N-alkylthio phthalimide (107) and Hunig’s base (diisopropylethylamine). The solution was refluxed at approximately 40°C for 24 hours under a nitrogen environment in order to provide sufficient transfer of the thio group from phthalimide to lactam. The N-protiophthalimide by-product was removed by aqueous solvents to yield a solid white crystalline product in 50-85% yield, depending on both R substituents present.

Scheme 2.7. N-thiolation of β-lactams 106 and 108.

While the N-alkylthio phthalimide reagent is commercially available, it was more cost effective to synthesize the reagent from the very inexpensive phthalimide reagent. An alkyl disulfide reagent, either methyl or sec-butyl disulfide, was added to a slurry of deprotonated phthalimide in CH₂CN and pyridine, then 1.2 equivalents of liquid bromine was cannulated into the flask to generate the N-thiolated phthalimide product in situ (Scheme 2.8). A crystalline product was produced after crystallization of the oily product in a solution of ethyl acetate and hexanes.

Scheme 2.8. Preparation of N-alkylthio phthalimide reagent.
2.2.3 Synthesis of $N$-Alkylthio C$_3$-Acrylated $\beta$-Lactams

While encapsulation is a well known means for incorporation of water insoluble drug monomers into a liposomal or nanoparticle-based drug delivery system, covalent attachment of these drug monomers had not yet been fully explored. Recently our lab has shown that drug monomers are able to be co-polymerized in a microemulsion polymerization in order to obtain drug-conjugated nanoparticles where the drug monomer is covalently bound to the polyacrylate backbone. This is accomplished through the addition of an acrylate or acrylamide moiety to the drug monomer, and both water soluble and water insoluble drug monomers can be co-polymerized in this fashion. This new and unique way to incorporate drug monomers into the nanoparticle means that not only can the water insoluble drug monomers possibly exhibit controlled release, but also that water soluble drug monomers can be incorporated to increase efficacy or stability.\textsuperscript{13, 14} This is accomplished through the acrylation of a free amine or alcohol present on the antibiotic structure, and in some instances, an amide moiety, if other structures present in the molecule are not sensitive to a strong base like sodium hydride. Other factors influence the ability of the antimicrobials to be chemically modified in order to permit covalent attachment of the drug to the nanoparticle, including the number of alcohol or amine moieties present in the structure as well as the location of the moiety within the structure. Presented in Scheme 2.9 is the synthetic route for modifying the $N$-thiolated $\beta$-lactams in order to incorporate an acrylate moiety at either the C$_3$ position of the lactam ring or \textit{para} to the lactam ring on the C$_4$ phenyl substituent.

**Scheme 2.9.** General synthetic route for formation of acrylated $N$-alkylthio $\beta$-lactams.
2.2.3.2 Acrylation of β-lactam monomers

Deprotection of the acetyl protecting group in lactam \(105a\) was performed under the same reaction conditions as described in Section 2.2.2.5. It was determined that acrylation of the C3 substituent was required prior to removal of the anisyl protecting group due to possible acrylation at the lactam amide, and also because it was determined that acrylation after thiolation of the lactam ring caused the thio group to be hydrolyzed from the lactam ring under the basic conditions required for hydroxyl deprotection and subsequent acrylation. Therefore, the lactam was more stable to acetyl hydrolysis at this stage of the synthesis, which afforded a more rapid and effective removal of the acetyl protecting group.

Acrylation of the free hydroxyl group located at the C3 position of the β-lactam ring yielded a yellow sticky solid, lactam monomer \(110\), in 45-60% yield after column purification. Acryloyl chloride purchased from Sigma Aldrich was used without further modification and was added dropwise to a solution of lactam monomer \(109\) in dry dichloromethane after deprotonation of the alcohol with sodium hydride. The reaction mixture was stirred at room temperature for 30 to 60 minutes, with progress continuously monitored via TLC.

**Scheme 2.10.** Preparation of C3-acrylate β-lactam monomer \(110\).

![Scheme diagram](image)

2.2.3.3. Attachment of C3 long chain acrylate substituent onto N-methylthio β-lactam \(9\)

In order to explore the effect of different lengths of the acrylate linker to the nanoparticle drug delivery system, lactam monomer \(9\) was synthesized and subsequently polymerized (Scheme 2.11). Due to the higher product yields observed for the N-methylthio β-lactam synthesis versus the N-sec-butylthio β-lactam synthesis, only the methylthiolated monomer was prepared using the long chain acrylate. Lactam monomer \(109\) was used as the starting material for synthesis of lactam monomer \(9\).
Scheme 2.11. General scheme for the preparation of C₃-long chain acrylate \(N\)-methylthio \(\beta\)-lactam 9.

Addition of the long acrylate chain to the free hydroxyl substituent at the C₃ position of the lactam ring was performed using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and 4-dimethylaminopyridine (DMAP) in dry dichloromethane. Deprotonation of the alcohol group was accomplished with the bulky DMAP base, followed by coupling of the deprotonated hydroxyl group with the acrylated succinate reagent.\(^{[7, 8]}\) This reaction yielded a colorless oil at 75-80% overall yield after column purification. The long chain acrylated \(\beta\)-lactam was then carried through the customary deprotection of the \(p\)-methoxyphenyl (PMP) group using ceric ammonium nitrate followed by thiolation using \(N\)-methylthiophthalimide 107a previously described to afford lactam monomer 9 in yields equivalent to the lactam monomers previously described.

2.2.3.4 Synthesis of C₄ acrylated \(N\)-methylthio \(\beta\)-lactam 5

It was hypothesized that the location of the acrylate linkage may also play a role in the observed antibacterial activity when incorporated in the nanoparticle drug delivery system.\(^{[14]}\) thus lactam monomer 5 was synthesized where the acrylate linker was placed at the \(para\) position of the phenyl ring located at the C₄ position of the lactam. Again, due to the ease in thiolation of the nitrogen present in the lactam ring when using \(N\)-methylthiophthalimide instead of \(N\)-sec-butylthiophthalimide, only the \(N\)-methylthio lactam was synthesized.
**Scheme 2.12.** Synthesis of \( C_4 \)-acrylated \( N \)-methylthio \( \beta \)-lactam monomer 5.\[^{[5, 7, 8, 14]}\]

Imine formation in the first step of this synthetic scheme was done under the same reaction conditions as used in Scheme 2.2, with camphorsulfonic acid as the catalyst. Protection of the \( p \)-hydroxy group of the imine was accomplished with acetyl chloride in 92% yield, and the imine was then cyclized using the Staudinger reaction under previously established conditions (Schemes 2.3) to afford the \( cis \) \( \beta \)-lactam 116 in 78% yield. Removal of the acetyl protecting group under basic conditions allowed the \( C_4 \) \( p \)-hydroxy phenyl \( \beta \)-lactam 117 to be subsequently acrylated with acryloyl chloride using the same reaction conditions as described in Scheme 2.10 to afford the \( C_4 \) acryloyl \( N \)-aryl protected \( \beta \)-lactam 118. Subsequent dearylation of the PMP protecting group followed by methylthiolation using \( N \)-methylthiophthalimide (107a) produced the white solid \( C_4 \) acryloyl \( N \)-methylthio \( \beta \)-lactam 5 in 89% yield.
2.3 Modification of Commercially Available Antibiotics

2.3.1. Penicillin-Based Antibiotics

Many of the commonly used commercial antibiotics for treating burn wound infections are water-soluble, such as the β-lactam antibiotics in Figure 2.4. Therefore, it was necessary to chemically modify these compounds in order to incorporate them into the nanoparticle framework. The choice of drug to be utilized was contingent on a few factors, but first and foremost was the observance of a chemical moiety in the drug structure that could be appropriately modified in order to add the acrylate or acrylamide moiety to the drug. Acrylation of the drug monomers was simplest when a single hydroxyl or amine group was present in the structure; therefore, the initial drugs chosen for acrylation were penicillanic acid, amoxicillin, ampicillin, and cefaclor (Figure 2.4).

![Penicillin G, Methicillin, Amoxicillin, Ampicillin, Penicillanic acid, Cefaclor](image)

Figure 2.4. Penicillin-based drug analogues considered for acrylation.

Penicillanic acid, ampicillin and cefaclor all possess a single free amino group that was easily accessible to the acryling agent, where penicillanic acid was the simplest of the drug monomers chosen and possesses only the single amine located at the C₃ position of the lactam ring. These drug monomers were also chosen because they do not contain any other structural moieties that would interfere with the acrylation procedure, such as an alcohol or a secondary amine group. Initial trials determined that, in some instances, the carboxylic acid moiety present on all of the lactam structures caused some problems with the acrylation reaction, and in those cases, a silyl protecting group was employed for temporary protection of the free acid (Scheme 2.13).
Penicillanic acid was the first penicillin analogue to be acrylated due to the bulk quantity available and that this penicillin structure is commercially available as the free acid instead of the water soluble potassium salt form, which would require acidification prior to silyl temporary protection. Synthesis of the penicillanic acid acrylamide 11 was accomplished through a two step process. First, the free carboxylic acid moiety was temporarily protected with the use of a silyl protecting group, employing N,O-bis(trimethylsilyl) acetamide in dry dichloromethane for 24 hours at room temperature. The protected acid (120) was then taken directly to the acrylation step without an intermediary work up, where the free amine located at the C₃ position of the lactam ring was acrylated using acryloyl chloride in the presence of a tertiary amine base (TEA). Aqueous work up of the reaction mixture afforded the deprotected penicillanic acid C₃-acrylamide monomer 11 in 91% overall yield. Due to observed self-induced polymerization of the acrylamide monomer at room temperature, the monomer was taken directly to the emulsion polymerization without further purification (Chapter 3).

The second generation cephalosporin antibiotic cefaclor was also commercially available in the free acid form, and thus followed the same acrylation procedure as was used for penicillanic acid (Scheme 2.14). The resulting cefaclor acrylamide monomer (17) was produced as a sticky solid that quickly self-polymerized under ambient conditions; therefore, purification processes were limited to an extractive work up of the reaction mixture prior to product isolation.
For the majority of the acylated penicillin-based antibiotics (monomers 11, 16, 17, 18, and 19), NMR analysis was difficult due to the insolubility observed in most deuterated solvent systems. Therefore, product confirmation relied almost solely on TLC analysis, which, when successful, showed spot to spot conversion of the commercially available drug monomer to the acrylated form in a solvent system of 5:1 dichloromethane to methanol.

**Scheme 2.15.** Acrylation of ampicillin sodium salt.

Ampicillin was the most difficult of the initial penicillin-based monomers to acrylate due to its low solubility in the organic media necessary for acrylation. Initial acidification of the ampicillin carboxylate salt was attempted in order to make the ampicillin more soluble in the organic solvent dichloromethane preferred for the acrylation reaction. However, due to the free primary amine present in the structure, acidification using dilute hydrochloric acid caused the amine to be protonated as well, which allowed the ampicillin to remain positively charged and readily soluble in the water system. Therefore, in order to perform the acrylation directly on the ampicillin sodium salt, a phase-transfer reagent was used in a two phase (water/dichloromethane) mixture in order to solubilize the drug in an organic solvent to permit silyl protection of the carboxylic acid (Scheme 2.15). N,O-bis(trimethylsilyl)acetamide was the silylating agent of choice for the acid protection step, and the reaction was carried out in a mixture of water and dichloromethane, with the use of tetra-N-butylammonium bromide (TBAB) as the phase transfer reagent. As has been previously established, once in the protected state, the ampicillin was taken directly to the acrylation step after removal of the initial mixed solvent system *in vacuo*. This procedure afforded spot to spot conversion of reactant to product via TLC analysis, and the ampicillin acrylamide 16 was produced as a yellow/brown solid in 52% yield.

The fourth drug chosen for polymerization was amoxicillin. Amoxicillin contains both a free phenolic hydroxyl group and a free amine, both located along the C₃ side chain away from the lactam ring. These moieties allowed the drug to be either mono-acrylated using one equivalent of acryloyl chloride to yield acrylamide 19, or di-acrylated to yield product 18 by providing two equivalents of the acryloyl chloride (Scheme 2.16). This aspect was intriguing because when monomer 18 was polymerized the polymer strands would likely undergo additional cross-linking, which may or may not affect the
antibacterial properties of the amoxicillin-based nanoparticle emulsion. These properties were later explored in Chapter 3.

**Scheme 2.16.** Acrylation and diacrylation of amoxicillin (18 and 19).

The final penicillin analogue chosen for this study was penicillin G, which is commercially available as the potassium salt. The drug was chemically modified in order to permit two acrylated forms of the drug (acylation at the C₃ amide to give acrylimide 13, or at the carboxylic acid moiety to give acrylate ester 12).

**Scheme 2.17.** Acrylation of penicillin G potassium salt.

In order for acrylation of the C₃-amide nitrogen on the lactam ring to be successful, the penicillin monomer must be able to dissolve in dichloromethane, which means that the drug must be acidified in order to convert the drug to its original free acid form (14). This was accomplished by titrating dilute hydrochloric acid into a solution of the penicillin
potassium salt in water. Addition of the acid was terminated upon precipitation of a sticky yellow solid, followed by decanting of the water solution from the precipitated free acid drug form and washing the solid 2-3x with water to remove excess HCl. The acidified penicillin was allowed to dry overnight in vacuo in order to obtain a solid crystalline powder (14), which was then protected with a trimethylsilyl group followed by acylation with acryloyl chloride. However, because the intended nitrogen for acylation is part of an amide system, a stronger base was needed than the previously used tertiary amine base. Here sodium hydride was employed to deprotonate the amide in order to make the amide nitrogen reactive towards the acryloyl chloride reagent. Aqueous work up of the reaction was performed to remove the silyl protecting group, leaving the water insoluble free acid acrylamide penicillin monomer 13 as a slightly yellow solid in 70% yield.

Scheme 2.18. Acrylation of penicillin G via the free acid.

Scheme 2.18 shows the synthesis of pencillin G acrylate ester 12. In order for the carboxylic acid on the penicillin to be acrylated, it was first converted from the sodium salt to the free acid form. The free acid was then reacted with ethyl chloroformate at 0°C for 30 minutes to yield the esterified acid 122, which was then directly reacted with 2-hydroxyethyl acrylate in the presence of triethylamine to yield the penicillin G acrylate as a yellow oil in 70% yield after column chromatography.
Scheme 2.19. Esterification of penicillin G free acid 14.

The long chain ester penicillin G analogue 15 was synthesized through EDCI coupling of penicillin G in the free acid form (14) with 2-hydroxyethyl propionate following the same reaction conditions as reported for Scheme 2.11 (Scheme 2.19).

2.3.2 Fluoroquinone-Based Antibiotics

The next commercially available antimicrobial examined for incorporation into the polyacrylate nanoparticles was ciprofloxacin (Figure 2.5). Ofloxacin, an analogue of ciprofloxacin, is sold as a racemic mixture of the chiral drug under the commercial names Floxin® and Ocuflon® for treatment of bacterial infections, including bronchitis, pneumonia, chlamydia, gonorrhea, skin infections, urinary tract infections, infections of the prostate, and infections of the eye. The biologically active (S)-enantiomer of ofloxacin, levofloxacin, has only recently been synthesized and has been approved for medical applications under the brand name Levaquin in the U.S. (Figure 2.5). Levofloxacin has shown to be twice as potent as the racemic ofloxacin due to elimination of the (R)-enantiomer, which has no biological activity.

Figure 2.5. Commercially available fluoroquinones considered for acrylation.

The second generation fluoroquinone, ciprofloxacin (cipro), is a potent broad spectrum antibacterial whose activity is expressed by inhibition of bacterial DNA replication. While this bacteriostatic antibiotic has been considered one of the work horses in bacterial infection treatment, resistance has been observed for some microbes, especially in hospital settings; therefore, this drug monomer was an excellent choice for incorporation into the nanoparticle. Cipro was chosen over levofloxacin and the more
readily available ofloxacin antibiotics due to the secondary amine located within the cyclohexyl ring system of the drug monomer, which is replaced by a tertiary amine in both ofloxacin and levofloxacin, making the amine unavailable for acrylation. Table 2.1 presents some of the microbes that are sensitive to cipro. Many of these microbes are also commonly observed colonizing burn wounds, making cipro an optimal choice for incorporation into the multi-drug conjugated nanoparticle delivery system for treatment of burn wound infections.

Table 2.1. Antibacterial activity of ciprofloxacin.

<table>
<thead>
<tr>
<th>Ciprofloxacin Antibacterial Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram Positive</strong></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em>: both MRSA and MSSA</td>
</tr>
<tr>
<td><em>Bacillus anthracis</em></td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
</tr>
<tr>
<td><strong>Gram Negative</strong></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
</tr>
<tr>
<td><em>Enterobacteriaceae</em> spp.</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td><em>Neisseria gonorrhoeae</em></td>
</tr>
<tr>
<td><em>Neisseria meningitidis</em></td>
</tr>
<tr>
<td><em>Campylobacter</em> spp.</td>
</tr>
<tr>
<td><em>Brucella</em> spp.</td>
</tr>
<tr>
<td><em>Moraxella catarrhalis</em></td>
</tr>
<tr>
<td><em>Vibrio</em> spp.</td>
</tr>
<tr>
<td><em>Haemophilus influenzae</em></td>
</tr>
<tr>
<td><em>Haemophilus ducreyi</em></td>
</tr>
<tr>
<td><em>Chlamydia trachomatis</em></td>
</tr>
<tr>
<td><em>Chlamydia pneumoniae</em></td>
</tr>
</tbody>
</table>

Ciprofloxacin was acrylated using two variations of the acryloyl chloride: the original acryloyl chloride and methacryloyl chloride (Scheme 2.20). This afforded two different analogues of the cipro acrylamide monomer to be used in the multi-drug conjugated nanoparticle emulsion (Chapter 4).
2.4 Antibacterial Activity of Drug Monomers

Antibacterial assays were performed for the majority of the drug monomers synthesized. Assays were performed against many *Staphylococcal* strains, including numerous strains of MRSA. MRSA was either purchased from ATCC (43300 and 33591) or was obtained from Lakeland Regional Medical Center as clinical isolates. The clinical isolates were arbitrarily labeled USF652-659, and the ATCC purchased strains 43300 and 33591 correspond to the USF-designated 920 and 919, respectively. Methicillin-sensitive *S. aureus* (MSSA) and *Pseudomonas aeruginosa* were purchased from ATCC as well (ATCC 25923 and 10145, respectively) and *Bacillus* species were either provided by Colorado Serum Co., Denver, CO. (*B. anthracis*-Sterne), purchased from ATCC (*Bacillus cereus*: ATCC 14579, *Bacillus subtilis*: ATCC 19569, *Bacillus megaterium*: ATCC 14581, *Bacillus thuringensis*: ATCC 10792), or provided by the Department of Defense Reagents Program (*Bacillus globigii*). Both the Kirby Bauer and broth-dilution minimal inhibitory concentration (MIC) antibacterial assays were performed for the drug monomers dissolved in DMSO or water. Both of these assays are reputable for their accuracy and ability to compare numerous antibiotics through the use of standardized techniques described in the National Committee for Clinical Laboratory Standards (NCCLS) handbook. All broths and agars used in the antibacterial assays were purchased from Fisher Scientific Company.
2.4.1 Kirby Bauer Assay

Kirby Bauer assays were performed on the drug monomers dissolved in DMSO in a 1mg/mL concentration, and were added to the agar wells in 20µL volumes in accordance with the guidelines recommended by the NCCLS handbook. Results of this assay are measured as the zone of bacterial growth inhibition (ZOI) in millimeters around the center of the well where the dissolved drug is placed. The diameter of the zone in millimeters is the value reported and partial growth inhibition zones are represented in parentheses. These assays rely on the ability of the substance being tested to diffuse from the well into the surrounding agar in a circular pattern in order for the drug to interact with the bacteria present on top of the agar surface. However, there are many inherent problems with this assay due to required diffusion of the drug monomer through the agar. Often times highly lipophilic drug monomers will not diffuse through the agar or have very limited diffusion due to positive interactions of the drug monomer with the agar preventing drug migration, therefore, a second assay was employed for accurate analysis of the antibacterial activity of the drug monomers synthesized here.

2.4.2 Broth Dilution Minimum Inhibitory Concentration (MIC) Assay

In order to establish the lowest drug concentration needed to prevent bacterial growth, the drug monomers were analyzed for antibacterial activity at various drug concentrations per mL of solvent (DMSO or water). Optical density measurements (540-550nm) were employed to determine at what lowest drug monomer concentration the antibiotic inhibits cell growth. The highest drug monomer concentration analyzed was 256µg/mL and reached the lower limit of 0.125µg/mL through serial dilution. Mueller Hinton broth was employed for the assay in accordance with the guidelines recommended in the NCCLS manual for antibacterial testings.

2.4.3 β-Lactam Monomers

Due to the high lipophilicity of the lactams, the monomers were dissolved in DMSO for both the broth-dilution MIC studies as well as in the Kirby Bauer assays. The commercially available drugs analyzed for antibacterial activity (penicillin G potassium salt and ciprofloxacin hydrochloride) were dissolved in nanopurified water for analysis.
Figure 2.6. Kirby Bauer assay of β-lactam monomers against *S. aureus*. All *N*-thiolated lactam monomers have anti-staphylococci activity, although lactam monomer 1 did not have antibacterial activity in this specific assay.

As seen in Figure 2.6, the anti-*Staphylococcal* activity of a few of the lactam monomers synthesized varied depending on the substituents present on the lactam molecule. The weakest drug monomer synthesized, according to Figure 2.6, was lactam monomer 1, which contained an acrylate moiety at the C₃ position of the *N*-methylthio β-lactam. Also, it was determined that the *N*-sec-butylthio lactam monomers (3 and 10) were more active than the *N*-methylthio lactam monomers (1 and 6). This was also observed for the anti-*Bacillus* activity of the lactams presented in Table 2.2.
**Table 2.2.** Zone of inhibition and minimum inhibitory concentration analysis of lactam monomers. A 20µL volume of a 1mg/mL solution of lactam monomer in DMSO was applied to the agar wells for ZOI analysis, and a 5.12mg/mL initial drug concentration was the starting point of the MIC analysis.

<table>
<thead>
<tr>
<th>Compound</th>
<th>B. anthracis- Sterne</th>
<th></th>
<th>S. aureus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ZOI* (mm)</td>
<td>MIC (µg/mL)</td>
<td>ZOI* (mm)</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>NA</td>
<td>NA</td>
<td>55 (61)</td>
</tr>
<tr>
<td>1</td>
<td>20</td>
<td>32</td>
<td>(12)</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>NA</td>
<td>(14)</td>
</tr>
<tr>
<td>3</td>
<td>(25)</td>
<td>4</td>
<td>17 (22)</td>
</tr>
<tr>
<td>4</td>
<td>33</td>
<td>NA</td>
<td>18</td>
</tr>
<tr>
<td>5</td>
<td>15 (20)</td>
<td>32</td>
<td>(25)</td>
</tr>
<tr>
<td>6</td>
<td>23 (26)</td>
<td>4</td>
<td>31</td>
</tr>
<tr>
<td>9</td>
<td>(21)</td>
<td>NA</td>
<td>(15)</td>
</tr>
<tr>
<td>10</td>
<td>38</td>
<td>0.5</td>
<td>41 (45)</td>
</tr>
<tr>
<td>Cipro</td>
<td>42 (45)</td>
<td>0.063</td>
<td>28 (34)</td>
</tr>
</tbody>
</table>

* ( ) = partial inhibition

Data presented in Table 2.2 shows that the lactam monomers display antibacterial activity against both *S. aureus* and *B. anthracis*-Sterne. It was determined that the most potent lactam analyzed was N-sec-butylthio lactam 10, where the MIC against *S. aureus* was 8µg/mL and 0.5µg/mL against *B. anthracis*. While none of the lactams were nearly as potent as the commercially available drug monomer used for treatment of these microbes in clinical settings (penicillin G or vancomycin for *S. aureus* and cipro for *B. anthracis*), the bioactivity of the lactams remained constant against resistant strains of the microbe (Figures 2.7, 2.8), which was not observed for the commercial drugs, specifically penicillin G.
Figure 2.7. Kirby Bauer assays comparing anti-\textit{Staphylococcal} activity of lactam 10 to penicillin G. Image to the left contains 100\,µg of β-lactamase spread over the agar. Image to the right does not contain β-lactamase.

Figure 2.8. Comparison of zone of inhibition data for lactams 4 and 6 versus penicillin G. Data presented for seven clinical isolates of MRSA (USF 652-659) and for MSSA (ATCC 25923).
As observed in Figures 2.7 and 2.8, lactams 4, 6, 10 and penicillin G all displayed excellent activity against methicillin-sensitive *S. aureus* (MSSA), however, in the presence of β-lactamase (Figure 2.7) or against MRSA (Figure 2.8), the activity of penicillin G diminished greatly. In Figure 2.8, the observed activity for penicillin G against the various strains of MRSA was measured as barely larger than the diameter of the agar well (7-8mm), therefore, it can be concluded that when in the presence of β-lactamase or against MRSA, for which penicillin G is not an effective antibiotic, the N-thiolated lactams are equally potent to these strains as they are towards MSSA.

### 2.4.2 Acrylated Monomers of Commercially Available Drugs

The penicillin G acrylamide monomer 13 displayed anti-*Staphylococcal* activity closest to the commercially available penicillin G. Since penicillanic acid is a less effective antibiotic than penicillin G, the activity of the penicillanic acid acrylamide 11 was expected to be weaker than the acrylated penicillin G monomers 12 and 13, which was observed in Figure 2.9.

![Figure 2.9. ZOI data for penicillin-based monomers against *S. aureus*.](image-url)
Table 2.3. Antibacterial activities of ciprofloxacin acrylamide monomers 7 and 8 against *B. anthracis*-Sterne, *S. aureus* (ATCC 25923) and *P. aeruginosa* (ATCC 10145). Drug monomers were applied as a 1mg/mL concentration in DMSO, with 20\(\mu\)L of this solution being added to each agar well for ZOI analysis.

<table>
<thead>
<tr>
<th>Sample</th>
<th><em>B. anthracis</em>-Sterne</th>
<th><em>S. aureus</em></th>
<th><em>P. aeruginosa</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ZOI* (mm)</td>
<td>MIC ((\mu)g/mL)</td>
<td>ZOI* (mm)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>42 (45)</td>
<td>0.063</td>
<td>28 (34)</td>
</tr>
<tr>
<td>7</td>
<td>40</td>
<td>0.125</td>
<td>26 (29)</td>
</tr>
<tr>
<td>8</td>
<td>35</td>
<td>0.5</td>
<td>25</td>
</tr>
</tbody>
</table>

* ( ) = partial inhibition

Ciprofloxacin acrylamide monomers 7 and 8 displayed nearly equipotent activity towards *B. anthracis* and *S. aureus* as the unmodified ciprofloxacin antibiotic, but were inactive against *P. aeruginosa*. This diminished activity was important because it enabled anti-*Pseudomonal* activity of the polymerized cipro acrylamide monomers (Chapter 3) to establish that there is release of the un-acrylated (free) drug from the polyacrylate backbone by the microbe.

2.5 Mode of Action of *N*-thiolated \(\beta\)-Lactams

The narrow scope of antimicrobial activity for the *N*-thiolated \(\beta\)-lactam monomers and the fact that the lactams are bacteriostatic suggests that a reversible interaction of the drug with a specific cellular enzyme in select microbes is occurring. There are many plausible reaction mechanisms that could be the cause of the observed antibacterial activity, as seen in Figure 2.10, however, recent studies in our lab have suggested that the majority of the lactams interact with the susceptible microbes through a sulfenylation reaction.[3]
Due to the unusual antimicrobial activity of these N-thiolated β-lactams,\cite{4, 6, 7, 9, 11} plus their antifungal activity against numerous *Candida* species, it seemed apparent that the lactam structure is acting through a unique mode of action against these microbes. Initial studies against *S. aureus* using a gram staining technique and SEM imaging of the treated and un-treated bacterial cells determined that the cell wall of the bacteria was not the target of this specific class of β-lactam drugs,\cite{3, 5} even though all other β-lactam antibiotics are well known inhibitors of cell wall biosynthesis for gram positive microbes. Bacteria were exposed to the antibiotics via a Kirby Bauer assay, and the microbes analyzed for imaging were taken from just within the edge of the zone of inhibition. Radioactive studies by Timothy Long also supported this finding, as well as ruled out other possible modes of action, such as DNA and RNA biosynthesis inhibition.\cite{3, 5}
Figure 2.11. Light microscopy images of *S. aureus* treated with various antibiotics. Penicillin G and ampicillin were both analyzed using methicillin-sensitive *S. aureus* (MSSA), while the mode of action of lactam monomer 6 was analyzed using methicillin-resistant *S. aureus* (MRSA). Images taken by Dr. Timothy Long.\[3,5\]

Gram staining studies using MSSA and MRSA strains of *S. aureus* were performed in order to elucidate the mode of action of the mono-cyclic thiolated β-lactam monomers. When the cell wall is intact for gram positive microbes, such as *S. aureus*, the microbial cells stain purple in the gram staining analysis. However, if the cell wall is damaged, or a gram negative bacterium is being analyzed, then the cells stain pink. The images in Figure 2.11 show that the cell wall of the MRSA cells was un-affected by lactam 6, observed by the purple gram staining. Penicillin G and ampicillin, two well-known antibiotics whose mode of action is through inhibition of cell wall biosynthesis for gram positive microbes, which caused the cells to stain mostly pink in color, as seen in Figure 2.11. Some of the penicillin-treated and ampicillin-treated cells were observed retaining the gram stain, indicating that they were unaffected by the antibiotics or that the antibiotics had not reached these microbes prior to harvesting for imaging. SEM analysis also confirmed this finding, where the images showed intact *S. aureus* cells that did not express any damage to the exterior of the cell wall.\[3,5\]
This gram staining study was also repeated with the spore-forming gram positive microbe *B. anthracis*. This microbe is able to produce a highly potent toxin when in its natural state; therefore, an attenuated strain of the microbe (Sterne) was used for this study. *B. anthracis* is a rod-shaped, gram positive microbe that forms long chain-link structures where the bacilli rods are attached to one another head-to-foot (Figure 2.12). This bacterium is also able to form a highly impenetrable spore coating that protects it from heat and allows the microbe to exist for years in environments with little to no nutrients.[17] When sporulation occurs, the bacterium becomes highly insensitive to normal antibiotic treatment, making it extremely difficult to treat.[19] The combination of the highly toxic Ames strain of *B. anthracis* with the ability of the microbe to sporulate makes it a potent and very dangerous microbe that can be used as a biological weapon. Therefore, antibiotics powerful enough to eliminate the endospore form of the microbe or antibiotics that can prevent sporulation are in dire need.

![Ampicillin](image1.png) ![Pen G](image2.png)

![B. anthracis](image3.png) ![Lactam Monomer 6](image4.png)

**Figure 2.12.** Light microscopy images of *B. anthracis* treated with various antibiotics. All assays performed here used the attenuated Sterne strain of *B. anthracis*.

In Figure 2.12, it is clearly distinguishable that the *Bacillus* rods treated with either ampicillin or penicillin G antibiotics exhibited damage to the bacterial cell wall apparent by the pink staining of the rods. The untreated anthracis cells stained purple after gram
staining analysis, indicating that when the bacilli are not treated with sub-lethal amounts of antibiotics there are no rods that stain pink in color. The bacilli treated with lactam 6, however, displayed a mixture of pink and purple rods, where in some instances, a long chain of rods would start out staining purple, but towards the end of the chain, stained pink in color (Figure 2.12). Also observed were definitive regions of all pink-stained bacilli as well as all-purple bacilli (Figure 2.13). One possible explanation for the observed gram-negative staining result for bacilli (but not for staphylococci) is that the drug is affecting the biosynthesis of the bacterial membrane, which is allowing intracellular contents to leach out of some of the cells and, therefore, produce negative gram staining in those affected cells. This hypothesis was supported by studies performed by Dr. Seyoung Jang, that found that the N-thiolated β-lactams inhibit fatty acid biosynthesis,\textsuperscript{[3,5]} however the exact stage of this biosynthetic pathway where the lactams exhibit their activity has yet to be elucidated. A secondary explanation for the gram stain results could be related to the ability of the anthrax to sporulate, which would also affect the gram staining results.

![Figure 2.13](image)

**Figure 2.13.** *B. anthracis* treated with C₃-methoxy N-methylthio β-lactam monomer 6. A) *Bacillus* rods observed staining purple = gram positive. B) *Bacillus* rods observed staining pink = gram negative.

Aside from the superficial effects the lactams imposed on the bacterial cells, little was known of how the anatomy of the microbial cells was affected by the antibiotic. Therefore, TEM imaging was performed on three different microbes sensitive to the N-thiolated β-lactams using lactam monomer 6, including the two gram-positive microbes previously discussed (*B. anthracis*-Sterne and MSSA) and the fungal strain *Candida albicans*, which is the leading cause of fungal infections colonizing burn wounds. The broth culture containing the microbe was treated with lactam 6 for a 30 minute incubation period (bacterial) or 1 to 4 hour period (fungi) at 37°C, and the cells were then cross-linked using gluteraldehyde in order to stop all cellular functions without damaging any of the cellular components. This allowed the cells to be imaged in their natural state after only a short antibiotic exposure.
Figure 2.14. TEM images of *B. anthracis* cells. A) Untreated *B. anthracis*-Sterne cells. A-Cell wall, B-Cell membrane, C-cytoplasm. B) Cells treated with *N*-methylthio β-lactam monomer 6. C and D) Cells treated with penicillin G potassium salt. Arrow in C points to a break in the peptidoglycan cell wall. Arrow in D points to a hydrated endospore surrounded by many other encapsulated endospores and germinating bacilli.

The spore coat, the internal spore cortex, cellular membranes and the spore core were all readily identified in the TEM images in Figure 2.14A of untreated bacilli through negative staining (2% uranyl acetate). All of the bacilli cultures were stained with osmium tetroxide coupled with lead citrate after sectioning of the embedded cells for enhanced visibility of these structures, yet this caused the black metal precipitation observed in some of the images. It has been noted that when bacilli and clostridial (another sporulating microbe) cells are in an endosporic, dormant state, they exhibit ultrafine (5-10nm) filamentous appendages on the exterior of the endospore that appear hair-like in structure (Figure 2.14A). These appendages were observed for many of the bacilli imaged by TEM analysis, confirming the presence of the endospores in the culture. The interior of the endospores, termed the protoplast, was clearly visible in many of the bacilli imaged.
Unlike the vegetative bacilli cells, the endospores shrink to less than 1µm in size upon sporulation and are distinguished as small rounded cells containing numerous “rings,” which represent the various layers of the spore coating anatomy.

Figure 2.15. TEM of *B. anthracis* treated with lactam monomer 6. Both images depict deterioration of the bacterial cells and break down of the exosporium coatings of the cells.

TEM imaging of the bacilli cells treated with lactam monomer 6 (Figure 2.14B, Figure 2.15) resulted in the observance of very few sporulated cells, where the cell culture that was not exposed to lactam 6, or that was exposed to penicillin G possessed many sporulated bacilli, as well as the observance of encapsulated endospores. This indicates that the presence of the lactam monomer inhibits spore formation. In some instances, the TEM images exposed a peptidoglycan outline of where the cellular contents used to be housed, which further indicates that the lactams do not have a direct effect on the cell wall of the microbes. It was assumed that the cellular contents were able to leach out of the cell wall through interruption of the cellular membrane. Also, very few germinating bacilli cells were observed in the lactam 6 treated cell culture, indicating that the antibiotic prevents both endospore formation and blocks cellular germination of the bacilli. One of the few spores observed in the lactam-treated culture (Figure 2.15, left) showed damage to the inner and outer membranes of the cell (white arrow) and to the spore cortex and sloughing off of the exosporium (black arrows) or external protective coating of the bacilli spore.

As seen in Figure 2.14C, penicillin G caused many of the bacilli observed to have breakdowns in the peptidoglycan cell wall, which caused cellular spillage visible in the TEM. However, there were numerous bacilli endospores and germinating cells observed for the penicillin-treated cell culture, indicating that until cell wall rupture occurs for the bacilli, the cells are able to undergo normal cellular function (Figure 2.14D). The round white spots located in the center of some of the cells in Figure 2.14D are due to hydration.
present within the bacterial cell that was not removed during the dehydration processing of the cells prior to embedment in plastic for sectioning. When scanning the plastic sections containing the penicillin-treated bacilli, there was a high degree of free-floating peptidoglycan surrounding many of the cells, presumably having been degraded and sloughed off the bacterial cell.

Figure 2.16. Overview of many bacilli present in penicillin-treated and untreated cell cultures. Left image is untreated cells, and right image is cells treated with penicillin G.

Mesosomes, which have been observed in both gram positive and gram negative microbes, are invaginations of the cellular plasma membrane that can form into vesicles within the bacterial cell, and are thought to play some role in DNA replication and/or cell division. As observed in Figure 2.16, both the penicillin-treated and untreated cells displayed black clusters within the cells, mainly in the germinating cells, which are most likely the mesosomes present within the prokaryotic cells. These black clusters were not observed in the cells treated with lactam 6, further indicating that the N-thiolated β-lactams inhibit fatty acid synthesis, which comprises the bacterial membranes present within the cells and the aforementioned mesosome vesicles observed in the untreated and penicillin-treated bacilli.

Imaging of S. aureus cells treated with β-lactam monomer 6 was also performed and presented in Figure 2.19. While many TEM images were captured of the treated cellular culture, very few cells were observed in an intact form. The majority of the cells observed looked like ghost cells, with little cellular contents intact within the cell.
Figure 2.17. *S. aureus* cells treated with lactam monomer 6. A and B) Cells treated with lactam 6 for 30 minutes. C and D) Control cell culture that was not exposed to any antibiotics.

The *S. aureus* cells treated with lactam monomer 6 showed a high degree of intracellular damage, observed by the lack of intracellular mass within the bacterial cell wall. Even though it seems that most of the cellular contents have been destroyed and spilled into the surrounding area, the bacterial cell wall appears to still be intact surrounding the ghost cells. This was also observed in Figure 2.14B for the bacilli cells treated with this lactam monomer *in vitro*. The untreated *S. aureus* cells appear to have intact intracellular mass depicted by the gray matter within the intact cell wall, as seen in Figure 2.17C and Figure 2.17D. Figure 2.17C shows germinating *S. aureus* cells that are in the process of splitting, while the image in Figure 2.17D is of an intact vegetative cell. In both images, the cell walls are whole and there is no observance of cellular spillage or deformation of the cell morphology, indicating that the cellular destruction observed for the lactam 6 treated cells is a direct result of the drug’s antibacterial activity.

*Candida albicans*, the causative microbe for fungal infections such as athlete’s foot, is also sensitive to the *N*-thiolated β-lactam antibiotics. Since fungi are eukaryotic organisms, they possess many complex internal structures similar to mammalian cells,
including a defined nucleus, mitochondria, cytoskeleton, and a network of internal membranes for compartmentalization. Therefore, TEM analysis of these microbes was performed so that a more complete mode of action for the drug monomers might be defined due to the diverse ultrastructures present within the microbe, where many of these structures are surrounded by phospholipid membranes.

**Figure 2.18.** *Candida albicans* cells treated with lactam monomer 6. A) Untreated control cells. B and C) Cells treated with β-lactam 6 for 1 hour. D) Cells treated for 4 hours with lactam 6. Arrows point to cell membrane invaginations or disruptions due to lactam treatment.

The images in Figure 2.18 show the fungal cells of *C. albicans*, where these cells are much larger than the previously observed bacilli and staphylococci cells. Also seen in these images are intact nuclei and mitochondrial membranes for both the lactam-treated and untreated cells. However, arrows in Figure 2.18C and Figure 2.18D point to cell membrane invaginations and disruptions due to lactam 6 exposure. The TEM and light microscopy studies support previous works performed that suggest the N-thiolated β-lactam antimicrobials act through inhibition of fatty acid biosynthesis, which in turn,
disrupts the cellular membranes that are formed from the lipid bilayers that hold all of the intracellular contents in place.

### 2.6 Conclusions

Antimicrobials were synthesized and acrylated to determine their antibacterial activity and for subsequent incorporation in a nanoparticle delivery system (Chapter 3). \(N\)-thiolated \(\beta\)-lactam monomers were synthesized for both encapsulation and for covalent attachment to the nanoparticle. Also, commercially available water soluble antibiotics were chemically modified in order to afford an acrylate or acrylamide derivative, which permits co-polymerization later in Chapter 3. All of the drug monomers synthesized here displayed antibacterial activities against \(S.\) \(aureus\) and \(B.\) \(anthracis\), and the \(N\)-thiolated \(\beta\)-lactams synthesized had equipotent activity against methicillin-sensitive \(S.\) \(aureus\) as well as methicillin-resistant \(S.\) \(aureus\). It was also determined that acrylation of ciprofloxacin removed its anti-\textit{Pseudomonal} activity.

The mode of action of the \(N\)-thiolated \(\beta\)-lactams has recently been investigated by Revell et al.\(^5\) which established that the lactams act on the fatty acid biosynthetic pathway in microbes that have coenzyme A-based thiol-redox buffer systems. In this chapter, various imaging techniques observed that the \(N\)-methylthio \(\beta\)-lactam (6) disrupted the cellular membranes of \(S.\) \(aureus\), \(B.\) \(anthracis\), and \(C.\) \(albicans\), and in many cases, entire cellular contents were destroyed leaving behind only the bacterial cell wall around ghost cells. \(N\)-Thiolated \(\beta\)-lactams also exhibit antimicrobial activity against the gram positive microbe \(S.\) \(aureus\), even in the presence of the \(\beta\)-lactamase enzyme, as well as MRSA, and acts through a fatty acid biosynthesis pathway that causes microbial cell membrane deterioration.

### 2.7 References


CHAPTER 3

NANOPARTICLE MICROEMULSION POLYMERIZATION FOR DRUG DELIVERY

3.1 Introduction

New classes of antibacterial agents and alternative therapy protocols are in dire need in this era of escalating drug-resistance among pathogenic bacteria. One of the most recent forays in this area is the use of nanotechnology for both drug delivery platforms and as therapeutic agents themselves. Nanoparticle-based antibiotics have provided anti-infectives with improved performance, sustained or controlled release, improved solubility and stability, lower cytotoxicity, and in some cases, targeted drug delivery. Since the first application of nanoparticles in therapeutics in 1976 by Kreuter and Speiser,\(^1\) nanotechnology has been applied to many areas in the medical field, including imaging, anti-cancer therapies, and also antimicrobial therapy, although little effort has been put towards improving antimicrobial efficacy thus far. With the increasing capacity of microbes to develop resistance to the currently prescribed antibiotics, the need to explore this form of advanced drug delivery for antimicrobials is paramount in overcoming current bacterial resistances and also possibly improving the efficacies of current antibiotics. That is the focus of this chapter, where the use of polyacrylate nanoparticles synthesized through a microemulsion polymerization technique is explored in order to increase the bioavailability of antimicrobials and also to rejuvenate some of the current antibiotics such as penicillins that have become increasingly obsolete due to the development of bacterial resistance mechanisms.

3.2 Penicillins and Bacterial Resistance

Since the initial discovery of penicillin by Alexander Fleming in 1928, and the first synthesis of the antibiotic for pharmaceutical use, antibiotics have played a major role in infection treatment. The penicillin antibiotics, and many of the other β-lactam-based classes of antibiotics subsequently discovered, act by inhibiting the synthesis of the bacterial cell wall. This thick cell wall, consisting mainly of cross-linked peptidoglycan polymer chains, is found only in prokaryotic cells and is considered to be a very thick protective layer in gram positive bacteria. While this layer is also present in gram negative microbes, it is limited in its thickness and fortification.
This cross-linked cell wall is essential for microbes to retain their rigid cell structure and morphology. When severely damaged, cytoplasmic leeching is observed, ultimately killing the microbe (Figure 3.2). The penicillin class of antibiotics acts on cell wall biosynthesis by preventing the inter-polymer strand cross-linking that gives the cell wall its rigidity, through interactions with the bacterial enzyme glycopeptide transpeptidase. As seen in Figure 3.2, *S. aureus* cells treated with penicillin G via a Kirby Bauer diffusion assay caused the cocci to become wrinkled in appearance, concaved from their inherent spherical morphology (darkened areas in center of cocci), and altered in size and cohesiveness.\(^2\)

However, with every great new discovery come new challenges and the establishment of penicillin as an antibacterial drug was certainly no different. Over-usage and a lack of public conscientiousness on how to properly administer this drug in terms of dosage

\(^2\)
regimens sparked a new phenomenon in bacterial infections and treatment, the era of drug-resistant microbes. Almost as soon as penicillin was made available for use in hospitals and clinics in the 1940s, bacterial resistance formed, especially in the gram positive species *Staphylococcus* and *Streptococcus*, ultimately creating one of the most dangerous microbes associated with human infection, methicillin-resistant *S. aureus* (MRSA). In burn patients the risk of acquiring an MRSA-based infection is 45%, and is one of the primary microorganisms contributing to infections associated with high mortality rates in burn victims.[3] MRSA’s antibiotic defense works by exuding the enzyme β-lactamase in the area immediately surrounding the microbe, which in turn protects the microbe from β-lactam antibiotics. This enzyme, as seen in Figure 3.3, works by effectively causing the ring opening of the β-lactam, which inactivates the drug’s antibacterial activity.

![β-lactamase](image)

**Figure 3.3.** Inactivation of penicillin G by the β-lactamase enzyme.

Many new analogs of penicillin, including cephalosporins, carbapenams, penams, nocardicins, and monobactams, have all been synthesized since the initial synthesis of penicillin for antibacterial usage, and with each new class of antibiotics, an increase in drug potency and broadening of the antibacterial activity towards gram negative microbes was observed. However, none of these antibiotics were able to successfully resist the enzymatic activity of the β-lactamase enzyme (Figure 3.3). However, clavulanic acid, a low molecular weight molecule was later discovered that, when delivered in combination with certain penicillins such as ampicillin and amoxicillin was capable of blocking the activity of the β-lactamase in order for the antibiotic to reach the bacteria and evoke its antibacterial activity. Since clavulanic acid has no antibacterial activity, only its ability to enhance the activity of penicillin antibiotics, it is an important advance against drug resistance. Nowadays, when penicillin antibiotics are prescribed, the drug often contains clavulanic acid for this specific purpose. However, there are some disadvantages to this, including further escalation of resistance from selective pressure on the microbe to over-express the gene for penicillinase production (Figure 3.4). Also, since most antibiotics are administered orally, poor stability and/or permeability in the digestive tract results in sub-therapeutic concentrations of the antibiotic reaching the infection site. Since patients often do not follow the recommended dosing regimen for oral antibiotics and stop treatment once they begin to feel better, an even greater threat exists for development of bacterial resistance to the drug. This is certainly the case for ear infections in young children where use of clavulanic and (or other β-lactamase inhibitors) in combination with a β-lactam antibiotic has led to severe antibiotic resistance.
Figure 3.4. Antimicrobial resistance trends in pathogens found in burn wounds. A) Resistance of *S. aureus* strains to antibiotics over time of treatment. B) Resistance of *Pseudomonas aeruginosa* strains to antibiotics over time of treatment. Data reprinted from *Burns*, Vol. 30, by Altoparlak et. al. Pages 660-664, Copyright 2004, with permission from Elsevier.\[^{[3, 4]}\]
Figure 3.5. Chemical structures of common antibiotics used to treat burn wound infections.
Studies have shown that, especially in burn treatment centers, patients who are administered a regimen of β-lactam antibiotics have a nine-fold increase in the chance of developing an MRSA infection over patients treated with the newer classes of glycopeptide and fluoroquinone antibiotics.\textsuperscript{[5]} However, glycopeptide antibiotics, such as vancomycin and teicoplanin, are considered a last resort for treating \textit{S. aureus} infections since resistance has already been observed for these antibiotics as well. Also, while the use of fluoroquinone antibiotics have been very effective broad spectrum antibiotics, resistance has been observed for these antibiotics as well including in the gram negative microbe \textit{Pseudomonas aeruginosa} which is the most frequent pathogen found colonizing burn wounds (Table 3.1).\textsuperscript{[6-11]} Therefore, this work set out to establish a new and unique drug delivery system that has the ability to shield antibiotics from resistance factors so that they can maintain full antibacterial activity.

\textbf{Table 3.1.} Microorganisms commonly found in burn wound infections.\textsuperscript{[6-11]}

<table>
<thead>
<tr>
<th>Microbe Classification</th>
<th>Pathogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram Positive</td>
<td>\textit{S. aureus}: MRSA, MSSA</td>
</tr>
<tr>
<td></td>
<td>\textit{S. epidermidis}</td>
</tr>
<tr>
<td></td>
<td>\textit{Streptococcus} spp.</td>
</tr>
<tr>
<td></td>
<td>\textit{Pneumococcus} spp.</td>
</tr>
<tr>
<td></td>
<td>Haemolytic streptococci</td>
</tr>
<tr>
<td></td>
<td>\textit{Bacillus} spp.</td>
</tr>
<tr>
<td></td>
<td>Vancomycin-resistant \textit{Enterococcus}</td>
</tr>
<tr>
<td></td>
<td>\textit{Enterococcus faecalis}</td>
</tr>
<tr>
<td></td>
<td>\textit{Corynebacterium} spp.</td>
</tr>
<tr>
<td></td>
<td>\textit{Neisseria} spp.</td>
</tr>
<tr>
<td>Gram Negative</td>
<td>\textit{P. aeruginosa}</td>
</tr>
<tr>
<td></td>
<td>\textit{Escherichia coli}</td>
</tr>
<tr>
<td></td>
<td>\textit{Klebsiella} spp.</td>
</tr>
<tr>
<td></td>
<td>\textit{Enterobacter cloacae}</td>
</tr>
<tr>
<td></td>
<td>\textit{Serratia marcescens}</td>
</tr>
<tr>
<td></td>
<td>\textit{Proteus mirabilis}</td>
</tr>
<tr>
<td></td>
<td>\textit{Acinetobacter calcoaceticus}</td>
</tr>
<tr>
<td></td>
<td>\textit{Bacteroides} spp.</td>
</tr>
<tr>
<td></td>
<td>\textit{Citrobacter fruendi}</td>
</tr>
<tr>
<td>Fungi</td>
<td>\textit{Candida} spp.</td>
</tr>
<tr>
<td></td>
<td>\textit{Aspergillus} spp.</td>
</tr>
<tr>
<td></td>
<td>\textit{Fusarium} spp.</td>
</tr>
<tr>
<td></td>
<td>\textit{Alternaria} spp.</td>
</tr>
<tr>
<td></td>
<td>\textit{Rhizopus} spp.</td>
</tr>
<tr>
<td>Viruses</td>
<td>Herpes simplex virus</td>
</tr>
<tr>
<td></td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td></td>
<td>Varicella-zoster virus</td>
</tr>
</tbody>
</table>
3.3 Nanoparticles as New Drug Delivery Vehicles

As detailed in Section 3.2, there are numerous antibiotics available to treat pathogens commonly associated with burn wound infections. However, many of the potent antimicrobials mentioned are water-soluble, which limits their use when the area of infection is found within highly lipophilic regions of the body such as the skin and soft (fatty) tissue. The level of antibacterial activity of the drugs is greatly diminished for such applications due to the antimicrobials unable to penetrate into the tissue. While these drugs can be successful for the initial superficial infections observed upon injury, they cannot reach the deep fascia where bacteria typically colonize. Therefore, in order to diversify the applications of these potent water soluble antibiotics, new drug delivery vehicles must be explored. Previous work has made use of creams and ointments to deliver multiple water insoluble antibiotics to skin and soft tissue abrasions and even higher complexity burn wounds. These creams and ointments make use of numerous types of drug delivery vehicles, including lyposomal and colloidal drug conjugates, as well as emulsification processes and cross-linked polymer micelles.\[12-18\] While these processes have yielded many commercial products, newer technologies (i.e. nanotechnology) exist that may improve upon these drug carriers and make them not only more versatile in application, but have also been shown to increase antibacterial efficacy.\[21\]

Currently, nanoparticles have become one of the most heavily researched areas of drug delivery. Many improvements have been made in this area, especially in cancer therapy, targeted delivery of therapeutics compounds, and medical imaging,\[18, 22-26\] yet up to now little effort has been made towards applying nanoparticles to antimicrobial drug therapy.\[19-21, 27-35\] Antibiotic-coated gold and silver-based metallic nanoparticles,\[30, 32, 35\] antibiotic encapsulated polymeric nanoparticles and liposomes,\[28, 34\] and biodegradable nanospheres\[29, 35\] have all been explored, yet little commercial development has been seen thus far. Couvreur et al. developed a series of poly(alkyl cyanoacrylate) nanoparticles which are bioresorbable and are currently being used as surgical glues\[22\] and more recently as carriers of antibiotics (specifically ciprofloxacin).\[28, 29, 31\] This drug delivery system provide the antibiotics incorporated within the particles with the ability to overcome problems associated with oral administration, including stabilizing the drug in the gastrointestinal tract and providing adequate adsorption into the blood stream. Couvreur et al. has also shown that encapsulation of the ciprofloxacin monomer within the poly(cyanoacrylate) nanoparticle has heightened the \textit{in vitro} activity of this antibiotic against \textit{S. aureus}.

Some \textit{in vivo} studies have looked into the effects of various nanoparticles, including chitosan and metal-based nanoparticles, for burn wound treatment, and have found positive results that show increased wound healing and enhanced cosmetic results.\[27, 36\] Also, the commercially available Acticoat\textsuperscript{TM} and Silveron wound dressings have utilized nanocrystalline silver for topical application to burn wounds.\[6\] These dressings consist of two sheets of high density polyethylene for antibacterial mesh that is coated with the nanocrystalline silver, which affords moderate penetration of unexcised burn eschar, a
highly lipophilic and extremely difficult tissue to penetrate, and controlled release over a prolonged time frame.[6] Advances in nanotechnology have allowed these topical antimicrobials to provide burn treatments with deep penetration of antimicrobial agents and also extend activity of the antimicrobials. This has not only had benefits in excavating infections below burn eschar prior to surgical debridement procedures, but has also decreased the occurrence of nosocomial infections due to a decrease in the number of dressing changes. Therefore, the work explored herein was established in order to build upon the current advances and proven positive effects of nanotechnology coupled with topical antimicrobial treatments for burn wound infections and create a new and unique multi-drug containing nanoparticle delivery system for administration to burn wounds immediately after thermal injury.

A previously described microemulsion polymerization process was chosen in order to synthesize polyacrylate nanoparticles in a single step (Figure 3.6). Due to the highly lipophilic nature and small size of the polyacrylate nanoparticles produced by emulsion polymerization, the hope is that utilization of these nanoparticles as a drug delivery system will allow the drug conjugate to penetrate deep into a wound bed and be able to treat all of the infected tissue present, which most topical antimicrobials (even when formulated in the currently used creams and ointments) are unable to achieve.

**Figure 3.6.** Nanoparticle containing emulsion, CNP5. This image depicts a freshly prepared drug-free nanoparticle emulsion (20% solid content) in a 25mL glass vial.

### 3.4 Emulsion Polymerization

Emulsion polymerization has been utilized for many years for industrial development of latex materials, adhesives, paints, paper and textile coatings.[37] The discovery of microemulsion polymerization in 1943 by Hoar and Schulman allowed this technology to expand towards drug delivery applications due to the formation of smaller, more stable droplets suspended within the emulsion.[38, 39] The emulsion polymerization technique
offers many advantages over other polymerization techniques such as reversible addition–fragmentation chain transfer (RAFT) and block polymer formation, including the use of water as the solvent system, formation of high molecular weight polymers with the ability to limit the molecular weight range to a narrow distribution, and a resulting low viscosity emulsion similar to water. Both the macroemulsion and the microemulsion polymerizations utilize surfactants to combine an aqueous phase (water) with water insoluble liquid acrylate monomer(s) to produce the oil-in-water emulsion and also to stabilize the polymeric particles that form from coagulating in the solution. Radical initiation is the means for polymerization of the acrylate monomer(s) after the initial micelle formation through the use of heat to generate the initial radical. For the polymerization to be successful, free oxygen present in the emulsified solution must be purged prior to addition of the radical initiator, since oxygen is a well known radical scavenger that will inhibit polymerization.

In emulsion polymerizations, the choice of surfactant and radical initiator inevitably dictates many of the physical properties of the resulting emulsion, including particle size and morphology, surface charge, emulsion stability, and molecular weight of the polymer chains. An anionic surfactant, dodecyl sulfate sodium salt (SDS), was chosen for these specific emulsion polymerizations which established an overall negative charge for the emulsion and subsequently, a low pH of 2 to 2.5. In order for the size distribution and particle morphology to be uniform, polymerizations were carried out at or below the critical micelle concentration, which is usually achieved by the addition of 1-6% (w/w) surfactant to monomer in the emulsion formulation. All of the drug-free emulsions synthesized here contained 1, 3, or 5% SDS in the formulation, and either 0.5 or 1% radical initiator (Figure 3.7).

![Sodium Dodecyl Sulfate and Potassium Persulfate](image)

**Figure 3.7.** Chemical structure of the surfactant, sodium dodecyl sulfate, and the radical initiator, potassium persulfate, used in the emulsion polymerization.

In the instance of microemulsions, the micelles are subsequently transformed into solid polyacrylate particles in the nanometer size range through radical-initiated polymerization of the acrylate monomers within the micelles. The resulting emulsion is typically optically transparent, isotropic, and thermodynamically stable, and contains uniformly spherical particles of a narrow size distribution. Figure 3.8 is a representative schematic of the microemulsion polymerization process, showing the emulsification stage of micelle formation.
Figure 3.8. Schematic of initial micelle formation during an emulsion polymerization.

3.4.2 Synthesis and Chemical Analysis of Drug-Free Nanoparticle Emulsions

In order to create the most optimal drug delivery system, numerous combinations of co-monomers, surfactant, and radical initiator were used in the emulsion formulation. Four different acrylate monomers were investigated in various combinations: butyl acrylate (BA), styrene (Sty), methyl methacrylate (MMA), and ethyl acrylate (EA). The surfactant used in the emulsion polymerizations was dodecyl sulfate sodium salt (SDS) and the radical initiator was potassium persulfate. However, the concentration of these two reactants used in each polymerization was varied from 0.5% to 5% of the total solid content.

Scheme 3.1. Microemulsion polymerization of butyl acrylate and styrene co-monomers. These two co-monomers were used in the drug-free emulsion formulations CNP5, CNP7, CNP9, CNP10, CNP15, and CNP16.

Six different (drug-free) control nanoparticle (CNP) emulsion were formulated using BA and Sty as the liquid acrylate monomers. Two different ratios of these monomers were used for the emulsions, an 8:2 ratio of BA:Sty (CNP7, CNP10), and a 7:3 ratio (CNP5, CNP9, CNP15, CNP16). These emulsions are further distinguished from one another by the content of surfactant and radical initiator in their formulations. CNP5 and CNP7 contained 3% SDS and 0.5% radical initiator, while CNP9 and CNP10 contained 5% SDS and 1% radical initiator in the formulation. Both CNP15 and CNP16 nanoparticle
emulsions contained only 1% SDS in their formulations, however, CNP15 contained 1% radical initiator and CNP16 contained only 0.5% radical initiator.

Scheme 3.2. Microemulsion polymerization of butyl acrylate and methyl methacrylate co-monomers. These two co-monomers were used in the drug-free emulsion formulations CNP6, CNP12, CNP13, CNP14, and CNP17.

Five different (drug-free) control nanoparticle emulsions were formulated using BA and MMA as the liquid acrylate monomers. Two different ratios of these monomers were used for the emulsions, an 8:2 ratio of BA:MMA (CNP6, CNP14), and a 7:3 ratio (CNP12, CNP13, CNP17). These emulsions are further distinguished from one another by the content of surfactant and radical initiator in their formulations. CNP12 and CNP13 contained 3% SDS and 0.5% radical initiator, while CNP6 and CNP14 contained 5% SDS and 1% radical initiator in the formulation. The nanoparticle emulsion CNP17 contained only 1% SDS in its formulation and 0.5% radical initiator.

The drug-free nanoparticle emulsions (CNP5s) were analyzed by analytical and chemical means in order to characterize their physical, chemical, and mechanical properties. Dynamic light scattering and imaging techniques were used to elucidate the physical properties of the emulsions, nuclear magnetic resonance (NMR) spectroscopy and Fourier transformed infrared (FTIR) spectroscopy and zeta potential analysis was used for chemical analysis of the emulsions, and uniaxial tension analysis was performed to determine the mechanical properties of the polymer films. NMR analysis was performed on selected emulsions by pipetting between 1 and 1.5mL of concentrated (20% solid content) nanoparticle emulsion onto a solid surface (glass or Teflon) and allowing the solution to form a film through coagulation of the emulsion. The film was subsequently placed in deuterated chloroform (CDCl3) in a thin glass NMR tube for NMR analysis. Even though the polymer films were unable to completely dissolve, the films did swell into a translucent gel-like material that was analyzed by proton NMR. Figure 3.9 provides a proton NMR spectrum obtained for the drug-free polyacrylate film sample CNP5.
3.4.3 Stability of the Nanoparticle Emulsions

Studies were performed on the nanoparticle emulsions in order to determine their stability to changes in temperature (room temperature, physiological temperature, and refrigeration storage temperature), changes in pH (from 1-12), aging, and stability to blood serum. Results of these studies were recorded as visual observations by particle size analysis of the emulsions by dynamic light scattering (DLS) analysis and by antibacterial activity (Figures 3.10, 3.11, and 3.12). The BA:Sty emulsions and the BA:MMA emulsions were visibly stable at pH 1 to pH 12, where the original concentrated nanoparticle emulsions (20% solid content) exhibited pHs between 2 and 3 (Figure 3.10A). Stability of the emulsions over a wide pH range suggests that the emulsions could tolerate post-polymerization chemical modification in order to add additional functionality to the nanoparticle surface for biomedical applications. This was not investigated further in this particular study.
Figure 3.10. Stability of nanoparticle emulsion CNP5 at various temperatures and pH changes. Data represents the average particle size for the emulsion at various pH values (A) and for each solid content concentration at different temperatures for 3 days (B). In each case, particle size was determined by dynamic light scattering.

As seen in Figure 3.10B, these nanoparticle emulsions are stable at low temperatures consistent with refrigeration storage (5°C), as well as at physiological temperatures
The emulsions were also stable over extended periods of time, where in Figure 3.11 it was observed that the average particle size for all solid content concentrations analyzed (1.5% to 10%) was the same when freshly prepared as it was after being stored at room temperature for one month.

![Figure 3.11. Stability of nanoparticle emulsion CNP16 upon aging for 30 days at room temperature. Data represents the average particle size for each solid content concentration. Samples were stored at room temperature while aging.](image)

The nanoparticle emulsion CNP16, containing a 7:3 ratio of BA:Sty, 1% SDS and 0.5% radical initiator, was stable over a minimum of one month. Moreover, visual observations established that all of the emulsions containing 3% SDS or less showed no polymer precipitation or surfactant settling for up to 2 years at room temperature. Figures 3.10 and 3.11 show that the nanoparticle size does not change upon dilution (from 20% to 1.25% solid content concentration). It was expected that the particle size should not be influenced by addition of more water to the emulsion, since the surfactant SDS should be closely associated to the nanoparticles and feel no dilution effect.

The stability of the nanoparticle emulsion systems towards blood serum was analyzed using the penicillin-containing nanoparticle emulsions NP13 and NP15 (described in Section 3.5). NP13 contained encapsulated penicillin G in the free acid form, and NP15 was formulated with penicillin G acrylamide as one of the co-monomers for the emulsion polymerization. Both emulsions have shown in vitro activity against S. aureus and MRSA. Here, the emulsions were diluted to the appropriate concentration (5.12mg drug/mL of emulsion) for minimal inhibitory concentration (MIC) serial dilution assays using
fetal bovine serum (FBS) in place of aqueous buffer. The emulsions were incubated with
the serum for 24 hours at room temperature prior to antibacterial analysis. Table 3.2
provides the MIC values for the emulsions against *S. aureus* and MRSA before and after
exposure to the blood serum.

**Table 3.2.** MIC data for penicillin-containing emulsions NP13 and NP15 before and after
24 hour exposure to fetal bovine serum. MIC values are reported in µg of drug per mL of

<table>
<thead>
<tr>
<th>Penicillin Samples</th>
<th><em>S. aureus</em></th>
<th>MRSA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Untreated</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penicillin G</td>
<td>0.012</td>
<td>16</td>
</tr>
<tr>
<td>NP13</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>NP15</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td><strong>Treated</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NP13</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>NP15</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

The data in Table 3.2 show that the antibacterial activity of the penicillin G acrylamide-
containing nanoparticle emulsion (NP13) does not change upon exposure to the various
proteins, enzymes, and salts present in blood serum. This indicates that it is highly
probable that this emulsion formulation will be stable upon intravenous administration.
Visual observations for both emulsions diluted with FBS concluded that sedimentation
did not occur after 24 hours of exposure, nor did the appearance of either emulsion change after initial dilution, which caused the emulsion to take on the brownish color of
the FBS. However, the MIC value for the penicillin G free acid encapsulated nanoparticle
emulsion NP15 drastically changed upon exposure to FBS, from 64 to 8µg/mL. The
dramatic increase in antibacterial potency for this emulsion could be the result of particle
degradation by biological molecules in the blood serum. When in the free polymer form,
the surfactant is not bound to the surface of the particles, which could then be allowing
the surfactant to exhibit toxicity against the microbes. A secondary explanation is that the
enzymes present in the blood serum promote the release of the drug from within the
nanoparticle more efficiently than when the nanoparticles are only exposed to the
microbial esterase and lipase enzymes. However, for a definitive answer, other studies
would need to be performed. Regardless of the variance in antibacterial activity for the
encapsulated penicillin nanoparticles, the covalently bound penicillin G acrylamide
nanoparticle emulsion was determined to be stable towards the blood serum, and thus was
used in the *in vivo* toxicology study defined in Chapter 8.
3.5 Drug-Containing Nanoparticle Formation by Emulsion Polymerization

Polymeric drug delivery was established almost 30 years ago and is still a heavily researched area in drug design and delivery (Gombotz and Pettie, 1995; Sinha and Khosla, 1998; Langer, 1998). Polyacrylates have been a large part of this effort, due to their lack of toxicity and optimal solubilizing properties. Polyacrylates have been used in medicine in various forms, including polymer film coatings, gels, and in liquid emulsions. Polyacrylate emulsions, and also lipid and other polymer-based emulsions and colloidal drug carriers, have been investigated for many years to deliver insoluble drug monomers to target sites and are frequently administered intravenously or topically due to their high stability, ability to stabilize drug monomers for delivery, and decrease irritation and pain at the application site. The emulsions have shown numerous benefits when used for drug delivery, including delivering the dissolved drug solely to its intended destination in the body, providing the drug with controlled release, and increasing the uptake and efficiency of the drug. Microemulsion polymerization was explored in this study as a means for encapsulation of many water insoluble antimicrobials, including the broad-spectrum antimicrobials amphotericin B, erythromycin, and doxycycline, and also for covalent attachment of acrylated drug monomers to the nanoparticle delivery vehicle. The application of these antimicrobials in the nanoparticle emulsion was to improve upon the already established benefits of emulsified drug delivery with the added benefit of the nano-size of the delivery vehicle as a means to increase the overall efficiency and possibly provide the drug with new medicinal applications.

Since incorporation of the drug monomers into the nanoparticle is essential for antimicrobial activity, the co-monomers chosen must fully dissolve the acrylated drug monomer prior to emulsification with water and surfactant. For most drug-containing nanoparticle emulsions synthesized in this current study, BA and Sty were the co-monomers of choice (Scheme 3.1). These two co-monomers were the best choice for such polymerizations when used in a 7:3 ratio of BA:Sty because of the stability and particle size of the resulting nanoparticle emulsion and also because this combination of co-monomers dissolved the acrylated drug monomers being explored. Also, the initial amount of surfactant used for these systems was 3% (w/w) because of the resulting emulsion stability and particle size determined from the drug-free nanoparticle emulsion systems. However, this amount of surfactant was later lowered to 1% (w/w) due to observed cytotoxic effects of the 3% SDS emulsions on human dermal fibroblast cells (Chapter 7). The amount of radical initiator utilized for this system was 0.5% (w/w).

3.5.2 Covalently Bound Drug-Conjugated Nanoparticle Formation by Emulsion Polymerization

For a drug monomer to be incorporated covalently into the nanoparticle, it must contain a chemical moiety similar to the ones present in the liquid acrylate co-monomers.
Therefore, all of the drug candidates were converted to an appropriate acrylate or acrylamide form. Schemes 3.3 through 3.9 depict the emulsion polymerization for each drug acrylate monomer. These emulsions were subsequently tested for antibacterial activity *in vitro*, along with their desiccated/coagulated polymer film.

**Scheme 3.3.** Emulsion polymerization of β-lactam monomer 1.

Proton NMR analysis was performed by J.-Y. Shim in his dissertation research for NP1a and compared the spectra obtained to that from lactam monomer 1 as well as poly(ethyl acrylate) in order to establish the degree of lactam incorporation into the emulsion. Integration of the peaks of the NMR spectrum from NP1a in Figure 3.12 showed that the 1:7 ratio of lactam 1 to EA was found in the solid content of the emulsion (polymer film), indicating that all of the lactam monomer is covalently incorporated into the nanoparticle polymer backbone. Figure 3.12 also shows the progression of lactam incorporation into the nanoparticle emulsion as the amount of lactam is increased in the emulsion formulation of NP1a from 20:1 EA: lactam up to 2.5:1 EA:lactam.
Proton NMR analysis showed signals at 2.6ppm, 5.6ppm and 6.1ppm that were assigned to SCH$_3$, C$_3$-H and C$_4$-H on the β-lactam respectively. The olefin protons of ethyl acrylate and acrylated lactam monomer in the range of 5.6-6.1ppm were not visible in the spectra indicating that all of the monomeric lactam acrylate and ethyl acrylate was converted into polymeric particles. The aromatic protons from the ortho-chlorophenyl C$_4$ substituent on the lactam ring was observed at 7.3ppm and the intensity of this signal increased as the amount of lactam monomer 1 in the emulsion formulation increased. While all of these respective signals for the acrylate monomers were observed, minus the acrylate proton signals, the peaks presented by the polymer films were much broader than the non-polymerized monomers individually, which is commonly associated with polymerized samples.

**Figure 3.12.** NMR spectra of NP1a and poly(ethyl acrylate). NMR analysis showed that the lactam monomer was incorporated into the nanoparticle emulsion, and that as the amount of lactam monomer is increased in the emulsion formulation, signals from the monomer become more apparent by NMR. NMR analysis performed by J.-Y. Shim.$^{[20,40]}$
Table 3.3. Formulation for emulsion polymerization NP1a.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Mass</th>
<th>Percent (%)</th>
<th>Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactam monomer 1</td>
<td>80 mg</td>
<td>2</td>
<td>NA</td>
</tr>
<tr>
<td>Ethyl acrylate</td>
<td>0.28g</td>
<td>15.5</td>
<td>0.3</td>
</tr>
<tr>
<td>Water</td>
<td>3.6g</td>
<td>80</td>
<td>3.6</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate</td>
<td>7.2mg</td>
<td>2</td>
<td>NA</td>
</tr>
<tr>
<td>Potassium persulfate</td>
<td>1.8mg</td>
<td>0.5</td>
<td>NA</td>
</tr>
</tbody>
</table>

The formulation for NP1a is defined in Table 3.3. NP1a was the first emulsion polymerization formulated in this study and are the same as those previously established by Dr. Jeung-Yeop Shim, where the acrylated β-lactam monomer 1 was co-polymerized with ethyl acrylate. It was later determined that the physical and chemical properties of these emulsions were not optimal in terms of particle size and emulsion stability, antibacterial activity and biomedical application, thus the formulation was changed to the one used for NP1 (Scheme 3.4, Table 3.4). The obvious difference between the two emulsion formulations is the liquid acrylate monomers used for the new emulsion formulation were butyl acrylate and styrene in a 7:3 ratio.

Scheme 3.4. Improved emulsion polymerization of β-lactam acrylate monomer 1.
Table 3.4. Formulation for nanoparticle emulsion NP1 and all other drug-containing emulsions.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Mass</th>
<th>Percent (%)</th>
<th>Volume (mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactam monomer 1</td>
<td>60mg</td>
<td>3</td>
<td>NA</td>
</tr>
<tr>
<td>Butyl acrylate</td>
<td>1.4g</td>
<td>13.5 or 15.5</td>
<td>1.57</td>
</tr>
<tr>
<td>Styrene</td>
<td>0.6g</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>Nanopure Water</td>
<td>8.0g</td>
<td>80</td>
<td>8.0</td>
</tr>
<tr>
<td>Dodecyl sulfate sodium salt</td>
<td>60mg</td>
<td>3 or 1</td>
<td>NA</td>
</tr>
<tr>
<td>Potassium persulfate</td>
<td>10mg</td>
<td>0.5</td>
<td>NA</td>
</tr>
</tbody>
</table>

The emulsion formulation represented in Table 3.4 was employed for every other drug-containing emulsion in this entire study (NP2-NP25), however, the amount of surfactant was varied during the study with the initial surfactant concentration being 3% SDS and later the concentration was reduced to 1%. A radical initiator concentration of 0.5% of the solid content was also held constant throughout the entire study for all drug-containing nanoparticle emulsions. The amounts of BA, Sty and radical initiator were held constant at a 7:3 ratio and 0.5%, respectively, as seen in Table 3.4. Initial toxicity analysis performed on the 3% SDS formulated emulsions resulted in slightly elevated levels of fibroblast cytotoxicity (Chapter 7), which lead to the development of the 1% SDS formulated emulsions. Herein, all of the physical, chemical and antibacterial data for this drug-containing lactam monomer 1 is presented for the emulsion containing 1% SDS.
Both of the ortho-chlorophenyl acrylate β-lactam monomers synthesized in Chapter 2 possess the same basic lactam structure and side chains at the C₃ and C₄ position of the ring, yet they differ in the alkyl chain attached to the N-thiolated portion of the molecule which is responsible for antibacterial activity. The difference in the alkylthio chain length greatly influences antimicrobial activity, with these two derivatives showing, as well as did changes to the C₃ and C₄ substituents (Chapter 2). Also polymerized individually were lactam monomers 5 and 9 where the acrylate linker for both monomers was much longer than for β-lactam monomers 1 and 3. Monomer 5 contained the acrylate linkage at the C₄ position on the lactam ring, as a para-substituent on the aromatic ring. Monomer 9 possessed a longer acrylate linkage at the original C₃ position on the lactam ring. Both of these lactam monomers were polymerized using the same formulation as described in Table 3.4 to provide nanoparticle emulsions NP5 and NP9.

The acrylated form of several commercially available drugs (11, 12, 16, 17, and 19) were also polymerized in order to define the antibacterial properties of the drugs when incorporated in the nanoparticle delivery system. This was done to examine if incorporation of various β-lactams in the nanoparticle could afford them with activity against resistant microbes including MRSA. In order to analyze this, the acrylated form of five different β-lactam drug monomers, ampicillin, amoxicillin, penicillanic acid, cefaclor, and penicillin G were all individually polymerized with BA and Sty. All of the drug-conjugated emulsions were formulated with 3% SDS (Scheme 3.6), except for the penicillin G emulsions that contained only 1% SDS (Scheme 3.8).
Scheme 3.6. Polymerization of penicillin-based acrylate monomers 11, 12, 16, 17 and 19 separately to afford NP11, NP12, NP16, NP17, and NP19.

\[ \text{Scheme 3.6. Polymerization of penicillin-based acrylate monomers 11, 12, 16, 17 and 19 separately to afford NP11, NP12, NP16, NP17, and NP19.} \]

*Staphylococcal* resistance has been observed for all of the commercially available drugs used to make the monomers chosen for polymerization; therefore, these antibiotics were optimal choices for testing the ability of the nanoparticle to shield the incorporated drug from \( \beta \)-lactamase produced by the microbe. All of these monomers were individually polymerized using 3% SDS surfactant and 0.5% radical initiator in the emulsion formulation, as well as a 7:3 ratio of BA:Sty.

As seen in Chapter 2, the commercially available penicillin-based antibiotic amoxicillin was chemically modified to possess two acrylate moieties; therefore, both the monacrylate and di-acrylate forms of this drug were individually polymerized to assess any differences in antibacterial activity should the nanoparticles have more highly cross-linked matrices (Scheme 3.7).
Scheme 3.7. Polymerization of amoxicillin diacrylate monomer 18 with BA and Sty.

As seen in Scheme 3.7, having the di-acrylate moiety present in the drug monomer provides cross-links between polymer strands. The heightened degree of cross-links over the already present cross-links within the nanoparticles due to impurities within the liquid acrylate monomers could indicate that the bacteria may have a harder time breaking down the acrylate bonds with lipase and esterase enzymes, thereby making it more difficult for the free drug to be released from the delivery system. The increase in amount of cross-linking present could also cause the nanoparticles formed to be smaller in diameter because they are being held together more tightly than when a mono-acrylated drug monomer is incorporated. The antibacterial data may answer how the di-acrylate moiety will affect the antibacterial activity of the drug-conjugated system.
Scheme 3.8. Polymerization of penicillin G acrylamide monomer (13) with butyl acrylate and styrene to give nanoparticle emulsion NP13.

Penicillin G acrylamide monomer was likewise co-polymerized with the BA and Sty liquid acrylate monomers using only 1% SDS in the emulsion formulation.

Scheme 3.9. Polymerization of acrylamide ciprofloxacin monomers 7 and 8 with butyl acrylate and styrene (7:3) to give nanoparticle emulsions NP7 and NP8, respectively.

Both ciprofloxacin acrylamide monomers 7 and 8 were polymerized using two different formulations. These monomers were first polymerized using 3% SDS, then subsequently polymerized with a decreased amount of 1% SDS. In both formulations, the amount of BA, Sty, and radical initiator were held constant at a 7:3 ratio and 0.5%, respectively. Initial toxicity analysis performed on the 3% SDS formulated emulsions resulted in slightly elevated levels of fibroblast cytotoxicity (Chapter 7), which led to the development of the 1% SDS formulated emulsions. Herein, all of the physical, chemical,
and antibacterial data is presented for the emulsions containing 1% SDS for these two drug monomers.

3.5.3 Drug-Encapsulated Nanoparticles by Emulsion Polymerization

There are many commercially-available antimicrobials that are water insoluble or highly lipophilic including the widely used erythromycin and the potent antifungal amphotericin B. While these antimicrobials have been effective in treating infections topically, they have seen little use as prophylaxis treatments due to their lack of solubility in aqueous systems. Therefore, in an attempt to diversify the use of these antimicrobials, experiments were conducted to try to incorporate them into the polyacrylate nanoparticles as an aqueous emulsion in an attempt to make these potent drugs accessible to blood borne infections and other infections where only water-solubilized drugs can reach. Due to the highly lipophilic nature of many of these drugs, no chemical modifications were necessary prior to emulsion polymerization. Other commercially available antibiotics required modifications due to manufacturing processes that have made them more water soluble (i.e. penicillin G is sold as a potassium salt). Four $N$-thiolated $\beta$-lactam monomers were chosen for nanoparticle encapsulation, along with two different variations of penicillin G, and a few other commercially available antibiotics that are often associated with treatment of burn wound infections or possess drug resistance (Figure 3.13 and 3.14).

![Chemical structures](image)

**Figure 3.13.** $N$-Thiolated $\beta$-lactam monomers encapsulated in the nanoparticle through emulsion polymerization.

Of all of the $N$-thiolated $\beta$-lactam monomers synthesized in this study, including the acrylate monomers intended for covalent attachment to the nanoparticle polymer backbone, lactam monomers NP6 and NP10 displayed the most potent antimicrobial activity against all of the microbes analyzed (Section 3.8), thus were chosen for encapsulation in the nanoparticle delivery system. Lactam monomer 4 was also chosen for encapsulation as a comparison of antibacterial activity to the covalently bound lactam monomers 1. Should bacterial esterases or lipases cleaves the ester bond between the nanoparticle matrix and the drug monomer in NP1, the active drug monomer released is expected to be hydroxy lactam 4.
Figure 3.14. Penicillin G monomers encapsulated in the nanoparticle through emulsion polymerization.

Of all the commercially available antimicrobials, a select few were chosen due to their known potency against the most common pathogens found in burn wounds, or their possession of drug resistance. This property of the drug monomers was considered because, as was performed for the covalently bound drug monomers, this study looked to establish whether or not the nanoparticle delivery system could circumvent the already formed bacterial resistance and protecting the antibiotic from resistance factors (i.e. β-lactamases). Both of the water insoluble penicillin G monomers used in the polymerizations of NP14 and NP15 represent structures similar to the covalently bound penicillin G acrylated monomers after bacterial cleavage of the monomers from the polymer backbone. Therefore, these two nanoparticle emulsions were polymerized in order to determine whether or not covalent attachment of the penicillin analogue to the polymer backbone is required for overcoming bacterial resistance, or if simple encapsulation of the monomer within the nanoparticle delivery system can accomplish this task.
Figure 3.14. Commercially available drug monomers encapsulated in the nanoparticle through emulsion polymerization.

The drug monomers presented in Figure 3.14 represent antibiotics commonly used to treat burn wound infections due to their broad spectrum of, antifungal properties, and water insolubility. All emulsions of encapsulated drug monomers were formulated with 7:3 ratio of BA:Sty, 3% SDS, and 0.5% radical initiator. Antibacterial activity and particle size and stability data were obtained for these nanoparticle emulsions as well as later cytotoxicity analysis using healthy human dermal fibroblast cells (Chapter 7).

3.6 Particle Size Analysis for Nanoparticle Emulsions

Particle size data was obtained for all of the drug-free nanoparticle emulsions synthesized using a Honeywell Microtrac UPA, assuming a refractive index of 1.51 for the emulsions and uniform particle size and morphology throughout the emulsion, as was observed by TEM and SEM analysis. Data for each emulsion was obtained by averaging data from 3 runs of 180 seconds per run per sample. The lengthy time requirement for each run correlates to the length of time needed for the particles to travel a sufficient amount in the solution during analysis to obtain a series of data points for comparison and accurate
calculation of the particle size. Analysis of a select few drug-conjugated nanoparticle emulsions was also performed by Torrey DeLuca at Malvern Instruments using a Malvern Zetasizer Nano ZS instrument for both particle size and zeta potential. This additional testing on a secondary instrument was performed as a quality control and unequivocally proved the accuracy of the Honeywell data.

### 3.6.1 Theory Behind Dynamic Light Scattering

Dynamic light scattering (DLS) has been used for analysis of particle size and distribution for emulsions and polymerizations for many years and utilizes the principle of light diffraction for measurement of particles in a solution. When particles are suspended in a solution, they undergo random fluctuations through Brownian motion defined by random collisions of the particles with one another, which inevitably causes random migration patterns of the particles within the solution. Since the particle size is inversely proportional to its migration (smaller particles migrate farther than large particles), the average size of particles in a solution can be calculated, as seen in Equation 3.1. The diffusion coefficient (D) of the solution is used to correlate the particle size to the migration aptitude of the particle, which is what the DLS instrument is capable of measuring from light scattered from particles migrating through the solution under the influence of Brownian motion.

**Equation 3.1.**

\[
R_H = \frac{k_B T}{6 \pi \eta D}
\]

- $R_H$ = Hydro-dynamic radius
- $D$ = Diffusion Constant
- $k_B$ = Boltzmann’s constant ($1.38 \times 10^{-23}$ JK$^{-1}$)
- $T$ = Temperature in Kelvin (K)
- $\eta$ = Viscosity

DLS instruments used for this study were able to calculate the average particle size present in a solution and was given as the mean volume ($m_v$), mean area ($m_a$), and mean number ($m_n$) for the solution. Two different light diffractions were taken into account by the instrument for each sample analyzed. Rayleigh scattering, where light is diffracted uniformly after passing through a solution of particles smaller than the light beam and Mie scattering, where larger particles (greater than 250nm diameter) cause an intense beam diffracted by the particles at independent angles.\cite{20} Due to the limited ability of these instruments to take into account more than one particle size range and morphologies, a solution must contain particles of a uniform size and morphology. If
various sized particles are present in the solution, then the average particle size obtained from this type of instrument will not be accurate, which is why imaging analysis must also be performed in conjunction with this technique to verify the constituents present in the solution.

3.6.2 Analysis of Drug-Free Nanoparticle Emulsions

For the drug-free polyacrylate emulsions presented in this chapter, the particle sizes are within a narrow size range. However, at high concentrations of solid content, the emulsions are viscous since they contain both the spherical nanoparticles and long chain polyacrylates that are not incorporated into the particles. This makes particle size analysis at high concentrations of solid content very difficult, which is why the emulsions were diluted prior to analysis. Dilution of the emulsion to approximately 1% solid content (one drop of concentrated emulsion into 10mL water) allowed accurate analysis of the particle size range. At this concentration, the data obtained from this instrument showed a narrow range of particle sizes present in the emulsions (Figure 3.16, Table 3.5).

Table 3.5. Average particle size for the drug-free nanoparticle emulsions, determined by dynamic light scattering.

<table>
<thead>
<tr>
<th>Nanoparticle Emulsion</th>
<th>Mean number ( (m_n) )</th>
<th>Mean average ( (m_a) )</th>
<th>Mean volume ( (m_v) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNP5</td>
<td>42.0</td>
<td>42.45</td>
<td>46.8</td>
</tr>
<tr>
<td>CNP6</td>
<td>65.6</td>
<td>72.2</td>
<td>76.9</td>
</tr>
<tr>
<td>CNP7</td>
<td>29.3</td>
<td>33.6</td>
<td>35.9</td>
</tr>
<tr>
<td>CNP8</td>
<td>48.1</td>
<td>52.7</td>
<td>55.1</td>
</tr>
<tr>
<td>CNP9</td>
<td>49.8</td>
<td>52.5</td>
<td>53.8</td>
</tr>
<tr>
<td>CNP10</td>
<td>45.1</td>
<td>49.1</td>
<td>51.3</td>
</tr>
<tr>
<td>CNP11</td>
<td>42.6</td>
<td>45.5</td>
<td>47.0</td>
</tr>
<tr>
<td>CNP12</td>
<td>64.0</td>
<td>67.1</td>
<td>68.7</td>
</tr>
<tr>
<td>CNP13</td>
<td>50.4</td>
<td>59.7</td>
<td>61.4</td>
</tr>
<tr>
<td>CNP14</td>
<td>50.2</td>
<td>59.6</td>
<td>62.3</td>
</tr>
<tr>
<td>CNP16</td>
<td>78.1</td>
<td>86.3</td>
<td>91.5</td>
</tr>
<tr>
<td>CNP17</td>
<td>75.6</td>
<td>84.4</td>
<td>89.2</td>
</tr>
</tbody>
</table>
Figure 3.16. Particle size analysis for drug-free nanoparticle emulsion CNP5. Data presented is for the $m_v$ calculation for the emulsion, where the average particle size reported here is 46.8nm.

The data for the nine drug-free nanoparticle emulsions is reported in Table 3.5, where traditionally, only the mean volume particle size diameter is reported in the literature. This data showed that the ratio of the co-monomers (whether it was BA:Sty or BA:MMA) affected the particle size distribution, as did the concentration of surfactant. Figure 3.16 distinguishes the difference in particle size between the 3% surfactant emulsions (CNP5 and CNP7) and the 5% SDS emulsions (CNP9 and CNP10). Emulsions having 3% SDS contained particles averaging between 30 and 40nm, whereas emulsions formulated with 5% SDS contained particles around 50nm in diameter. The data in this figure also determined that as the amount of styrene was increased, the average particle sizes increased. Comparison between the 7:3 ratio of co-monomers (CNP5 and CNP9), with styrene being the lower constituent, and the 8:2 ratio of co-monomers, showed a slight decrease in average particle size (approximately 5nm) as the amount of styrene was reduced. In fact, even though CNP8 was formulated with 3%
surfactant, because of the ratio of co-monomers (5:5 BA:Sty), the average particle size was on par with emulsions formulated with 5% SDS (approximately 52nm).

**Figure 3.17.** Effect of varying the ratios of butyl acrylate: styrene co-monomers and the amount of surfactant on particle size.

### 3.6.3 Particle Size Analysis for Drug-Conjugated Emulsions

Particle size analysis was performed using DLS methodology on many of the drug-containing nanoparticle emulsions synthesized. The average particle size for the covalently bound drug-containing nanoparticle emulsions (NP1a, NP7, NP8, and NP11) was a compilation of the data obtained for up to 3 separate batches of the emulsions, where the data represented for NP7 and NP8 in Table 3.6 is the average for both the 3% SDS and 1% SDS nanoparticle emulsions. Averaging of this data was possible since the particle size ranges were parallel for both the 3% and 1% SDS formulated emulsions, which then gave a more statistically significant particle size range for the emulsions. The encapsulated nanoparticle emulsions containing either \(\beta\)-lactam drug monomers (NP2 and NP6) or commercially available drug monomers (NP23, NP24, and NP25) were also analyzed for their average particle size, with the data presented in Table 3.6.
Table 3.6. Average particle size data for drug-containing nanoparticle emulsions as determined by dynamic light scattering.

<table>
<thead>
<tr>
<th>Drug-Containing Nanoparticle Emulsion</th>
<th>Mean number (mₙ)</th>
<th>Mean average (mₐ)</th>
<th>Mean volume (mᵥ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP₁a</td>
<td>87.5</td>
<td>123.9</td>
<td>147.2</td>
</tr>
<tr>
<td>NP2</td>
<td>49.3</td>
<td>53.7</td>
<td>56.3</td>
</tr>
<tr>
<td>NP6</td>
<td>47.7</td>
<td>53.5</td>
<td>56.4</td>
</tr>
<tr>
<td>NP7</td>
<td>55.6</td>
<td>61.4</td>
<td>64.4</td>
</tr>
<tr>
<td>NP8</td>
<td>51.7</td>
<td>57.3</td>
<td>60.5</td>
</tr>
<tr>
<td>NP₁₁</td>
<td>58.8</td>
<td>67.2</td>
<td>71.7</td>
</tr>
<tr>
<td>NP23</td>
<td>63.2</td>
<td>67.3</td>
<td>69.4</td>
</tr>
<tr>
<td>NP24</td>
<td>69.8</td>
<td>77.3</td>
<td>81.4</td>
</tr>
<tr>
<td>NP₂₅</td>
<td>101.9</td>
<td>140.9</td>
<td>157.3</td>
</tr>
</tbody>
</table>

Particle size analysis was performed on both covalently-bound and encapsulated drug-containing emulsions. Also analyzed was the drug-conjugated emulsion NP₁a that was formulated with EA as the only liquid acrylate monomer along with acrylate lactam 1. The average particle size (mₙ) for the EA:lactam emulsion was much higher than was observed for the same monomer co-polymerized with liquid BA and Sty (NP1). The other β-lactam containing nanoparticle emulsions also exhibited smaller particles on average than NP₁a as did the penicillin-containing emulsion NP₁₁.

The encapsulated β-lactam nanoparticle emulsions NP2 and NP6 were within the same particle size range as the covalently bound β-lactam nanoparticle emulsions (data not shown). However, when the commercially available drug monomers amphotericin B, erythromycin, and doxycycline were encapsulated within the nanoparticle emulsion (NP₂₅, NP₂₃, and NP₂₄, respectively) using the same 7:3 ratio of BA:Sty as the β-lactam containing nanoparticle emulsions, the average particle size dramatically increased from approximately 48nm to up to 102nm in diameter (Table 3.6). Encapsulated amphotericin B drug monomer created the largest nanoparticles, with the size ranging from 101.9nm for the mₙ value, to 157.3nm for the mᵥ value. The smaller of these commercially available encapsulated drug monomer nanoparticle emulsions was erythromycin, with doxycycline only slightly larger. The average particle size for both of these emulsions was only slightly higher than those observed for the commercially available drug monomers that are covalently bound to the nanoparticles (NP₁₁). This observed increase in particle size, even though the emulsion formulation remained exactly the same for all emulsions presented in Table 3.6 (aside from NP₁a), is most likely the result of the nanoparticle forming around the drug monomer, and therefore, the
size, shape, and bulkiness of the drug incorporated is having a large effect on the resulting particle size.

3.7 Zeta Potential Analysis for Nanoparticle Emulsions

Zeta potential analysis was performed for many of the nanoparticle emulsions synthesized in order to establish the stability of the emulsions and also the surface charge of the emulsions. Typically, a colloidal solution or one containing particles is considered stable when its zeta potential is greater than the absolute value of 30mV (30mV and above or -30mV and below). All of the emulsions that were analyzed were diluted to solid content concentrations of 2.5% for standardized testing, which was a requirement of the ZetaPlus Zeta Potential Analyzer manufactured by Brookhaven Instruments Corporation. At this solid content concentration, all of the emulsions analyzed exhibited numbers at or greater than -30mV.

3.7.1 Fundamental Aspects in Zeta Potential Measurements

Zeta potential is the measure of a solution’s overall charge and is most often determined for aqueous solutions or solutions of moderate electrolyte concentrations. This measurement is calculated from Henry’s equation represented in Equation 3.2, which converts a solution’s electrophoretic mobility into zeta potential by taking into account a solution’s dielectric constant (ε) and its viscosity (η).

Equation 3.2

\[
U_e = \frac{2\varepsilon z f(K_a)}{3 \eta}
\]

\[
\begin{align*}
z & = \text{zeta potential} \\
U_e & = \text{electrophoretic mobility} \\
\varepsilon & = \text{dielectric constant} \\
\eta & = \text{sample viscosity} \\
f(K_a) & = \text{Henry’s function}
\end{align*}
\]

The analysis of the nanoparticle-based emulsions was performed under the assumption that the emulsions act under the conditions of the Smoluchowski model. The instruments used for these zeta potential measurements utilize the described equation and other
external properties input into the software (such as dielectric constant, pH, and viscosity) in order to produce the average zeta potential for the emulsions.

3.7.2 Zeta Potential Analysis of Drug-Free Nanoparticle Emulsions

Zeta potential analysis was performed for all of the drug-free polyacrylate nanoparticle emulsions synthesized. Prior to testing, all of the emulsions were diluted to either 2.5% or 1.25% solid content in order to obtain the most accurate data that is comparable for all emulsions tested. However, many of the drug-free emulsions were also tested at other solid content concentrations in order to establish the occurrence of trends that may form as a result of diluting the emulsions, which subsequently alters the bulk pH of the emulsion.

![Graph showing zeta potential data for drug-free poly(butyl acrylate-styrene) nanoparticle emulsions at various solid content concentrations. Data was obtained as an average of two-10 cycle runs per sample. The dispersant viscosity of all emulsions analyzed was 0.8872cP, the material refractive index was 1.59, the dielectric constant for the emulsion medium was 78.55, and the zeta measurement was performed at 40V using the Smoluchowski model.](image)

The pHs of the nanoparticle emulsions were determined to be inversely proportional to its solid content concentration where the highest solid content concentration (20%) had a pH of approximately 2 and low solid content concentrations of 0.625% displayed pH in the...
range of 4 to 4.5. Although pH was taken into account for the calculations performed by the ZetaPlus instrument, there still existed a distinct difference in zeta potential for the emulsions at various solid content concentrations. However, in every instance analyzed, the emulsions were most stable (displayed the largest zeta potential) at a solid content concentration of 2.5%. This established the need to perform all zeta potential analysis at this specific concentration, since the manufacturer’s guide for this particular instrument suggests concentrations anywhere from 1% to 0.1%.

In Figure 3.18, the data suggests that the drug-free poly(butyl acrylate-styrene) emulsion CNP7, formulated with only 3% surfactant, was much more stable that the two other drug-free poly(butyl acrylate-styrene) emulsions CNP9 and CNP10, which both contained 5% surfactant. This data clearly shows a significant increase in emulsion stability in relation to a decrease in surfactant concentration. However, data obtained for the drug-free poly(butyl acrylate-styrene) emulsion CNP16 that contains only 1% surfactant was recorded as having an average zeta potential of -48.99mV +/- 1.29mV at 2.5% solid content. This data is similar to the data for the emulsion CNP9 that was formulated with 5% surfactant and the same 7:3 ratio of co-monomers. This indicates that the 3% surfactant in the emulsion formulation is the most optimal for stability purposes. This trend was also observed for the drug-conjugated nanoparticle emulsions. When ciprofloxacin acrylamide was covalently bound to the polymer backbone using 3% surfactant in the emulsion formulation, the zeta potential was much larger (-83.91mV +/- 1.00) then when only 1% surfactant (-31.79mV +/- 1.75) was employed. At 2.5% solid content only 0.75mg/mL of drug was present in the drug-containing emulsions.

Table 3.7. Zeta potential data for all of the drug-free nanoparticle emulsions analyzed. Data presented is an average of 2-10 cycle runs for each sample analyzed and the values obtained for each sample are in millivolts (mV).

<table>
<thead>
<tr>
<th>Nanoparticle Emulsion</th>
<th>Zeta Potential (mV)</th>
<th>Standard Deviation (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNP5</td>
<td>-34.29</td>
<td>0.22</td>
</tr>
<tr>
<td>CNP7</td>
<td>-90.20</td>
<td>6.89</td>
</tr>
<tr>
<td>CNP8</td>
<td>-84.45</td>
<td>11.74</td>
</tr>
<tr>
<td>CNP9</td>
<td>-55.82</td>
<td>21.32</td>
</tr>
<tr>
<td>CNP10</td>
<td>-73.81</td>
<td>5.44</td>
</tr>
<tr>
<td>CNP16</td>
<td>-48.99</td>
<td>1.29</td>
</tr>
<tr>
<td>CNP17</td>
<td>-39.71</td>
<td>2.27</td>
</tr>
</tbody>
</table>

The data in Table 3.7 shows a trend in zeta potential for the drug-free nanoparticle emulsions. The data showed that as the amount of styrene in the emulsion formulation was increased from 7:3 to 8:2, the zeta potential increases in negativity, following the
trend of CNP5 and CNP9 < CNP7 and CNP10. Also, it was observed that an increase in percent surfactant in the nanoparticle formulation from 3% to 5% SDS increased the zeta potential substantially as was expected (CNP5 to CNP7, and CNP9 to CNP10), yet when the amount of surfactant was decreased from 3% to 1% SDS (CNP5 to CNP16), the zeta potential did not decrease in negativity as might be expected, but increased slightly. Since data presented in Chapter 7 showed that at 5% SDS, there is a high percentage of surfactant that is not coordinated to the surface of the particles, and is therefore often observed sedimenting in the concentrated emulsion, the increase in negativity for the 5% SDS formulated emulsion (CNP9 and CNP10) could be the result of the excess surfactant, which is anionic in nature affecting the zeta potential and is therefore not a direct measure of the emulsion’s stability. However, the data here shows that the drug-free nanoparticle emulsions containing 1 or 3% SDS possess zeta potentials that indicative that they are highly stable.

Table 3.8. Comparison of the stability of drug-conjugated nanoparticle emulsions. Values were obtained for each emulsion after dilution to 2.5% solid content.

<table>
<thead>
<tr>
<th>Drug-Containing Nanoparticle Emulsion</th>
<th>Zeta Potential (mV)</th>
<th>Standard Deviation (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP1a</td>
<td>-41.93</td>
<td>1.55</td>
</tr>
<tr>
<td>NP2</td>
<td>-76.27</td>
<td>1.88</td>
</tr>
<tr>
<td>NP6</td>
<td>-82.52</td>
<td>2.73</td>
</tr>
<tr>
<td>NP7</td>
<td>-45.49</td>
<td>2.86</td>
</tr>
<tr>
<td>NP8</td>
<td>-60.96</td>
<td>1.77</td>
</tr>
<tr>
<td>NP23</td>
<td>-44.47</td>
<td>2.59</td>
</tr>
<tr>
<td>NP24</td>
<td>-50.11</td>
<td>2.04</td>
</tr>
<tr>
<td>NP25</td>
<td>-40.84</td>
<td>3.01</td>
</tr>
</tbody>
</table>

The data in Table 3.8 shows that all of the drug-containing nanoparticle emulsions are stable by means of zeta potential measurement. However, a correlation was observed that directly linked the influence of particle size to the average zeta potential measured for an emulsion. The nanoparticle emulsions containing larger particles (NP1a, NP23, NP24, and NP25), are all on the lower end of the zeta potential spectrum, and that emulsions containing smaller particle sizes (NP2, NP6, NP7, and NP8) all produced highly stable emulsions in terms of zeta potential. Therefore, from this data, it appears that the smaller the particle size, the more stable that emulsion is.
3.8 Imaging and Analysis of Bacterial Interactions with Nanoparticles

3.8.1 Microscopy Imaging of Nanoparticle Emulsions

Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) studies were performed on many of the nanoparticle emulsions synthesized. In order to visualize individual nanoparticles using these instruments, the emulsions were diluted 3,000 fold for SEM and $10^{10}$ for TEM analysis.

![SEM and TEM images of β-lactam conjugated nanoparticle NP1a.](image)

**Figure 3.19.** SEM and TEM images of β-lactam conjugated nanoparticle NP1a. Particles range between 40-80nm in diameter. SEM image (left) taken by Dr. Jeung-Yeop Shim.³

SEM and TEM microscopy analysis of all emulsions analyzed confirmed that the nanoparticles present in the emulsions are uniformly spherical with a small size range distribution. However, for many of the nanoparticle emulsions analyzed, the particle sizes were smaller by imaging analysis than they were via dynamic light scattering, which is further explained below. Atomic force microscopy analysis was performed on the second nanoparticle emulsion formulation for this lactam (NP1) that was formulated with a 7:3 ratio of BA:Sty co-monomers instead of only EA. Figure 3.20 shows that the average particle size for this nanoparticle emulsion is much smaller than the average particle size observed for NP1a.
Figure 3.20. AFM analysis of NP1 nanoparticle emulsion. Images obtained using tapping mode of AFM. Nanoparticles depicted displayed an average height between 15 and 26nm from the surface of the glass slide. NP1 used in this analysis contained 1% SDS in the formulation.

Figure 3.20 shows the array of data produced for nanoparticle NP1 by AFM analysis. The original spectrum obtained for this emulsion (top left) contains a red line through the center where the height for all of the particles which this line circumvents is plotted in the bottom image in Figure 3.20. Also, a 3D image can be produced using the AFM software of a selected number of particles, which produces a replication of the original image at various angles.

AFM analysis of all of the emulsions presented herein displayed particles that were significantly smaller than was determined by DLS analysis, with an approximate 20-30nm difference in diameter between the two measurements. Figure 3.21 provides some possible explanation for this observed difference in particle size analysis. Due to the observance of thin polymer strands present in the emulsions via AFM and TEM analysis, this would inevitably throw off the measurements by DLS. This is due to the nature in which the DLS instrument performs its measurements, i.e. light diffraction. If there are
other objects present in the emulsions that are not uniform with the nanoparticles’ morphology, it is very difficult to accurately calculate the average particle size by DLS. While many of the DLS measurements were determined to be similar to the TEM and SEM particle size analysis, AFM analysis showed much smaller nanoparticles than was determined by these techniques. However, because AFM is a direct contact microscopy analysis that has been shown to be able to detect objects at the molecular level, the data obtained from AFM analysis was considered the most accurate performed for these nanoparticle emulsions.

In order to explore the effect of the long chain polyacrylate strands on the dynamic light scattering analysis, two drug-conjugated nanoparticle emulsions (NP1 and NP8) were diluted to 1% solid content then centrifuged using a benchtop centrifuge at 14,500rpm for 50 minutes. A portion of the supernatant was then removed for DLS and AFM analysis.

**Table 3.9.** Comparison of DLS and AFM data before and after purification processes. All of the data presented here is the diameter or height of the particles presented in nanometers.

<table>
<thead>
<tr>
<th>Sample</th>
<th>DLS-Before</th>
<th>DLS-After</th>
<th>AFM</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP1</td>
<td>NA</td>
<td>25.3</td>
<td>15-26</td>
</tr>
<tr>
<td>NP8</td>
<td>51.7</td>
<td>9-10</td>
<td>7-16</td>
</tr>
</tbody>
</table>

As seen in Table 3.9, the purification process performed on the two nanoparticle emulsions drastically reduced the diameter measurement of the particles by DLS analysis. By doing so, the measured diameters of the particles via DLS were within the observed particle size range determined by AFM. These findings corroborate the initial theory that the presence of long polymer strands in the emulsions causes the DLS measurements to be inaccurate and by the data presented in Table 3.9 for the nanoparticle emulsion NP8, the presence of the polymer strands caused an apparent inflation in the DLS data, by four times the actual particle size. This shows that the nanoparticles present within the emulsions are within the range of 10 to 40nm in diameter, instead of the original 40-80nm particles previously described.\[^{20,40}\]
**Figure 3.21.** TEM image of drug-free nanoparticle emulsion CNP5. Particle sizes ranged from 30-45nm in diameter by visual measurement. Arrows in image to right point to polymer strands observed in the emulsion.

**Figure 3.22.** AFM image of drug-free nanoparticle emulsion CNP16. Both images are 3D replications of the original AFM image obtained, where the image to the right provides an accurate height scale for the nanoparticles measured.

The average particle size measured by AFM for the drug-free BA:Sty emulsion CNP16 was 15.8 +/- 1.77nm (Figure 3.22). Images taken of the drug-free nanoparticle emulsions showed that the emulsion contains both small nano-sized spherical polymer balls as well as long chain polymer strands (Figure 3.21). These strands were also observed by AFM analysis of the multi-drug conjugated nanoparticle emulsion MDNP2 (Chapter 4). These visual observations of the emulsions confirmed the original notion that the polyacrylate polymer films formed upon water loss from the emulsion is more likely due to the presence of polymer strands interacting with one another than the aggregation of only nanoparticles. This notion was built also upon the observed mechanical properties of these polymer films, which is later defined in Chapters 5 and 6.
Figure 3.23. TEM image of β-lactam encapsulated nanoparticle emulsion NP6.

TEM image of nanoparticle emulsion NP6 containing the encapsulated N-thiolated β-lactam monomer 6 depicts nanoparticles that are not uniformly spherical (Figure 3.23). In fact, the particles appear to be slightly oblong. This morphology was observed for many of the drug-encapsulated nanoparticle emulsions, which could possibly be the result of the nanoparticles forming around the drug monomer being incorporated, thus is more likely to be non-uniform in nature.

Figure 3.24. TEM and AFM image of ciprofloxacin acrylamide conjugated nanoparticle emulsion NP7. AFM image (right) was produced from the emulsion purified by continuous extraction, as described in Section 3.7.2. Images were performed on emulsions formulated with 3% SDS.
As seen in Figures 3.24 and 3.25, both the TEM and AFM images for the ciprofloxacin-conjugated nanoparticle emulsions NP7 and NP8 display a higher variability in particle size than was observed for other emulsions. This variability is very pronounced in the AFM image for NP7 (Figure 3.24) where there appears to be a few particles much larger than the majority present within the emulsion, where the majority of the nanoparticles for this emulsion were 23.7 +/- 2.7nm. The average particle size range for NP8 was also within this same range, whereas the few sporadic larger particles present in the emulsion are between 28 and 35 nm.

![AFM Image of NP8](image)

**Figure 3.25.** AFM image of NP8. Images are between 7 to 16nm in height, with some observed larger particles between 28 to 35nm.

### 3.8.2 AFM Imaging of Polymer Films

In order to gain perspective on the anatomy of the polymer films formed from the nanoparticle emulsions, AFM imaging was employed. A drop of the concentrated emulsion was drop cast onto a glass coverslide and the polymer film was allowed to form on the glass surface at room temperature prior to AFM analysis. AFM was performed under tapping mode by dry imaging.
Figure 3.26. AFM image of polymer film resulting from NP8 nanoparticle emulsion. Image of the left is the original image obtained from the AFM. The right image is a 3D replication of the original AFM showing the full topography of the film. The NP8 emulsion used for these images was formulated with ciprofloxacin methacrylamide and used 1% SDS for the polymerization.

The AFM images taken from the polymer film sample NP8 shows large craters in the topology of the polymer film. In the image on the left side in Figure 3.26, the white areas are the raised portions of the polymer film, and the darker areas represent the lulls in the film. This film was formed from the concentrated (20% solid content) emulsion, and therefore, no nano-sized particles were observed due to the heavy degree of particle coalescing into a solid polyacrylate film. However, in Figure 3.27, the polymer film was formed using a more dilute sample of the drug-free poly(BA:MMA) nanoparticle emulsion CNP17, and therefore, individual particles were observed in the film. This indicates that when a reduced amount of solid content is used for the film formation, the film becomes more of a compilation of particles closely packed together than it is a solid film structure. This finding also concurs with the visual observations of the polymer film samples formed from diluted emulsion samples where the film does not seem whole and is very flimsy and weak. It also does not express the ability to withstand any deformation as is observed for the films formed from the concentrated emulsions (Chapter 5).
Figure 3.27. AFM images of CNP17 polymer film. Film was formed using 1% solid content concentration of the poly(BA:MMA) emulsion.

In Figure 3.27, there is an obvious difference in the polymer film topology from that of the NP8 film in Figure 3.26. Also, there is a large observed difference in the concentration of the particles in Figure 3.27 versus Figure 3.25, prepared from a more highly diluted sample of the nanoparticle emulsion NP8. Obviously, upon dilution, the amount of nanoparticles present in solution is greatly reduced, along with the ability of the emulsion to form a solid polymer film.

3.8.3 TEM Analysis of Nanoparticle Interactions with S. aureus

In Section 3.9, the antibacterial activity of these drug-conjugated nanoparticle systems was explored and potent antibacterial activity \textit{in vitro} was established for many of the drug-containing systems. However, it was not clearly understood how the nanoparticles were interacting with the bacterial cells in order to obtain this antibacterial activity. Two possible concepts for the nanoparticle’s mode of action were originally visualized in the schematic shown in Figure 3.28. The first idea was that the nanoparticles would collapse upon initial contact with the cell wall and the antibiotic incorporated in the nanoparticle delivery system would be released directly into the cell through the enzymatic activity of enzymes present on the outside of the cell membrane (Figure 3.28). The second concept relied more upon the bacteria cells to interact with the particles. Due to the small size of the particles, it was surmised that the particles could be internalized by the bacteria cell in order to release the bound or encapsulated drug through either endocytosis or phagocytosis, depending on the particle size and its interactions with the macromolecules present within the cell membrane. Therefore, the schematic drawing in Figure 3.27 was formed for the potential modes of action of the nanoparticles.
In order to verify this, a TEM study was performed using *S. aureus* cells treated with the drug-free nanoparticle emulsion **CNP5**. Cells were incubated with a the diluted emulsion for 30 minutes then the solution was cross-fixed with gluteraldehyde to freeze all of the metabolic and cellular functions. TEM imaging was previously performed to confirm that the presence of gluteraldehyde in the emulsion would not affect the nanoparticles. The cellular suspension was then analyzed “as is” (Figure 3.29) in order to observe the external interactions of the nanoparticles with the bacteria cells, and after cross-sectional preparation of the cell pellet embedded in plastic (Figure 3.30). This study confirmed that both of the original modes of action concepts depicted in Figure 3.28 may be considered for the drug-free nanoparticle emulsion **CNP5**.

**Figure 3.28.** Schematic image of nanoparticle interaction with the bacteria cells.
Figure 3.29. TEM images of whole *S. aureus* cells. A) TEM image of **CNP5** nanoparticles. B and C) Images of *S. aureus* whole cells not treated with **CNP5**. D) *S. aureus* cell with a **CNP5** nanoparticle adhered to surface of the cell. Bacterial cells were not dehydrated prior to TEM visualization; therefore, the whitening in the center of the cells was caused by effervescing of the internal water by the electron beam.

In Figure 3.29A, the particles appear to be of normal size and morphology after treatment with gluteraldehyde for fixation. As observed in Figure 3.29D, the nanoparticle is found sticking to the surface of the bacteria cell, which confirms the initial concept of the nanoparticle mode of action. However, in order to establish whether or not the nanoparticles are internalized by the bacterium prior to particle degradation, cross-sectioning of the bacterial cells after interaction with the nanoparticles was required.
**Figure 3.30.** TEM images of sectioned *S. aureus* cells interacting with CNP5 nanoparticles. A-C) Cross-sections of *S. aureus* cells treated with CNP5. D) Cross-section of *S. aureus* cells untreated. All images were taken of cells without any staining of the nano-thin cross-sections of the cells embedded in plastic to avoid metal precipitation on the images.

TEM analysis of the cross-sections of *S. aureus* cells embedded in plastic showed that the drug-free nanoparticles were found both adhered to the surface of the cells (Figure 3.30A and B) and also within the cell wall (Figure 3.30C). No nanoparticle-sized materials were observed in images of the cells that were not treated with the nanoparticle emulsion (Figure 3.30D). Also, particles were not observed inside the cells further than just within the cell membrane indicating that the bacteria may be able to degrade the particles once within the cytoplasm of the cell. This study established that the bacteria cells are able to internalize the nanoparticles, where once inside, could degrade the polymer matrix of the particles thereby releasing the bound or encapsulated drug directly into the bacterium. This essentially provides direct delivery of the drug to the bacterium without encountering any degradation prior to internalization. This study was also performed using *N*-thiolated β-lactam encapsulated nanoparticle emulsion NP6, however, upon cross-sectional TEM visualization of the culture, most of the *Staphylococcal* cells were
completely destroyed by the drug-containing nanoparticles and the few cells that were observed by TEM did not show any nanoparticles within or adhering to the bacterial cells. This observation indicates that drug cleavage and subsequent antibacterial activity is extremely rapid following bacterial interaction with the nanoparticles. Since the bacterial cultures were exposed to the nanoparticles for only a short period of time (30 minutes), many of the intact nanoparticles were observed in sections of the TEM grid far removed from the few remaining bacterial cells indicating that these nanoparticles had not yet interacted with the bacterial cells.

3.9 Antibacterial Activity of Nanoparticle Emulsions

Both the Kirby Bauer and MIC antibacterial assays were performed to determine antibacterial properties of the nanoparticle-containing emulsions. The subsequent polyacrylate films formed from the emulsions by air evaporation were analyzed only by Kirby Bauer ZOI assays for determination of antibacterial activity. Both of these assays, while being reputable for their accuracy and able to compare numerous antibiotics through standardization of these assays, presented a challenge for accurate assessment of the antibacterial activity for the drug-conjugated emulsions. However, this was overcome by using the agar dilution MIC assay, described in NCCLS.\textsuperscript{[50]}

3.9.1 Kirby Bauer Assay

Kirby Bauer assays were performed on the drug-conjugated nanoparticle emulsions and the resulting drug-conjugated polymer films in accordance with the guidelines recommended by the National Committee for Clinical Laboratory Standards (NCCLS).\textsuperscript{[28]} These assays rely on the ability of the substance being tested to diffuse from the well into the surrounding agar in a circular formation in order for the drug to interact with the bacteria present on top of the agar surface. However, there are many inherent problems with this assay due to required diffusion. Often observed for these assays was the appearance of a white ring surrounding the walls of the well where the emulsion was added. This ring is presumably the long-chain polyacrylate strands present in the emulsion that cause film formation. Since purification methods have shown that the large polymer strands make up approximately half of the solid content of the emulsion, this assay is only able to analyze the antibacterial activity of the nanoparticles and small drug-bearing oligomers present in the emulsion indicating that the activity observed does not fully represent the activity of the emulsion.

3.9.2 Agar Dilution Minimum Inhibitory Concentration Assay

In order to establish the lowest drug concentration needed to prevent bacterial growth, the emulsions and drug monomers were analyzed for antibacterial activity at various drug concentrations in µg drug per mL of emulsion or solvent (DMSO). Three different agars
were employed here for these assays, the original Mueller Hinton agar that is described in the NCCL manual for antibacterial testings\[50\] and a pH indicator-containing agar. All agars used were purchased from Fisher Scientific and used without modification. The two pH indicators used here were either bromothymol blue or phenol red. The phenol red-containing agar was used for the *Staphylococcus* strains analyzed, and the bromothymol blue-containing agar was used for the *P. aeruginosa*, *E. coli*, and *B. anthracis* assays. These unconventional agars were used in order to provide a clearer identification of bacterial growth inhibition since many of the samples analyzed, especially the β-lactam and penicillin-containing emulsions, are bacteriostatic in nature. Therefore, they cannot be analyzed for the presence of living microbes by secondary culturing on TSA agar plates overnight as described in NCCL\[50\]. The pH indicator was employed to provide a color change in the agar associated with the growth of bacteria present on the agar. These color-changing MIC assays were compared with the traditional Mueller Hinton agar-based assay as a secondary experiment in order to verify the observed results.

### 3.9.3 β-Lactam-Containing Nanoparticle Emulsions

Antibacterial testing was performed on many of the drug-containing emulsions synthesized in this chapter. Assays were performed against many *Staphylococcus* strains, including ten strains of MRSA. MRSA strains were either purchased from ATCC (43300 and 33591) or were obtained from Lakeland Regional Medical Center as clinical isolates. The clinical isolates were arbitrarily labeled 652-659 and the ATCC purchased strains 43300 and 33591 correspond to the USF-designated 920 and 919, respectively. Methicillin-sensitive *S. aureus* (MSSA) was purchased from ATCC as well (ATCC 25923) and *Bacillus* species was either provided by Dr. Daniel Lim (*B. anthracis*-Sterne) or purchased from ATCC.
Figure 3.31. Kirby Bauer data for the β-lactam containing nanoparticle emulsions against various clinical and ATCC strains of MRSA. Zone data is determined by measuring the diameter in millimeters of the zone of bacterial growth inhibition.

Figure 3.31 compares the zone of inhibition (ZOI) data of three drug-containing (two encapsulated and one covalently bound) nanoparticle emulsions, NP1a, NP2, and NP6 against 10 strains of MRSA. Interestingly, the anti-MRSA activity was drastically different between these strains for β-lactam containing emulsion NP1a while the other two β-lactam-containing nanoparticle emulsions (NP2 and NP6) displayed fairly consistent zones of inhibition against all of the isolates and ATCC strains analyzed, except for MRSA clinical isolate strain 653, which NP2 caused a much larger zone of inhibition than was observed for any of the other MRSA strains.
Table 3.10. MIC values for \(\beta\)-lactam-containing nanoparticle emulsions against various bacteria. MIC values are reported in µg drug per mL of emulsion.\(^{[51]}\) (ND= not done)

<table>
<thead>
<tr>
<th>Nanoparticle Emulsion</th>
<th>(S.\ aureus) (ATCC 25923)</th>
<th>MRSA (ATCC 43300)</th>
<th>(B.\ anthracis)-Sterne</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNP5</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>NP1a</td>
<td>256</td>
<td>256</td>
<td>256</td>
</tr>
<tr>
<td>NP1</td>
<td>16</td>
<td>16</td>
<td>64</td>
</tr>
<tr>
<td>NP2</td>
<td>64</td>
<td>64</td>
<td>ND</td>
</tr>
<tr>
<td>NP5</td>
<td>16</td>
<td>16</td>
<td>ND</td>
</tr>
<tr>
<td>NP6</td>
<td>8</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>NP9</td>
<td>8</td>
<td>8</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data presented in Table 3.10 showed that, when acrylate lactam monomer 1 is co-polymerized with only ethyl acrylate as the sole liquid acrylate monomer, the antibacterial activity of the monomer is almost completely lost (MIC is 256µg/mL). However, when the monomer is formulated with butyl acrylate and styrene as the co-monomers, the antibacterial activity is consistent with the lactam monomer dissolved in DMSO. It was also observed that some of the lactam monomers’ activity was increased when applied to the bacterial cultures in the nanoparticle delivery system (NP5 and NP9). This supports the hypothesis that when the drug monomers are enzymatically cleaved from the polymer backbone, the active drug monomer released is often more active than the acrylate drug monomer. This was also observed in Chapter 2, where the lactam monomers 6 and 10 were much more active than the acrylated versions 1 and 3. However, since upon enzymatic cleavage of the acrylate moiety, the drug monomer is reverted back to the free alcohol precursor 4. This is also supported by the fact that all of the \(\beta\)-lactam containing emulsions displayed MIC values within the same range of activity against \(S.\ aureus\) strains, including MRSA, except for lactam encapsulated emulsion NP2, which contains the less active acetate moiety at the C3 position and is not a precursor to the lactam monomers 1 or 3.
Figure 3.32. Results of Kirby Bauer assay for NP1. Drug-conjugated emulsion NP1 is formulated from acrylated lactam monomer 1 with ethyl acrylate.

Next, NP1 was tested against seven species of Bacillus as an aqueous emulsion. This N-thiolated β-lactam conjugated nanoparticle emulsion showed very good zones of inhibition across the board for all of the Bacillus species tested in vitro. This agrees with the MIC value of 16μg/mL reported for this emulsion in Table 3.10. The data in Table 3.11 shows that the other drug-containing lactam nanoparticle emulsions NP1a, NP2, NP5, and NP6 displayed no or only moderate activity against B. anthracis via Kirby Bauer analysis.
Table 3.11. Kirby Bauer assay data for β-lactam-containing nanoparticle emulsions against *B. anthracis*-Sterne. Data is reported as the diameter of the zone of inhibition in millimeters.

<table>
<thead>
<tr>
<th>Sample</th>
<th>ZOI* (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP1a</td>
<td>0</td>
</tr>
<tr>
<td>NP2</td>
<td>12 (17)</td>
</tr>
<tr>
<td>NP5</td>
<td>(21)</td>
</tr>
<tr>
<td>NP6</td>
<td>25</td>
</tr>
</tbody>
</table>

*() = partial inhibition

Again, as seen in Table 3.11, C₃-acetoxy N-methylthio β-lactam encapsulated nanoparticle emulsion NP2 displayed diminished activity against *B. anthracis* from the other β-lactam emulsions. This was consistent with the *Staphylococcal* results as well, indicating that this drug monomer encapsulated in the nanoparticle delivery system is not a very potent antibacterial. The *Bacillus* data was also consistent for the C₃-acrylate N-methylthio β-lactam conjugated emulsion NP1a, where no zone of inhibition was observed for this emulsion against the majority of the MRSA and *S. aureus* strains analyzed. Both the C₃-long chain acrylate and the C₃-methoxy N-methylthio β-lactam containing nanoparticle emulsions (NP5 and NP6 respectively) displayed excellent ZOI data against *B. anthracis* (Table 3.11).

3.9.4 Nanoparticle Emulsions Containing a Commercially Available Drug

3.9.4.1 Penicillin-containing emulsions

All of the penicillin-containing nanoparticle emulsions were tested for anti-*Staphylococcal* activity *in vitro* by both the Kirby Bauer and minimum inhibitory concentration assays. The data is summarized in Table 3.12.
Table 3.12. Anti-*Staphylococcal* activity of penicillin-based nanoparticle emulsions. MIC values are reported as µg of drug per mL of emulsion. ZOI data is recorded for the emulsions at 100µg drug in 100µL of emulsion.

<table>
<thead>
<tr>
<th>Sample</th>
<th><em>S. aureus</em> (ATCC 25923)</th>
<th></th>
<th><em>MRSA</em> (ATCC 43300)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ZOI* (mm)</td>
<td>MIC (µg/mL)</td>
<td>ZOI* (mm)</td>
<td>MIC (µg/mL)</td>
</tr>
<tr>
<td>CNP16</td>
<td>0</td>
<td>&gt;256</td>
<td>0</td>
<td>&gt;256</td>
</tr>
<tr>
<td>NP11</td>
<td>9 (11)</td>
<td>64</td>
<td>(18)</td>
<td>64</td>
</tr>
<tr>
<td>NP12</td>
<td>45 (50)</td>
<td>16</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>NP13</td>
<td>(11)</td>
<td>2</td>
<td>ND</td>
<td>2</td>
</tr>
<tr>
<td>NP14</td>
<td>ND</td>
<td>64</td>
<td>ND</td>
<td>64</td>
</tr>
<tr>
<td>NP15</td>
<td>(15)</td>
<td>64</td>
<td>0</td>
<td>64</td>
</tr>
<tr>
<td>NP16</td>
<td>13</td>
<td>256</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>NP17</td>
<td>0</td>
<td>128</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>NP18</td>
<td>11 (14)</td>
<td>256</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>NP19</td>
<td>11</td>
<td>128</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>55 (61)</td>
<td>0.012</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>Cefaclor</td>
<td>20</td>
<td>ND</td>
<td>(14)</td>
<td>ND</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>41</td>
<td>ND</td>
<td>13</td>
<td>ND</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>45</td>
<td>ND</td>
<td>15</td>
<td>ND</td>
</tr>
</tbody>
</table>

*( ) = partial inhibition, ND= not done

Many of the commercially available β-lactam drug monomers displayed no activity *in vitro* when in the nanoparticle drug delivery system (NP16-19). The penicillin-containing emulsions (NP11 through NP15) all displayed equal activity against MRSA as they did against MSSA, where the penicillin G acrylamide nanoparticle emulsion NP13 was the most potent emulsion analyzed in the MIC agar and broth studies, despite very unimpressive zones of inhibition in the Kirby Bauer assay. This fact established the use of this penicillin G acrylamide monomer in the multi-drug conjugated nanoparticle emulsion MDNP1. The initial multi-drug conjugated nanoparticle system contained the drug monomers 15-19, however, since these monomers did not display strong antibacterial activity *in vitro* when in the nanoparticle delivery system, it was determined that any antibacterial activity observed for this emulsion (MDNP2) was due to the ciprofloxacin monomers present in the emulsion. This prompted the re-formulation of the multi-drug conjugated system (Chapter 4), along with the need to decrease the amount of surfactant present in the emulsion from 3% to 1% (w/w) due to observed toxicity (Chapter 7).

Also observed in Table 3.12 is that the amount of surfactant present in the emulsion did not cause the antibacterial activity observed. This is due to the fact that the most potent anti-*Staphylococcal* emulsion, NP13, was formulated with only 1% SDS, whereas all of
the other drug-containing nanoparticle emulsions presented in Table 3.12 were formulated with 3% SDS. Therefore, the antibacterial activity would have been higher for the other emulsions if the activity was due to toxicity caused by the surfactant present in the emulsions.

**Figure 3.33.** Zone of inhibition assay for NP11 against *S. aureus* +/- 100µg β-lactamase. Image on the left contains 100µg of β-lactamase, while the image of the right was not treated with β-lactamase. R-11 represents NP11, and was added to the agar wells in either 20, 50, or 100µg of drug monomer in equal amounts of emulsion. Penicillin G was added to the wells as 20µg drug monomer in 20μL water.

The Kirby-Bauer assay in Figure 3.33 shows that when 100µg of β-lactamase is spread evenly over the agar plate prior to addition of *S. aureus* and subsequent well drilling, the anti-*Staphylococcal* activity of penicillin G in the potassium salt form was completely destroyed. However, the penicillanic acid acrylamide-conjugated nanoparticle emulsion (NP11) displayed zones even at the reduced concentration of 20µg drug monomer in 20μL of emulsion. Since penicillanic acid normally displays weaker activity than the penicillin G monomer against methicillin-sensitive *S. aureus* (MSSA), this indicates that the nanoparticle delivery system is not only protecting the drug monomer from the destructive β-lactamase, but is also enhancing the activity of the drug monomer *in vitro.*
3.9.4.2 Ciprofloxacin-conjugated nanoparticle emulsions

Table 3.13. ZOI and MIC data for ciprofloxacin acrylamide nanoparticle emulsions NP7 and NP8. ZOI data performed on 20μg of drug monomer in 20μL of emulsion per well. MIC data also based on the amount of drug monomer present per mL of emulsion analyzed.

<table>
<thead>
<tr>
<th>Compound</th>
<th>B. anthracis- Sterne</th>
<th>S. aureus</th>
<th>MRSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ZOI (mm)</td>
<td>MIC (µg/mL)</td>
<td>ZOI (mm)</td>
</tr>
<tr>
<td>NP7</td>
<td>38</td>
<td>2</td>
<td>26</td>
</tr>
<tr>
<td>NP8</td>
<td>31</td>
<td>1</td>
<td>28</td>
</tr>
<tr>
<td>Cipro</td>
<td>42 (45)</td>
<td>0.063</td>
<td>28 (34)</td>
</tr>
</tbody>
</table>

*ND* = not done

Table 3.13 reports both the zones of inhibition and the MIC values for the acrylated cipro-conjugated nanoparticle emulsions NP7 and NP8, as well as the antibacterial activity of the unmodified cipro drug. It was observed here that the cipro-conjugated emulsions were highly potent towards S. aureus and B. anthracis, where ZOI data was observed to be almost exactly the same for the cipro-conjugated nanoparticles as it was for the commercially available cipro analogue. The MIC for cipro against S. aureus was within the same range as the cipro-conjugated nanoparticle emulsions, indicating that almost no antibacterial activity is lost for this drug when incorporated into the nanoparticle delivery system. However, against B. anthracis, the original cipro drug has a much lower MIC than either of the cipro-conjugated emulsions.
Figure 3.34. Anti-Staphylococcal activity of ciprofloxacin-conjugated nanoparticle emulsions. KG13 written on the plates represents NP7, KG11 represents NP8, and KRG15 represents CNP15. Three amounts of drug were tested: 100µg, 50µg, and 20µg.

Figure 3.34 shows this pictorially for the zones of inhibition for NP7 (left) and NP8 (right) at different drug loading amounts. Both the cipro-conjugated nanoparticle emulsions (Figure 3.34) and the polymer films (Figure 3.35) displayed excellent activity against both MSSA and MRSA equally. The cipro-conjugated nanoparticle emulsions yielded large ZOIs that were comparable to the free cipro drug and MIC values of 2µg/mL and 1µg/mL against B. anthracis for NP7 and NP8, respectively (Table 3.13). Interestingly the activities (zone sizes and MICs) are only slightly diminished for MSSA and MRSA.

Figure 3.35. Anti-Staphylococcal activity of the cipro-conjugated polymer films. A 0.5mL aliquot of each concentrated (20% solid content) emulsion was also used to form the polymer films for ZOI analysis. KG13 written on the plates represents NP7, KG11 represents NP8, and KRG15 represents CNP15.
Figure 3.35 shows a similar study for the drug-conjugated polyacrylate films from the cipro-conjugated emulsions NP7 and NP8. A 0.5mL aliquot of concentrated (20% solid content) emulsion was used to form the polymer films for ZOI analysis. The emulsions were added on top of the already inoculated agar, and the polyacrylate films were allowed to solidify in the biosafety cabinet prior to incubation for bacterial growth, thereby allowing the solid polymer film to be interacting with the proliferating bacteria and not the emulsion. The data presented in Figure 3.35 shows that the polymer films formed from the cipro-conjugated nanoparticle emulsions (NP7 and NP8) displayed anti-\textit{Staphylococcal} activity, which was not observed for the drug-free polymer film formed from the emulsion CNP15. Therefore, the observed antibacterial activity was not due to leaching of the excess surfactant or radical initiator into the surrounding agar, but was due to the direct interaction of the microbes with the polymer film formed from the emulsion, since no bacterial growth was observed under the polymer film. The observed zones of inhibition surrounding the polymer films may have been caused by diffusion of the nanoparticle constituents present in the emulsions prior to film formation instead of a direct interaction of the polymer film. Regardless of the reason for the observed zones of inhibition, the films were determined to possess antibacterial capabilities \textit{in vitro}. This conclusion is also supported by the bacterial permeability studies and carbon source assays described in Chapter 5.

![Figure 3.35](image)

**Figure 3.35.** Anti-MRSA activity of cipro-conjugated nanoparticle emulsions NP7 and NP8. ZOI measurements are recorded for 20\(\mu\)g of cipro monomer in 20\(\mu\)L of emulsion.
Figure 3.36 plots the measured zone sizes for the cipro-conjugated nanoparticle emulsions NP7 and NP8 against different strains of MRSA. The MRSA strains marked 652-659 are all clinical isolates obtained from Lakeland Regional Medical Center, and strain 919 was the ATCC strain 43300. Interestingly, five of the clinical MRSA strains were completely resistant to NP7, while were still slightly susceptible to NP8. The remaining three clinical isolates (657-659) and the ATCC strain (919) were all highly sensitive to the cipro-conjugated nanoparticle emulsions, displaying zone sizes equal to those observed for cipro in its commercially available form.

The cipro-conjugated nanoparticle emulsions NP7 and NP8 displayed potent activity in vitro against the most common pathogen found in burn wound infections, P. aeruginosa (Figure 3.36). In fact, the activity of the emulsions was comparable to that of the commercially available ciprofloxacin, which is one of the most commonly prescribed antibiotics for Pseudomonal infections. However, the acrylated ciprofloxacin monomers alone did not appear to be very effective anti-Pseudomonal agents. This fact provides further proof that the microbes are able to cleave off the drug (cipro) from the polymer matrix, presumably inside the cell, in order for antibacterial activity to be manifested.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Trial 1</th>
<th>Trial 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cipro</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>256</td>
<td>128</td>
</tr>
<tr>
<td>NP7</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>128</td>
<td>128</td>
</tr>
<tr>
<td>NP8</td>
<td>8</td>
<td>4</td>
</tr>
</tbody>
</table>

**Figure 3.37.** MIC and Kirby Bauer data for ciprofloxacin-conjugated nanoparticle emulsions against *P. aeruginosa* (ATCC 10145). MIC values are reported as the µg of drug monomer per mL of emulsion or DMSO solvent. KG13 in the image represents NP7, and cipro acrylate represents monomer 7.

In order to analyze the effect of purification processes on the antibacterial activity of the drug-conjugated nanoparticle emulsions, Kirby Bauer assays were performed on equal volumes and concentrations of the emulsion NP7 both before and after continuous extraction for 72 hours of organic impurities present in the emulsion using cyclohexane. Upon evaporation of the cyclohexane extract, it was determined that 33.3mg of impurities was extracted from 10mL of the 10% solid content emulsion (2g total solid mass). The
emulsion’s physical appearance was unchanged by the purification process, as determined by AFM imaging, and the emulsion retained its ability to form a film.

Figure 3.38. Kirby Bauer assay using both the concentrated and diluted nanoparticle emulsion NP7 before and after purification against *S. aureus*. The concentrated emulsion used in the assay on the right contained 10% solid content (3mg ciprofloxacin acrylamide monomer per 1mL emulsion), whereas the assay on the left represents the diluted emulsion at 1mg drug monomer per 1mL emulsion. The zones on the top half of both plates represent the purified emulsion (extracted with cyclohexane) and the zones on the bottom half of the plates represent the original unpurified emulsion. (KG13: NP7).

For the zone of inhibition assay performed on the purified (top half) and unpurified (bottom half) NP7 emulsion (Figure 3.37), the standardized assay (left) which incorporates a 1mg drug per 1mL emulsion concentration at 20µL in each well was supplemented with a more concentrated (3mg drug/mL) sample of emulsion (right). This was done in order to magnify any observed differences in the measurements.

The zones of inhibition for the purified emulsion was 14-15% smaller than the zones observed for the unpurified original emulsion at both 3.33% and 10% solid content (1mg/mL and 3mg/mL drug concentration respectively). This indicates that some of the acrylated drug monomer is not polymerized with the other two co-monomers and is most likely remaining in suspension by either encapsulation within the nanoparticles or by sticking to the surface of the nanoparticles. NMR analysis of the evaporated organic layer after continuous extraction for 3 days displayed low intensity peaks in the region where the acrylate peaks for the liquid acrylate monomers and the cipro acrylate monomer 7 are normally found, between 5.9 and 6.5ppm. This indicates that a small percentage of the acrylate monomers added to the emulsion formulation (33.3mg of the nominal 4.0g) was not polymerized and remained suspended in the emulsion. Since the cipro acrylate
monomers alone showed anti-Staphylococcal activity in vitro, the change in ZOI data could be due to removal of the un-polymerized cipro acrylate monomer from the emulsion. However, due to the small percentage of un-polymerized drug monomer observed in the extracted organic layer, this may not be the only cause of the change in antibacterial activity, and other issues (surfactant levels, etc) in changes to the nanoparticles upon purification can not be ruled out.

3.10 Conclusions

The antibacterial containing nanoparticle emulsions synthesized here all contained particles in the nano-size range. The emulsion formulation of a 7:3 ratio of BA:Sty liquid acrylate monomers was the most optimal combination for both complete dissolution of the drug monomers and also produced the most stable nanoparticle emulsions with a small size range. Stability studies showed that this emulsion formulation was stable to many different physical and chemical conditions, including a wide range of temperature and pH changes, and also stable towards many biological molecules present in blood serum. Purification processes showed that longer polymer strands present in the emulsions can be removed through mild centrifugation processes, and that continuous extraction of the emulsion using an organic solvent (cyclohexane) removes excess acrylate monomers that were not polymerized from the emulsion. Encapsulation of larger antibiotic monomers, such as amphotericin B and erythromycin, caused the resulting nanoparticles to be larger in size and morphologically distorted, and encapsulation of even the smaller β-lactam monomers resulted in particles of oblong morphology as opposed to the uniformly spherical nanoparticles observed for the drug-conjugated and drug-free nanoparticles.

Antibacterial and TEM analysis suggested that the drug-conjugated to the polymer backbone must be cleaved in order for antibacterial activity to be observed, and also the nanoparticles can deliver the bound or encapsulated drug monomers to the bacterium either by collapsing upon interaction with the bacterial cell wall, or after incorporation into the bacterium’s cytoplasm through either phagocytosis or endocytosis bacterial invagination methods. The three most potent modified commercially available drug monomers incorporated into the nanoparticle system were penicillin G acrylamide monomer 13 and the two ciprofloxacin acrylamide monomers 7 and 8. Also, the most potent N-thiolated β-lactam acrylated monomers synthesized that produced the most optimal nanoparticle emulsions in terms of particle size, stability, and antibacterial activity were 1 and 3. Therefore, these five acrylated drug monomers were chosen for the multi-drug conjugated nanoparticle emulsion described in the next chapter (Chapter 4).
3.11 References


CHAPTER 4

MULTI-DRUG CONJUGATED NANOPARTICLES FOR BURN WOUND TREATMENT

4.1 Introduction

Burn wound infection sites are complex environments containing numerous pathogenic microbes, many of which are commensal with the human body or comprise the normal skin flora, such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Streptococcus pyogenes*.\[^{1-4}\] When an abrasion or disruption of the skin barrier occurs, these persistent bacteria transform into opportunistic pathogens, causing many problems that may delay healing and increase the risk of sepsis. Other bacteria and fungi that are not part of the normal skin flora have been reported in burn wound infections, with *Pseudomonas aeruginosa* and *Escherichia coli* topping the list, which has 22 different strains and species of pathogens, including bacteria, fungi, and viruses.\[^{1,2,5}\] Because of the extremely nutrient-rich environment of a burn wound, especially prior to burn wound excision when the burn eschar is still intact, it is an easily colonized environment that calls many forms of microbes to immediately after thermal injury.\[^{2}\] Some of the more common infections associated with burn wounds are burn wound cellulites, burn wound impetigo, open burn-related surgical wound infections, and invasive infections in unexcised burn wounds.\[^{5}\] All of these infection types have been known to contribute to the more complicated bacterial infections, including bacteremia, sepsis, and toxic shock syndrome if left untreated.\[^{2,5}\] These bacteria can originate from various other regions in the body, including the nasal passages, the gut, and the blood stream, and can also be transmitted to the wound through nosocomial contact with hospital staff. While many precautions are taken at hospitals today to prevent such person to person spread of pathogens, nosocomial infections still remain ubiquitous. In fact, nosocomial contact is the number one cause of MRSA infections in hospitals and in burn wounds, where transference of MRSA from the patient’s nasal passage to the burn wound is second.\[^{4,6-8}\] Due to the highly drug-resistant nature of this pathogen, as well as the ability of *Pseudomonal* strains to rapidly form resistances, application of more than one antibiotic provides the best treatment and diminishes the likelihood for the microbe to develop further resistance.\[^{9,10}\]

Also observed in burn wounds and in the underlying soft tissue is the presence of biofilms. Biofilms are complex environments of microorganisms that work together in a synergistic fashion in order to facilitate their growth and survival. These communities are often found colonizing surfaces and are able to form a protective polymeric matrix around the film that is extremely difficult for antimicrobials to penetrate. However, this biofilm community has also been observed in burn wounds even though there is no solid surface for the bacteria to adhere to in order to originate the biofilm formation. This is most likely due to the high number of various microbes present in the wound
environment and the endless supply of nutrients present in the site. *P. aeruginosa*, *S. aureus*, and *S. epidermidis* are the leading microbes involved in biofilm formation in burn wounds (Table 4.1).\(^2\) \(^3\) Since burn wounds provide an optimal environment for microbial growth and colonization, it is not surprising that biofilm communities develop here. Microbiological testing of burn wounds has shown that mature biofilms have been observed in burn wounds after only 10 hours post-trauma.\(^2\) The presence of such bacterial organizations makes the infection even more difficult to treat with a single antimicrobial, and is often even difficult to treat with multiple antibiotics, especially when the drugs are not able to penetrate the outer layers of the protective film barrier. Also, the entire biofilm takes on the characteristics of its strongest bacterium present, therefore, if one microbe within the colony expresses multiple drug resistance, the entire biofilm will experience this resistance. If only a single microbe or a few microbes survive the initial antimicrobial treatment, those microbes, coined persister cells, can re-create the entire biofilm community, where now every cell will express this multiple resistance.\(^2\) Therefore, a drug delivery vehicle is needed that can deliver more than one antimicrobial deep into the tissue where the pathogenic microorganisms are cultivating, and penetrate biofilms that may have already been established within the wound or surrounding tissue.
Table 4.1. Common microbes found in many biofilms causing human infection. This data was reprinted with the permission of the publisher.\cite{[3]}

<table>
<thead>
<tr>
<th>Infection or Disease</th>
<th>Common Biofilm-Causing Microbes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dental caries</td>
<td>Acidogenic Gram-positive cocci (e.g., <em>Streptococcus</em>)</td>
</tr>
<tr>
<td>Periodontitis</td>
<td>Gram-negative anaerobic oral bacteria</td>
</tr>
<tr>
<td>Otitis media</td>
<td>Non-typable strains of <em>Haemophilus influenzae</em></td>
</tr>
<tr>
<td>Musculoskeletal infections</td>
<td>Gram-positive cocci (e.g., staphylococci)</td>
</tr>
<tr>
<td>Necrotizing fasciitis</td>
<td>Group A streptococci</td>
</tr>
<tr>
<td>Biliary tract infection</td>
<td>Enteric bacteria (e.g., <em>Escherichia coli</em>)</td>
</tr>
<tr>
<td>Osteomyelitis</td>
<td>Various bacterial and fungal species</td>
</tr>
<tr>
<td>Bacterial prostatitis</td>
<td><em>E. coli</em> and other Gram-negative bacteria</td>
</tr>
<tr>
<td>Native valve endocarditis</td>
<td>Viridans group streptococci</td>
</tr>
<tr>
<td>Cystic fibrosis pneumonia</td>
<td><em>P. aeruginosa</em> and <em>Burkholderia cepacia</em></td>
</tr>
<tr>
<td>Meliodosis</td>
<td><em>Pseudomonas pseudomallei</em></td>
</tr>
</tbody>
</table>

**Nosocomial infections**

| ICU pneumonia                         | Gram-negative rods                                                                               |
| Sutures                               | *Staphylococcus epidermidis* and *S. aureus*                                                      |
| Exit sites                            | *S. epidermidis* and *S. aureus*                                                                 |
| Arteriovenous shunts                  | *S. epidermidis* and *S. aureus*                                                                 |
| Schleral buckles                      | Gram-positive cocci                                                                              |
| Contact lens                          | *P. aeruginosa* and Gram-positive cocci                                                           |
| Urinary catheter cystitis             | *E. coli* and other Gram-negative rods                                                           |
| Peritoneal dialysis (CAPD) peritonitis| A variety of bacteria and fungi                                                                   |
| IUDs                                  | *Actinomyces israelii* and many others                                                           |
| Endotracheal tubes                    | A variety of bacteria and fungi                                                                   |
| Hickman catheters                     | *S. epidermidis* and *C. albicans*                                                               |
| Central venous catheters              | *S. epidermidis* and others                                                                      |
| Mechanical heart valves               | *S. aureus* and *S. epidermidis*                                                                 |
| Vascular grafts                       | Gram-positive cocci                                                                              |
| Biliary stent blockage                | A variety of enteric bacteria and fungi                                                           |
| Orthopedic devices                    | *S. aureus* and *S. epidermidis*                                                                 |
| Penile prostheses                     | *S. aureus* and *S. epidermidis*                                                                 |
4.2 Multiple Antimicrobials for Treatment of Burn Wound Infections

There is a well-known precedent for the use of multiple broad-spectrum antimicrobials for topical treatment of burn wound infections, and has been shown to be the most optimal choice due to the observed therapeutic value.\[^1\] Topical creams, such as the newest to the market, Flamazine, are now utilizing multiple antimicrobials in their formulations in an attempt to combat the high variability of bacterial and fungal species present in burn wounds. The leading antimicrobial currently used in such multi-drug formulations is polymixin B due to its broad-spectrum antimicrobial activity and water insoluble nature. Other anti-infectives used for these topical treatments are amphotericin B, due to its potent antifungal activity, and many sulfa-containing antimicrobials. Antimicrobial drugs for combination in topical anti-infective treatments must cover both facultative and anaerobic microbes present in the intestinal and skin flora, which commonly migrate into burn wounds after injury.\[^1\] Skin and tissue banks often employ the same mixture of antimicrobials as is used for mammalian cell culturing media to treat incoming donated tissued to prevent contamination, including various combinations of penicillin, streptomycin, kanamycin, gentamicin, nystatin, tobramycin, co-trimoxazole, vancomycin, and amphotericin B, to name a few.\[^9\] The majority of these antimicrobials are water-soluble, and thus are used to treat the excised tissues by soaking the tissues in the solution. However, for topical burn wound treatment, this practice is not sufficient due to the inability of the water soluble antimicrobials to penetrate the burn wound eschar or reach deep into the wound bed, where the majority of the colonizing microbes lie. Therefore, the currently used topical treatments for burn wound infections are limited to the water insoluble antimicrobials, which may not be the most effective antimicrobials for the highly resistant microbes often colonizing and forming biofilms in these wound beds. Also, while the combination of multiple antimicrobials can have a synergistic effect of the antibacterial activity, unwanted interactions have also been observed when drugs are combined into a single antimicrobial formulation.\[^10\] If not formulated properly, resistance to the antibiotics can form faster than when using only a single antimicrobial in the formulation.\[^10\] Therefore, there is a dire need for a drug delivery system that will allow the combination of both water-soluble and water-insoluble antimicrobials where the antibacterial activity for the formulation has been optimized for synergistic activity.

Hospital studies have shown that gender, age, and also the geographic location of the hospital heavily influences the strains and species of bacteria and fungi present in a burn wound infection. However, in almost all studies performed, the two most predominant pathogens found in nearly every burn wound examined were \textit{Staphylococcus aureus} and \textit{Pseudomonas aeruginosa}. These two pathogens, while differing by gram distinction, are both highly opportunistic and are able to cause severe infection, sepsis, and toxic shock syndrome, and each produces high rates of morbidity when untreated. Both of these microbes are also well known for their ability to rapidly develop resistance to antibiotics, especially \textit{S. aureus}, whose infamous antibiotic-resistant strain methicillin-resistant \textit{S. aureus} (MRSA) has been the cause of nearly 100,000 deaths each year in the United States. Therefore, to develop a drug delivery system that possesses potent anti-MRSA and anti-\textit{Pseudomonas} activity our laboratory is examining nanoparticle polymers that
contain at least one or more antibiotics active against each of these microbes that work synergistically to optimize the antibacterial activity against these microbes.

4.3 Synthesis of Multi-Drug Conjugated Polyacrylate Nanoparticles

4.3.1 MDNP2

As discussed in Chapter 3, numerous single drug-conjugated nanoparticle emulsions were successfully synthesized and their antibacterial activity assessed via zone of inhibition (ZOI) and minimum inhibitory concentration (MIC) analysis. Many of the drug-conjugated emulsions displayed antibacterial activity, even against microbes that were inherently resistant to the antibiotic. However, in many instances, the ZOI assay was not an effective means of antibacterial analysis. Ciprofloxacin-conjugated emulsions gave zones of inhibition only slightly smaller in diameter than the original ciprofloxacin monomer, yet the β-lactam conjugated nanoparticle emulsions (NP16-NP19) did not show any antibacterial activity through ZOI analyses. It was unclear why this difference in Kirby Bauer results occurred between the β-lactam and cipro-conjugated emulsions.

The antibacterial results shown in Chapter 3 promoted the incorporation of the penicillin-based antimicrobials in the first multi-drug conjugated nanoparticle formulation, due to the observed activity against resistant microbes when in the nanoparticle delivery system. Since this was the first of the multi-drug formulation emulsions, all of the acrylated commercially available drug monomers were incorporated into the delivery system in order to begin optimization of the system (Figure 4.3). This would establish whether the individual emulsions synthesized or the combination of all the monomers in one formulation would yield the most optimal antimicrobial activity in vitro.

![Acrylated drug monomers](image1)

**Figure 4.1.** Acrylated drug monomers incorporated in MDNP2 nanoparticle emulsion.
Figure 4.1 shows the structures for the six acrylated antibiotics used to prepare the multi-drug conjugated nanoparticle emulsion MDNP2. Theoretically, the anti-*Staphylococcal* activity of the first multi-drug conjugated system, which was formulated with four different penicillin-based drug monomers, showed be extremely potent if all of the drug monomers work in a synergistic fashion. The two ciprofloxacin acrylamide monomers (7 and 8) were chosen due to their exceptional antibacterial activity against *P. aeruginosa*. The difference between cipro acrylamide monomers 7 and 8 was in the acryl side chain (acrylamide versus methacrylamide), which allows for potentially different physical properties of the resulting nanoparticle polymer. Therefore, the anti-*Pseudomonal* activity of the multi-drug conjugated system MDNP2 should outweigh that observed for the individual ciprofloxacin-conjugated emulsions if the two ciprofloxacin (cipro) acrylate monomers are able to work synergistically against the microbe in the multi-drug formulation. In addition, the four β-lactam analogues (11, 16, 18 and 19) were studied due to their common utility as broad-spectrum antibiotics.

**Table 4.2.** Formulation of MDNP2 nanoparticle emulsion. The total weight of all the drug monomers combined that were added to the emulsion polymerization was 132.9mg, which contributed to 3.2% of the solid content of the emulsion (second table).

<table>
<thead>
<tr>
<th>Acrylated Drug Monomer</th>
<th>Weight (mg)</th>
<th>% of Total Drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>40.0</td>
<td>30.10</td>
</tr>
<tr>
<td>8</td>
<td>20.0</td>
<td>15.05</td>
</tr>
<tr>
<td>11</td>
<td>10.0</td>
<td>7.52</td>
</tr>
<tr>
<td>16</td>
<td>40.0</td>
<td>30.10</td>
</tr>
<tr>
<td>18</td>
<td>20.0</td>
<td>15.05</td>
</tr>
<tr>
<td>19</td>
<td>2.9</td>
<td>2.18</td>
</tr>
<tr>
<td>All 6 Drugs</td>
<td>132.9</td>
<td>100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mass (g)</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Drug</td>
<td>0.133</td>
<td>3.2</td>
</tr>
<tr>
<td>Butyl acrylate</td>
<td>2.59</td>
<td></td>
</tr>
<tr>
<td>Styrene</td>
<td>1.11</td>
<td>12.8</td>
</tr>
<tr>
<td>Water</td>
<td>16.00</td>
<td>80</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate</td>
<td>0.12</td>
<td>3.0</td>
</tr>
<tr>
<td>Potassium persulfate</td>
<td>0.04</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Table 4.2 shows the amounts of each antibiotic acrylate and formulation conditions. The multi-drug conjugated emulsion MDNP2 was based on that of the drug-free poly(butyl acrylate-styrene) emulsion CNP5 (page 69, Chapter 3), with the same ratio and amount of co-monomers, surfactant, and radical initiator. Sodium dodecyl sulfate (SDS) and potassium persulfate were the surfactant and radical initiators used for the polymerization, where 3% surfactant and 1% radical initiator was employed in the emulsion formulation. However, this amount of surfactant was found to be less than optimal during initial \textit{in vitro} cytotoxicity analyses (Chapter 7). Also, some of the acrylated drug monomers utilized in this formulation were found to not exhibit antibacterial activity when tested \textit{in vitro} (Chapter 3), and therefore, the multi-drug formulation was reformatted in order to include a less cytotoxic amount of SDS (1%) and also include drug monomers that have shown better antibacterial activity when individually polymerized within the nanoparticle delivery system.

AFM imaging analysis was performed for the resulting multi-drug conjugated nanoparticle emulsion as previously described in Chapter 3. Figure 4.2 shows that the resulting nanoparticles present in MDNP2 are uniformly spherical with a small size range distribution, averaging 14 to 21nm in diameter.

![AFM image of MDNP2](image)

\textbf{Figure 4.2.} AFM image of MDNP2. Image to the left is a full 5x5 \( \mu m \) scan of the deposited emulsion. Image to the right is a focused scan of an individual particle observed followed by 3D transformation of the scan using the IGOR software system.

AFM imaging was first performed on the multi-drug conjugated nanoparticle emulsion over all of the other emulsions previously synthesized (Chapter 3), therefore, various deposition techniques were performed for this emulsion in order to optimize imaging. Imaging was performed for the deposited emulsion under both liquid and dry conditions, and also after various deposition times and concentrations. Figure 4.2 shows one of the original AFM images taken of the deposited emulsion on the glass coverslip, where a
longer deposition time was employed, along with no rinsing of the deposit prior to nitrogen or air drying and imaging.

![AFM Image](image.png)

**Figure 4.3.** AFM of individual particle and polymer strands present in MDNP2. The particle size is 14.7nm and the polymer strands (arrow) are approximately 0.8nm tall. Dry imaging of the deposited emulsion using the AFM tapping-mode was utilized for these images.

In Figure 4.3, both the long chain polymer strands (arrow) and the solid spherical nanoparticle were observed in the emulsion. This data substantiated the TEM observations reported in Chapter 3 for the drug-free emulsion, where the observed polymer strands were poorly visible but still present in the emulsion. Here, the polymer strands are clearly visible and well defined for the emulsion, indicating that both constituents are present in the emulsions. Varying the deposition technique utilized for AFM imaging allowed only the nanoparticle constituents to be visualized in the later emulsions, where a short deposition time followed by rinsing of the deposition surface afforded only the anionic nanoparticles adhering to the glass surface for imaging.

### 4.3.2 MDNP1

After analysis of MDNP2 by antibacterial assays *in vitro* (Section 4.4) and analysis of the individual drug-conjugated nanoparticle emulsions in Chapter 3, it was determined that this antimicrobial emulsion system was not working synergistically to afford heightened antibacterial activity against the majority of the bacteria analyzed. Instead, what was observed was a diminished activity when compared to the individually polymerized cipro-containing emulsions, although the activity was greater for the multi-drug conjugated emulsion than was observed for the individual penicillin-containing emulsions. Also, the antimicrobial activity of the penicillin G acrylimide 13 was more potent than all of the other acrylated penicillin analogues present in MDNP2, and
therefore, 13 replaced all of the other penicillin analogues in MDNP2 to afford the new multi-drug conjugated nanoparticle emulsion formulation MDNP1 (Figure 4.4).

![Chemical structures](image)

**Figure 4.4.** Acrylated drug monomers used to prepare multi-drug nanoparticles MDNP1.

Due to the inherent anti-MRSA activity of the $N$-thiolated β-lactam monomers synthesized in Chapter 2, it was determined that incorporation of at least two of these acrylated drug monomers in the new multi-drug formulation was necessary in order to provide the emulsion with established anti-MRSA activity that does not rely solely on the protective nature of the nanoparticle delivery system for activity, as was observed for the penicillin G acrylamide monomer. These two β-lactam monomers were also chosen for the second nanoparticle formulation due to their activity against the fungal species, *Candida*, which is commonly found invading burn wounds and adding to the bacterial infection load. These lactams have also proven to have activity against many of the gram positive microbes found in burn wounds, including *Staphylococcal* species and some *Streptococcal* species (Chapter 2).
Table 4.3. Formulation for multi-drug conjugated nanoparticle emulsion MDNP1. The total amount of all the drug monomers incorporated into the emulsion (120mg, first table) totaled 3% (w/w) of the solid content of the emulsion (second table).

<table>
<thead>
<tr>
<th>Acrylated Drug Monomer</th>
<th>Weight (mg)</th>
<th>% of Total Drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10.0</td>
<td>8.33</td>
</tr>
<tr>
<td>3</td>
<td>20.0</td>
<td>16.67</td>
</tr>
<tr>
<td>7</td>
<td>40.0</td>
<td>33.33</td>
</tr>
<tr>
<td>8</td>
<td>30.0</td>
<td>25.00</td>
</tr>
<tr>
<td>13</td>
<td>20.0</td>
<td>16.67</td>
</tr>
<tr>
<td>All 5 Drugs</td>
<td>120.0</td>
<td>100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mass (g)</th>
<th>Percentage %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Drug</td>
<td>0.120</td>
<td>3</td>
</tr>
<tr>
<td>Butyl acrylate</td>
<td>2.67</td>
<td></td>
</tr>
<tr>
<td>Styrene</td>
<td>1.15</td>
<td>15.5</td>
</tr>
<tr>
<td>Water</td>
<td>16.00</td>
<td>80</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate</td>
<td>0.04</td>
<td>1</td>
</tr>
<tr>
<td>Potassium persulfate</td>
<td>0.02</td>
<td>0.5</td>
</tr>
</tbody>
</table>

The multi-drug conjugated emulsion MDNP1 was formulated after the drug-free poly(butyl acrylate-styrene) emulsion CNP16, with the same ratio and amount of co-monomers, surfactant, and radical initiator (Table 4.3). SDS and potassium persulfate were again chosen as the surfactant and radical initiators, where only 1% surfactant was employed in the emulsion formulation and 0.5% potassium persulfate. Further analysis of this emulsion formulation showed optimal antibacterial activity in vitro and no toxicity in either in vitro or in vivo settings.

AFM analysis was also performed for this multi-drug conjugated emulsion system and yielded the same results as the MDNP2 nanoparticle emulsion, with uniformly spherical nanoparticles within the same size range (Figure 4.5). Therefore, changing the amount of surfactant (3% to 1% w/w) and radical initiator (1% to 0.5% w/w) did not alter the
physical characteristics of the nanoparticles, nor did reducing the number of drug monomers incorporated from six to five.

Figure 4.5. AFM image of MDNP1. The average diameter of the particles present in this emulsion are 21.0nm +/- 5.3nm. The subset image to the right of a single particle is 36nm wide and depicts a particle of 18nm in height.

AFM analysis of the multi-drug conjugated nanoparticle emulsion MDNP1 displayed uniformly spherical nanoparticles present in the emulsion, with an average particle size of 21nm. This finding is comparable to the nanoparticle size range determined for MDNP2 by AFM analysis. Also, it was determined through this assay that the particle size range for the multi-drug conjugated nanoparticles was equivalent to the size range observed for the single drug-conjugated nanoparticle emulsions determined by AFM (Chapter 3). Therefore, the addition of multiple antimicrobials to the nanoparticle drug delivery system when the drug monomers are covalently bound to the polymer backbone does not cause an increase in the average particle size. It is presumed here that if multiple antimicrobials are added to the nanoparticle delivery system via encapsulation of the drug monomer within the particle, the average particle size would be drastically increased due to the data presented for the encapsulated drug monomer nanoparticles in Chapter 3. Therefore, the choice of using the acrylated bound drug monomers for the multi-drug containing nanoparticle system instead of opting for encapsulation of the monomers was the most optimal.
4.4 Determination of Microbiological Activity of Multi-Drug Conjugated Nanoparticles MDNP1 and MDNP2

In order to determine the effectiveness of the multi-drug conjugated nanoparticle emulsions MDNP1 and MDNP2 and the resulting polymer films, antibacterial assays were performed against a variety of bacterial strains and species, by varying the amounts of total drug concentration. Prior to analysis, the emulsions were diluted to 1mg total drug weight per 1mL of emulsion with nano-pure water. This allowed equivalent amounts of the total drug present in the multi-drug conjugated emulsion to be compared to that of the single-drug conjugated emulsion, and also to the individual drug monomer dissolved in DMSO or water. Although this is not a direct comparison, since it is the total amount of drug being calculated in the dilution and not one individual drug, this still provides the relationship necessary for accurate comparative analysis of antibacterial activity.

4.4.1 Introduction

Antibacterial testing of the multi-drug conjugated nanoparticle systems was performed against various strains of *S. aureus*, including numerous strains of MRSA. Other microbes analyzed included *Bacillus* spp. and *Pseudomonas aeruginosa*, both of which have been identified as two of the causative microbes in burn wound infections. Since *P. aeruginosa* is the most consistently found and often the most deadly pathogen colonizing burn wounds, it was imperative that sufficient activity be observed against this microbe in order to consider the emulsion for topical application of burn wound infections. It has been established that many of the pathogens found in burn wounds are able to form complex cooperative environments known as biofilms within the wound in less than 24 hours post thermal trauma (Section 4.1), which are extremely difficult to treat; therefore it was paramount that the emulsion be able to eliminate these microbes from the wound as early as possible. Also, it is proposed that the polymer film that forms upon application of the aqueous emulsion on the wound will protect the burn wound from these and other invading pathogens. Therefore, the polymer films were analyzed for *in vitro* antibacterial activity against these same microbes.

4.4.2 Antibacterial Activity of MDNP2

The original multi-drug conjugated nanoparticle emulsion formulated was the most thoroughly analyzed of the two multi-drug systems, and therefore, more data is presented here for this emulsion than is for MDNP1. This was due to the fact that the nanoparticle formulation for MDNP2 was only later determined to be slightly cytotoxic to human dermal fibroblast cells longer after the original nanoparticle formulation was synthesized and analyzed. However, aside from this fact, which is explored later in Chapter 7, the initial multi-drug conjugated nanoparticle emulsion (MDNP2) did not display the antibacterial activity that was expected of it. This was due to the lack of antibacterial
activity observed for the penicillin-based drug monomers that were incorporated into this nanoparticle emulsion. As seen in Chapter 3, these acrylated monomers displayed little to no antibacterial activity against the *Staphylococcal* strains when individually polymerized in the nanoparticle emulsion system. Therefore, the observed antibacterial activity for MDNP2 was the result of the ciprofloxacin acrylamide monomers present in the emulsion. As seen below in Figure 4.6, activity was expressed for the emulsion and polymer films against many of the bacterial species present in burn wounds at both the onset of infection and also later in the infection development, but this activity was not nearly as potent as the individually polymerized ciprofloxacin acrylamide nanoparticles.

**Figure 4.6.** Antibacterial activity of multi-drug conjugated polymer film and emulsion against *S. aureus* (ATCC 25923). In the plate shown to the left, 500µL of emulsion (20% solid content) was added to the surface of the agar, and allowed to dry to form a thin white film. KG32 represents MDNP2 in the assays, and KRG15 represents CNP15.

For the Kirby Bauer assays presented in Figure 4.6, the emulsion was added to the agar wells based on the amount of total drug incorporated. A 1mg/mL concentration of total drug in the emulsion was obtained by dilution of the concentrated emulsion. 100µg, 50µg, and 20µg of total drug in equal volumes of emulsion (100µL, 50µL, 20µL) was analyzed by ZOI in order to present enough emulsion to the assay in order to observe the antibacterial activity. This was done due to the inherent problems mentioned in Chapter 3 that go along with analyzing the nanoparticle emulsions by this assay. As previously stated, due to the vast degree of long chain polyacrylate polymer strands present in the emulsion that are not part of the nanoparticle, and since these long strands are not able to diffuse through the agar apparent by the white ring coating the inside of the agar well in Figure 4.6 only a portion of the drug content is diffusing into the agar.

A 0.5mL aliquot of concentrated (20% solid content) emulsion was used to form the polymer films for ZOI analysis. The emulsion was added on top of the already inoculated
agar, and the polyacrylate films were allowed to solidify in the biosafety cabinet prior to incubation for bacterial growth, thereby allowing the solid polymer film to be interacting with the proliferating bacteria and not the emulsion. The data present in Figure 4.6 shows that the polymer film formed from the multi-drug conjugated nanoparticle emulsion (MDNP2) displayed very good anti-*Staphylococcal* activity, which was not observed for the drug-free polymer film formed from the emulsion CNP15. Therefore, the observed antibacterial activity was not due to leaching of the excess surfactant or radical initiator into the surrounding agar, but was due to the direct interaction of the microbes with the both the nanoparticles that appear to have diffused into the agar prior to film formation and from the polymer film, since no bacterial growth was observed under the polymer film and since a zone of inhibition was formed surrounding the polymer film. The bacterial permeability studies described in Chapter 5 also contributed to the understanding of the antibacterial nature of the polymer films, as did the carbon sources assays presented in that chapter as well. Therefore, it was concluded that both the nanoparticle emulsion and the resulting polymer films possessed antibacterial activity.

![Figure 4.7. Antibacterial activity of MDNP2 against *P. aeruginosa*. KG32 in the image represents MDNP2. A 1mg/mL concentration of total drug in the emulsion was obtained by dilution of the concentrated emulsion. 100µg, 50µg, and 20µg of total drug in equal volumes of emulsion (100µL, 50µL, 20µL) was analyzed by ZOI.](image)

The zones of inhibition measured for the multi-drug conjugated emulsion against *P. aeruginosa* are described in Table 4.4, along with the MIC values for the emulsion, plus the values of the ciprofloxacin acrylamide monomers that provide the emulsion with the anti-*Pseudomonal* activity. Interestingly, the degree of anti-*Pseudomonal* activity for the nanoparticle emulsion at 20µL volume did not produce a very large ZOI, whereas for the staphylococci-based assays, the ZOI measurements for the 20µL volumes of emulsion
were only slightly smaller than were observed for the higher volumes of emulsion in the agar well.

Table 4.4. Anti-\textit{Pseudomonal} activity of MDNP2 and ciprofloxacin acrylamide monomers and emulsions. ZOI data is presented for 20\(\mu\text{g}\) of cipro monomer in 20\(\mu\text{L}\) of emulsion or DMSO.

<table>
<thead>
<tr>
<th>Sample</th>
<th>ZOI (mm)</th>
<th>MIC ((\mu\text{g/mL}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cipro</td>
<td>40</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>15</td>
<td>128</td>
</tr>
<tr>
<td>NP7</td>
<td>22</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>NA</td>
<td>128</td>
</tr>
<tr>
<td>NP8</td>
<td>21</td>
<td>4</td>
</tr>
<tr>
<td>MDNP2</td>
<td>11 (13)</td>
<td>4</td>
</tr>
</tbody>
</table>

The data in Table 4.4 makes a bold statement towards the ability of the \textit{Pseudomonal} bacterium to cleave the cipro drug monomer from the backbone of the polymer chain, thereby releasing the active cipro monomer. Obviously, the commercially available form of cipro has potent activity against \textit{Pseudomonal} species and is one of the most commonly prescribed antibiotics for these infections. However, after chemical modification of this drug monomer into the acrylamide form (7 and 8), the antibiotic loses almost all of its antibacterial activity against this microbe. Yet when co-polymerized with butyl acrylate and styrene, the drug regains its activity against this microbe and shows potent activity \textit{in vitro}. This finding is the most persuasive data yet to support the occurrence of bacterial cleavage of the drug monomer from the polymer backbone, thereby releasing the active form of the drug monomer. This data also aids in the confirmation that the observed antibacterial activity in the Kirby Bauer assays is not due to the non-polymerized acrylate drug monomer dissolved in the emulsion, but that the drug must be being cleaved off the polymer in order for the antibacterial activity to be detected. The data in Table 4.4 and the ZOI assay shown in Figure 4.7 was repeated a minimum of 3 times in order to verify these findings, and in each assay performed, these findings were further solidified as accurate and reproducible. Therefore, in terms of \textit{in vitro} anti-\textit{Pseudomonal} activity, the microbe must cleave the covalently bound cipro monomer from the polymer backbone in order to release the active drug monomer.
In Figure 4.8, it appears that there is a diminished degree of antibacterial activity against MRSA for the multi-drug conjugated emulsion **MDNP2** (KG32) versus the ciprofloxacin-only conjugated emulsions **NP7** and **NP8** (KG13 and KG11 respectively). This decrease in activity was attributed to the lowered amount of ciprofloxacin acrylamide monomers present in the multi-drug conjugated emulsion versus the high concentration of the monomers in the single-drug conjugated emulsions **NP7** and **NP8**. This also helped establish the fact that the other drug monomers in the emulsion (**11, 16, 18, and 19**) were not contributing to the anti-*Staphylococcal* activity to a large extent. Since the single-drug containing emulsion **NP11, NP16, NP18, and NP19** did not produce large zones of inhibition against *S. aureus*, as shown in Chapter 3, this further solidified this hypothesis. While the MIC data for these single drug-containing emulsions did show some anti-*Staphylococcal* activity, the MICs were not very strong, indicating
that the ciprofloxacin acrylamide monomers present in MDNP2 are the cause of the observed anti-\textit{Staphylococcal} activity for this emulsion.

Due to the diminished antibacterial activity observed for the MDNP2 nanoparticle emulsion as compared to the individual ciprofloxacin-conjugated emulsions, it was determined that the acrylated monomers incorporated into this delivery system should be adjusted in order to provide a synergistic effect of the drugs present in the nanoparticle formulation, rather than the observed diminished antibacterial activity. Also, in Chapter 7, it was determined that the nanoparticle emulsions formulated with 3% surfactant and 1% radical initiator inflict some toxic effects on human dermal fibroblast cells, even when in the polymer film form, formulations containing more than 1% surfactant were non-favorable in terms of cytotoxicity. Therefore, the need for a new multi-drug conjugated nanoparticle formulation with no cytotoxic effects and a heightened antibacterial activity against microbes commonly found in burn wounds was addressed.

\subsection*{4.4.3 Antibacterial Activity of MDNP1}

MDNP1, formulated with 1% surfactant and 0.5% radical initiator, was analyzed for antibacterial activity \textit{in vitro} against some of the common pathogens present in burn wounds. Prior to analysis, the emulsion was diluted to 1mg total drug weight per 1mL of emulsion. This allowed the emulsion to be tested \textit{in vitro} at the same drug concentrations as the monomers dissolved in DMSO were previously tested, providing comparable results to both the drug monomers, but also to the single drug-conjugated emulsions and MDNP2.

![Figure 4.9. Anti-\textit{Staphylococcal} activity of MDNP1. Activity was tested against both \textit{S. aureus} (ATCC 25923) and MRSA (ATCC 43300). A 1mg/mL concentration of total drug in the emulsion was obtained by dilution of the concentrated emulsion. Volumes of the diluted emulsion (100µL, 50µL, 20µL) were analyzed by ZOI.](image)
The images in Figure 4.9 show that the multi-drug conjugated nanoparticle emulsion MDNP1 is very potent even at a low total drug concentration of 20µg in 20µL emulsion. This is most likely due to the potent effect of the multiple drugs on the bacteria, where all of the drugs present in the emulsion have activity against *S. aureus*.

![Image of Kirby Bauer assay](image)

**Figure 4.10.** Kirby Bauer assay for MDNP1 against *B. anthracis*-Sterne.

The anti-*Bacillus* activity of the multi-drug conjugated emulsion was considered to be extremely potent, and the degree of ZOI variance among the different volumes of emulsion analyzed was almost none. This indicates that there is a higher degree of nanoparticles present in the emulsion than there is the long polyacrylate strands. However, these polymer strands are still present in the emulsion, as observed by the white polymer rings surrounding the inside of the agar wells and also observed slightly diffused into the surrounding agar. Since the non-particle based polymer strands are required for the formation of the protective film for burn wound and other topical wound applications, the presence of the white polymer precipitate in these Kirby Bauer assays is a positive observation, regardless that this diminishes the ability of the assay to provide an accurate antibacterial activity analysis.
Table 4.5. Zone of inhibition data for MDNP1 nanoparticle emulsion. Emulsion is applied to the agar wells in a 1mg of total drug per mL emulsion.

<table>
<thead>
<tr>
<th>Volume of Emulsion (µL)</th>
<th>B. anthracis-Sterne</th>
<th>S. aureus</th>
<th>MRSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>31 (33)</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>50</td>
<td>27 (30)</td>
<td>27</td>
<td>28</td>
</tr>
<tr>
<td>20</td>
<td>25</td>
<td>24</td>
<td>25</td>
</tr>
</tbody>
</table>

*( ) = partial inhibition

ZOI analysis of the multi-drug conjugated nanoparticle emulsion MDNP1 displayed equal activity against S. aureus and MRSA, indicating that the penicillin-inactivating enzyme β-lactamase has no effect on the activity of the antibiotics when in this delivery system. It was determined here that the ZOI data observed for the emulsion at 20µL was comparable to the ZOI data for the emulsion at 100µL against all 3 bacterial species analyzed, indicating that only a small volume of the emulsion is needed to observe potent antibacterial activity. Also, the data in Table 4.5 for the anti-Staphylococcal ZOI assay showed that the emulsion MDNP1 was more potent than either of the individual acrylated N-thiolated β-lactam monomers when applied to the assay dissolved in DMSO (Chapter 2), therefore, addition of these monomers to the nanoparticle emulsion increased the observed activity in vitro. The activity for MDNP1 against the various staphylococci strains was much more potent than was observed for MDNP2, and was also more potent than the individually polymerized cipro acrylamide emulsions (Chapter 3), indicating that the mixture of antimicrobials present in MDNP1 possessed a synergistic effect in the treatment of S. aureus and MRSA in vitro.

4.5 Conclusions

The multi-drug conjugated nanoparticle emulsion system MDNP1 appears to be an optimal treatment for burn wound infections. This drug delivery system contains antibiotics that are synergistic and provide overlapping activity against the pathogens commonly found colonizing burn and other skin and soft tissue wounds and abrasions. The equipotent activity in vitro of the emulsion against both MSSA and MRSA strains of S. aureus makes this drug delivery system optimal for treatment of infections where MRSA may be present. Also, the protective nature of the nanoparticle delivery system and the fact that all of the antibiotics present in the nanoparticle emulsion are active against S. aureus and MRSA should prevent the development of bacterial resistance to one or more of the antibiotics present in the emulsion. Due to the highly potent anti-
Pseudomonal activity of the ciprofloxacin monomers present in the emulsion, and the anti-fungal activity of the acrylated N-thiolated β-lactam monomers, this multi-drug conjugated nanoparticle delivery system has the potential to eradicate the majority of the microbes commonly found in burn wounds. This delivery system also has the potential to treat biofilms formed within the wounds due to the highly lipophilic nature of the nanoparticles, as well as the multiple antimicrobial treatment presented in the nanoparticles that would be able to combat the various pathogens present in the biofilms. Therefore, the multi-drug conjugated nanoparticle emulsion system has the potential to revolutionize the treatment of burn wound infections and needs to be further explored for in vivo efficacy and toxicity.

4.6 References

CHAPTER 5

MECHANICAL PROPERTIES OF SMART POLYACRYLATE POLYMER FILMS FOR BIOMEDICINAL APPLICATIONS

5.1 Introduction

Biocompatible polymer films are of great utility in the medical field, including for prostheses, sensors, and artificial skin development. These polymers have been researched and optimized for many years in order to improve current biomedical devices, and are now being applied to new biotechnologies such as sensors, microarrays, lab-on-a-chip technologies, immunoassays, protection from foreign body response, and as new detection materials. Some of these biocompatible films are protein-based, as seen in spider silk films,[1] and some are synthetic-based, such as polycarbonates, polyethylene glycol (PEG), ε-caprolactone, poly (L-g lactonic acid) (PLGA), porous poly (D,L- lactic acid) (PDLLA), polyactic acid (PLA), polyglycolide (PGA) and the copolymer PGLA, multilayer films, and poly (L-lactic acid) (PLLA).[2-7] Some of these synthetic polymers can be made absorbable or biodegradable upon secondary chemical modifications, and other bioabsorbable polymer materials have also been studied for medicinal uses, including natural collagen-based sutures, and chitosan- and hyaluronic acid-based polymers.[8]

![Structure of PLGA and PLA polymers.](image)

**Figure 5.1.** Structure of PLGA and PLA polymers.

Other well-known synthetic polymers, such as polyacrylates, have been used in medicine in many applications since the 1960’s due to their lack of toxicity, especially in prosthetics, bone cement, wound dressings, and absorbent materials. [9-15] Various forms of polyacrylate polymers have been studied for biomedical applications, including polymer films and coatings, gels, and liquid emulsions, and in delivery of insoluble drug monomers to target sites within the body.[16-29] Since polyacrylates have been one of the most widely used polymers for both commercial and biomedical applications to date, and
is therefore of considerable interest to our laboratory for future developments in these areas.

![Bar chart showing tensile strengths of various biological materials](image)

**Figure 5.2.** Comparison of the tensile strengths of various biological materials.\textsuperscript{[30, 31]}

### 5.1.2 Synthetic Polymers

Tissue engineering has been a widely growing area of research in the field of biotechnology and has become the mainstay of bioengineering; a promising new discipline that has already been integrated into many universities’s engineering colleges. This interdisciplinary area of research revolves around the use of various cell types with natural and/or synthetic materials to engineer matrices for partial or whole replacement or improvement of biological tissues.\textsuperscript{[47]} The products of this type of research play a major role in advancing and optimizing burn wound treatment, heart valve and other vascular replacements, and organ replacement systems, making this a major area of interest in the biomedical engineering field. This field often requires biological and synthetic-based systems to be combined in an effort to obtain scaffolds with equivalent properties as the tissue it wishes to replace, therefore, an understanding of both systems individually and in combination is critical.\textsuperscript{[32-39]} Many of the aforementioned polymers have been investigated for roles in tissue engineering, especially semi-synthetic polymers that are bioabsorbable. However, for these polymers to be considered for tissue replacement, the mechanical properties of the polymers must be known and be compatible with the tissue in question. Many factors must be analyzed for a material prior to its use in a tissue scaffold, including the length of time prior to complete biodegradation, when the material loses its mechanical properties, and the point at which the material is completely resorbed by the body.\textsuperscript{[6]} Complete lack of cytotoxicity must
also be a property of the polymer for such an application, since target cells also constitute the scaffold, and it must promote cellular adhesion from the surrounding cells to aid in scaffold integration, and the polymer must not be chewed apart by the body’s immune system, as is often seen in breast implant materials.

Some of the most commonly used polymer structures for biomedical applications, including tissue scaffolds, are aliphatic polyesters such as those containing lactic acid, galactone, or glycolide as the base monomer (PGA, PLLA, PDLLA, PLGA, PLA, PEG), or in a copolymer (PGLA).\[3\-5, 7\] This is because these monomers, and in some instances the polymer form as well, are often naturally occurring in the body, which makes them non-toxic to mammalian systems, biocompatible, and often bioabsorbable.\[3, 6\]

Polycarbonate and ε-caprolactone polymer films have also been incorporated for biomedical purposes, including tissue scaffolds due to the elastomeric property of the polymer;\[2, 6\] however, the optimal mechanical properties of these films is highly dependent on the molecular weight of the polymer, and is therefore difficult to maintain batch-to-batch and in vivo.\[2\] The biocompatible nature of the polyesters has allowed them to be incorporated into numerous sensor and microarray systems, as well as considered for tissue scaffolds, implants, and grafts, and research is still looking at improving these films for optimization in such systems. The film structures are often added to such systems using lithography, doping, or electrospinning techniques,\[3, 7, 40\] and can be formed in a variety of different three dimensional patterns and with various degrees of porosity on a surface of a membrane, sensor, array, scaffold, or other platform. This provides even further utility of these types of synthetic-based polymer films in biomedical applications.

Once added to the surface of the intended system, these polymer films can often be further modified to provide additional utility to the system. This is often seen when these polymer films are used as detection interfaces for biophysical and biochemical applications.\[7\] When designing a microarray or other form of biological detection system, films are frequently modified after application to the system to include the addition of an enzyme or other biomolecule that binds to the surface of the film and interact with what the system is trying to detect. These biomolecules are usually a permanent addition to the film through either covalent or ionic bonding, and are able to retain their native biological activity. Specific polymers can also be chemically modified after synthesis to make them biodegradable, which allows them to be developed for tissue engineering, or gives them additional utility in the system they are bound to.\[2, 6\]

However, the mechanical properties of the polymers must be in synch with the application in order for it to be used. Many of the biodegradable polymers mentioned above (PGA, PLA, PEG) do not have optimal mechanical properties for such applications as tissue engineering due to their brittle nature,\[6\] indicating that they not function equal to the native tissue. Other issues for these polymers come from the quickness with which they are degraded in vivo, thus not making them the optimal choice for a long term in vivo application, but a good choice for microarray detection materials due to their ability to bind biological entities post-synthetically. Other issues arise when a polymer requires cross-linking in order for it to retain the shape required or to maintain optimal mechanical
properties. Cross-linking, either through chemical or photochemical cross-linking, can have very detrimental affects when in vitro and in vivo, and thus most research tries to avoid this whenever possible. Therefore, there is a great need for improvement still in this area of research, and optimal synthetic materials still have yet to be found for applications in tissue engineering and other biomedical applications.

5.1.3 Biorubbers

Within nature, there are many materials that are integral to life that behave as rubbers, or elastic materials. These pliant materials, often referred to as biorubbers, work in conjunction with many vital systems to support life through their elastic properties that allow vital tissues to expand and retract continuously. There are three key biorubber materials in living organisms: abductin in mollusks, resilin in arthropods and elastin in echinoderms and mammals, and in each organism, proper function of this material is critical for sustaining life.

The first type of naturally occurring biorubber belongs to the class of animals deemed mollusks, specifically the bivalve shellfish. These animals possess a protein-based material called abductin that is present in the ligament that acts as a hinge for the mollusk. This hinge action provides the mollusk with the ability to open and close its bivalve shell system rapidly, which allows the animal to not only feed itself, but also provides rapid protection from invading predators by allowing the animal to close itself off from its environment quickly through the elastic nature of the ligament. In winged arthropods, the resilin protein is a major part of the ligament involved in flight. The resilin component allows the elastic ligament to expand and contract rapidly, which in turn expands and contracts the thorax of the arthropod. This movement is what indirectly causes the wings that are attached to the thorax to move, creating the motion necessary for flight. While these biorubbers obviously play a crucial role in sustaining life for these animals, they appear to be involved in only one part of the animal’s anatomy, typically involved in facilitating movement of the animal, making their usefulness limited to those sole applications. However, in mammals, the biorubber material present constitutes many of the animals’ vital organs and is involved in numerous physiological aspects of life for mammals.

Elastin is a major component of human and mammalian tissue where elasticity is a requirement, such as in elastic ligament, blood vessels, lungs, skin, and heart aorta tissues. Its ability to undergo cyclic loading of stress and relaxation gives it its unique viscoelastic property that is exploited by the mammalian anatomy. Elastin is an extra cellular matrix protein that exists as an array of cross-linked fibers in the specified tissues and is formed from self-assembled tropoelastin proteins under physiological conditions. The physical properties and arrangement of these fibers within the tissue it comprises varies from tissue to tissue, depending on the tissue’s need
for elasticity and the amount of elastin present in the tissue. The degree of elasticity in a single tissue (i.e. aortic heart tissue) can vary from animal to animal in mammals due to the animal’s physiological requirements; however, in all animals, elastin fibers are insoluble proteins that have been observed as having a half life of approximately 70 years, making it one of the most stable proteins present in mammalian systems.

The mechanical properties of elastin and the other biorubber proteins describe a highly viscoelastic material with a unique biphasic stress/strain curve that consists of an initial linear region followed by an amorphous region. Elastin itself has shown tensile strengths on the range of 0.5MPa and elastic moduli of approximately 1.5MPa. However, the biological tissues that elastin comprises have shown tensile strengths up to eight orders higher when tested, due to the presence of collagen in the tissue that provides strength for the tissue. Also, when elastin is present in fibrilar formation, such as seen in elastic ligaments, the fiber is not only comprised of cross-linked elastin protein but also contains microtubules, which in turn, alter the stress/strain curve characteristics for the fiber yet do not affect its tensile strength. In terms of tissue engineering, elastin has been able to be combined with numerous synthetic polymer systems and also various solvents in order to increase its tensile strength in order to mimic the mechanical properties of biological tissues where elastin is found. This same type of biocomposite engineering has also been applied to collagen, where collagen is the biological material and a synthetic polymer is incorporated into a material that is an attempt to emulate the mechanical properties of elastin. In this study, the polymer film samples synthesized and analyzed displayed mechanical properties equal to those observed for both elastin, and elastin fibers, where viscoelastic stress/strain data and similar tensile strength and elastic moduli are observed. Also, the polyacrylate films constructed herein display the same ability to undergo numerous stress/retraction cycles as the elastin fibers, with deformations observed as high as 1000%, which is one order higher than observed for elastin fibers that undergo only 150% deformation naturally.

5.2 Emulsion Polymerization

Polyacrylates have seen much use in industry and in the medical field since the late 1960’s, yet improvements in the polymer composition need to be made in order to optimize the utility of the polymers in the medical field. Reducing foreign body responses to the polymers, eliminating biofilm formation at the surface of the polymer, and eliminating host-encapsulation of polymer-coated device or host cellular aggregation at the surface of the polymer in vivo are just some of the goals of new medicinal polymers.

In order for polymers to be developed that meet the needs of these new medicinal applications, they must maintain properties that are optimal for such uses, including possessing properties parallel to the three goals previously listed. The polymers must also abide by other parameters as well to be optimal for such uses, including displaying appropriate pore sizes and physical and mechanical properties that cater to each specific use. In the present study, a variety of polymer films formed through a single step
Emulsion polymerization process containing differing ratios of butyl acrylate (BA) to styrene (Sty) and BA to methyl methacrylate (MMA) were synthesized. The physical and mechanical properties of these films were analyzed in order to identify the most optimal films for various medicinal applications. A total of 15 different films were formed and the physical and mechanical properties were assessed, resulting in the development of numerous new and unique elastomeric polymer films for various medicinal applications that display unique properties consistent with biologically-based pliant materials. Typically, this elastomeric property is seen when BA and Sty individual block or graft polymers are blended together, where, as the amount of styrene is increased in the mixture, the viscoelastic nature and adhesive properties of the films is lost, yet the tensile strength of the films increases as does the elastic modulus.\[^9\] Here, the polyacrylate films are formed through a single step emulsion polymerization process, which provides more control over the consistency and homology of the resulting emulsion and polymer films, as well as easier handling that equates to lower costs associated with the material production. The elastomeric property observed for the blended BA and Sty polymer films is also found for the films obtained from the single-step emulsion polymerization, and equal tensile strength and elastic moduli are also observed for these films as the blended block polymer films, indicating that similar mechanical properties are observed for the polymer films regardless of the polymerization technique utilized. The polymer films were analyzed by uniaxial tension analysis, as shown in Figure 5.3 for determination of the film’s tensile strength, elastic modulus, and maximum strain.

**Table 5.1.** Formulation of emulsion polymerizations containing butyl acrylate (BA) and styrene (Sty) or methyl methacrylate (MMA).

<table>
<thead>
<tr>
<th>Emulsion</th>
<th>Co-Monomer</th>
<th>BA :Co-monomer ratio</th>
<th>Surfactant (%)</th>
<th>Initiator (%)</th>
<th>Max Stress (MPa)</th>
<th>Young’s Modulus (MPa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNP5</td>
<td>Sty</td>
<td>7:3</td>
<td>3</td>
<td>0.5</td>
<td>1.165</td>
<td>0.269</td>
</tr>
<tr>
<td>CNP6</td>
<td>MMA</td>
<td>8:2</td>
<td>3</td>
<td>0.5</td>
<td>0.449</td>
<td>0.365</td>
</tr>
<tr>
<td>CNP7</td>
<td>Sty</td>
<td>8:2</td>
<td>3</td>
<td>0.5</td>
<td>0.146</td>
<td>0.102</td>
</tr>
<tr>
<td>CNP9</td>
<td>Sty</td>
<td>7:3</td>
<td>5</td>
<td>1</td>
<td>0.643</td>
<td>0.615</td>
</tr>
<tr>
<td>CNP10</td>
<td>Sty</td>
<td>8:2</td>
<td>5</td>
<td>1</td>
<td>0.594</td>
<td>0.423</td>
</tr>
<tr>
<td>CNP12</td>
<td>MMA</td>
<td>7:3</td>
<td>3</td>
<td><strong>0.5</strong></td>
<td><strong>1.671</strong></td>
<td><strong>0.771</strong></td>
</tr>
<tr>
<td>CNP13</td>
<td>MMA</td>
<td>7:3</td>
<td>5</td>
<td>0.5</td>
<td>0.984</td>
<td>0.647</td>
</tr>
<tr>
<td>CNP14</td>
<td>MMA</td>
<td>8:2</td>
<td>5</td>
<td>1</td>
<td>0.296</td>
<td>0.326</td>
</tr>
<tr>
<td>CNP15</td>
<td>Sty</td>
<td>7:3</td>
<td>1</td>
<td>1</td>
<td>0.605</td>
<td>0.451</td>
</tr>
<tr>
<td>CNP16</td>
<td>Sty</td>
<td>7:3</td>
<td>1</td>
<td><strong>0.5</strong></td>
<td><strong>0.675</strong></td>
<td><strong>0.458</strong></td>
</tr>
<tr>
<td>CNP17</td>
<td>MMA</td>
<td>7:3</td>
<td>1</td>
<td>0.5</td>
<td>1.105</td>
<td>0.478</td>
</tr>
</tbody>
</table>
Figure 5.3. Images of polymer film before and during mechanical testing. Initial film length placed between the clamps is approximately 10mm, and the film is stretch to 100mm, approximately a 1000% deformation.

5.3 FTIR Data and Analysis

5.3.1 Introduction

FTIR spectroscopic analysis was performed on poly(butyl acrylate-styrene) (CNP16) and poly(butyl acrylate- methyl methacrylate) (CNP17) polymer films, and polymer films containing BA, Sty, and covalently bound acrylated drug moieties (NP7, NP8, MDNP1). Characteristic bands of all three co-monomers utilized in the emulsion polymerization were present in all the spectrums; however, some of these peaks were weakened or overshadowed in the polymerized form. Also, peaks from the drug moieties were not observed, however, intensified peaks were observed when the drug possessed similar moieties as the polymeric co-monomers. Spectra for the BA and Sty-containing polymer film CNP16 were similar to that of the blended block polymer films described by Cerrada et. al.[9]

5.3.2 Drug-Free Polymer Films
**Figure 5.4.** FTIR spectra for polymer film samples **CNP16** and **CNP17**.

**CNP16** displays an obvious extra set of peaks in the 800-650 cm\(^{-1}\) region of the spectrum from the C-H and ring bending absorption, indicative of the presence of an aromatic constituent,\(^{[40]}\) namely styrene. However, the commonly observed aromatic humps normally observed for styrene in the 2500 cm\(^{-1}\) region are absent when the styrene monomer is co-polymerized as the smaller side of the ratio with another acrylate monomer.\(^{[9]}\) Both samples display a strong, sharp signal in the 1700 cm\(^{-1}\) region, characteristic of a strong C=O stretching, and a sharp peak at 1150 cm\(^{-1}\) representing a C-O stretching,\(^{[9, 40]}\) where both of these peaks appear even stronger for **CNP17**, which has not one but two sets of acrylates in the sample, thereby causing the stronger absorption of signals. The C=C stretching that would be observed for the vinyl group of the acrylate and styrene monomers at 1640 cm\(^{-1}\) is absent, indicating a successful polymerization of the monomers.
5.3.3 Drug-Containing Polymer Films

![FTIR spectra for NP7 and NP8.]

**Figure 5.5.** FTIR spectra for NP7 and NP8.

The spectra in Figure 5.5 show the difference between the polymer films of NP7 and NP8. While both of these emulsions contained acrylated ciprofloxacin drug monomers and the same polymer formulation (7:3 BA:Sty, 1% surfactant, 0.5% radical initiator), the difference between the two is that NP7 is straight ciprofloxacin acrylamide and NP8 is ciprofloxacin methacrylamide. NP7 has a double peak at 2870 cm\(^{-1}\) while NP8 has only a single, sharper peak in this region. The C=O and C-O stretching peaks at 1700 cm\(^{-1}\) and 1150 cm\(^{-1}\), and all of the peaks for that matter below 2000 cm\(^{-1}\) are stronger and sharper for NP8 than they are for NP7, approximately a 5% transmittance difference in most cases. However, these differences are minimal and difficult to detect due to the low concentration of the drug moiety in the polymer film (15% of the solid polymer film). Since the amount of drug is so small, there should be no difference between the two spectra, which was the general results of the FTIR analysis, even though slight differences were observed for peak intensities. Also, signals for the free carboxylic acid moiety present on both cipro drug monomers incorporated in the polymers are missing from the FTIR spectra in the 3300 cm\(^{-1}\) region, further confirming that the amount of drug present in the films was too low to be detected.
Figure 5.6. Acrylated ciprofloxacin and β-lactam monomers polymerized in MDNP1. This emulsion is formulated with the base acrylate monomers BA and Sty in a 7:3 ratio and 1% surfactant. Monomers 1, 3, 7, 8, and 13 were individually used in the polymerization of NP1, NP3, NP7, NP8, and NP13 respectively.

Figure 5.7. FTIR analysis of MDNP1.

In the FTIR spectrum (Figure 5.9) for the multi-drug conjugated polymer film MDNP1, the peaks observed are the same as seen for NP7 and NP8, which is expected since MDNP1 contains both ciprofloxacin drug monomers 7 and 8, yet it also contains two
different β-lactam monomers (1 and 3) and the penicillin acrylamide monomer 11. Therefore, since all 5 drug monomers (Figure 5.6) contain the acrylate moiety, the C=O stretching peaks are much stronger and sharper than was observed for NP7 and NP8. The split peak in the region of 2870cm⁻¹ that was observed for NP7 is not seen here, and only a sharper single peak of weak intensity was observed.

Since the amount of drug available in the polymer film samples is between 15-25% of the solid content, it is reasonable that there would be a lack of drug monomer peaks observed for the films analyzed. Since all five of the drug monomers contain aryl rings, these moieties should exhibit overtones in the 1800-2200cm⁻¹ range of the spectrum, yet this was not observed in Figure 5.6, indicating that the drug monomers were not contributing to the FTIR spectrum. However, intensified signals were observed for the drug-containing films in the regions where C=O and C-O stretching is often found (1700cm⁻¹ and 1150cm⁻¹), therefore, the presence of additional carbonyl and/or acrylate structures is likely in this analysis.

5.4 Tensile Strength Analysis

5.4.1 Stress versus Strain Data

Under uniaxial tension, many of the films analyzed here were able to withstand maximum deformations of 100mm from originally 10mm long films, approximately 10 times their original length, without failure. On average, all films demonstrated a weak tensile strength relative to other synthetic and natural polymers (Figure 5.2), with the mean maximum stress observed for all films being around 0.7MPa. All films demonstrated reversible extensibility in these experiments, retracting to approximately 150% more than their original lengths (~15mm) after full 1000% deformation, and retracting to 100% of their original lengths (~10mm) after between 500-700% deformation, on average. Also, all films were observed retracting to 100% of their original lengths immediately after failure.
Figure 5.8. The average elastic modulus at up to 10% deformation and maximum tensile strength for each film based on an average sample size of 5 samples per film. Elastic modulus is calculated for each film from the first 10% strain.

Figure 5.8 shows for 11 drug-free nanoparticle emulsion films the elastic properties and maximum stress capabilities. Films containing 3% sodium dodecyl sulfate (SDS) as the surfactant and a ratio of 7:3 with BA being the heavier side of the ratio (CNP5 and CNP12) proved to be the strongest films, where the BA:MMA film (CNP12) was the strongest of all of the films analyzed. On average, the BA:MMA films were stronger than the BA:Sty films, with 3 of the top 5 film samples being BA:MMA copolymers. These films often failed prior to maximum 100mm displacement, yet retracted to their original shape upon failure faster than most films, especially CNP12 which retracted the fastest of all the films analyzed. Films containing 1% SDS and the same amount of radical initiator as CNP12 and CNP5 (0.5%) were the second strongest set of films (CNP16 and CNP17). CNP15, which contained 1% SDS but also 1% radical initiator, was weaker than CNP16, where the only difference between the two was the amount of radical initiator present in the formulation, indicating that varying even the smallest constituent in the emulsion formulation has an impact on the tensile strength of the resulting film. The weakest films (CNP7 and CNP14) both contained 5% SDS, 1% radical initiator, and a ratio of 8:2 BA to co-monomer. The elastic modulus did not follow the same trend observed for the maximum tensile strength of the films, where many of the intermediary films in terms of maximum tensile strength all displayed stress versus strain curves exhibiting the same slope at 1% strain, which defines the Young’s Modulus of the film sample analyzed.
Figure 5.9 plots the stress-strain curves for films of CNP6, 12, 13, 14, and 17, as a result of a single 100mm uniaxial deformation analysis. CNP12 possessed the highest degree of tensile strength in this series of polymer films synthesized, at a maximum of 1.671MPa. All other films displayed a maximum tensile strength within 0.2-0.4MPa of each other. The stronger films in this series (CNP12 and CNP13) both broke after being displaced approximately 50mm uniaxially on average. The weaker two films (CNP6 and CNP14) either did not break or broke very close to the 100mm displacement mark, thus displaying a higher tolerance for strain, but an overall lower tensile strength. CNP17 appears to be a blend of the four other samples analyzed, displaying a high maximum stress yet without premature failure. CNP12, CNP13, and CNP17 all were polymerized from a 7:3 ratio of BA:MMA, whereas CNP6 and CNP14 contain an 8:2 ratio of the same monomers.
The second set of films analyzed in the same fashion as the BA:MMA films (Figure 5.10) contained co-monomers BA and Sty, in ratios of either 7:3 or 8:2 BA:Sty. The strongest of this series of polymer films was \textbf{CNP5}, with a maximum stress of approximately 1.2MPa. \textbf{CNP17} was the second strongest film in this series, with an average maximum stress of approximately 0.90MPa, a value greatly reduced from \textbf{CNP5}. These two films both contained a 7:3 BA:Sty ratio, however, their concentrations of surfactant used in the initial micelle formation step varied from 3\% for \textbf{CNP5}, to 1\% for \textbf{CNP17}. The weakest film analyzed was \textbf{CNP7}, which was determined to be very sticky and flimsy when handled. However, this film was able to withstand the full 100mm displacement in every sample analyzed.

\textbf{Figure 5.10.} Quasi-static testing of BA:Sty polymer films during a single uniaxial 100mm deformation.
Overall analysis of the 11 samples analyzed and compiled into a single stress-strain chart (Figure 5.11) determined that CNP12 possessed the highest maximum stress of all the polymer films synthesized, with CNP5 at a close second, yet both films displayed the lowest maximum strains withstood. This indicated that the stronger the film is, the lower the degree of strain it can withstand. However, not withstanding a high degree of strain does not indicate that a film has a high maximum stress, as is observed for CNP13, even though this film sample is one of the 5 strongest films analyzed. The two weakest films analyzed were CNP14 and CNP7, both of which were synthesized using an 8:2 ratio of co-monomers and underwent 1000% deformation without failure, consistently. Curiously, both of these films were able to be displaced the full 100mm without failure, however, their ability to retract was greatly weakened by this lengthy displacement.
Figure 5.12. Stress-relaxation of CNP12. Stress was recorded for a 30mm displacement and measurement of stress relaxation was recorded over one minute relaxation time. Time points are arbitrary numbers corresponding to the point when that stress value was obtained from the instrument.

Figure 5.12 shows a plot of the stress withstood by a control nanoparticle film, CNP12, during two individual single-cycle analyses where the maximum deformation was 30mm and the film was given a resting period of one minute between cycles. Many films displayed similar maximum strains, which was due in part to a limitation in the instrument’s ability to displace the films over 100mm. However, the BA:Sty films on average were able to withstand a higher strain, yet displayed a lower tensile strength on average than the BA:MMA films. Even with these variances, all films displayed a consistent viscoelastic property, as depicted in Figure 5.12, and were able to exert a memory-type property after initial and repeated displacements, and were able to retract fully or, if extended the full 1000% deformation, to a close range from their nominal lengths, usually within 50% of the original dimensions.

5.4.2 Cyclic Analysis of Film Samples

Cyclic analysis was then performed on each of the polymer film samples formed, using an N of 5 for each sample. Data points were collected, and a moving average of these points was plotted for the original 5mm ramp, followed by the 10mm cyclic analysis for cycles 5, 10, 15, 20, 25, and the terminating 100mm ramp to failure. The tensile strength for each film was compared before and after the 25 cycle analysis (Figure 5.9) in order to determine how fatigue would affect the film’s mechanical properties. Also, the presence of hysteresis was determined for certain films, and the observed degree of hysteresis was analyzed at various strain velocities in order to determine the extent of hysteresis for the film samples.
Figure 5.13 plots the stress-strain curve for drug-free nanoparticle polymer **CNP17** before and after 25 cycles of stress/relaxation testing. The cyclic analysis caused the polymer film to slightly change its stress/strain curve; however, the viscoelastic nature of the curve remained after cyclic analysis. Figure 5.14 expands on this to show cyclic stress/relaxation experiments on four drug-free films, those of CNP5, 12, 16, and 17. Each cycle provided the film with a 10mm stretch/relaxation, causing 100% deformation at each cycle, followed by a final 100mm uniaxial deformation, causing 1000% deformation. Analysis determined that the maximum stress or tensile strength of the polymer sample was decreased for some samples after a 25 cycle analysis of 10mm stretch/relaxation; however, when observed, this loss was minimal compared to the high degree of maximum stress obtained by the films. All films displayed an active ability to return to the original size and shape between each cycle, and most samples analyzed retained the memory characteristic after all 25 cycles and the final ramp to failure, thus making them prime materials for inclusion in such medical devices as artificial skin and heart valve systems, where the elastomeric properties of the material is constantly taxed and must not lose its original strength or ability to do so with time.
Figure 5.14. Cyclic analysis of polymer films. Analysis of select films over a 25 cycle period of 10mm deformation. Only cycles 1, 5, 10, 15, 20, and 25 were selected to be graphed, along with the initial 5mm strain and the final ramp-to-failure. Time points are arbitrary numbers corresponding to the point when that stress value was obtained from the instrument.

All film samples analyzed in Figure 5.14 showed a slight decrease in force after each cycle, but the strength at failure appears to be similar to that of an untested film sample, as was also observed in Figure 5.13. It was also determined that the tendency of a film to break prior to 1000% deformation is random; however, failure almost never occurs before 700% deformation. CNP5 appeared to be affected the most by the continual cyclic strain, where its stress dropped below 0MPa at about the fifth cycle and continued to do so until the final ramp to failure. Therefore, this film formulation, while having a high tensile strength, is easily fatigued and loses some of its elastomeric ability upon continual strain/relaxation. CNP12 however does not appear to become fatigued between cycles and keeps the same viscoelasticity throughout the cyclic analysis, making this formulation optimal for applications such as artificial heart valve and skin tissue replacement, although the final tensile strength of the film appears to have been lessened by the cyclic analysis, making it equivalent to the tensile strength of its 1% SDS counterpart CNP17. CNP17 on the other hand, while having a lesser tensile strength than CNP12, did not lose any of its elastomeric properties upon cyclic analysis, nor was its final tensile strength weakened by the cyclic fatigue. Therefore, CNP17 appears to be the best overall film formulation for biomedical applications where the elastomeric property of the film is continuous exploited.

In Figure 5.15, the occurrence of hysteresis was examined for CNP12 over numerous cycles during the cyclic analysis of the film sample. All films analyzed by cyclic means
displayed hysteresis to some extent; therefore none of the films are 100% elastic by definition.

**Figure 5.15.** Hysteresis of CNP12 during cyclic analysis. A) Hysteresis at various cycles during a 25 cycle analysis. B) Hysteresis at various strain velocities during a single cycle analysis.
Figure 5.15A shows that the relative degree of hysteresis displayed by **CNP12** during a 25 cycle analysis stays consistent throughout the cycles, however, the maximum stress observed after each cycle slightly decreases as the analysis proceeds while the maximum strain endured remains constant. This was also determined from Figure 5.14A for **CNP12** where the relaxation portion between each strain cycle progressively decreased throughout the analysis. The gradual decrease in stress appears to halt once reaching a certain point somewhere between the 10\textsuperscript{th} and 15\textsuperscript{th} cycle, where the cycle’s high point does not decrease any further. The overall decrease in maximum stress observed in Figure 5.15 between each set of 5 cycles appears to be approximately 0.1MPa, which is minimal in relation to the observed maximum stress of 1.8MPa for the film upon uni-axial single strain analysis.

Figure 5.15B is a graph of a cyclic analysis of **CNP12**, where the relative velocity of the strain and relaxation was adjusted to decrease between each cycle. In each cycle, the film was stretched 30mm then returned to the original 10mm length, and the film was allowed to rest between each cycle. This analysis showed that the observed hysteresis was not eliminated by decreasing the relative velocity in which the cyclic analysis was performed. Instead, the hysteresis reaches a “plateau” of sorts at 0.01Hz, where the observed loop created upon cyclic analysis does not become any smaller as the frequency is reduced during the cyclic analysis. This showed that the viscoelasticity observed cannot be overcome and that the films are not able to achieve a pure elastic state, indicated by a lack of hystoresis, even at reduced frequencies as low as 0.001Hz.
5.4.3 Conditional Analysis of Tensile Strength

Figure 5.16. Comparison of the mechanical properties of CNP15 when the film is freshly formed versus when it has been formed a month earlier.

CNP15 is not one of the strongest films analyzed in terms of tensile strength, and therefore, it was hypothesized that age and exposure to a laboratory environment over a certain amount of time would inflict a larger change in the maximum stress observed for the film than it would for a film such as CNP5 that has a higher tensile strength. Visual analysis of the film showed fibers and debris adhered to the surface of the aged film, and some of its stickiness was also lost, most likely due to long term handling of the film sample. Figure 5.16 shows a stress/strain curve for a freshly prepared sample versus an aged sample (one month). The maximum tensile strength of the film was reduced by 0.25MPa after being exposed to the environment over one month time span. When the original maximum stress is only 0.65MPa though, this degree of loss is quite significant, with approximately one third of the original strength lost. This determined that in order for a film to maintain a high tensile strength, handling prior to application must be kept at a minimum. Also, the film should not be left on a bench top for extended periods of time prior to application where work is being done and the occurrence of particulates in the air is high.
Figure 5.17. Effect of hydration on the mechanical behavior of CNP12.

Many of the biomedical applications proposed for these film samples involves the films being exposed to a moist or wet environment. Therefore, it was necessary for the film samples’ tensile strength to be analyzed after being fully hydrated. CNP12 was chosen for this analysis due to its observed tensile strength being relatively high for these film samples, which would hopefully allow any change in maximum stress to be more easily observed. The tensile strength for the film sample was analyzed before and after being hydrated in distilled water. Two sections of the polymer film sample were cut from the original film formed and submerged under water for either 30 minutes or 48 hours (2 day), then analyzed for their maximum stress and strain (Figure 5.17).

Upon short term hydration, the tensile strength of the film increases significantly to above 2.0MPa. However in Figure 5.17, the maximum stress for CNP12 when dry (1.3MPa) was less than that observed previously for this film formulation by approximately 0.3MPa. Upon hydration for an extended period of time (48 hours) there appears to be a drop in tensile strength by approximately half of the maximum stress observed for the dry film sample. Also interesting was the effect hydration inflicted on the maximum strain for the film samples. The maximum strain increased slightly for the film upon hydration for 30 minutes from 4.3 to 5.6 strain, as shown for the yellow curve. However, the maximum strain returned to slightly below it original dry film value after 48 hours of hydration. The structure of the stress versus strain curve was the same for the wet and dry samples, indicating that the viscoelasticity of the film samples is not altered upon hydration, and all of the samples have failed well before 100mm displacement.
5.5 Mechanical Analysis of Drug-Containing and Other Polyacrylate Films

![Chemical structures of drugs](image)

**Figure 5.18.** Chemical structures of the drugs present in the films analyzed for mechanical properties.

Figure 5.19 shows the stress/strain curve for some drug-containing polymer films. **NP1a, NP7** and **NP11** all contained drugs that were covalently bound to the polymer backbone, whereas **NP2** and **NP24** contained drug monomers that were encapsulated in the nanoparticles. **NP1a** and **NP2** contained N-thiolated β-lactam drug monomers and **NP11** contained the penicillanic acid acrylamide β-lactam monomer. All of these resulting polymer films displayed very low maximum stress values (approximately 0.4MPa), although **NP1a** was not able to withstand nearly as great of a maximum strain as the other two β-lactam containing films. **NP1a**, in this instance, was formulated using only ethyl acrylate as the base polymer, which may explain the shortened maximum strain value. All of the other drug-containing polymer films were formulated after **CNP5**, with a 7:3 ratio of BA:Sty.
Figure 5.19. Stress versus strain curve for drug-containing polymer films. All emulsions were formulated with 3% SDS and 0.5% radical initiator.

NP7, which contained covalently bound ciprofloxacin acrylamide, displayed the highest maximum tensile strength of the covalently bound drug-containing films, which was equivalent to the maximum stress observed for CNP16 (Table 5.1). Since NP7 and NP11’s polymer formulation followed the formulation of CNP5 (Table 5.1), this data indicates that the presence of a covalently bound drug moiety to the nanoparticles decreases the maximum stress of the resulting polymer film sample. However, since all of the drug-conjugated films were not analyzed, it cannot be determined whether or not this is true for all of the films, or whether this is also the case for the multi-drug conjugated polymer films.

The doxycycline encapsulated polymer film sample NP24 displayed the highest maximum stress of the samples analyzed in Figure 5.19 at approximately 1.4MPa, which was also seen for the erythromycin encapsulated film sample NP23 (data not shown) and for the control film CNP5 (Table 5.1). Therefore, when commercially available drug moieties are encapsulated in the nanoparticles without chemical modification, the resulting polymer films retain the same maximum stress as the control film that the films were formulated after. Interestingly, NP1a and NP7 were the only two film samples that did not withstand the entire 100mm displacement and reached failure at approximately half this distance.
Figure 5.20. Cyclic analysis of polymer films containing drug monomers. Time points are arbitrary numbers corresponding to the point when that stress value was obtained from the instrument.

All of the drug-containing samples analyzed followed the emulsion formulation of CNP5 and, subsequently, all of their behaviors upon cyclic analysis were similar to that of CNP5’s where a decrease in stress was observed between each cycle (Figure 5.20). NP24 lost some of its tensile strength after cyclic analysis, where its maximum stress at failure was slightly below 1.2MPa. A non-fatigued sample of NP24 has a tensile strength of just above 1.4MPa; therefore, the film sample loses approximately 0.2MPa upon cyclic fatigue. All of the other drug-containing film samples analyzed for behavior upon cyclic fatigue possessed similar maximum stresses before and after cyclic analysis. This could possibly indicate that when a film sample has a low tensile strength, as is seen for NP5, NP6, and NP7, and was previously seen for CNP17, that the film does not lose a significant amount of tensile strength after cyclic fatigue and also retains the same degree of stress between each cycle. However, film samples that originally have a larger tensile strength tend to lose some of this strength upon cyclic fatigue. This is most likely due to limitations of the instrument since the weaker film samples display maximum stress values that are within the lower limits of the MTS machine’s capabilities. Therefore, it is more difficult to distinguish changes at such a low value range than it is for the instrument to detect changes at the higher tensile strength values seen for the stronger film samples.
5.6 Analysis of the Protective Capabilities of Polymer Film Samples

5.6.1 Introduction

When deciding upon a specific biomedical application for polymer films, there are numerous factors and physical properties that must be clearly understood for the best fit. Aside from the mechanical properties, which was first examined for the polymer films, and the cytotoxicity of the films, other attributes of the film must also be established, including pore size and diffusibility of the polymer films. One of the major reasons for using the drug-conjugated and multi-drug conjugated nanoparticle emulsions for topical treatment of skin/soft tissue and burn wound infections is the subsequent formation of a polymer film over the wound. Therefore, the main objective of this study was to establish whether or not the polymer films are able to prevent bacterial invasion of the wound, either through its antibacterial activity or by providing a physical barrier for the burn wound from its surrounding environment. If bacterial migration was observed, the next step was to establish if this was due to large pores present in the films that permitted the bacterial migration, or if it was the result of the bacteria’s ability to degrade the polyacrylate strands, essentially eating their way through the films. This study also determined if any of the microbes commonly found in burn wound infections were able to utilize the polymer films as a carbon source for food and energy, which would essentially be promoting bacterial growth in the wound bed. Also analyzed was the ability of small molecular weight compounds to diffuse through the polymer films, and it necessary to clarify what type and size ranges of the compounds are able to penetrate these polyacrylate films.

Three different assays were developed and carried out under in vitro conditions to address these issues. The first assay was developed to determine if motile bacteria (P. aeruginosa, Escherichia coli, Citrobacter freundii, Proteus mirabilis, Enterobacter cloacae, and B. anthracis-Sterne) were able to penetrate the drug-free and drug-containing polymer film samples. The second assay was developed in order to test, if bacterial penetration of the film does occur, whether the bacteria are penetrating through pores in the polymer films or if they are able to eat their way through the films by degrading the polyacrylate strands within the films. The final assay was based on the same premise of the initial assay, except instead of analyzing bacterial penetration of the polymer films, small molecular weight dyes were used.

5.6.2 Bacterial Penetration Study

In order to accurately test the ability of motile bacteria to penetrate any form of material, especially one formed from evaporation/coagulation of a liquid solution, in an in vitro setting, an assay was developed using multiple layers of agar in either sterile test tubes or Petri dish plates commonly used for Kirby Bauer assays, as illustrated in Figure 5.21.
Figure 5.21. Representative of the multi-layered agar assay formed in a small Petri dish (left) versus a test tube (right).

In each container, the nutrient free agar layer was inoculated with 5-10 µL of saline solution containing $10^5$ CFU/mL of all six bacterial strains to be analyzed. The nutrient rich agar used for this assay (Bacto Acetate Differential Agar, purchased from Difco Company and used without modification) contained the pH indicator bromothymol blue (3,3-dibromothymolsulfone phthalein, MW: 624.38g/mol), which when bacteria are present in the agar and utilize the acetate nutrients for food the pH of the agar rises, which turns the agar from green (neutral pH of 6.1 to 7.5) to blue (alkaline pH of 7.6 and above). In the presence of the acidic emulsions (pH of approximately 2-2.5), the pH indicator-containing agar turns yellow in color. The use of this agar allows the assay to provide a visual detection of bacterial penetration without the need for analysis of the agar or microscopic visualization for microbial detection.
Figure 5.22. Images of multi-agar layered test tube-based polymer film permeability assay. A) Test tubes prior to bacterial inoculation in top nutrient-free agar. B) After 4 days of incubation at 37°C of P. aeruginosa inoculated test tubes. C) After 7 days of incubation at 37°C of P. aeruginosa inoculated test tubes.
As seen in Figure 5.22, the bottom agar containing the nutrient rich differential agar remained green in color for all polymer films analyzed. In many of the test tubes, the top agar (nutrient free M9 agar) either remained clear in color, as expected, or took on a bluish tint after incubation of the inoculated test tubes. This development established the ability of small molecules, including both the pH indicator and the acetate nutrients, to pass through some of the polymer films analyzed. The agars which remained clear could be the result of one of two things: either the pH indicator was unable to penetrate the polymer film, or the acetate was not able to penetrate. Due to the relative molecular weights of these two molecules (624.38 g/mol indicator versus 82.02 g/mol sodium acetate), it is more likely that it is the pH indicator that was unable to penetrate all of the films analyzed. This assay not only showed that some films are highly penetrable to even small molecular weight molecules, but also that motile bacteria were not observed penetrating the polymer films to get to the nutrient rich agar at the bottom. A positive control where no polymer film was applied between the nutrient-rich and nutrient-free agar layers resulted in bacterial presence throughout both agar layers, indicating that when no obstructive material is placed between the two agar layers the microbes are able to migrate freely throughout the agars.

Unfortunately, due to the aerobic nature of all the microbes analyzed here, it was very unlikely that the microbes would wish to migrate deep into the agar where molecular oxygen is limited; therefore, the assay was repeated using the small Petri dish plates and the agar layers were reversed. Due to the ability to provide a larger surface area for the agar layers using the Petri dish and the subsequent thinner layers formed, it is assumed that the bacteria present in the lower layers of agar are more likely to encounter elemental oxygen for respiration than they would in a test tube setting. This was an important aspect of the assay since the bacteria need to be viable for motile activity to be expressed, and therefore, for polymer film penetration to be observed. In this assay, the bottom layer of nutrient free agar was inoculated with the bacterial solution by stabbing the agar with a thin metal rod previously dipped in a mixed microbial-containing saline solution. The emulsion was then added to the surface of this agar and allowed to transform into the solid polymer film in a biosafety cabinet for 1-2 hours, then the nutrient-rich agar was added to the surface of the solidified polymer film. After the top agar layer solidified, the plates were incubated for up to 10 days to observe if the bacteria were able to penetrate the polymer films over an extended period of time. Results from this assay confirmed the original findings in the test tube-based assay, that none of the bacteria could pass through the film barrier separating the agar layers.

A third assay was then performed using only two specific nanoparticle emulsions: CNP15 and the multi-drug conjugated nanoparticle emulsion MDNP2 (described in Chapter 4). This assay utilized a multi-diffusion chamber (Figure 5.23) to analyze ten different locations throughout the film for bacterial penetration.
Figure 5.23. Multi-diffusion chamber for polymer film diffusion/penetration studies. The clear plastic lid closes over the top wells to seal in the microbial solution, and the polymer film is placed between the single large bottom chamber and the multiple top chambers.

Also employed for this broth-based assay was a Franz diffusion cell (Figure 5.24), where a single film was placed over an opening in the cell below the solution containing a bacterial culture. A sampling arm extending from the bottom chamber cibtaububg a standard TBS solution (initially containing no bacteria) allows for sampling of the culture daily to determine bacterial concentration. Swabs of the bottom chamber culture were taken on days 1, 2, 3, 4, and 7 (final) from the Franz diffusion cell and only on day 7 for the multi-diffusion chamber, and streaked onto the same Bacto acetate differential agar as was used in the initial two assays for the presence of bacteria.

Figure 5.24. Franz diffusion cell. The top chamber is removable and the polymer film (previously formed) is placed over the hole in the bottom chamber and is sealed in place by the top chamber, thereby exposing the film to both the bacterial-free and bacterial-containing solutions. (TSB: tryptic soy broth)
Results of this study showed that for the **MDNP2** polymer film, which was analyzed using the Franz diffusion cell, no bacteria were present in the bottom chamber at any of the days inspected, nor on the final day of the study (day 7). The lack of bacterial penetration and presence in the bottom chamber solution could be the result of the antibacterial activity of the polymer film (Chapter 4), or could be that the presence of pores, if any, in the film were too small for bacterial penetration. In either scenario, this assay, along with the initial two multi-layered agar assays, proved that the multi-drug conjugated polymer films are able to prevent bacterial invasion of nutrient-rich environments.

In the case of the drug-free polymer film **CNP15**, bacteria were present in the bottom chamber for the multi-diffusion chamber on day 7. Since this chamber does not permit sampling of the bottom solution prior to termination of the assay, the exact day of bacterial infiltration could not be established in this scenario. This observed bacterial penetration could indicate that either the chamber is not the most optimal apparatus for this assay, and that some of the top chamber’s solution could have come in contact with the bottom chamber’s solution when terminating the assay, or that the bacteria present in the solution were able to penetrate the film either through pores or by microscopic degradation of the film. The latter question was further explored in section 5.6.3 of this chapter.

### 5.6.3 Carbon Source Assay

In order to establish whether a microbe can utilize a specific chemical, compound, or material in order to create the energy it needs to sustain life, the bacteria must be grown on media that contains no nutrients or carbon sources, i.e. minimal media. For this study, a minimal media (M9 minimal salts agar) was purchased from Difco Company and used without any modifications. Small Petri dish plates were filled with this agar, and after hardening, 1.00mL of emulsion was added to the surface of the agar. After polymer film formation was complete, a saline solution containing $10^5$ CFU/mL was added to the surface of the films by placing 5-6 drops of the solution on the film. Incubation was performed at 37°C for all bacteria analyzed and the plates were incubated up to 7 days, depending on the observance of bacterial growth. A positive control using tryptic soy agar (TSA) was used to ensure that bacterial growth would occur when exposed to typical nutrients, and a negative control using only the M9 minimal salts agar was also employed to confirm a lack of bacterial on this media alone. The data reported in Table 5.2 is the average of the results from three separate batches of emulsions per sample.
Figure 5.25. Carbon source assay using *P. aeruginosa* (left side of plates) and *E. coli* (right side of plates). A) The control plate showed no growth of either bacterial strains, nor did the agar plate coated with a thick white film formed from the β-lactam conjugated nanoparticle emulsion NP2 (KG9). B and C) *P. aeruginosa* displayed growth on all agar plates coated with the more translucent drug-free polymer films (plates 1, 2, 3, and 4) and no observable growth of *E. coli*. (Plate 1: CNP6, Plate 2: CNP12, Plate 3: CNP13, Plate 4: CNP14)

The results represented in Figure 5.24A showed that when no polymer film is added to the minimal agar, no bacterial growth was observed after a 10 day incubation period, and that no bacterial growth was also observed when a drug-conjugated polymer film was
added to the agar surface. However, when poly(butyl acrylate: methyl methacrylate) films free of any drug monomers was added to the agar, the *P. aeruginosa* was able to utilize the polyacrylate films as a carbon source and displayed moderate to heavy colonization on top of the films. Interestingly, since the *N*-thiolated β-lactam monomers show absolutely no antibacterial activity against *P. aeruginosa*, and since all of the β-lactam containing polymer films did not permit growth of this microbe, it appears that this lack of growth is not due to the antibacterial nature of the drug-containing films, which one would assume, but rather a change in the matrix of individual polymer strand entanglement within the film formed, which appears to prevent the microbe from utilizing the polymer as a carbon source. Another explanation for these results is that when the drug monomer is present within the polymer film, either through co-polymerization of the drug with the liquid acrylate monomers or through encapsulation within the nanoparticles, the drug is able to block whatever interactions the microbe utilizes in order for it to degrade the drug-free polymer film that allows it to be used as nutrients. In either case, this assay has established that the drug-containing and therefore the multi-drug conjugated polymer films cannot be utilized by *P. aeruginosa* as a carbon source, and that *E. coli* cannot use any of the polymer film samples formulated with BA and MMA. This is shown in Table 5.2 on the next page, with nine of the drug-free control nanoparticle films and five of the drug-containing films being examined. Most microbes do not grow on any of the films, but only the five drug-containing films prevent *P. aeruginosa* from growing.
Table 5.2. Carbon source assay data for all microbes and polymer films analyzed over 7 day incubation. M9 minimal agar was the negative control employed, and TSA agar was the positive control. (+ represents observed bacterial growth, NG:no growth.

<table>
<thead>
<tr>
<th>Microbe</th>
<th>Polymer Film Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CNP5</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>+</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>+</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>NG</td>
</tr>
<tr>
<td><em>Coagulase-negative SA</em></td>
<td>NG</td>
</tr>
<tr>
<td><em>Klebsiella oxytoca</em></td>
<td>NG</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>NG</td>
</tr>
<tr>
<td><em>Proteus mirabilis</em></td>
<td>NG</td>
</tr>
<tr>
<td><em>Citrobacter freundii</em></td>
<td>NG</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td>NG</td>
</tr>
<tr>
<td><em>Bacillus anthracis</em></td>
<td>NG</td>
</tr>
<tr>
<td><em>Bacillus coagulans</em></td>
<td>NG</td>
</tr>
<tr>
<td><em>Bacillus niger</em></td>
<td>NG</td>
</tr>
<tr>
<td><em>Acinetobacter calcoaceticus</em></td>
<td>NG</td>
</tr>
<tr>
<td><em>Candida albicans</em></td>
<td>NG</td>
</tr>
<tr>
<td><em>Candida kruesi</em></td>
<td>NG</td>
</tr>
<tr>
<td><em>Candida parapsilosis</em></td>
<td>NG</td>
</tr>
</tbody>
</table>
Many other microbes commonly detected in burn wounds were analyzed in this fashion. Here, it was determined that almost all of the microbes tested were unable to use the poly(butyl acrylate:methyl methacrylate) films as a carbon source. All three of the Candida fungal species tested were completely unable to utilize any of the polymer film samples as a carbon source, along with Acinetobacter calcoaceticus, Bacillus anthracis, Proteus mirabilis, Klebsiella oxytoca, S. aureus, and coagulase-negative S. aureus. Not a single microbe was able to utilize any of the drug-conjugated poly(butyl acrylate: styrene) polymer films as a carbon source, even though some of the microbes were able to utilize the poly(butyl acrylate: styrene) polymer films when no drug was present in the formulation, including E. coli and P. aeruginosa. Klebsiella pneumoniae, Citrobacter freundii, and Enterobacter cloacae all showed some sporadic, but reproducible, growth on a few drug-free films analyzed.

5.6.4 Molecular Weight Cutoff Analysis

Two drug-free polymer films CNP6 and CNP7, formulated with either MMA or Sty respectively, and two drug-encapsulated polymer film samples (NP23 and NP25) containing encapsulated erythromycin and amphotericin B, respectively, were subjected to these permeability studies in order to determine any differences between drug-containing and drug-free films, and also any differences between films containing Sty or MMA co-monomers. A capillary zone electrophoresis (CZE) instrument was used to detect the disappearance of the various dyes from the top well of the diffusion chambers (Figure 5.23), indicating their penetration through the polymer film samples. The CZE instrument was used to analyze the retention time of each dye individually, followed by analysis of all five dyes in a single solution, in order to provide CZE readings of each dye prior to interaction with the polymer films. Thereafter, the multiple dye-containing solution was added to the top of the individual chambers above the polymer film in the multi-well diffusion chamber depicted in Figure 5.23 and after 24 hours, an aliquot from each top chamber was removed and analyzed by the CZE for percentage of dye remaining in the wells.
Figure 5.26. Chemical structure of five pH indicators used for molecular weight cutoff assays.

The dyes analyzed were all between 404 and 844 g/mol, where the order of increasing molecular weight is: cresol red, thymol blue, bromothymol blue, bromocresol green, and methyl thymol blue. CZE analysis showed that the fastest dye to move through the capillary was thymol blue, followed by bromothymol blue, then cresol red, bromocresol green, and finally methyl thymol blue. Therefore, the size of the dyes was not the single distinguishing factor for movement through the capillary, but also charge and perhaps other interactions between the electrophoresis instrument and the dye ultimately determined the rate at which each dye flowed through the capillary.
Figure 5.27. Percentage of each dye analyzed that penetrated polymer film samples. Dialysis tubing was used as a positive control, and a solid plastic material was used as a negative control. Dyes are arranged from first to be read by the CZE instrument to last.

The data presented in Figure 5.27 shows that size, in terms of molecular weight, was not the sole influence in molecule penetration. Methyl thymol blue, the largest dye analyzed, had the highest percentage of film penetration for drug-containing poly(butyl acrylate-styrene) film samples NP23 and NP25, where NP25 showed penetration as high as 85%, yet all of the other dyes analyzed for these two films were permeable at only 40-50% penetration. However, this does show that the drug-encapsulated polymer film samples are permeable to small molecular weight molecules, but the percentage of penetration is highly dependent on the interactions between the molecule and the polymer film. This property is highly favorable for applications such as wound dressings and artificial skin and lung tissue grafts, where small molecular weight molecules, especially very small molecules such as oxygen, carbon dioxide, and water, must penetrate the tissue as would naturally occur. The ability to combine antimicrobial therapy to these artificial tissues has yet to be explored, and the favorable properties of these drug-conjugated polymer films seem to be an optimal prototype for such applications. The drug-free polyacrylate films analyzed (CNP6 and CNP7) showed little to almost no penetration of the various dyes after 24 hours of exposure. These film samples behaved more like the generic plastic material used than a penetrable material. The plastic film used for the negative control was approximately 1-2mm thick and was malleable, however, it was designed to be used...
as a containment plastic, therefore, should have been practically impenetrable by any and all molecules. Since the percentages observed for dye penetration for the drug-free polymer films was statistically similar to those observed for the plastic material, this indicates that without the presence of drug, the poly(butyl acrylate-styrene) and poly(butyl acrylate-methyl methacrylate) films are virtually impenetrable towards low end molecular weight molecules. Further investigation should be performed to determine the ability of essential small molecules, such as water, O₂, and carbon dioxide, to penetrate the films. Biomedical applications where no degree of molecular penetration would be beneficial are areas along the lines of coatings for prosthetics and bone cement and other biological glue-like materials that can hold in place biological sutures or replacement materials. Thus, it is important to know which materials would be useful for that versus as a burn wound treatment.

5.7 Conclusions

Mechanical analysis determined that the polyacrylate polymer films possess “memory,” indicated by their ability to undergo large degrees of deformation followed by self-induced retraction to their initial dimensions during a relaxation period. This property is likely due to physical cross-linking between the single polymer strands tangled within the film matrix that aid in film retraction, along with the presence of nanoparticles that allow the strands to slide along side each other, aiding in the extensibility/retractability of the films. The polymer films possess a viscoelasticity, evidenced by a high degree of hysteresis when cyclically analyzed and a low maximum tensile strength (between 0.1 and 2.0MPa), and with minimal deformation observed upon cyclic fatigue. This observed viscoelasticity relates these smart polymer films to materials that are often associated with a class of pliant materials called protein rubbers or biorubbers, which includes elastin, resilin, and abductin. Since elastin is a major constituent of elastic biological tissues, such as elastic ligaments, skin, lung, and blood vessel tissue, this property may allow many of these polymer film formulations to be used as the elastin constituent in biocomposite material.

The polymer films, both drug-containing and drug-free, were able to physically prevent the movement of bacterial migration into nutrient-rich media. Carbon source analysis determined that the majority of the microbes commonly found in burn wounds were unable to utilize these polyacrylate films for a source of energy, and that all microbes analyzed were unable to grow in the presence of the drug-containing poly(butyl acrylate: styrene) films in this assay. The degree of molecule penetration through the films will ultimately determine the optimal emulsion formulation for each medical application. Since the maximum stress that CNP12 and CNP5 are able to withstand is on par with that of aortic tissue, which is mechanically similar to intact skin, this allows these polyacrylate films to be considered as a new prototype for development of artificial tissues. Thus, these new smart polyacrylate films offer a promising new material
formulated from a single step emulsion polymerization process for numerous medical applications ranging from treatment of burn wounds to tissue engineering and repair.

5.8 References

42. Snodgrass, R.E., Annual Reports of the Smithsonian Institution. 1929. 1930.
CHAPTER 6

MECHANICAL PROPERTIES OF COLLAGEN-NANOPARTICLE EMULSION BIOCOMPOSITES

6.1 Introduction

In the past few years, collagen has been heavily studied for its application in tissue engineering and is currently the most popular material being researched for such applications.\textsuperscript{[1-5]} Other naturally-occurring materials have also been explored, including elastin, gelatin, chitosan, and hyaluronic acid.\textsuperscript{[6-9]} All of these natural products have been combined with various synthetic materials including various polymers and also numerous organic solvents for artificial tissue construction. Additions of these biological-based structures to currently used synthetic biomaterials have been studied for increasing biocompatibility as well as improving the mechanical properties of the materials.\textsuperscript{[7]} Therefore, it is now common practice to use synthetic materials in combination with collagen and other natural macromolecules to form biocomposites for tissue engineering applications. In such cases, the most common natural products used for these biocomposites are collagen fibers, fibrils, or individual macromolecules, where the collagen is usually present in a lower quantity than the synthetic polymer constituent.\textsuperscript{[3]}

When attempting to synthesize materials to emulate elastic tissues, such as ligaments, blood vessels, lungs, skin, and heart aorta tissue,\textsuperscript{[7, 8, 10, 11]} the collagen constituent must be combined with a synthetic material that possesses elastomeric-like mechanical properties to obtain an artificial tissue that closely resembles the elastic tissue.\textsuperscript{[1-5]} The biocomposite constituents can also be reversed, where the elastic constituent is provided through a natural product like elastin, and the structural strength and integrity of the material is provided through a mechanically strong synthetic material.\textsuperscript{[7-9]} In either scenario, the most optimal biocomposite must possess the strength of the collagen constituent and the elasticity of an elastin constituent, which are both found in the representative tissue. The aim is that the combination of the two materials in the biocomposite will provide the mechanical and physical properties needed in order to closely resembles the natural material being replaced.

Most of the recent biocomposites use collagen as the natural material, since this polymer is present in most biological tissues, including the skin, ligaments, and tendons, which often require artificial replacements when damaged. Therefore, the purpose of this study was to combine one of the polycrylate polymer film samples with native sea cucumber collagen fibrils in order to develop a biocomposite fibrous material that has the elasticity of the synthetic films combined with the high tensile strength of the collagen fibrils. Sea cucumber collagen fibrils were chosen over native bovine collagen as a proof of principle since we could obtain native intact collagen fibrils from the dermis of sea cucumbers.
Also, sea cucumber dermal collagen fibrils possess a high aspect ratio making them paramount in forming linear and various other arrays of smooth collagen fibers. Sea cucumbers and sea urchins are the only two animals from which intact native collagen fibrils can be extracted without damage to the collagen fibril, making sea cucumbers an optimal and readily available source of collagen. The formation of collagen fibers from fibrils has been established by Koob et. al. for both sea cucumber collagen and bovine collagen,[12-15,18] and the sea cucumber fibers have been tested and proven to possess similar mechanical properties as bovine collagen fibers; therefore, this was the best choice as a model system for these initial exploratory experiments.

Table 6.1. Formulation for CNP3 nanoparticle emulsion.

<table>
<thead>
<tr>
<th>CNP3</th>
<th>Percent (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl acrylate (EA): MMA, (4:1)</td>
<td>18.5%</td>
</tr>
<tr>
<td>Water</td>
<td>80%</td>
</tr>
<tr>
<td>Sodium Dodecyl Sulfate</td>
<td>1%</td>
</tr>
<tr>
<td>Potassium Persulfate</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

6.2 Quasi-Static Analysis of Biocomposite Fibers

Uniaxial tension experiments on films formed from the poly(ethyl acrylate-methyl methacrylate) nanoparticle emulsion CNP3 showed that this film formulation was stronger and stiffer than all of the butyl acrylate: styrene and butyl acrylate: methyl methacrylate polymer films synthesized in Chapter 5. This is due to the fact that when polymerized, ethyl acrylate (EA) polymer films exhibit greater tensile strengths than butyl acrylate polymer films, and since films containing methyl methacrylate were stronger than ones containing styrene (Chapter 5), it was assumed that this formulation would provide the strongest films so that the addition of the emulsion to the fiber formulation does not diminish the tensile strength of the fibers too dramatically. Also, since the intended use of this film formulation was to interact with collagen material for use as artificial tissues, only 1% surfactant (SDS) was used in order to avoid unnecessary toxicity.
Figure 6.1. Uniaxial tension analysis of the poly(ethyl acrylate-methyl methacrylate) film CNP3.

Stress/strain analysis of the polyacrylate film sample CNP3 (Figure 6.1) shows mechanical properties very similar to those observed for elastin.\textsuperscript{[7, 8, 10, 11]} However, the average tensile strength of elastin itself is on the range of 0.5MPa and its elastic modulus is approximately 1.5MPa.\textsuperscript{[9, 11]} Therefore, the CNP3 polymer is slightly stronger than elastin, yet, the elastic modulus is approximately equivalent, indicating that the behavior of the two materials is the same. This data indicates that the polyacrylate film formulation is equivalent if not better than the natural polymer, and thus is an optimal material for combination with collagen in order to form a biocomposite material for elastic tissue replacement.

The mechanical data presented in Figure 6.2 will be further explained in Section 6.3 of this chapter. However, it should be mentioned here that the data presented in Figure 6.2, and the data reported for all mechanical analyses in this chapter, are the result of the averaged data for 3 fibers per formulation. This provided smooth lines for the graph, and also explains the jumps in the graph of some of the formulations.
Figure 6.2. Average of the uniaxial tension data for the biocomposite fibers formed from concentrated CNP3 nanoparticle emulsion (20% solid content) and sea cucumber collagen fibrils. Data is presented as the average of 3 samples per fiber formed up to failure of the first fiber.

In order to establish the behavior of these biocomposite fibers, various volumes of concentrated (20% solid content) CNP3 were employed in the fiber formulation. The volume of sea cucumber collagen fibrils suspended in solution was also altered through numerous dilutions with sterile water to determine if decreasing the amount of collagen in the fiber formulation would improve the mechanical properties of the fiber construct and possibly provide stronger elasticity. Therefore, the biocomposite fibers were formed from either mixing a constant volume of the emulsion with percentages of collagen fibril suspension, or mixing a constant volume of the fibrils with varying amounts of the emulsion to provide alternative combinations of the two constituents.
Figure 6.3. Cyclic analysis of dry sea cucumber collagen fibers (A) and collagen/emulsion biocomposite fibers containing 100µL of the nanoparticle emulsion CNP3 (B).
Both the control collagen-only fibers and the biocomposite fibers were analyzed by a 25-cycle stress/relaxation experiment, followed by a 10mm ramp to failure for the fiber (Figure 6.3). Upon close analysis, the control fibers in Figure 6.3A displayed stress relaxations below 0MPa as the number of cycles increased. The tensile strength of the control fibers was not diminished upon fatigue, indicating that repeated uniaxial tension on the collagen fiber did not affect the tensile strength of the fibers. However, no elasticity was observed for these fibers, and when undergoing relaxation, the fibers were observed lagging towards the center of the fiber. This property was not seen for the biocomposite fibers, where the fiber was able to undergo stretching during the tension portion of the cycle, and then retracted to the fibers original length upon relaxation. This is why the cyclic stress/relaxation data for the biocomposite fiber in Figure 6.3B does not show a decrease in stress upon relaxation throughout the cycles. However, the lull between cycles did increase as the cycles progressed, which was also observed for the control fibers, indicating that the fiber took progressively longer to react to the tension being placed on the fiber at the onset of each cycle as the experiment progressed, for both the collagen control fibers and CNP3.

The maximum tensile strength for the biocomposite fiber containing 100µL of CNP3 was in the same range as the un-fatigued fiber (102 for the fatigued and 112MPa for the un-fatigued fibers); therefore, cyclic fatigue did not alter the overall strength of the fiber (Figure 6.2 and 6.3B). Little deformation was observed on the fiber by the repeated cyclic displacement, and no lagging in the actual fiber was observed during the relaxation period of the cycles. This is indicative of the elasticity of the biocomposite fibers, and is also how the elastic biological tissues react towards fatigue, making the biocomposite fiber optimal for such an application.

6.3 Mechanical Properties of the Biocomposite Fibers

Mechanical properties (average force, stress, strain, elastic modulus) were determined for all the fiber formulations constructed, where 5 samples per formulation were analyzed. Data for both the emulsion dilution fiber set and the collagen dilution fiber set are presented in Table 6.2. The “control fibers” for the CNP3 emulsion dilution set of fibers contained no CNP3 in the formulation and were formed from only sea cucumber collagen fibrils. The fibers formulated with 100% collagen in the collagen dilution set of fibers contained 100µL of CNP3 and 10mL of collagen fibril suspension, therefore, the mechanical properties of these fibers should be equal to those seen in the emulsion dilution set for the fibers containing 100µL of CNP3.
In Figure 6.4, there is an observed increase in the diameter of the fibers as the amount of CNP3 emulsion in the fiber formulation is increased. The fibers also appear smoother when formulated with the CNP3 emulsion and appear more durable. In Table 6.2, the control values are representative of sea cucumber collagen fibers without any emulsion present. The volumes given are for the amount of concentrated CNP3 (20% solid content) added to 10mL of collagen fibril solution prior to fiber formation. Once it was determined that the most optimal amount of concentrated CNP3 emulsion added to the fibril solution was 100µL, this volume was then used to determine if altering the percentage of the collagen suspension would provide better mechanical properties than the concentrated suspension. Dilutions of the collagen fibril suspension with distilled water were made where collagen makes up 100%, 50%, 25%, 10% of the total suspension, then was mixed with 100µL of concentrated CNP3 nanoparticle emulsion prior to fiber formation and subsequent mechanical analysis.
Table 6.2. Mechanical data for the collagen-CNP3 biocomposite fibers. The volume of CNP3 was varied for each fiber formulation and the control fibers did not contain CNP3.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>25µL</th>
<th>50µL</th>
<th>100µL</th>
<th>150µL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wet</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Force (N)</td>
<td>0.1311</td>
<td>2.2953</td>
<td>4.5260</td>
<td>4.2305</td>
<td>3.5169</td>
</tr>
<tr>
<td>Stress (MPa)</td>
<td>1.1545</td>
<td>7.4585</td>
<td>36.0398</td>
<td>41.7555</td>
<td>32.9256</td>
</tr>
<tr>
<td>Strain</td>
<td>0.1874</td>
<td>0.7077</td>
<td>0.49052</td>
<td>0.4210</td>
<td>0.3703</td>
</tr>
<tr>
<td>Young's Modulus (MPa)</td>
<td>37.27773</td>
<td>3.485523</td>
<td>68.5623</td>
<td>87.22434</td>
<td>91.11033</td>
</tr>
<tr>
<td>% Swell</td>
<td>102.930</td>
<td>36.8514</td>
<td>56.7766</td>
<td>33.5782</td>
<td>23.2172</td>
</tr>
<tr>
<td><strong>Dry</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Force (N)</td>
<td>5.3695</td>
<td>3.0522</td>
<td>5.0477</td>
<td>3.7748</td>
<td>4.8905</td>
</tr>
<tr>
<td>Stress (MPa)</td>
<td>222.972</td>
<td>44.1657</td>
<td>172.500</td>
<td>114.480</td>
<td>59.3254</td>
</tr>
<tr>
<td>Strain</td>
<td>0.3564</td>
<td>0.3785</td>
<td>0.4395</td>
<td>0.3720</td>
<td>0.4966</td>
</tr>
<tr>
<td>Young's Modulus (MPa)</td>
<td>1392.754</td>
<td>83.94695</td>
<td>788.621</td>
<td>439.5076</td>
<td>199.127</td>
</tr>
</tbody>
</table>

Table 6.2 provides all of the mechanical properties observed for the biocomposite fibers formulated from 10mL of concentrated collagen fibril suspension. Each of the individual mechanical properties reported here are described in more detail below. However, the most important finding presented in this table is that the mechanical properties for the biocomposite fibers (containing 25-150µL of the emulsion) were not nearly as affected by hydration as was observed for the control collagen-only fibers, indicating a higher degree of mechanical strength retention for these biocomposite fibers.

Figure 6.5A shows that when dry, all of the biocomposite fibers containing CNP3 and sea cucumber collagen fibrils displayed relatively similar maximum forces to fibers containing only collagen (control) when analyzed under dry condition. However, when hydrated, the control (collagen) fibers displayed almost no force tolerance, whereas the biocomposite fibers displayed almost equivalent maximum forces as their dry counterparts. This indicates that the presence of only a slight amount of nanoparticle emulsion provides the collagen fibers with the strength it needs to be able to withstand forces when hydrated similar to those observed for the dry fibers.
Figure 6.5. Average force in Newtons for CNP3-collagen fiber biocomposites. Data is presented as an average of five samples per fiber formulation. A) Data obtained from fibers containing 10mL of collagen suspension and varied amounts of CNP3 nanoparticle emulsion. B) Data obtained from fibers containing 100µL of CNP3 nanoparticle emulsion and various dilutions of the collagen suspension.
The maximum force withstood by the collagen fibers in Figure 6.5B decreased as the dilution ratio of collagen to water increased per fiber formulation. There was an observable difference in the maximum force withstood by the dry and hydrated fibers formulated with only 9% collagen, however, due to the high degree of deviation for the average maximum force of the dry fibers, this difference is not statistically relevant. All other fibers tested did not show any significant differences in the maximum force of the hydrated and dry fibers, however, all fibers formed from diluted collagen suspensions were not able to withstand forces nearly as high as those obtained from the undiluted collagen suspensions. Also, the maximum force for the fibers formulated with only 9% collagen resembled the maximum force observed for the control collagen-only fibers in Figure 6.5A when hydrated (approximately 0.5N difference).
Figure 6.6. Average stress in megapascals for biocomposite collagen/emulsion fibers. A) Data obtained from fibers containing 10mL of collagen suspension and varied amounts of CNP3 nanoparticle emulsion. B) Data obtained from fibers containing 100µL of CNP3 nanoparticle emulsion and various dilutions of the collagen suspension.
In Figure 6.6A, the collagen fibers formulated with 50 and 100µL of CNP3 nanoparticle emulsion appeared to possess the highest tensile strength properties, except for the control collagen fibers. The 100µL CNP3/collagen fibers appeared to have more consistent strength than the corresponding 50µL sample, even though the 50µL sample displayed a higher maximum stress value. Figure 6.6A also shows that as the volume of CNP3 nanoparticle emulsion was increased (25 to 150µL) in the fiber formulation, the closer the maximum stress values were for the wet and dry fibers.

Analysis of the data in Figure 6.6B provided a clear observation for the tensile strength trend for the fibers formed from diluted collagen suspensions that as the amount of collagen present in the biocomposite fiber formulation decreases, the average tensile strength for both the wet and dry fibers decreases. The non-diluted fibers formulated with 100% collagen and 100µL of CNP3 exhibited the highest average tensile strength for both the wet and dry fibers analyzed, however, these fiber samples also displayed the highest degree of deviation in the average tensile strength for the three fiber samples analyzed under both wet and dry conditions. This high degree of deviation was also observed in Figure 6.6A for the dry fibers containing 100µL of CNP3, but was not observed for the wet fibers. All of the other fibers analyzed in Figure 6.6B did not show a significant difference in tensile strength between the hydrated and dry fibers.
Figure 6.7. The maximum strain observed for the biocomposite fibers. A) Data obtained from fibers containing 10mL of the collagen suspension and varied amounts of the CNP3 nanoparticle emulsion. B) Data obtained from fibers containing 100µL of the CNP3 nanoparticle emulsion and various dilutions of the collagen suspension.
The observed maximum strain for both the wet and dry biocomposite fibers were statistically similar (Figure 6.7A), except for the fibers formulation with 25μL of CNP3, in which case the hydrated fiber samples withstood greater maximum strains than the dry fibers. All of the biocomposite fibers analyzed (fibers containing both collagen and nanoparticle emulsion in the formulation) displayed strains significantly higher than those observed for the control, collagen-only fibers. Also, Figure 6.7B shows that as the amount of collagen in the fiber formulation was reduced, the maximum strain withstood by the fibers increased. The fibers containing only 9% collagen in the formulation withstood maximum strains three times greater than those withstood by the fibers containing 100% collagen in the formulation (Figure 6.7B), and up to 4 times greater than was observed for the control collagen-only fibers in Figure 6.7A. This indicates that the elastic properties of the polymer film constituent are being incorporated into the biocomposite fibers, which is allowing the fibers to endure higher degrees of deformation than they would when formulated with only collagen fibrils. Also, the fibers were observed displaying elastic stretching upon uniaxial tension, and were also observed retracting back to their original length upon failure, indicating that memory property observed for the polymer film samples analyzed in Chapter 5 was also incorporated into the biocomposite fibers. This elastic property was not observed for the collagen-only fibers, where the fibers did not stretch in any way under uniaxial tension. Also, the degree of elasticity for the biocomposite fibers visibly increased as the amount of collagen present in the fiber formulation decreased, which supports the data presented in Figure 6.7B.

The difference in strains observed between the wet and dry emulsion-containing fibers was variable; however, this variance observed was not statistically significant. Thus, neither the wet nor the dry fibers show predominantly higher maximum strains in Figure 6.7. The maximum strain measured for the biocomposite fibers containing 100%, 50%, and 33% collagen in the uniaxial tensile test appeared to be statistically similar, with maximum strains between approximately 0.4 and 0.8. The maximum strain observed for the biocomposite fibers containing only 20% or 9% collagen were statistically similar as well, with strains ranging between approximately 1.5 and 2.0. These results indicate that there was a drastic change in the maximum strain withstood by the fibers between the 33% and 20% collagen dilutions. This drastic change in mechanical property may be indicative of a change in the dominating constituent in the biocomposite, where the mechanical properties expressed are originating more from the CNP3 nanoparticle emulsion constituent in the biocomposite when only 20% collagen is used in the formulation.
Figure 6.8. The elastic modulus of the biocomposite fibers. A) Data obtained from fibers containing 10mL of collagen suspension and varied amounts of the CNP3 nanoparticle emulsion. B) Data obtained from fibers containing 100µL of CNP3 nanoparticle emulsion and various dilutions of the collagen suspension.
The elastic modulus or the stiffness of a given material observed for the biocomposite fibers in Figure 6.8A is much lower than that of the dry, collagen-only control fibers, but is substantially higher than the elastic modulus of the wet control fibers. Fibers containing 50 or 100µL of CNP3 emulsion produced the highest modulus for the biocomposite fibers, but 100µL displayed the least amount of variance between hydrated and dry fibers analyzed. This drastic difference between the collagen fibers and the biocomposite fibers when dry indicates that the fibers are displaying stress/strain curves more closely resembling those observed for the CNP3 polymer films than they are for the collagen fibers, since the elastic modulus is determined as the rate of change of stress with strain for a given material. This data also shows that when hydrated, the fibers are able to maintain much higher structural integrity than is observed for the collagen-only fibers. This is most likely the result of the nanoparticle-based emulsions coating the fibrils within the fiber construct and providing some degree of protection from water damage for the fiber. Intriguingly, when 150µL of nanoparticle emulsion CNP3 was used in the biocomposite formulation, the elastic modulus was nearly equal for the wet and dry fibers. This observation sheds some light on which constituent is responsible for the majority of the mechanical properties observed for this biocomposite fiber, since the polymer film resulting from the nanoparticle emulsion CNP3 also expresses similar elastic moduli when dry and upon short term hydration.

Statistically speaking, there is no difference between the elastic modulus for each of the wet and dry fibers analyzed in Figure 6.8B. This also demonstrates that more of the emulsion’s characteristics are being expressed by these fibers than the collagen’s. Again, the dilution ratio of 1:2 for collagen appears to be the turning point where almost none of the collagen’s mechanical properties are being expressed in the fiber constructs, and the elastic modulus for these fibers is within only a few megapascals greater than the CNP3 films. Based on the data presented in Figure 6.8, the ratio of 10mL of undiluted collagen fibril suspension to 100µL of CNP3 nanoparticle emulsion in the fiber formulation appears to yield biocomposite fibers that express the strongest properties of each constituent (high strength of collagen and elasticity of polymer).

### 6.4 Swelling Retardation of Collagen Fibers

One of the reasons that collagen has been more heavily studied when in combination with other materials than it has alone for tissue engineering is due to the fact that when hydrated, fibers formed from reconstituted collagen loses its structural integrity and the maximum stress of the fibers dramatically decreases from 222MPa to less than 2MPa. This is because during the extraction process, especially from mammalian sources, the cross-linking and ionic interactions between the native collagen fibrils is broken, which is what provides the structural integrity of the mammalian collagen in vivo\textsuperscript{113, 16, 18}. In the case of native sea cucumber dermal collagen, the structural integrity of the fibers is thought to be due to the ionic interactions of anionic chondroitin chains bound to the native fibrils interacting with the cationic sulfate molecules bound to the exterior of the
collagen fibrils opposing the chondroitin.\cite{18} Since any biocomposite material will be hydrated when placed in a biological setting, this is an obvious problem that needs to be addressed. In order to overcome this, research has focused on two different solutions: 1. cross-linking the collagen fiber using a chemical cross-linking agent,\cite{15-17} or 2. combining the collagen with a synthetic material for support.\cite{2-5} This study has chosen the latter of the two options to try to overcome this hydration problem.

![Figure 6.9](image)

**Figure 6.9.** Images of biocomposite and collagen fibers when wet and dry. A and B) Dry and wet (respectively) biocomposite fiber formulated with 100µL of **CNP3** nanoparticle emulsion. C and D) Control collagen-only fiber, dry and wet (respectively).

Figure 6.9 shows a comparison of collagen versus collagen-**CNP3** composites when wet versus dry. Data represented in Figure 6.6A showed that the dry control (collagen) fibers depicted in Figure 6.9C are twice as strong as the dry collagen-**CNP3** fibers depicted in Figure 6.9A, but after hydration, the wet biocomposite fibers (Figure 6.9B) are up to 40 times stronger than the wet collagen fibers (Figure 6.9D). The mechanical properties of the collagen fibers change drastically when hydrated, and a large degree of swelling (100%) is observed for the fibers (C to D). When the collagen fibrils are combined with any volume of **CNP3** nanoparticle emulsion between 25 and 150µL prior to fiber formation, the observed swelling is reduced drastically. Figure 6.10 shows that the largest degree of swelling (60%) was observed for the fibers formulated with 50µL of **CNP3**.
emulsion and 10mL of undiluted collagen fibril suspension, and the lowest amount of fiber swelling (2%) was observed for the biocomposite fibers formulated with only 9% collagen fibril suspension diluted in water with 100μL of CNP3 emulsion in the formulation. The degree of fiber swelling was determined by Equation 6.1 for each fiber analyzed.

The diameter of the fibers was determined by measuring the width of the fiber at two locations in the center of the fiber when dry or after being hydrated for one hour and averaging these two measurements. The percent swelling for the fiber is then calculated based on the following Equation 6.1.

**Equation 6.1**

\[
\text{Swelling \%} = \frac{\text{Average diameter of dry fiber}}{\text{(Diameter of wet fiber - Diameter of dry fiber)}} \times 100\%
\]

As seen in Table 6.2, there are little to no differences observed in the mechanical properties of the biocomposite materials when wet versus when dry, indicating that the emulsion is creating an interaction between the fibrils in the fiber construct which is unaffected by hydration. An explanation could be that the emulsion is actively blocking the majority of the ionic interactions between the chondroitin molecules and the collagen fibrils, which provides the native fibers with the observed structural strength, by coating the fibrils within the construct. This would explain why the biocomposite fibers are much weaker than the collagen-only fibers when dry. Also, if the emulsion is taking the place of the inter-fibril ionic bonding, which is degraded upon hydration,[18] and the polymer film is only minimally affected by hydration, this would explain why there is little difference observed in the mechanical behavior of the dry versus hydrated biocomposite fibers. In either case, the biocomposite is able to retain its mechanical properties in a moist environment and is able to retard swelling of the fiber.
Figure 6.10. Degree of fiber swelling upon hydration for the control collagen fibers and the biocomposite fibers. A) Data obtained from fibers containing 10mL of collagen suspension and varied amounts of CNP3 nanoparticle emulsion. B) Data obtained from fibers containing 100µL of CNP3 nanoparticle emulsion and various dilutions of the collagen suspension.
All of the “collagen-only” fibers swelled to double their original diameter, which is reported as 100% swell. The 25µL and 50µL biocomposite fibers produced a wide range of hydration, indicated by the large deviation in the results. This is most likely due to the variance observed for diameter of the fibers constructed from these formulations, where some areas along the fibers were larger than others, providing a greater difference in the diameter of the wet and dry fibers analyzed. Therefore, no conclusions can accurately be drawn from the data presented in Figure 6.10A for the fibers formulated with 25µL or 50µL of CNP3. Fibers formulated with 100µL or 150µL of CNP3 sufficiently retarded hydration of the collagen fibers over a one hour time period, producing between 28-35% or 15-24% hydration swelling, respectively, as compared to the 100% hydration swelling observed for the control collagen fibers.

![Image](image1.png)

**Figure 6.11.** Display of the variance along the length of the biocomposite fiber formulated with 100µL of CNP3 nanoparticle emulsion and 9% collagen in the fibril suspension. Images of dry pieces of fiber (top) and hydrated pieces of fiber (bottom), all cut from the same parent fiber.

None of the fibers in Figure 6.10B swelled significantly after one hour of hydration. Fibers formulated with very low concentrations of collagen fibrils in the suspension (22% and 9% collagen-containing fibers) were not homogeneously spherical, as was the case with the collagen fibers, which was due to the heightened emulsion content present in the fiber. This caused the fiber to take on a flatter dimensional conformation upon drying, which significantly altered the cross-sectional area of the fibers, as seen in Figure 6.11. Therefore, the higher variability in swelling percentage for these fibers was more likely due to this variance in fiber diameter throughout the length of the fiber than it was in the
swelling difference of the fibers. The images of the single fiber formed from 9% collagen in the fiber formulation showed that not only was no swelling observed for the fibers (bottom images), but that throughout the length of the fiber, the morphology was somewhat random due to the uneven distribution of emulsion throughout the length of the fiber composition. Since the polyacrylate films take on whatever conformation they are formed on, and since the biocomposite fibers are formed by hydraulically lifting the fibers from the fiber-forming solution, the fibers do not take on a constant morphology when air drying in the formulations with low collagen concentrations, hence the inconsistent morphology. The fibers containing the original 100% collagen formulation and the fibers containing 50% collagen in the formulation, as seen in Figure 6.10B, displayed a consistent shape throughout the fiber, and thus the lack of swelling observed for these fibers was significant and not a product of imperfections in the fiber formation.

This data suggests that, when combined with a polyacrylate nanoparticle emulsion, collagen fibers can potentially be used for various biomedical applications without the concern of structural degradation by the moist \textit{in vivo} environment. This is due to the observed retention of mechanical properties when the fibers are hydrated, as well as the observed swelling retardation, yet the fibers still appear to be able to absorb some degree of water. Also, for certain biomedical applications, such as ligament replacement, swelling of the fiber can be useful when regulated so that swelling is not instantaneous, but rather occurs over an extended period of time to allow for surgical manipulations. In this case, these biocomposite fibers may be optimal because they will eventually swell to a certain degree, but this does not occur upon immediate hydration, and even when hydrated, these fibers do maintain mechanical properties. Applications where this property would be useful is when artificial ligaments are being utilized for ligament replacement, and instead of using a bone cement or glue to adhere the artificial ligament to the bone, hydraulic fixation is utilized, where the fiber swells within a small hole created in the bone and this swelling locks the fiber in place. This is just one example of where regulated swelling/hydration of the fiber construct would be useful, however, in each biomedical application proposed here, mechanical soundness of the biocomposite fiber upon hydration or within a moist environment is paramount.

\section*{6.5 TEM Analysis of Biocomposite Fibers}

In order to further understand the nature of the interactions between the collagen fibrils and the nanoparticle emulsion, TEM analysis was performed on chemically fixed cross-sections of the control collagen and biocomposite fibers (Figure 6.12). Fibers sections were stained with both uranyl acetate and lead citrate in order to observe the individual fibrils within the construct and their spatial orientations.
Figure 6.12. TEM images of the cross-sections of control collagen and collagen/CNP3 biocomposite fibers. A) Collagen-only control fiber polymerized from sea cucumber collagen fibrils. B) Biocomposite fiber containing 10mL of sea cucumber collagen fibril solution and 100μL of CNP3 nanoparticle emulsion. C) Outer edge of control fiber. The black arrow is pointing to a horizontally-oriented fibril in the fiber. Black dots within the images are a product of stain precipitation during processing.

The control fiber appears to contain mainly linearly-arranged fibrils tightly packed in the fiber in a longitudinal orientation, as evidenced from the “packing” of circular gray areas in Figure 6.12A and C. This provides the fibrils with the orientation necessary for interfibrillar interactions between the anionic chondroitin and the cationic collagen fibril that provides the fiber with the high observed tensile strength when dry. However, the tensile strength of the reconstituted fibrils is not completely on par with the native fibrils in vivo, which is mostly likely due to the small percentage of fibrils in the fiber that are
not in the same longitudinal arrangement as the majority of the fibrils are (Figure 6.12C arrow). When hydrated, especially with phosphate-buffered saline or saline solutions, it is likely that these ionic interactions are disrupted, possibly depleting the high tensile strength previously observed for the dehydrated fiber (Figure 6.12C). Also, when hydrated, it is presumed that the water molecules take up space in between the fibrils in the fiber, interacting with the collagen fibrils, and thereby causing the observed swelling.

The emulsion-treated fibrils do not appear to have a linear arrangement; in fact, they seem to be randomly oriented within the fiber cross-section in Figure 6.12B. The collagen fibrils that are present are very loosely arranged within the fiber, indicating that the emulsion is perhaps intertwining with the fibril and forming a protective film around the fibrils. The random orientation of fibrils within the biocomposite fiber is most likely a factor of the mixing performed when the emulsion is introduced to the fibril suspension prior to fiber formation. The increased spatial distribution between the collagen fibrils in the fiber composite does not permit the fibrils to interact with one another to the extent that they do in the control fibers, which helps illustrate why there is an observed difference in tensile strengths between the collagen and biocomposite fibers when dry. Moreover, the variability observed for the individual fibrils’ spatial orientation within the fiber explains the elasticity observed for the biocomposite fibrils and why the elastic properties of the polymer film sample are also expressed in the biocomposite fiber. Since most of the collagen fibrils present in the biocomposite fiber appear to be protected from the environment by the emulsion/polymer, this could rationalize the observed retention of mechanical properties upon hydration.

### 6.6 Calcium Deposition Analysis

It has been established since the 1960’s that the dermis of echinoderms is neurally regulated, and that extracellular concentrations of calcium (Ca\(^{2+}\)) has a large influence on the stiffening of the dermis, which is a form of protection for these animals.\(^{36}\) While the mechanism of this calcium interaction is still heavily debated, it has been established that as the concentration of calcium presented to the dermis from the extracellular matrix increases, so does the stiffness of the dermis. Therefore, since it has not yet been established whether the stiffening is a result of a calcium-dependent cellular interaction, or a direct calcium effect on the viscosity of the extracellular matrix,\(^{12}\) the effect calcium doping may have on these biocomposite materials was explored. The biocomposite fibers formulated with 10mL of undiluted collagen fibril suspension and 100µL of CNP3 emulsion were fully submerged in a prepared solution containing 10mM calcium chloride (CaCl\(_2\)) or treated with ddi H\(_2\)O for 24 hours. The diameter for both the treated and untreated fibers was then measured and the fibers were characterized by uniaxial tension analysis.
Figure 6.13. Effect of calcium doping on the biocomposite fibers. A uniaxial tension experiment was performed in order to establish the presence of a calcium-induced stiffening effect on the biocomposite fibers containing 100µL of CNP3.

In Figure 6.13, both the calcium-doped (yellow line) and the undoped (green line) fibers appear to display relatively similar mechanical properties, including maximum stresses within 2-3MPa of each other and maximum strains within 0.05 of one another. The stress/strain curves for the calcium-treated and the untreated control fibers were comparative, however, the calcium-treated fiber (yellow line) did possess a larger elastic modulus due to the slightly more linear stress/strain curve. Also observed was inconsistent maximum strains for the calcium-treated biocomposite fibers, where failures occurred between 0.3 and 0.6, which was not observed for the untreated biocomposite fiber in Figure 6.13. While the calcium-doped fibers appeared to be slightly stiffer (less strain observed), the maximum tensile strength for each set of fibers was equivalent, thus the calcium treatment had minimal effects on the fiber’s overall stiffness. Further analysis comparing the mechanical properties of the calcium-doped biocomposite fibers to the collagen-only control fibers is needed to further appreciate any observed differences presented here for the treated versus untreated biocomposite fibers.

6.7 Conclusions

The addition of nanoparticle emulsion CNP3 to the reconstituted collagen fiber formulation ultimately established the development of a new biocomposite material that may be ideal for artificial elastic tissue replacement. The biocomposite fibers expressed
properties of both the emulsion and collagen constituents, and the expression level of each was able to be regulated based on the relative amounts of emulsion and collagen suspension in the fiber formulation. The most optimal biocomposite fiber formulation was ultimately determined to contain 10mL of the sea cucumber collagen fibril suspension and 100µL of CNP3 nanoparticle emulsion, as determined by both the mechanical properties and the observed consistent morphology of the fibers formed from this formulation. These fibers possessed the most consistent mechanical properties that were closest to the control collagen fibers for the majority of the mechanical properties defined in this study, which was a desirable feature. Also, these fibers displayed a limited degree of fiber swelling upon hydration and minimal degree of variance observed for the mechanical properties of the fibers when hydrated versus when in the original dry state, another favorable outcome that the fibers formulated only from collagen fibrils did not possess.

TEM analysis showed that the biocomposite fibers actively incorporated the emulsion into the fiber formation, presumably in the solidified polymer film state, and that the presence of the polymer surrounding the collagen fibrils and the random spatial orientation of the fibrils within the biocomposite fiber ultimately diminished tensile strength for the biocomposite fibers (when dry). However, upon hydration, these interactions and spatial differences observed for the biocomposite fibers allowed them to retain their full mechanical properties and retard or possibly even prevent swelling of the fibers upon hydration, whereas the control, collagen-only fibers lost all of their stiffness and observed tensile strength upon hydration and swelled to more than twice their nominal cross-sectional area. The presence of the polymer in the fiber also provided the biocomposite fibers with an elastic property that allowed the fibers to undergo cyclic fatigue without any observed effect on the tensile strength of the fiber. It has been shown that in the native echinoderm animal Cucumaria frondosa, from which the collagen fibrils were extracted, calcium (Ca²⁺) influences dermal stiffening and creates a dramatic increase in the stiffness of the dermal collage. However, calcium doping of the biocomposite fibers appeared to have little effect on the tensile properties of the fibers, where the only observed difference was slight variability observed for the maximum strain withstood by the fibers.

In conclusion, the biocomposite collagen/emulsion fibers synthesized displayed properties similar to many natural elastic tissues present in the body, including lung, skin, blood vessel, and aortic heart valve tissue. Therefore, this new biocomposite material would appear to be ideal for use in artificial tissue constructs for treatment of such medical problems as artificial burn wound skin grafts or artificial ligament/tendon replacement to name a few.
6.8 References

CHAPTER 7

CYTOTOXICITY OF DRUG MONOMERS, NANOPARTICLES AND FILMS

7.1 Introduction

In order for any drug to be established as a form of treatment outside of the lab, toxicity analysis must be performed. Toxicity can be determined in a variety of different ways; however, in order for any drug to be non-toxic, it must not have detrimental effects at the cellular level. Therefore, in this chapter, cytotoxicity is analyzed for drug monomers used in preparing nanoparticle emulsions, as well as the drug-conjugated and drug-free nanoparticle emulsions, and drug-conjugated and drug-free smart polymer films. For the toxicity experiments, human dermal fibroblast cells are used. Two different assays were employed in this study to analyze the number of viable fibroblast cells after exposure to a sample, a CyQUANT DNA binding assay, and an MTT colorimetric assay. Both assays detect the number of viable cells through fluorescence absorption caused by interaction of a colorimetric dye with viable cells. These assays not only give quantitative analysis of cytotoxicity, but they can also be used to measure drug sensitivity, response to growth factors, and cell activation. While these assays were chosen as the most optimal for the present study, there are numerous others available for this type of analysis.

7.1.2 Trypan Blue Assay

There are a variety of cell growth and viability assays that quantify the amount of living cells in a culture, some of those are spectrophotometric assays that require the use of a spectrophotometer, and others use dyes that require only a light microscope for analysis. One of the simplest cell viability assays is the trypan blue assay, where the solubilized dye is added to the culture media and allowed to interact with the cells. Cells that are viable have intact cellular membranes and do not take up the dye, thus displaying a blue ring around the outside of the cells. In cells where the cellular membrane has been compromised, indicating cell death, the dye will penetrate the membrane and bind to intracellular contents, making the cells appear completely blue under the microscope. While this assay is a relatively simple method that requires minimal instrumentation and is fairly inexpensive, it is extremely difficult to differentiate between viable and non-viable cells, especially if the microscope being used for such an assay is not high resolution. It is also a solely qualitative method of analysis and therefore can only tell the user whether a cell is alive or dead, and indicates nothing about cellular function. Also, this assay requires a person to count the number of viable cells and each test must be read individually, which ultimately produces a higher degree of variability than other instrument-based assays and does not permit high throughput screening.
7.1.3 Radioactive H\(^3\) or C\(^{14}\) Thymidine Uptake Assay

There are numerous colorimetric and radioactive assays that require high powered detection instruments that calculate the number of viable cells in a culture based on comparison to a standardized curve. These assays are much more reliable, permit high throughput screening due to the use of 96 well plates and versatile spectrophotometers, and provide real-time quantitative analysis. The radioactive cell growth and viability assay uses the radioactive agent H\(^3\)-thymidine for detection of viable cells. Thymidine uptake is not only a useful tool for cellular viability analysis, but it can also be used with tissue cultures and to measure mRNA concentrations in vitro.\(^{[1]}\) This assay requires cellular manipulation in the form of cell harvesting and media changes for detection to occur.\(^{[1]}\) This inevitability increases the degree of variability in the results and the possibility of error. However, recent advances by companies have made this assay much more user friendly and reproducible.

Previously, cellular cultures were required to undergo harvesting in order to determine the thymidine uptake levels, and also required the use of scintillation fluid for radioactivity detection. However, recent alterations to the commercially available kits for this assay provide the user with base plates that incorporate scintillants, therefore eliminating the need for separation steps in the assay and expensive scintillation fluid for detection, which is even more expensive to dispose of than it is to purchase. These assays are now able to be read on microplate readers, which provide this assay with high throughput capabilities. However, the fact still remains that this assay requires the use and handling of radioactive materials, which most people would rather not use if at all possible, and also calls for additional permits and safety approval for a lab. Therefore, if cellular toxicity can be analyzed through the use of a safe dye instead of a radioactive substrate, most researchers would probably choose the non-radioactive means of measurement.

7.1.4 Cellular Toxicity Assays

There are also commercially available assays that determine the amount of cellular death within a culture. These assays commonly use a dye whose fluorescence emissions can be read by a spectrophotometer and that binds to an intracellular component of the cells. If cells are necrotic due to interaction with a substrate, the cells will rupture, thereby releasing the intracellular components into the media. Through the use of a standardized calibration curve with known cell numbers that are lysed with a known cellular lysis agent, the number of dead cells can be obtained through this assay. However, the number of cells that are initially added to each experiment must be known, and a control well must also be used to determine how many cells there would be after overnight culture in order to obtain an accurate toxicity analysis. This, however, indicates that a simple viability assay, such as the trypan blue assay, must be used in conjunction with this type of assay in order to correctly analyze cellular toxicity.
One example of this kind of kit is the lactate dehydrogenase cytotoxicity assay kit, which detects the biochemical activity of the cytoplasmic enzyme after release into the surrounding media following rupture of a cell’s plasma membrane. In this assay, an aliquot of the media that the culture is grown in is centrifuged to remove any cellular debris, and then the supernatant is combined with the enzyme in solution, followed immediately by spectrophotometric analysis. This assay is a very good tool for monitoring the progress of a cell culture over a long time period because the cellular media can be sampled above where the viable cells are attached to determine cell death. When determining cytotoxicity, it is also important to analyze the substrates interaction with the cells over extended periods of time, and thus this assay is optimal for such a study since the cells are not disrupted by the assay, and toxicity can be monitored daily so that accurate analysis of when cellular death is initiated or at a maximum can be determined.

7.1.5 MTT Colorimetric Assay

The MTT assay has recently been considered one of the best assays available for fast and reliable detection of cellular growth and viability. Developed in 1983 by Mosmann, this assay was originally used to measure proliferation of lymphokines, mitogen stimulations and complement-mediated lysis. The success of this assay in current research practices has largely been due to the spectrophotometric procedure used that can detect slight changes in cell metabolism, making it much more sensitive than trypan blue staining, and the colorimetric reagent used for the assay instead of a radioactive one, which makes this procedure much safer than the older radioactive procedures.

This colorimetric assay measures the amount of an insoluble formazan crystal that is formed through the biochemical reduction of a yellow tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT), by the mitochondria of viable cells. Cell cultures are incubated with the MTT reagent for 2-4 hours, then the cells are lysed and any of the formazan crystal product is solubilized for detection. The samples can then be analyzed by any spectrophotometer or multiplate reader that has filters at a wavelength of 570nm. The amount of purple color produced by the dissolved formazan crystal product is directly proportional to the number of viable cells. Detection levels for this assay range from as few as 1,000 cells to numbers as high as 5,000 cells per well. The reduction of tetrazolium salts is a safe and accurate alternative to radiometric testing that provides a linear relationship between cell number and absorbance, enabling accurate, straightforward quantification of changes in proliferation.

The MTT system is a quantitative, more sensitive test than basic trypan blue or toxicity assays because there is a linear relationship between cell activity and absorbance, thereby allowing the growth or death rate of cells to be measured. This assay has also been
extended to cytotoxicity analysis of new drug therapies for detection of anticancer activity,\textsuperscript{4, 6} making this assay a key tool for numerous kinds of drug development.

### 7.1.6 CyQUANT DNA Binding Assay

Another form of the colorimetric assay is the CyQUANT Assay. This assay analyzes cell viability from a different perspective than the MTT assay because it does not measure whether a cell is alive or dead, but measures the amount of DNA present in a culture media, and has been shown to be more sensitive than both the trypan blue and the MTT assays and also faster, requiring only one hour incubation with the cells or cellular lysates. This assay has the capability of measuring 100 to 20,000 cells per well, thereby giving it a wide detection range.\textsuperscript{7} The CyQUANT assays are a fast and sensitive means for counting cells in a population and measuring proliferation. However, its use is limited to a microplate format due to the principle behind the assay. When fibroblast, and many other cells types, are grown in culture, they have extracellular proteins and components that allows them to attach to a substrate (i.e. the bottom of a microplate well). This assay is built upon this principle and uses it to determine viable cell numbers in a culture. After a culture of cells has been allowed to attach to the microplate and is subsequently exposed to a sample of interest over a certain time frame, the media in each well is removed from the culture, leaving behind only the cells that remained attached to the well after exposure to the substrate.

After lysis of attached cells remaining after 24 hours of exposure to the substrate, the lysate is combined with the DNA-binding dye, and fluorescence can be read at the excitation wavelength of 485nm and at the emission wavelength of 530nm, and values obtained can be compared to a standardized curve in order to estimate the absolute number of viable attached cells in a well. Since only viable cells undergo cellular attachment to surfaces, it is concluded that the number of cells measured by this assay indicate the number of viable cells present in the culture. However, this is not a good measure of the overall number of viable cells since cellular attachment can be disrupted by some drugs, and yet the cells remain viable. Therefore, this assay is an indicator of the overall biocompatibility of a substrate (i.e. whether a substrate has any negative effect on a cell population) and does not determine the extent of toxicity or cell death, which is why the MTT assay is often employed as a secondary assay.

As is often seen with fluorescence and absorbance measurements, which both the MTT and the CyQUANT assays utilize, there is a high degree of variability in readings; therefore it is necessary to perform multiple well readings for each sample. Also, if dead or apoptosing cells or cellular DNA adheres to the attached cells and is not removed with the culture media, then this will also skew the numbers obtained. Also, since this assay can only measure the amount of DNA from cells that remain adhered to the well after 24 hours, this method is not recommended for cellular suspension analysis. This is why in
this study, both the MTT and the CyQUANT assays were used to determine cytotoxicity of the drug monomers, nanoparticle emulsions, and smart polymer films.

Figure 7.1. Standardized calibration curve for translation of fluorescence readings from a CyQUANT assay to real-time cell numbers.

7.2 Cytotoxicity of Drug Monomers

To test the cytotoxicity of any drug monomer, it must be solubilized in a solvent in order for it to interact with the cells on a molecular level. The most favorable solvent used for such assays is water, but since all of the acrylated drug monomers synthesized are water insoluble, another solvent was chosen that is the next best thing, dimethyl sulfoxide (DMSO). DMSO is the second most common solvent for drug testing in antimicrobial, anticancer, and cytotoxicity studies, yet it is also known to be cytotoxic at high concentrations. Any concentration of DMSO above 10% in the culture medium is considered toxic and can cause inhibitory effects on healthy mammalian cells, as well as antimicrobial effects. Therefore, the amount of drug that can be tested in both the antimicrobial studies and the cytotoxicity studies is limited by the amount of drug that can be dissolved in 20% DMSO. Drug monomer fluorescence readings should be compared to those obtained for cells treated with DMSO only and not with the control cells.

In this study, acrylated drug monomers were tested against human dermal fibroblast cells isolated from full thickness skin specimens obtained from the US Skin Bank, Cincinnati, OH. Drug monomers were dissolved in DMSO at 1mg/mL concentrations, and tested against the fibroblast cells at either 10 or 20µg/mL concentrations. Since most drug monomers analyzed possess antimicrobial activity at or below 16µg/mL, these
concentrations were deemed appropriate for cellular toxicity analysis, and samples were obtainable at this concentration in aqueous DMSO.

7.2.1 β-Lactam Monomers

**Figure 7.2.** Cytotoxicity assay for β-lactam monomers in DMSO. A) CyQUANT assay performed on 20µg of lactam monomer in 20% DMSO. B) MTT assay for either 10µg/mL or 20µg/mL lactam monomer in DMSO.
The data obtained for lactams 1, 3, 4, 6, and 10 are plotted in Figure 7.2. The CyQUANT assay was repeated numerous times for the β-lactam drug monomers over a six month period. Upon initial assay, fibroblast cells were added to the wells at the same time as the lactam drug monomers. Results showed that the lactams prevented the cells from adhering to the bottom of the well, and the cells remained spherical in morphology. Since the CyQUANT assay calls for cellular attachment in order for conclusive results to be obtained, the assay was repeated with a slight modification in the procedure. For the subsequent assay, the cells were allowed to grow and attach to the wells overnight prior to exposure to the lactam monomers. Here, it was found that age of the monomer, purity levels, and length of time in DMSO all influenced the toxicity of the monomers (Figure 7.2 and 7.3). This was illustrated by the difference observed for the toxicity results for lactam 10 in Figure 7.2A and B, where in the CyQUANT assay, the lactam appeared to cause a toxic effect, yet in Figure 7.2B, this was not the case, and treated cells appear to be as numerous as was observed for the DMSO-treated cells. In Figure 7.2B, it was determined that the lactam monomers yielded equal results for both the 10 and 20µg/mL drug concentrations.

![Figure 7.3](image-url)

**Figure 7.3.** Analysis of toxicity of β-lactam 10 in DMSO by MTT assay over a 24 month period. Control cells were exposed to only DMEM culture media.

Results of an MTT assay (Figure 7.3) showed that the older a drug sample was, or the longer it was dissolved in DMSO, the more the drug caused the fibroblast cells to become detached from the wells and revert back to the spherical shape. This spherical morphology was often observed for the cells throughout the study when exposed to
lactam monomers of low purity when not allowed to attach to the wells prior to exposure. If the cells were allowed to attach prior to exposure, many would become slender and attempt to detach from the wells. It was found after a few trials that when the lactam monomer was prepared fresh and dissolved in DMSO only just before testing, the monomers did not cause any visible toxic effects on the fibroblast cells (Figure 7.4), and the cells remained attached and were able to proliferate equally to those of the control cells. Also, as seen in Figure 7.3, the difference in toxicity between one and two years after lactam 10 synthesis does not alter the toxicity of the drug, yet between one month post-synthesis and one year, there is a marked increase in toxicity of the drug. This indicates that drug monomers should be incorporated into nanoparticle emulsions promptly after synthesis.

Figure 7.4. Images of fibroblast cells treated with lactam monomers 3 and 4. When lactam monomers are prepared fresh and with high purity, fibroblast cells remain elongated and viable, actively proliferating equal to control cells.

Assays were also performed with the cells being exposed to the lactam monomers over a period of 7 days (Figure 7.5). However, this data may not be very accurate since media changes were not performed due to the risk of losing viable cells that had detached from the well surface and taken on a spherical morphology due to the lactam interaction with the cells, causing them to be in the media suspension. Even in control wells where no test substrates were added, cellular debris was present throughout the well and appeared settled on top of the still viable cells, indicating that the cells were more vulnerable to toxicity by the end of the study. The heightened toxicity observed for this assay was therefore likely due to increased sensitivity of the cells to the drug monomers due to a lack of fresh nutrients present for the cells. Also, in this assay, the fibroblast cells were not allowed to attach to the wells prior to exposure to the lactam monomers, increasing the likelihood of the cells to remain in the suspension.
**Figure 7.5.** Fibroblast cells after treatment with β-lactam monomers over 7 days. Control wells with only media present contained attached fibroblast cells (left arrow) and cells detaching from the well (right arrow). Cells treated with lactam 1 mostly became spherical and detached from the well, but some (arrows) remained attached and viable. Lactam 6 produced no such effects and showed no toxicity.
In the extended exposure assay (Figures 7.5 and 7.6), lactam monomers 1 and 10 were considered cytotoxic to the cells and caused the cells to remain in their spherical morphology, whereas lactams 3 and 6 allowed the cells to attach to the well similar to the cells exposed to DMSO. In this assay, lactams 1 and 10 were older samples that must not have been of high purity, since numerous other assays showed these two monomers to be non-cytotoxic to the fibroblast cells (Figures 7.2 and 7.3). Lactam 6 was freshly prepared for this assay and was considered to be the purest sample analyzed for this monomer thus far. The heightened number of control cells determined by the CyQUANT assay is most likely due to the excess cellular debris caused from cellular lysis attaching to the attached cells, thereby causing higher numbers from the fluorescence readings than were viable cells. Also, since the cells exposed to 1 and 10 remained in suspension in the spherical form, these cells were not able to be included in the CyQUANT assay since they were not attached to the wells. Therefore, this data set is not as accurate as the other data sets, and for an extended exposure time assay to be performed, many issues need to be resolved, including changing the assay to the MTT assay, which was unavailable at the time of this experiment.

**Figure 7.6.** CyQUANT cytotoxicity analysis of fibroblast suspension mixed with lactam monomers over 7 day incubation period.
7.2.2 Acrylated Commercially Available Monomers

All of the acrylated drug monomers analyzed, except for the β-lactams, were prepared from commercially available drug compounds whose toxicity has already been established and deemed non-cytotoxic. Upon acrylation, the toxicity of the drug entity may change. Therefore, it was necessary to analyze the cytotoxicity of the drug monomers, even though it is believed that through the mode of action of the nanoparticle delivery system the in its original commercially available form and thus should be non-toxic when used for infection treatment.

Figure 7.7 showed that all of the acrylated drug monomers, except the acrylate penicillin G, were non-sytotoxic to the human dermal fibroblast cells. Interestingly, the fluorescence results for the MTT assay shown in Figure 7.7B conclude that the ciprofloxacin methacrylamide 8 was cytotoxic, yet the CyQUANT assay in Figure 7.7A and visual observation of the treated fibroblast cells (Figure 7.8 and 7.9) indicate that no toxic effects were caused by this drug. The batch of cipro monomer 8 used for the MTT assay may have contained additional contaminants including remaining solvent or unreacted reagents that was not present in the majority of the other batches of cipro methacrylamide 8 synthesis, and therefore, is the drug is not cytotoxic when thorough work up and evaporative processes are employed.
Figure 7.7. Cytotoxicity assays for acrylated commercially available drug monomers 7 and 8. A) CyQUANT assay data for 40μg of drug in 20μL DMSO. B) MTT assay data for both 20μg and 10μg drug samples in DMSO.
Figure 7.8. Images of fibroblast cells treated with acrylated commercially available drugs. All cells imaged either resemble control cells or cells treated with DMSO only, and therefore, all appear healthy and viable.
**Figure 7.9.** Close up images of fibroblast cells treated with ciprofloxacin acrylated monomers 7 and 8.

Images of the cells in Figures 7.8 and 7.9 that were treated with acrylated drug monomers 7 and 8 dissolved in DMSO showed that data obtained from the CyQUANT and MTT assays is accurate and that the drug-treated fibroblasts were healthy and viable. Close up images in Figure 7.9 depict healthy elongated fibroblast cells with intact nuclei present in all of the cells. Although some cells took on a shortened, stubbier morphology, as was seen with for the cells treated with DMSO in Figure 7.8, they were still viable, proliferating cells that were attached to the wells. Therefore, all of the acrylated drug monomers analyzed in either the MTT or CyQUANT assays were deemed non-cytotoxic towards human dermal fibroblast cells.

### 7.3 Cytotoxicity of Nanoparticle-Based Emulsions

#### 7.3.1 Drug-Free Polyacrylate Emulsions

Drug-free emulsions were analyzed for cytotoxic affects against the human dermal fibroblast cells at various concentrations of solid content in order to determine at what concentration, if any, the emulsions become cytotoxic. Since there were no drug monomers in these emulsions to contend with, this gave the opportunity to determine if the emulsion had inherent toxicity. All of the emulsions were first diluted prior to
addition to the cells using nano-purified water, and all emulsions were analyzed at 20µL volumes per well, regardless of the original solid content concentration.

![Bar graph showing fluorescence absorbance (485/535nm) for control nanoparticles (20µL)](image)

**Figure 7.10.** CyQUANT cytotoxicity data for drug-free emulsions at 10% solid content.

Initial assays were performed on emulsions at 10% solid content, in which instance most of the emulsions created cytotoxic effects on the fibroblast cells (Figure 7.10). This was not easily determined due to the high amount of polymer and surfactant precipitation on top of the fibroblast cells, creating almost an intact film covering the cells (Figure 7.11). This sedimentation/film formation completely inhibited visualization of any fibroblast cells that may have remained attached and viable, and thus accurate analysis of the number of viable fibroblast cells in each well via the CyQUANT assay was not possible. Also, when the films were able to be removed from the wells, fibroblast cells may have adhered to the films, and would have been removed prior to assay analysis. Therefore, accurate assessment of toxicity for these emulsions having a solid content of 10% or greater is not feasible with either of the assays.

Although many of the control (non-drug containing) emulsions were not able to be tested then, two samples (**CNP16** and **CNP17**) were accurately tested since no precipitation was observed in the treated cell-containing wells to interfere with the fluorescence assays and these emulsions did not display any cytotoxicity at 10% solid content (Figure 7.10). This is mainly because they contain only 1% SDS in their formulation, which resulted in less surfactant and polymer sedimentation in the wells, presumably due to a higher degree of nanoparticles present within the emulsion over the longer chain polyacrylate strands.
Figure 7.11. Interaction of CNP12 at various solid content concentrations with human dermal fibroblast cells. The emulsion, when applied to the cells at solid content concentrations above 2.5%, precipitated on top of the cells, most likely along with excess surfactant, thereby blocking accurate fluorescence measurements and visualization.
Figure 7.12. CyQUANT assay analysis of dilution scheme for drug-free nanoparticle emulsions CNP5 and CNP12. For both emulsions, 20% and 10% solid content concentrations appear to be cytotoxic when applied to cultured fibroblast cells in a well.

The images in Figure 7.11 correlate well with the CyQUANT data obtained in Figure 7.12. The high variability for CPN12 at 5% solid content concentration is consistent with the images of cellular debris and polymer and surfactant precipitate skewing the data. Also, some cells may have still been attached to the wells under the precipitated material in the case of the 10% and 20% solid content wells, which would explain the CyQUANT data. The fact that any fluorescence was observed for cells treated with these concentrations is more likely due to fluorescence being emitted by the polymer itself and not from cellular DNA. In later experiments, this observed fluorescence was nulled from the experimental data by analyzing the fluorescence emission observed for the emulsion in the culture media when no cells are present in the wells and subtracting these values from the values obtained for the actual cytotoxicity assays.
Figure 7.13. MTT assay of multiple drug-free emulsions at various solid content percentages.

In Figure 7.13, three different formulations of drug-free emulsions are analyzed at various solid content concentrations for cellular toxicity via the MTT assay. CNP3 was formulated with ethyl acrylate: methyl methacrylate, CNP16 was formulated with butyl acrylate: styrene, and CNP17 was formulated with butyl acrylate: methyl methacrylate. All three control emulsions analyzed contained only 1% SDS and 0.5% radical initiator, and therefore, should be non-cytotoxic at solid content concentrations as high as 10% in vitro. Figure 7.13 shows that the emulsions having a solid content concentration of 0.75% to 2.5% were non-toxic. The data in Figure 7.10 corroborates this hypothesis, since in this figure only the CNP16 and CNP17 formulations contained 1% SDS, whereas all of the other emulsions contained either 3% or 5% SDS. Overall analysis of the data concluded that all emulsions, regardless of the ratio and type of co-monomers used, ideally should contain 1% SDS and 0.5% radical initiator for the most favorable properties in vitro.

7.3.2 Single Drug-Conjugated Emulsions

While it was established in the previous study that drug-free emulsions are non-cytotoxic at concentrations at or below 5% solid content, this may not be the case when drug monomers are covalently bound to the nanoparticles. Since drug monomers also can display cytotoxicity at high concentrations, the concentration of the drug in the emulsion must also be taken into account. Therefore, seven drug-conjugated emulsions were
prepared and diluted to 20µg/mL or 10µg/mL (2.5% or 1.25%) solid content prior to cytotoxicity analysis by CyQUANT and MTT assays \textit{in vitro}.

\textbf{Figure 7.14}. CyQUANT and MTT assays for single drug-conjugated nanoparticle emulsions. A) CyQUANT assay performed at 2.5% solid content. B) MTT assay performed using 2.5% versus 1.25% solid content concentrations.
All of the drug-containing nanoparticle emulsions analyzed in Figure 7.14 were considered non-cytotoxic. The only nanoparticle formulation that showed some cytotoxicity was \textbf{NP1}, but this was not a significant degree of variance from the control data due to the high standard deviation observed when analyzed at 2.5% solid content. Heightened fluorescence readings above those for the control cells could be a result of the antibiotics present in the nanoparticle emulsions removing any bacteria that may have infiltrated the cultures. Also, since the standard deviations for many of the emulsions that produced high fluorescence readings are fairly large, the data falls within the same range as the control readings. Other possible explanations for the increased fluorescence readings for the 2.5% solid content emulsions could be due to small amounts of the emulsion being analyzed by the spectrophotometer. Since the emulsions contain the aromatic co-monomer styrene, plus some of the drug monomers express fluorescent moieties, they could also be contributing slightly to the fluorescence readings, which would produce values higher than cells not exposed to any emulsion or test substance (control cells). Since previous data has shown that the higher the solid content concentration, the more likely it is that some of the polymer/surfactant will precipitate in the cell-containing wells, this explains why the fluorescence readings for \textbf{NP8} and \textbf{NP11} are higher for the cells exposed to the 2.5% solid content emulsions than they are for the cells exposed to the 1.25% solid content emulsions.

### 7.3.3 Multi-Drug Conjugated Emulsions

The two different formulations, \textbf{MDNP1} and \textbf{MDNP2}, were analyzed for cytotoxicity by the MTT and CyQUANT assays. This was deemed necessary since the two formulations not only contain different drug monomers in different concentrations, but also because \textbf{MDNP1} was formulated similarly to \textbf{CNP16}, where only 1% surfactant and 0.5% radical initiator were used (Figure 7.15). \textbf{MDNP2} was formulated after \textbf{CNP5}, with 3% surfactant and 0.5% radical initiator, therefore, a difference in toxicity levels due to the surfactant levels was expected (Figure 7.17).

![Figure 7.15. Acrylated drug monomers incorporated in MDNP1. The total amount of all drugs incorporated in the emulsion is 5% of the solid content.](image)
Figure 7.16. CyQUANT assay for various solid content concentrations of MDNP1.

Figure 7.17. Acrylated drug monomers incorporated in MDNP2 emulsion formulation. Emulsion was also formulated with a 7:3 ratio of butyl acrylate: styrene, with 3% surfactant and 0.5% radical initiator.
Figure 7.18. Toxicity assays for both multi-drug conjugated nanoparticle emulsions. A) CyQUANT assay at 2.5% solid content only. B) MTT assay of emulsions at both 2.5% and 1.25% solid content concentrations.
Figure 7.18B shows that the multi-drug nanoparticle formulation MDNP1 is the least cytotoxic of the two formulations made, and displays almost no cytotoxicity at 2.5% solid content concentration (Figure 7.16). This is most likely due to two factors: higher purity of the drug monomers incorporated into MDNP1 and the fact that only 1% surfactant was used for this emulsion as opposed to 3% that was used for MDNP2. The CyQUANT assay showed no difference in toxicity levels between MDNP1 and MDNP2, but this assay was only performed on the emulsions at 2.5% solid content, where at this concentration in the MTT assay (Figure 7.18B), the fluorescence readings were also equal. This indicates that if the CyQUANT assay was performed on the emulsions at 1.25% solid content, then a difference in cytotoxicity may have been observed in this assay as well.

7.4 Cytotoxicity of Polymer Films

7.4.1 Drug-Free Polymer Films

Polymer films were able to be analyzed for toxicity in two ways: by formation of the film on the bottom of the well prior to addition of fibroblast cells, and by addition of small pieces of film samples to attached fibroblast cells in media. Both of these routes were explored for the CyQUANT assay, however, only the addition of the small polymer film pieces to the attached cells was analyzed for the MTT assay. This was due to some experimental problems encountered when the polymer film was formed in the wells before the assay was initiated.
Figure 7.19. CyQUANT assay for drug-free polymer films. All films weigh approximately 80mg. A) Initial assay using all polymer film samples. B) Assay on least cytotoxic polymer films samples, all containing 3% surfactant or lower.
In Figure 7.19, in the CyQUANT assay data, the films were not washed with ethanol prior to addition to the fibroblast cells for removal of any bacteria or other debris that can adhere to the films due to them being exposed to the environment for a long period of time prior to analysis. Therefore, the CyQUANT assay was repeated where all the films were dipped in 70% ethanol then allowed to air dry overnight in a biosafety class 2A cabinet in order to remove all of the excess ethanol prior to addition to the assay. Also, in the initial CyQUANT assay, the polymer films were formed on the surface of the wells by addition of 50μL emulsion to the well then allowing it to dry overnight in the biosafety cabinet. Since the CyQUANT assay determines the amount of attached cells, the low fluorescence readings may be due to the fibroblast cells not being able to adhere to the polymer films when greater than 1% surfactant is used in the emulsion. If this were the case, then this would indicate that the fibroblast cells may not have been damaged by any toxic effects from the films, but they were unable to adhere to the films, thus the low fluorescence readings obtained from the assay; therefore, the second set of data shown in Figure 7.19B is more reliable and was determined to be reproducible.
As seen in Figure 7.20, the polymer film samples containing only 1% SDS did not appear to have visibly affected the cells’ viability or altered their confluency. This supports the data obtained in Figure 7.19B, which showed fluorescence readings for CNP16 and CNP17 equal to those of the control cells. Polymer films that contained 5% SDS in their formulation were only analyzed in the initial CyQUANT assay due to the observed toxicity, and in many wells precipitated excess surfactant onto the fibroblasts. This was most likely the cause of the observed toxicity, and therefore, only formulations containing 3% SDS were further analyzed by the MTT assay. The films containing 3% SDS (CNP7 and CNP12) in Figure 7.19 show viable cells present in the wells, but not as
dense or confluent as the control cells or cells treated with CNP16 or CNP17. These cells appear fatter than the control cells; however, they are all attached to the wells, indicating cellular viability. These findings support the data obtained from the CyQUANTA assay in Figure 7.17B.

The data presented in this section supported the data in Section 7.3.1, that emulsion polymerization formulations that contained 3% SDS or less are non-cytotoxic to dermal fibroblast cells. This was not only true for the emulsions, but also for the polymer films. The films displayed even less cytotoxicity than the parent emulsion, most likely due to no chance of intercellular interactions with the polymer films, unless surfactant or radical initiator is leached from the films, as was seen for the 5% SDS films (CNP 9, 10, 13, 14). Overall, when pieces of polymer film samples that are formulated with less than 5% SDS are in direct contact with healthy human dermal fibroblast cells, no cytotoxic effects are observed.

### 7.4.2 Drug-Conjugated Polymer Films

![Bar Chart](image)

**Figure 7.21.** CyQUANTA assay of drug-conjugated polymer films. All samples weigh approx 80mg and are a mix between 3% SDS and 1% SDS films.

Figure 7.21 shows that many of the drug-conjugated films were non-cytotoxic at 80mg pieces of film. The film samples were all washed for 2 hours with 70% ethanol then allowed to air dry overnight in a biosafety class 2A cabinet to kill any microbes adhered
to the film samples prior to analysis. Polymer films containing acrylated penicillins (NP11, NP12, and NP15) and ciprofloxacin acrylated monomers (NP7 and NP8) all displayed lower than normal fluorescence readings, while the lactam-conjugated film NP1 displayed no cytotoxic affects, according to the data. However, visual observations of the fibroblast cells (Figure 7.22) did not concur with the data in Figure 7.21, since, upon observation, many of the wells containing these film samples possessed healthy elongated fibroblast cells, and cells exposed to NP1 were not nearly as healthy as some of the other treated cells (Figure 7.22).

![NP1 and NP7](image-url)

**Figure 7.22.** Image of fibroblast cells exposed to NP1 and NP7 film samples.

Some samples caused a sediment to form on the cells, as was seen for many of the 3% surfactant-containing films, yet the cells below the sediment all appeared viable and attached to the wells. Cells exposed to NP7 and NP8 appeared to have the same density of cell population as the control cells, and all were elongated and attached to the well, with a few spherical but attached cells also observed (Figure 7.22), which is in complete contrast to the data in Figure 7.21. This is just one of the inconsistencies observed with data from this particular CyQUANT assay, which was not observed for any of the other experiments using this assay. However, this assay also posed a problem when analyzing the lactam monomers due to the spherical morphology observed for some of the treated cells. Unfortunately, the MTT assay was not able to be used for the drug-conjugated and multi-drug conjugated film samples, which may have most likely supported the visual findings over the CyQUANT data. Therefore, no real conclusions can be made from the presented data in this section due to the inconsistency of the findings. However, since emulsions of these drug-conjugated nanoparticles were non-cytotoxic, and the drug-free polymer film samples were non-cytotoxic (Section 7.4.1), it is reasonable to surmise that the drug-conjugated polymer films are also non-cytotoxic.
7.4.3 Multi-Drug Conjugated Films

![Graph showing fluorescence readings for different polymer films](image)

**Figure 7.23.** CyQUANT assay on multi-drug conjugated polymer film samples.

Some cellular lysis debris was observed for cells exposed to MDNP2, but also in these wells were healthy elongated cells, as well as some spherical cells, which are not incorporated into the CyQUANT data since they do not adhere to the wells. This indicates that the 3% surfactant in the MDNP2 formulation is slightly cytotoxic when analyzed in vitro against healthy fibroblast cells. Therefore, the MDNP1 formulation, which contains only 1% SDS, is the better choice than MDNP2 for biomedical applications since it has been shown to be non-cytotoxic. Also, the data presented in Figure 7.23 for MDNP1 did not concur with the visual observations of the cells exposed to this film sample. The fibroblasts exposed to MDNP1 maintained an attached, elongated morphology and the cell population in the wells appeared dense. While some of the cells appeared fatter than the control cells, similar to cells exposed to DMSO in Figure 7.8, these cells were still viable and should have been detected in the CyQUANT data. Since cellular debris was observed in the control wells, the actual cell count measured in these wells may be artificial and higher than expected due to the DNA from the lysed cells contributing to the CyQUANT assay data. The data obtained for the control cells and the cells exposed to MDNP1 are similar, indicating that the multi-drug conjugated polymer film was non-cytotoxic to the healthy dermal fibroblast cells.
7.5 Toxicity Analysis of Surfactant

After analysis of the drug-free emulsions and the drug-free polymer films, it became apparent that the amount of surfactant was a contributor to the observed toxicity for some of the formulations. CyQUANTA analysis was therefore done on the free surfactant used, dodecyl sulfate sodium salt (SDS), in order to determine the degree of toxicity. The hope of this study was to determine at what concentration the SDS could be used in the emulsions that would not produce a cytotoxic affect on the fibroblast cells.

Various concentrations of SDS dissolved in sterile saline were added to attached fibroblast cells and allowed to incubate overnight. CyQUANT assay was then performed on the attached fibroblast cells. Results showed that all levels of SDS were highly toxic to the human dermal fibroblasts (Figure 7.24). While one percent surfactant displayed the least amount of toxicity, the number of healthy attached cells for this concentration was still drastically lower than the cells in the control wells.

![Figure 7.24](image)

**Figure 7.24.** CyQUANT analysis of SDS added to attached fibroblast cells in culture media. Control cells were exposed only to the culture media, and SDS was applied to the cells dissolved in double distilled water.

Since the drug-free emulsions did not exhibit toxic effects nearly as severe as the effects observed for SDS alone, it was determined that in order to understand the toxicity observed in the emulsions, the SDS should be analyzed in the presence of one of the least toxic emulsions to determine if any interactions between the nanoparticles and the SDS can decrease the observed toxicity.
Various amounts of SDS were mixed with 0.1mL of an emulsion made with 5% solid content to obtain the same concentrations as were tested for the SDS alone. At 5% solid content, the amount of inherent SDS in the emulsion is 0.75%, therefore, this concentration was adjusted by adding additional SDS to the emulsion to reach the concentrations previously analyzed. The new emulsions were then added to the attached fibroblast cells in 20µL volumes and incubated overnight. CyQUANT assays were then performed on the attached cells.

![Graph](image)

**Figure 7.23.** CyQUANT analysis of SDS toxicity when added to attached fibroblast cells in combination with CNP5 at 5% solid content. Control cells were exposed only to the culture media, and SDS was added to the CNP5 emulsion after being diluted to 5% solid content concentration to obtain the specified percentages of SDS in the emulsion.

Results in Figure 7.23 showed that when combined with control nanoparticles prior to addition to healthy fibroblasts, the surfactant was much less toxic than when added to the cells without the control nanoparticles, and showed only weak toxicity at 1%. At concentrations of 7% and 9% SDS, all fibroblast cells were killed by the treatment. These results for 1% SDS may be due to interactions of the surfactant with the nanoparticles, which may prohibit the surfactant from interacting with the cells. Upon dilution of the emulsion to 5% solid content, a great majority of the unused surfactant is removed, thereby leaving behind room for more surfactant to be added to the emulsion. However, it appears that the nanoparticles become saturated between 1 and 2% SDS, which would explain the regained toxicity at 3% SDS. Subsequently, it also appears that at 3 and 5% SDS, the surfactant is able to remain in solution for the most part, which would explain
why some viable cells were observed. Yet at 7% and higher, all of the excess SDS most likely precipitated onto the cells in culture, creating an extremely toxic environment for the cells.

This study has lead to the conclusion that 1% SDS in the emulsion polymerization process is optimal for in terms of minimizing cytotoxicity, and therefore, emulsions of CNP15, CNP16, and CNP17 were prepared containing only 1% SDS. Also, due to the results of this experiment, the multi-drug conjugated system MDNP1 made with 1% SDS displayed less cytotoxicity than the 3% SDS-containing MDNP2. As seen in sections 7.3 and 7.4, these emulsions and polymer films were completely non-toxic to the fibroblast cells, as determined by both CyQUANT and MTT assays. Therefore, this study was crucial to obtaining an optimal emulsion formulation for the multi-drug conjugated nanoparticles, as well as polymer film samples, for some of the intended biomedical applications.

7.6 Conclusions

It was determined from various toxicity assays that the β-lactam monomers are non-cytotoxic when prepared fresh, have not been dissolved in DMSO for a long period of time, and are of high purity. Otherwise, the lactams cause the fibroblast cells to either remain in the spherical morphology, or cause the cells to detach from the wells and return to their spherical morphology. This is most likely caused by impurities in the lactam samples interfering with the proteins and complexes involved in cellular adhesion. However, when purified lactam samples are used, cytotoxicity can be avoided.

As was expected, all of the acrylated commercially available drug monomers were non-cytotoxic based on both the MTT and CyQUANT assays. As was the case with the lactam monomers, purity of the monomer and the DMSO it is dissolved in can cause toxic effects on the fibroblast cells; however, when purified, and fresh DMSO is used, no cytotoxic effects were observed for any of the commercially available drug acrylates.

Initial toxicity assays with CyQUANT DNA binding dye and the control emulsions showed that the emulsions formulated with 5% weight percent of SDS caused toxic effects in the fibroblast cells, as did the emulsions formulated with 3% SDS to some extent. This was why the control emulsions CNP15, CNP16, and CNP17 were formulated with 1% surfactant and then analyzed for cytotoxicity. After issues with polymer precipitation in the cell culture wells were worked out by diluting the emulsions to at least 5% solid content prior to exposure to the fibroblast cells, it was determined that the emulsions containing 3% or 1% SDS were non-cytotoxic. It was concluded from this, and from the SDS concentrations analysis, that 5% SDS was excessive for the emulsion formulation since no detrimental effects are observed when all of the surfactant is utilized by the nanoparticles. Therefore, all of the remaining drug-containing emulsions were formulated with 1% SDS.
All of the polymer film samples analyzed that contained less than 5% SDS were completely non-cytotoxic to the fibroblast cells. Toxicity was observed for the polymer films only when excess ethanol remained on the films, or when polymer films where not allowed to fully form and some of the emulsion remained, which then precipitated in the culture media and on top of the fibroblasts. Polymer films containing 5% SDS caused the culture media to become cloudy white when added to the wells, further indication that this amount of SDS in the formulation is excessive and causes toxicity. While conclusive data was not obtained for any of the single or multi-drug conjugated polymer film samples, visual observations of the cells showed little to no cytotoxic effects on the fibroblast cells, depending on the surfactant concentrations of the films. Results from the drug-conjugated emulsion in vitro assays also support the conclusion that these drug-conjugated films are non-cytotoxic towards human dermal fibroblast cells when SDS levels are below 3%.

7.6 References

1. MTT Cell Proliferation Assay. 2007, American Type Culture Collection (ATCC).
CHAPTER 8

In vivo BIOCOMPATIBILITY OF PENICILLIN-CONJUGATED NANOPARTICLE TREATMENT FOR MRSA INFECTIONS

8.1 Introduction

Microbial drug resistance has become a widespread problem in hospital and non-hospital environments, and currently used treatments for these infections have become less effective. One of the most predominant and deadly of these drug resistant microbes is methicillin-resistant Staphylococcus aureus (MRSA), which is an opportunistic pathogen commonly invading skin and soft tissue abrasions and wounds causing bacteremia and eventually leading to sepsis and toxic shock syndrome, often resulting in morbidity for patients. In burn centers, it has been reported that patients treated with β-lactam antibiotics for wound infection are nine times more likely to develop an MRSA strain in the wound bed. This is due to the rapid formation of Staphylococcal resistance when exposed to this type of antibiotic. This problematic bacteria has been responsible for nearly 4,000 deaths each year in the United States alone, and antibiotic therapy has been reduced to only a select few approved therapies, such as glycopeptide-type drugs and oxazolidinones, due to rapidly formed resistance that continues to evolve each year.

As many bacteria, including MRSA, become increasingly resistant to commonly used antibiotics, a growing need for new drug design, discovery, and delivery methods has surfaced and requires immediate attention. While many water-soluble antibiotics are able to act on microbial infections once they have reached the blood stream, these drugs are not able to reach the root of the problem, which often occurs in areas where water-soluble drugs are ineffective. Yet lipophilic antimicrobials are also ineffective here as well due to a lack of initial uptake by the body in order to reach these sites. Often such systemic infections, frequently caused by drug resistant bacteria, are initiated in fatty tissue areas, such as skin and soft tissues or on the surface of implanted medical devices where highly drug resistant microbial biofilms often form. Being able to treat such infections not only within the blood stream but also at the site of initiation with multiple drugs would be optimal due to a reduction in the risk for cross-resistance that often occurs when using a single antibiotic.
As described in Chapters 3 and 4, novel drug-conjugated nanoparticles synthesized through a single step emulsion polymerization process are a promising new way to combat this solubility and bioavailability issue against drug-resistant bacteria, including MRSA.\textsuperscript{[12, 13]} This nanoparticle delivery system has proven to be an effective way of shielding the antibiotic from resistance factors prior to direct contact with the bacteria, thus rejuvenating the activity of the $\beta$-lactam-based antibiotics against $\beta$-lactamase producing bacteria such as MRSA.\textsuperscript{[13]} This drug delivery system has been established \textit{in vitro} for nanoparticle systems containing both individual covalently bound antibiotics, as well as with multi-drug conjugated nanoparticles, and has shown potent anti-MRSA activity, as well as potent activity against \textit{P. aeruginosa} and other common opportunistic pathogens known to invade skin wounds. The anti-MRSA activity was observed even when the usually inactive penicillin G is the bound drug, thereby providing a promising new method for combating microbial drug resistance and many forms of \textit{Staphylococcus} infections.\textsuperscript{[13]} The most common and dangerous route of infection for MRSA and other opportunistic pathogens has been through dermal abrasions, which often lead to bloodstream systemic infections if left untreated. The biocompatibility of the single and multi-drug-containing nanoparticle emulsions has been established \textit{in vitro} using human dermal fibroblast cells, and results have shown that the acrylated drug monomers and the resulting drug-containing emulsions have no toxic effects on the cells (Chapter 7). In order to establish the efficacy of the nanoparticle system in treating MRSA infections in an \textit{in vivo} setting, biocompatibility of the most promising penicillin-containing emulsion,

\textbf{Figure 8.1.} Representation of the emulsion polymerization with acrylated penicillin G monomer (13).
NP13, and the multi-drug conjugated emulsion MDNP1 was established in vivo. NP13 and its non-drug analogue, CNP16, were prepared as formulated in Table 8.1. NP13 has 5% by weight of penicillin G monomer 13.

Table 8.1. Formulations for CNP16 and NP13 tested in vivo.

<table>
<thead>
<tr>
<th>Monomers in Emulsion</th>
<th>CNP16</th>
<th>NP13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin G Acrylate Monomer (13)</td>
<td>0%</td>
<td>5%</td>
</tr>
<tr>
<td>Butyl Acrylate: Styrene (7:3 Ratio)</td>
<td>18.5%</td>
<td>13.5%</td>
</tr>
<tr>
<td>Nano-purified Water</td>
<td>80%</td>
<td>80%</td>
</tr>
<tr>
<td>Sodium Dodecyl Sulfate</td>
<td>1%</td>
<td>1%</td>
</tr>
<tr>
<td>Potassium Persulfate</td>
<td>0.5%</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

The biocompatibility of the drug-conjugated and drug-free emulsions (CNP16, NP13, and MDNP1) was established in vivo by either intraperitoneal injection or by topical application to a skin abrasion in mice models. For the multi-drug conjugated emulsion, MDNP1, biocompatibility was only determined by topical administration and served as an initial study for determining the efficacy of the emulsion for burn wound infection treatment. To determine if the penicillin-conjugated nanoparticle emulsion could be used as a prophylaxis treatment, systemic administration was one of the routes chosen for toxicity analysis. Also, since one of the most dangerous routes of infection for MRSA is through skin and soft tissue abrasions due to the high risk of sepsis and TSS forming,[2,5,8] topical administration on the emulsion was also chosen as a route of administration. However, since the emulsion would be applied to skin that is not intact and is therefore more sensitive and vulnerable to toxicity when treating this form of infection, an abrasion model was chosen to test the biocompatibility and/or toxicity of the emulsions when applied topically. The dose concentration and regimen were varied for all three nanoparticle-based emulsions administered, and saline controls were employed to provide a base for the cytokine levels in blood serum, as well as to observe any variances in the skin/soft tissue wound healing process. Biocompatibility was ultimately established by determining cytokine levels in the blood serum of the mice at days 8 and 14 of the study, which translates into detection of an inflammatory response at those time points. Toxicity
and/or overall animal health and well being was determined visually by monitoring animals for signs of pain, irritation, inflammation, discomfort, and distress.

8.2 Effect of Nanoparticle on Systemic Application in Mice Models

8.2.1 Visual Analysis of Toxicity

All mice injected into the peritoneal cavity with high concentrations of the emulsions (>1.5 weight % of solid content), whether containing penicillin G or not, endured polymer precipitation within the peritoneal cavity. However, the amount of precipitated polymer observed decreased parallel to decreasing the solid content of the emulsions. Polymer was observed trapped in the subcutaneous region of the abdomen as well as attached to many vital organs, mucous membranes, and adipose tissue within the peritoneal cavity, yet all organs and internal systems seemed to be healthy and appeared consistent with normal size and coloring.

![Figure 8.2.](image)

**Figure 8.2.** Effects of systemic nanoparticle administration. A) Subdermal area of mouse treated with 5% solid content CNP16, unpurified. B) Polymer from 5% NP13 purified emulsion clustered on the inside of the peritoneal cavity lining and various internal organs. C) Polymer from 5% NP13 purified emulsion clustered in the fatty tissue covering the lower abdomen and within the mucous membranes affixed to the large and
small intestines. D) Peritoneal cavity of mouse treated with 0.25% NP13 unpurified. E) Peritoneal cavity of mouse treated with saline solution.

It was determined that the precipitated polymer concentrated in highly lipophilic areas, such as in the mucous membranes. At concentrations of 3% solid content and higher, some abdominal distension was observed, however, this was not the case with all mice receiving these concentrations, and no signs of pain were observed upon abdominal examination. Mice receiving emulsions below 3% solid content showed no physical signs of abdominal pain or distension, nor any signs of sensitivity or overall discomfort. At concentrations below 1.5% solid content, little to no polymer precipitation was observed.

8.2.2 Biochemical Analysis of Blood Samples

Cytokine levels in the blood samples taken on days 8 and 14 of the study were analyzed using an enzyme-linked immunosorbent assay (ELISA) kits for detection of interleukin 6 and tumor necrosis factor alpha. Both these cytokines are key factors in the innate inflammatory response of the mouse, and an increase or decrease in the levels of the cytokines in the blood relates directly to the toxicity of the treatment applied. Very little variance in the levels of IL-6 were observed for any of the mice treated with the emulsions, however, there were observed differences in the levels of TNF-α. At higher solid content concentrations (5% and 3%), unpurified samples produced very low concentrations of TNF-α as compared to the saline-treated mice. While a decrease in these levels does not indicate an inflammatory response, this could mean that the emulsions that precipitated in the peritoneal cavity may have affected other processes, which caused the levels of TNF-α to drop in the blood serum. However, since the levels of TNF-α were not raised, nor were the levels of IL-6, this implies that even with polymer precipitated in the peritoneal cavity, that the polymer itself is biocompatible and did not illicit an inflammatory response.
Figure 8.3. Comparison of the levels of TNF-α to IL-6 in mice when 0.1mL of unpurified NP13 emulsion is injected intraperitoneally twice a day for 8 days.
A purification process for the nanoparticle emulsion, prior to injection in mice, using low resolution centrifugation was employed in an attempt to reduce the degree of polymer precipitation once administered. As shown in Figure 8.2, this process did not drastically alter the amount of polymer precipitation upon intraperitoneal injection when analyzed at 5%, 3%, 2%, 1.5% solid content, although no polymer was observed outside the abdominal wall or attached to the peritoneum at 3% solid content and below. Purified solutions were more smoothly injected, however, and appeared to enter the peritoneal cavity easier than the unpurified counterpart emulsions. As seen in Figure 8.4, the TNF-α concentration for mice receiving 3% solid content was increased to match levels observed for saline-treated mice by this purification process but not for the IL-6 concentration, where the purified and unpurified emulsions produced concentrations comparable to that of saline-treated mice. At solid content levels below 3% (for the centrifuged nanoparticle sample), there was no statistical difference between the concentration of the cytokines for the saline-treated and the nanoparticle-treated mice.

**Figure 8.4.** Comparison of TNF-α concentrations for purified versus unpurified NP13 samples at various solid content concentrations.

Figure 8.5 illustrates that there was no difference in the levels of cytokines for mice treated with CNP16 versus NP13. Polymer precipitation occurred for both of the purified (centrifuged) formulations at the 5% and 2% solid content concentrations, regardless of whether drug was present in the emulsion or not.
Figure 8.5. Comparison of cytokine levels for purified NP13 and CNP16 emulsions.
8.2.3 Analysis of the Findings

Systemic application of the nanoparticle-containing emulsions proved to be somewhat problematic in terms of polymer coagulation upon injection into the mouse model. Coagulation consistently occurred in highly lipophilic regions of the peritoneal cavity, such as in the mesentery membrane of the intestines, the peritoneum, within adipose tissue in the lower abdominal region, and on the exterior surface of the stomach, liver, and pancreas. This is most likely due to the presence of non-SDS stabilized polyacrylate strands in the emulsion that precipitate upon contact with mucous membranes.

This issue was resolved through purification processes and dilution of the solid content concentration to below 1.5%. While these procedures reduced the amount of drug able to be administered in a single dose, higher dose volumes and more frequent applications can in principle be achieved at these lower concentrations. The presence of covalently bound penicillin to the polyacrylates did not affect whether or not the polymer precipitated upon intraperitoneal injection, and also did not appear to have any difference in cytokine levels upon administration. Thus, the drug-conjugated and drug-free emulsions behaved equally within the mouse model. Overall, the drug-conjugated nanoparticle system proved to be completely non-toxic and biocompatible upon intraperitoneal injection when at solid content levels below 1.5%, with or without post-polymerization purification.

8.3 Effect of Penicillin Nanoparticles upon Application to Dermal Abrasion

8.3.1 Visual Analysis of Inflammation or Irritation

All mice responded well to the nanoparticle treatments, with no observed inflammation or irritation on or around the abrasion throughout the study. Mice receiving only saline treatments showed extended periods of irritation and inflammation due to bacterial infection during the first few days of the study. These mice took an average of 3 days longer to heal from the dermal abrasion than mice receiving emulsion treatments, usually between days 6 and 8 of the study, with some taking even longer to heal.
Figure 8.6. Mouse treated with saline solution three times a day. An obvious inflammation appeared in the wound area on day 3, indicating a possible bacterial infection. Wound healing was setback an additional 2 days, and was still not fully healed by day 8.

The mouse in Figure 8.6 receiving saline treatments three times a day appeared to have contracted an infection by day 3, apparent by the sudden redness and inflammation on the abrasion area, thus the wound appears more prominent. The infection appeared to have cleared by day 5, where the wound again looks similar to its progress on day 2. This kind of setback in the wound healing process was not observed for mice receiving treatment with any of the nanoparticle emulsions at any level of solid content studied.

The mouse in Figure 8.7 was likewise treated with the nanoparticle emulsion NP13 receiving 9% solid content by spraying the emulsion onto the area. The images of the treated mouse showed healed abrasions as early as day 2 of the study when applied three times a day, and by day 4 when applied two times a day. Mice receiving lower solid content concentrations, (2% and 5% solid content) showed healed abrasions as late as day 5 and 6 of the study.
Figure 8.7. Mice treated three times a day with 9% solid content NP13 (0.1mL). The abrasion was almost fully healed by day 2, and fur re-growth was almost confluent by the final day of the study (day 14).
Figure 8.8. Mice treated two times a day with 9% solid content NP13 (0.1mL). The abrasion was fully healed by day 5 and fur re-growth was almost completely confluent by day 14.

Fur re-growth began on the final day of treatment for mice receiving nanoparticle emulsions three times a day and even earlier for mice who received nanoparticle treatments twice a day. All mice, including the saline-treated ones, displayed some fur re-growth by day 10 of the study. Polymer clumps formed on the surface of the abrasion region after the abrasion was fully healed, indicating that the emulsion treatment was no longer necessary. No precipitated polymer was observed below the dermis on the dorsal area or within the peritoneal cavity. Pathology performed on the treated animals confirmed that all internal organs appeared healthy and of normal size and color for all emulsions examined.

8.3.2 Biochemical Analysis of Blood Samples

The levels of IL-6 in mice treated with either CNP16 or NP13 were not elevated beyond those of mice treated with only saline. Since the earliest blood samples were taken on day 8 of the study, and any bacterial infections observed in saline-treated mice were cleared by day 5, it is practical to believe that the levels of pro-inflammatory cytokines such as IL-6 and TNF-α would be back to a normal level by day 8 for these mice.

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Figure 8.9. TNF-α concentrations in the blood serum of mice treated with various concentrations of CNP16 and NP13 two or three times a day.
Figure 8.10. IL-6 concentrations in the blood serum of mice treated with various concentrations of CNP16 and NP13 two or three times a day.
As observed in Figure 8.9, some variance was observed with the levels of TNF-α for the treated versus untreated mice, where CNP16 showed elevated levels at 7% and 9% solid content when treated 2x a day but not 3x a day, on the final day of the study as did NP13 at 9% solid content applied twice a day. However, since all of the treated and untreated dermal abrasions were fully healed and all of the fur re-grown over the abrasion area without any observance of redness or irritation, these elevated levels may have been caused more directly by an environmental or metabolic factor and is not directly related to the nanoparticle treatments. Also, since the nanoparticle treatments were stopped on day 8, where no elevated levels of TNF-α were observed, this also further points to an outside factor affecting the levels of TNF-α for these mice.

8.3.3 Analysis of the Findings

Application of the nanoparticle-containing emulsions to the dermal abrasions displayed not only minimal toxicity, but also great advantages for the healing wound. Results of this study showed that not only did the polymeric film that formed over the wound protect it from invading pathogens, but also became an integral part of the freshly forming skin layers. Removal of film-coated fur clumps stuck to the surface of the abrasion due to the film binding the fur to the abrasion surface caused fresh abrasions to appear within 8 hours, thus indicating infiltration of dermal fibroblast and epidermal keratinocyte cells into the polymer film matrix.

All of the results for every concentration of emulsion produced no problematic effects on the abrasion area. No redness, irritation, or inflammation was observed for any mice treated with the emulsion, and all mice appeared healthy and active throughout the study. Most mice did not appear to notice the film on the dorsal surface, and no mice were observed rubbing the area against other objects in an attempt to remove the film. This indicates that the mice were very tolerant of the film and did not feel any pain or discomfort associated with the treatment. No polymer clumps were observed in the subcutaneous region or below that, and no clumps were observed within the skin layers as well. However, this does not preclude the fact that the nanoparticles may have been able to migrate through some of the skin layers. Further studies need to be performed in order to determine the extent of nanoparticle infiltration into the epidermal and possibly the dermal layers or even if the nanoparticles in the emulsion are necessary.

As the polymeric film forms on the abrasion surface, the area does not dry out or become rigid. In fact, the polymer film blended in smoothly with the skin and maintained the same mechanical properties as the skin upon application, thereby allowing the animal to move naturally. The fact that the skin area did not dry out or become rigid, indicates that this treatment was able to provide the wound bed with the moisture and oxygen needed to heal properly, which could be one of the reasons for the enhanced healing effect. Had no oxygen or nutrients been able to penetrate the film and reach the wound bed, as needed for healing, a delayed healing process would have been expected, which was not
observed. With small abrasions, this is not that significant, but in the application of much larger or deeper wounds, having uninhibited access to oxygen and nutrients from the surrounding healthy tissue is vital for recovery.

These results demonstrate the biocompatibility of the emulsions and the ability of the resulting polymeric film to serve as a foundation for regeneration of skin layers. Films were never observed sloughing off the area, even upon complete wound healing, thus further indicating the incorporation of the emulsion into the wound healing process and absorption of the emulsion down into the freshly formed dermal and epidermal layers.

8.4 Affects of Multi-Drug Conjugated Nanoparticles on Dermal Abrasions

8.4.1 Visual Analysis of Inflammation and Irritation

The multi-drug conjugated nanoparticle emulsion MDNP1 was applied to dermal abrasions at 7% and 5% solid content three times a day for eight days. Mice receiving 5% solid content took longer to heal (approx 7 days) than the mice receiving 7% solid content. Neither emulsion caused any signs of inflammation or irritation in the abrasion region or surrounding areas, and all mice formed healthy pink skin where the abrasion was originated. After the abrasions fully healed, continued application of the emulsion to the abrasion region caused clumps of polymer film to form in the area of application, indicating that the emulsion treatment is no longer necessary (Figure 8.11, day 8). Mice who received 7% solid content MDNP1 healed faster than mice receiving 9% solid content of NP13 and CNP16 twice a day, and also faster than mice receiving 7% solid content CNP16 three times a day, and were fully healed by the end of day 4 (Figure 8.11). This further suggests that application of the emulsions three times a day is most optimal for fast wound healing and that the presence of antimicrobials does speed up wound healing probably by eradicating bacteria from the wound. However, the treatment should be discontinued upon complete wound healing in order to allow fur re-growth in the abrasion area since application of the emulsions three times a day appears to inhibit initiation of fur re-growth, while applications of emulsions only twice a day did not.
Figure 8.11. Effect of MDNP1 on skin abrasions. A) Mice treated three times a day with 7% solid content MDNP1. B) Mice treated three times a day with 5% solid content
MDNP1. Mice receiving 7% solid content are fully healed by day 4, and mice receiving 5% solid content were fully healed by day 7.

In Figure 8.10, the skin on the dorsal area is a healthy soft pink on days 8 for mice treated with either 5% or 7% solid content. By day 2, wound contraction was visible for all mice treated with MDNP1, and on day 3, slight scabbing was observed in the abrasion area for mice treated with 7% solid content. Fur re-growth began on day 9 and reached confluency by day 14 of the study.

8.4.2 Biochemical Analysis of Blood Samples

Biochemical analysis of the blood samples of topically treated mice further corroborates the visual observations from this study (Figure 8.12). Levels of both IL-6 and TNF-α were equal for mice receiving MDNP1 and saline only. The level of TNF-α on day 8 for mice receiving 5% solid content were much higher than those observed for mice receiving saline only or 7% solid content, but this is believed to be due to an infection in the ear hole punch the mice received and not from the emulsion treatment, since the cytokine concentration was dramatically reduced by day 14, long after the ear hole infection cleared. This is mostly likely the cause of the variance in trend seen between mice receiving 7% solid content and saline and mice receiving 5% solid content.
Figure 8.12. Comparison of TNF-α and IL-6 concentrations for mice receiving MDNP1 three times a day.
The levels of IL-6 were also slightly higher, on average, for mice receiving 5% solid content, yet the trend in levels was the same for all samples analyzed. This heightened cytokine level for mice receiving 5% solid content also supports the conclusions from the TNF-α concentration abnormalities.

8.4.3 Analysis of the Findings

The data suggests that the multi-drug conjugated nanoparticles are completely non-toxic to skin abrasions when applied topically at solid content concentrations as high as 7%. It also suggests that the nanoparticles are extremely beneficial to skin wounds in terms of eradicating bacteria from the wound and by protecting the wound from invading pathogens. The fact that cytokine levels for mice receiving 7% solid content were not significantly different from those in mice receiving sterile saline treatments further identifies this nanoparticle system as an optimal treatment for all forms of skin and soft tissue wounds, and appears to represent all of the optimal qualities of current topical antimicrobials without any observed side effects or drawbacks. However, this system must be evaluated in a basic wound infection model in order to determine the extent of antimicrobial activity \textit{in vivo} prior to any further explorations into treatment of multiple bacteria-contaminating burn wound infections.

8.5 Conclusions

A total lack of toxicity and high biocompatibility was found for both intraperitoneal and topical administration for the nanoparticle-containing emulsions. Despite the occurrence of polymer coagulation within the peritoneal cavity upon IP injection of the emulsions, cytokine levels remained statistically similar to those of saline-treated mice. However, coagulation was not a favorable attribute of the emulsion treatment and, therefore, the solid content concentration was lowered to levels below 1.5% to avoid this issue. When applied topically, concentrations as high as 9% solid content did not produce any toxicity or inflammatory responses and cytokine levels also remained similar to those of saline-treated mice. Beneficial properties were observed for the skin abrasion treatments as well, where the average time required for healing was decreased by 2-3 days, on average, with the application of the emulsion. The ability to treat numerous bacterial strains at once and also provide a protective covering over an open wound, through a sprayable administration route decreases the need for physical contact between the patient and medical professional and in turn decreasing the risk of nosocomial infection, the multi-drug conjugated nanoparticle system should be considered a potentially groundbreaking new treatment option for burn wound and other acute and chronic skin-based infections. Moreover, the data from this study suggests that the multi-drug conjugated nanoparticle emulsions are may be effective treatments for skin/soft tissue infections as well as for systemic-based infections. The next step to determine this is to examine the nanoparticle emulsions in an MRSA animal infection model.
8.6 References

CHAPTER 9

CONCLUSIONS AND FUTURE WORK

The work presented in this dissertation has investigated a potentially new approach towards the treatment of burn wounds. Many of the current techniques and treatments employed in hospitals around the world are leaving patients open to microbial infection at the early stages after thermal injury, where patients are most vulnerable to pathogenesis at the injury site. Chapter 1 looked at many of the current treatment options available (skin grafts and wound excision) for burn wound management, and found that many are only able to be performed once the patient has become stable, leaving the patient open to infection and a high risk of mortality. Topical antimicrobial mixtures are frequently applied to the wound site during this time where surgical treatment cannot be performed, however, due to the mode of application (direct application of creams and ointments to the wound) and the frequency required for continual treatment, this form of treatment tends to be very traumatic for the patient, and also heightens the threat of nosocomial infection.

In this work, a representative selection of antibacterial drugs were synthesized or obtained commercially and converted to acrylate or acrylamide analogues in order to incorporate them into a water-based nanoparticle emulsion. Eight N-alkylthiolated β-lactam monomers, as well as the commercially available drug monomers erythromycin, doxycycline, amphotericin B, ciprofloxacin, cefaclor, penicillanic acid, penicillin G, amoxicillin, and ampicillin were all incorporated into the nanoparticle drug delivery system individually and both the acrylated drug monomer and the drug-containing nanoparticle emulsions were analyzed \textit{in vitro} for antibacterial activity.

Initially, the nanoparticle emulsion was designed to provide a water-based delivery system for the water insoluble N-thiolated β-lactams. In Chapter 2, the mechanism of action of the N-thiolated β-lactams was studied, as well as the selective antibacterial nature of this class of antibiotics. The antibacterial activity of the acrylated commercially available drugs was also established here. The water soluble commercially available drug monomers chosen for this study (ciprofloxacin, cefaclor, ampicillin, penicillin G, penicillanic acid, and amoxicillin) were all chemically modified to incorporate an acrylate or acrylamide moiety prior to subjection to the emulsion polymerization, which allowed them to be co-polymerized with the liquid acrylate co-monomers and subsequently covalently bound to the polyacrylate backbone of the nanoparticle. While many of the acrylated drug monomers displayed antibacterial activity, some lost most their potency upon acrylation. However, after co-polymerization, the antibacterial activity of the original antibiotic was restored, indicating that the drug monomer was most likely being enzymatically cleaved from the polyacrylate backbone to an active drug form upon interaction with the microbe. It was also observed for many of the acrylate-bearing
β-lactam monomers incorporated covalently in the nanoparticle that the antibacterial activity of the drug monomers against both MSSA and MRSA strains increased after co-polymerization into the nanoparticle system. Further *ex vivo* studies need to be performed with these drug-conjugated emulsions using purified enzymes to confirm these findings on a biochemical level.

Another major discovery for the drug-conjugated nanoparticle emulsion system described in Chapter 3 was that upon incorporation of a penicillin, especially when modified to contain an acrylate moiety for polymerization rather than encapsulation, antibacterial activity against MRSA was recovered. This discovery led to the conclusion that the nanoparticle delivery system is able to protect the drug from degradative enzymes exuded by the microbe. This finding was supported by microscopy studies that analyzed the interaction of *S. aureus* with the drug-free nanoparticle emulsion CNP5, which ultimately determined that the nanoparticles can either collapse at the surface of the microbial cell wall to present the conjugated or encapsulated drug monomer to the microbe, or the nanoparticle is taken up by the microbe through either endocytosis or phagocytosis, where the particle is enzymatically degraded and the drug is then released directly into the cytoplasm of the microbe. Stability studies of the drug-conjugated nanoparticle emulsions determined that the particles are stable to normal mammalian biomacromolecules, including various enzymes, that are present within the blood serum, and therefore, only interaction with the microbe elicits the release of the drug monomer directly into the microbe itself.

In order to improve the current standards in treatment of burn wound infections, the possibility of incorporating more than one class of acrylated drug monomers into the nanoparticle delivery system was explored. This ultimately led to the formulation of two multi-drug conjugated nanoparticle emulsion systems, MDNP1 and MDNP2, where MDNP1 was preferred for biomedical applications. In this emulsion, two novel N-alkylthio β-lactam monomers were incorporated into the nanoparticle system along with an acrylated form of penicillin G and two analogues of the modified ciprofloxacin acrylamide monomer, accordingly the emulsion with a broad spectrum of antimicrobial therapy which is necessary for treating multiple microbes present in burn wound infections. These drugs were chosen based on their antimicrobial activity against the two most common pathogens found colonizing burn wounds, *S. aureus* and *P. aeruginosa*. Incorporation of the N-thiolated β-lactams also provided the emulsion with antifungal capabilities, especially against *Candida albicans*, the leading fungal species and strain present in burn wounds. Equivalent levels of activity against both methicillin-sensitive and resistant strains of *S. aureus* made this the best candidate for topical treatment of burn wound infections.

Chapters 5 and 6 analyzed the ability of the nanoparticle emulsions to form a film upon drying on any surface, making this ideal for topical applications that require both antimicrobial therapy and additional coverage for wound healing. The polyacrylate films formed from both the drug-containing and drug-free emulsions all exhibit viscoelastic
properties consistent with natural plant materials, such as the elastic constituent in skin and soft tissue, making them candidates for biomedical applications that require continuous stress/relaxation cycles. It was also determined that combination of the nanoparticle emulsions with native collagen fibrils created the formation of a biocomposite material applicable towards many biomedical applications that call for both strength and elasticity of the material. These films were also determined to exhibit antibacterial activity (Chapter 3) as well as the ability to prevent bacterial penetration into underlying nutrient-rich media while still permitting diffusion of nutrients and other small molecules such as oxygen, water (Chapter 5).

Chapter 7 looked at in vitro cytotoxicity studies performed using human dermal fibroblast cells. Here, it was determined that all of the acrylated commercially available drug monomers maintained their biocompatibility after chemical modification, and when incorporated into the nanoparticle emulsion drug delivery system using 1% surfactant and 0.5% radical initiator, no cytotoxic effects were observed. It was also determined that the polyacrylate polymer films formulated with 3% surfactant or less were non-cytotoxic as well. Chapter 8 focused on in vivo studies performed using a mouse model in order to determine if the drug-conjugated, multi-drug conjugated, or drug-free nanoparticle emulsions created any toxicity or inflammation upon systemic and topical application. The drug-free nanoparticle emulsion chosen for this study was CNP16, the single-drug conjugated nanoparticle emulsion analyzed was the penicillin G acrylamide nanoparticle emulsion NP13, and the multi-drug conjugated nanoparticle emulsion analyzed was MDNP1. CNP16 and NP13 were analyzed at various concentrations for systemic application of the emulsion, and it was determined that at 0.25% solid content, the emulsions do not precipitate within the intraperitoneal cavity of the body, yet at concentrations as high as 5% solid content, there were no raised levels of TNF-α or IL-6, indicating that no inflammatory response was caused by the emulsion. A dermal skin abrasion was inflicted on the dorsal surface of the mouse in order to establish compatibility of the nanoparticle emulsions for topical treatment of skin and soft tissue infections. Upon topical application of all three emulsions to the skin abrasion, no inflammatory response or visible irritation was observed for any of the emulsions at concentrations as high as 9% solid content. It was also established here that application of the emulsions aided the wound healing process by decreasing the observed time needed for complete healing of the abrasion, and also appeared to prevent bacterial infiltration into the wound bed. Therefore, the multi-drug conjugated nanoparticle emulsion has the potential to revolutionize burn wound treatment and provide optimal infection control and prevention immediately after thermal injury.

The evidence from these studies, taken collectively, indicates novel properties and potential applications of N-thiolated β-lactams, acrylated commercially available antibiotics, and polyacrylate nanoparticle emulsions in human therapy, most specifically, in the treatment and prevention of drug-resistant bacterial infections. The application of burn wound infection treatment and prevention is continuing to be investigated, and additional functionality of the multi-drug conjugated nanoparticles is being explored for
this specific application, as well as continued *in vivo* analysis of the treatment in treating and preventing infections. A variety of new studies are underway in the Turos laboratory to explore and understand these properties and future opportunities.
CHAPTER 10

MATERIALS AND METHODS

10.1 Synthesis of Drug Monomers

All reagents were purchased from Sigma-Aldrich or Acros Chemical Company and used without further purification. Solvents were obtained from Fisher Scientific Company. Thin layer chromatography (TLC) analysis was carried out using EM Reagent plates with a fluorescence indicator (SiO$_2$-60, F-254). Products were purified by flash chromatography using J.T. Baker flash chromatography silica gel (40 µm). NMR spectra were recorded in CDCl$_3$, unless otherwise noted. $^{13}$C NMR spectra were proton broadband decoupled. Dichloromethane was dried for many reactions by distillation at 40°C.

10.1.1 Synthesis of $C_3$-alkoxy $N$-thiolated $\beta$-lactam Monomers

10.1.1.1 $N$-Anisylimine Formation (103)

Commercially supplied $p$-anisidine reagent was recrystallized using water heated to 60°C and subsequently dried in vacuo. Aldehyde 101 and recrystallized $p$-anisidine (102) were dissolved in dry dichloromethane and a catalytic amount of camphorsulfonic acid was added to the mixture, which was then stirred at room temperature for 1-2 hours, with progression of the reaction monitored by TLC. The resulting imine was then recrystallized from cold methanol to give the pure final product, $N$-(4-methoxyphenyl) imine 103 in 95% yield.

(E)-$N$-(2-Chlorobenzylidene)-4-methoxybenzenimine (103): yellow crystalline solid, mp 51-52°C. $^1$H NMR (250 MHz, CDCl$_3$): $\delta$ 8.94 (s, 1H), 7.4-6.9 (m, 8H), 3.85 (s, 3H).
10.1.1.2 Acid Chloride Formation (104a and 104b)

**104a**: To a flame dried 250mL round bottom flask containing glycolic acid (10g, 0.13mol), acetyl chloride (20g, 0.26mol) was added in a dropwise fashion at 0°C. The ice bath was then removed and the resulting slurry dissolved within 30 minutes, followed by the formation of a white precipitate after 1 hour. Benzene (50mL) was then added to the mixture and the reaction stirred for an additional 12 hours. The resulting solid was then filtered and washed with benzene and cold methanol to give pure product 108a in 80% yield (mp 62-63°C). The solid product (108a) was then treated with thionyl chloride (31.8 g, 0.24 mol) by dropwise addition at 0°C while stirring, followed by removal of the ice bath. The slurry was then stirred for 24 hours at 30°C, followed by distillation of the resulting liquid *in vacuo* to give the acetoxyacetyl chloride (104a) in 70% yield.

\[
\begin{align*}
\text{HO} & - \text{COOH} \rightarrow \text{RO} - \text{COCl} \\
\text{RCI} & \rightarrow \text{SOCl}_2 \\
0{^\circ}\text{C} & \rightarrow 30{^\circ}\text{C} \\
75\% & \rightarrow 104a: R=\text{Ac} \\
104b: R=\text{Me} & \end{align*}
\]

**104b**: To a 250mL flame dried round bottom flask containing methoxyacetic acid (31.4g, 0.35mol), thionyl chloride (41.4 g, 0.35 mol) was added in a dropwise fashion while stirring over 30 minutes at 0°C. The ice bath was then removed and the mixture was stirred for an additional 12 hours at 30°C. The pale yellow product was then distilled to provide pure acid chloride 104a in 75% yield (bp 112-113°C).

10.1.1.3 Synthesis of *N*-4-Anisyl Azetidin-2-ones (105a and 105b)

Imine 103 (3.00g, 1.22mol) was dissolved in 75mL dry dichloromethane with triethylamine (TEA) (7.92mL, 6.11mol) at 0°C, followed by dropwise addition of the acid chloride (104a or 104b) (1.57mL, 1.47mol) and subsequent removal of ice bath. After 3-4 hours, the product was dried *in vacuo* and the lactam product was recrystallized in methanol to afford a white crystalline solid in 60-80% yield, depending on the alkoxy acid chloride used. TLC in 2:1 hexanes:ethyl acetate showed single product formation after 3.5 hours (R\(_f\) 105a≈ 0.38, R\(_f\) 103= 0.66)

\[
\begin{align*}
\text{Cl} & - \text{N} \rightarrow \text{Cl} \\
\text{OCH}_3 & \rightarrow \text{Cl} \\
0{^\circ}\text{C} & \rightarrow \text{RT} \\
\text{TEA, CH}_2\text{Cl}_2 & \\
105a: R=\text{Ac, 65\%} \\
105b: R=\text{Me, 74\%} & \end{align*}
\]
(±)-(3R,4S)-3-Acetoxy-4-(2-chlorophenyl)-N-(4-methoxyphenyl)azetidin-2-one (105a): white crystalline solid, mp 130-132°C. $^1$H NMR (250 MHz, CDCl$_3$) δ 7.43 (1H, d, $J = 8.9$ Hz), 7.32-7.23 (5H, m), 6.83 (2H, d, $J = 8.9$ Hz), 6.16 (1H, d, $J = 5.0$ Hz), 5.78 (1H, d, $J = 5.0$ Hz), 3.76 (3H, s), 1.76 (3H, s); $^{13}$C NMR (63 MHz, CDCl$_3$) δ 168.6, 161.3, 156.6, 133.8, 130.1, 129.9, 129.7, 128.6, 126.7, 118.5, 114.4, 75.4, 58.1, 55.3, 19.8.

(±)-(3R,4S)-3-Methoxy-4-(2-chlorophenyl)-N-(4-methoxyphenyl)azetidin-2-one (105b): white solid, mp 183-184°C. $^1$H NMR (250 MHz, CDCl$_3$): δ 7.47 (d, 1H, $J = 6.7$ Hz), 7.39-7.27 (3H, m), 6.97 (bs, 1H), 5.25 (d, 1H, $J = 4.5$ Hz), 4.83-4.82 (app m, 1H), 3.26 (s, 3H); $^{13}$C NMR (63 MHz, CDCl$_3$): δ 168.5, 133.7, 133.1, 132.9, 129.2, 128.2, 126.9, 86.9, 59.0, 55.9.

10.1.1.4 $N$-Dearylation of β-Lactam Monomers

Five hundred milligrams of lactam 105a or 105b (1.58mmol) was dissolved in 15mL of acetonitrile (0.03mL CH$_3$CN per mg lactam). Ceric ammonium nitrate (CAN) (2.60g) was dissolved in 23.6mL of d.i. H$_2$O (0.009mL H$_2$O per mg CAN), then the CAN solution was added dropwise to the lactam solution over 30-40 minutes at -15°C. Product was extracted with ethyl acetate, which was then washed with 5% NaHCO$_3$, 10% NaHSO$_3$, followed by 5% NaHCO$_3$ again and finally with brine solution and dried over sodium sulfate. Product was removed in vacuo to yield 80-95% pure product. TLC analysis using 1:1 hexanes:ethyl acetate revealed spot-to-spot conversion of reactant with no by-products observed after aqueous work up.

(±)-(3R,4S)-3-Acetoxy-4-(2-chlorophenyl)azetidin-2-one (106a): White solid, 93% yield, mp 100-101°C. $^1$H NMR (250 MHz, CDCl$_3$) δ 7.30-7.21 (4H, m), 5.85 (1H, d, $J = 5.1$ Hz), 5.46 (1H, d, $J = 5.1$ Hz), 2.16 (3H, s); $^{13}$C NMR (63 MHz, CDCl$_3$) δ 173.2, 165.2, 134.5, 129.2, 128.9, 128.7, 127.8, 126.7, 80.0, 60.8, 17.8.

(±)-(3R,4S)-3-Methoxy-4-(2-chlorophenyl)azetidin-2-one (106b): White solid, 89% yield (mp 116-118°C). $^1$H NMR (250 MHz, CDCl$_3$): δ 7.47 (1H, d, $J = 6.7$ Hz), 7.39-7.27 (3H, m), 6.97 (1H, bs), 5.25 (1H, d, $J = 4.5$ Hz), 4.83-4.82 (1H, d, $J = 4.5$ Hz), 3.26 (3H,
10.1.1.5 *N*-Thiolation of Lactam Monomers 106 and 108

Dissolved 100mg of *N*-protiolactam monomer (0.40mmol) in 10mL of dry dichloromethane and 0.2mL of Hunig’s base (1.2mmol) was added to the mixture. The solution was heated to 40°C, and after addition of 84.15mg *N*-alkylthiophthalimide (107a or 107b, 0.36mmol) and the reaction was refluxed at 40°C for 24 hours, with monitoring of the reaction progress via TLC. Column chromatography was performed using various solvent systems, depending on the lactam monomer synthesized, for final purification of the product.

\[
\begin{align*}
\text{Hunig's base} & \xrightarrow{\text{CH}_2\text{Cl}_2, \text{reflux}} \\
\text{106, 108} & \quad + \quad \text{107} \\
\text{2, 4, 6, 10} & \\
107a: R' &= \text{Me} \\
107b: R' &= \text{sec-butyl}
\end{align*}
\]

(±)-(3R,4S)-3-Acetoxy-4-(2-chlorophenyl)-*N*-methylthio-azetidin-2-one (2): Colorless crystalline solid, 87% yield, mp:174-176°C (Rf = 0.8 in 40:1 CH2Cl2:MeOH). \(^{1}\)H NMR (250 MHz, CDCl3) δ 7.30-7.21 (4H, m), 5.85 (1H, d, J = 5.1 Hz), 5.46 (1H, d, J = 5.1 Hz), 2.43 (3H, s), 2.10 (3H, s); \(^{13}\)C NMR (63 MHz, CDCl3) δ 173.2, 168.2, 135.5, 129.2, 128.9, 128.5, 127.9, 126.7, 80.0, 60.8, 22.0, 16.8.

(±)-(3S,4R)-4-(2-Chlorophenyl)-3-hydroxy-*N*-(methylthio)azetidin-2-one (4): White solid. \(^{1}\)H NMR (250 MHz, CDCl3) δ 7.44-7.24 (4H, m), 5.37 (1H, d, J = 5.0 Hz), 5.29 (1H, d, J= 5.0Hz), 2.51 (3H, s).

(±)-(3S,4R)-4-(2-Chlorophenyl)-3-methoxy-*N*-(methylthio)azetidin-2-one (6): White crystal, mp 71-73°C. \(^{1}\)H NMR (250 MHz, CDCl3): δ 7.35 (1H, d, J = 7.4 Hz), 7.24 (3H, m), 5.29 (1H, d, J = 4.9 Hz), 4.80 (1H, d, J = 4.9 Hz), 3.16 (3H, s), 2.40 (3H, s); \(^{13}\)C NMR (63 MHz, CDCl3): δ 170.4, 133.8, 131.4, 129.6, 128.9, 126.8, 86.7, 62.7, 58.9, 21.8.

(±)-(3S,4R)-*N*-sec-Butylthio-4-(2-chlorophenyl)-3-methoxyazetidin-2-one (10): Light yellow oil, 51% yield (Rf =0.7 in 2:1 hexanes:ethyl acetate). \(^{1}\)H NMR (250MHz, CDCl3)
δ 7.4 (1H, d, J = 7.4 Hz), 7.3 (3H, m), 5.3 (1H, d, J = 4.7 Hz), 4.9 (1H, d, J = 4.8 Hz), 3.2 (3H, s), 3.0 (1H, m), 1.48 (1H, m), 1.2 (3H, dd, J = 6.8, 4.9 Hz), 0.94 (3H, q, J = 6.0 Hz); 13C NMR (63 MHz) δ 171.0, 133.8, 131.4, 129.5, 128.9, 126.8, 86.3, 64.1, 58.8, 48.1, 28.1, 19.0, 18.6, 11.1.

10.1.1.6 Preparation of N-Alkylthiophthalimide Thiolating Agents (107a and 107b)

An alkyl disulfide reagent, either methyl or sec-butyl disulfide, was added to a slurry of N-protophthalimide in CH3CN and pyridine, then 1.2 equivalents of liquid bromine was cannulated into the flask to generate the N-thiolated phthalimide product in situ. A crystalline product was produced after recrystallization of the oil product in a solution of ethyl acetate and hexanes.

\[
\text{Br}_2, \text{CH}_3\text{CN, 0°C} \quad \text{NH} \text{, pyridine} \quad \rightarrow \quad \text{107a: } R = \text{Me} \quad \text{107b: } R = \text{sec-Butyl}
\]

2-(Methylthio)isoindoline-1,3-dione (107a): white crystalline product in 83% yield, mp 175-176°C. 1H NMR (250 MHz, CDCl3) δ 7.82 (4H, m), 3.77 (3H, s).

2-(Butylthio)isoindoline-1,3-dione (107b): crystalline solid, mp 43-45°C. 1H NMR (250 MHz, CDCl3): δ 7.83 (4H, m), 3.21 (1H, m), 1.55 (2H, m), 1.24 (3H, d, J = 6.8 Hz), 1.04 (3H, t, J = 7.3 Hz).

10.1.2 Synthesis of Acrylated N-Alkylthio β-Lactams

10.1.2.1 Deprotection of C3-Hydroxyl Group from N-Aryl Protected Lactam 105a

To a solution of 105a (838mg, 2.43mmol) in 10mL of acetone was added a solution of KOH (273mg, 4.86mmol) in 2mL of MeOH at 0°C. The hydrolysis was complete only 10 minutes after the addition of KOH/MeOH, indicated by TLC monitoring (2:1 hexanes:ethyl acetate). The reaction was quenched by adding an equal volume of water and product was extracted with ethyl acetate, dried over sodium sulfate, and isolated in vacuo to yield 700mg of the product in 95% yield. (Rf 109 = 0.38, Rf 105a = 0.56)
(±)-(3R,4S)-4-(2-Chlorophenyl)-3-hydroxy-N-(4-methoxyphenyl)azetidin-2-one (109): white solid, mp 193-194°C. \(^1\)H NMR (250 MHz, CDCl\(_3\)) \(\delta\) 7.48 (1H, d, J = 7.5 Hz), 7.33-7.22 (5H, m), 6.84 (2H, d, J = 8.9 Hz), 5.63 (1H, d, J = 5.1 Hz), 5.33 (1H, d, J = 5.1 Hz), 4.88 (1H, brs), 3.78 (3H, s); \(^{13}\)C NMR (63 MHz, DMSO-d\(_6\)) \(\delta\) 166.6, 156.1, 133.1, 132.9, 130.9, 129.7, 129.6, 128.9, 127.3, 118.6, 114.9, 77.2, 60.0, 55.6.

10.1.2.2 Acrylation of C\(_3\)-Hydroxy N-Aryl \(\beta\)-Lactam (109)

Lactam monomer 109 (500mg, 1.65mmol) dissolved in 20mL of dry dichloromethane was treated with 99mg of NaH (60% oil immersion, 2.48mmol) and stirred at 0°C for 10 minutes. Acryloyl chloride (0.16mL, 1.97mmol) was then added dropwise to the reaction mixture at room temperature and the reaction was stirred for an additional hour while monitoring via TLC (2:1 hexanes:ethyl acetate). Product was washed with water 2x (20mL each) and subsequently dried over sodium sulfate and evaporated \textit{in vacuo}. Column chromatography was performed on the product using 4:1 then 2:1 hexanes:ethyl acetate, which yielded 74% pure white crystalline product that precipitated from a dark yellow oil. (R\(_f\) 110 = 0.65)

(±)-(3R,4S)-4-(2-Chlorophenyl)-3-acrylate-N-(4-methoxyphenyl)azetidin-2-one (110): white crystalline solid, 74% yield, mp 180-182°C. \(^1\)H NMR (250 MHz, CDCl\(_3\)) \(\delta\) 7.45-7.22 (6H, m), 6.88 (2H, d, J = 8.8 Hz), 6.31 (1H, d, J = 4.8 Hz), 6.11 (1H, dd, J = 4.8, 15.2Hz), 5.85 (1H, dd, J = 4.9, 15.4 Hz), 5.81 (1H, d, J = 5.0 Hz), 5.71 (1H, dd, J = 4.8, 15.2 Hz), 3.72 (3H, s). \(^{13}\)C NMR (100 MHz, CDCl\(_3\)) \(\delta\) 163.9, 161.6, 159.9, 132.5, 130.5, 130.3, 130.0, 128.8, 127.8, 127.0, 126.7, 119.0, 118.9, 114.7, 114.4, 58.5, 55.6, 30.0.
10.1.2.3 Dearylation of N-Aryl C₃-Acrylate β-Lactam 110.

Procedure was followed as described in Section 10.1.1.4, with 84% yield of pure solid lactam product.

(±)-(3R,4S)-3-acryloxy-4-(2-chlorophenyl)azetidin-2-one (111): tan to white solid product. ¹H NMR (400 MHz, CDCl₃) δ 7.41-7.22 (4H, m), 6.59 (1H, bs), 6.21 (1H, d, J = 5.0 Hz), 6.08 (1H, dd, J = 2.4, 10.8 Hz), 6.04 (1H, dd, J = 2.4, 10.0 Hz), 5.80 (1H, dd, J = 2.4, 10.8 Hz), 5.39 (1H, d, J = 4.8 Hz). ¹³C NMR (100 MHz, CDCl₃) δ 164.8, 159.9, 132.5, 130.5, 128.8, 127.8, 127.0, 119.0, 118.9, 114.7, 58.5, 55.6.

10.1.2.4. N-Alkylthiolation of N-Protio β-Lactam 111.

Procedure was followed as described in Section 10.1.1.5.

(±)-(3R,4S)-3-acryloxy-4-(2-chlorophenyl)-N-methylthioazetidin-2-one (1): White solid in yield, mp:87-88°C. ¹H NMR (250 MHz, CDCl₃) δ 7.31-7.20 (4H, m), 6.15 (1H, d, J = 3.6 Hz), 5.98 (1H, dd, J = 2.4, 11.6 Hz), 5.75 (1H, dd, J = 2.2, 9.2 Hz), 5.62 (1H, dd, J = 2.4, 9.6 Hz), 5.47 (1H, d, J = 3.2 Hz), 2.45 (3H, s). ¹³C NMR (63 MHz, CDCl₃) δ 168.5, 163.6, 132.8, 132.5, 130.7, 130.0, 129.7, 128.8, 126.9, 126.8, 62.4, 55.4, 22.1.

(±)-(3R,4S)-3-acryloxy-4-(2-chlorophenyl)-N-sec-butylthioazetidin-2-one (3): Colorless oil in 82% yield (Rf=0.70 in 2:1 hexanes:ethyl acetate). ¹H NMR (250 MHz, CDCl₃) δ 7.31-7.20 (4H, m), 6.15 (1H, d, J = 3.6 Hz), 5.98 (1H, dd, J = 2.4, 11.6 Hz), 5.75 (1H, dd, J = 2.2, 9.2 Hz), 5.62 (1H, dd, J = 2.4, 9.6 Hz), 5.47 (1H, d, J = 3.2 Hz), 3.0 (1H, m), 1.48 (2H, m), 1.2 (3H, dd, J = 6.8, 4.9 Hz), 0.94 (3H, q, J = 6.0 Hz).
Synthesis of (±)-(3R,4S)-4-Acetoxyphenyl-N-(4-methoxyphenyl)imine (115): To a solution of p-anisidine (1.08g, 8.81mmol) in 15mL of CH₂Cl₂ was added p-hydroxybenzaldehyde (1.07g, 8.81mmol), and a catalytic amount of (+)-camphorsulfonic acid. The resultant mixture was stirred until TLC indicated the disappearance of starting materials. The resulting imine was used for the next step without further purification.

To the solution of 4-hydroxyphenyl-N-(4-methoxyphenyl)-imine (114, 1eq) with 2eq of TEA, acetyl chloride (1.5eq.) was added dropwise to the solution and the resulting solution was stirred at room temperature for 15 minutes. The product was washed with d.i. water (2×20 mL) and dried with sodium sulfate and the product was evaporated in vacuo to afford 115 as a light yellow solid in 92% yield (mp 95-96°C). ¹H NMR (250 MHz, CDCl₃) δ 8.39 (1H, s), 7.84 (2H, d, J = 8.5 Hz), 7.20-7.11 (4H, m), 6.86 (2H, d, J = 8.8 Hz), 3.77 (3H, s), 2.26 (3H, s).

(±)-(3R,4S)-4-(4-acetoxyphenyl)-3-methoxy-N-(4-methoxyphenyl)azetidin-2-one (116): White solid in 78% yield, mp 94-96°C. ¹H NMR (250 MHz, CDCl₃): δ 7.33 (2H, d, J = 8.4 Hz), 7.09 (2H, d, J = 8.4 Hz), 6.55 (1H, d, J = 17.3 Hz), 6.26 (1H, dd, J = 17.3,
10.4 Hz), 6.00 (1H, d, J = 10.4 Hz), 4.78 (1H, d, J = 4.5 Hz), 4.68-4.66 (1H, m), 3.11 (3H, s); $^{13}$C NMR (63 MHz, CDCl$_3$): δ 168.0, 164.5, 150.5, 133.2, 132.9, 128.8, 127.7, 121.5, 116.0, 86.6, 58.2, 57.5.

$(\pm)$-$(3R,4S)$-4-(4-acryloylphenyl)-3-methoxy-1-methylthioazetidin-2-one (5): White solid in 89% yield, mp 87-88°C. $^1$H NMR (250 MHz, CDCl$_3$): δ 7.29 (2H, d, J = 8.4 Hz), 7.08 (2H, d, J = 8.4 Hz), 6.50 (1H, d, J = 17.3 Hz), 6.21 (1H, dd, J = 10.3, 17.1 Hz), 5.92 (1H, d, J = 10.4 Hz), 4.75 (1H, d, J = 4.8 Hz), 4.69 (1H, d, J = 4.8 Hz), 3.05 (3H, s), 2.26 (3H, s); $^{13}$C NMR (63 MHz, CDCl$_3$): δ 170.3, 164.2, 150.9, 132.9, 131.1, 129.9, 127.7, 121.4, 86.5, 65.5, 58.3, 22.0.

10.1.2.6 Synthesis of C$_3$-Long Chain Acrylate Lactam (9)

\[
\begin{align*}
\text{HO} & \quad \text{O} \\
\text{Cl} & \quad \text{OCH}_3 \\
\text{N} & \quad \text{C} \\
\text{O} & \quad \text{OH} \\
\text{H} & \quad \text{O}
\end{align*}
\]

\[
\begin{align*}
\text{HO} & \quad \text{O} \\
\text{Cl} & \quad \text{OCH}_3 \\
\text{N} & \quad \text{C} \\
\text{O} & \quad \text{OH} \\
\text{H} & \quad \text{O}
\end{align*}
\]

\[
\begin{align*}
\text{HO} & \quad \text{O} \\
\text{Cl} & \quad \text{OCH}_3 \\
\text{N} & \quad \text{C} \\
\text{O} & \quad \text{OH} \\
\text{H} & \quad \text{O}
\end{align*}
\]

\[
\begin{align*}
\text{HO} & \quad \text{O} \\
\text{Cl} & \quad \text{OCH}_3 \\
\text{N} & \quad \text{C} \\
\text{O} & \quad \text{OH} \\
\text{H} & \quad \text{O}
\end{align*}
\]

\[
\begin{align*}
\text{HO} & \quad \text{O} \\
\text{Cl} & \quad \text{OCH}_3 \\
\text{N} & \quad \text{C} \\
\text{O} & \quad \text{OH} \\
\text{H} & \quad \text{O}
\end{align*}
\]

Synthesis of $(\pm)$-$(3R,4S)$-3-Acryloyloxy ethyl succinate-4-(2-chlorophenyl)-N-(4-methoxyphenyl) azetidin-2-one (112): To the solution of hydroxyl lactam 109 (500mg, 1.65mmol) in 5mL of CH$_2$Cl$_2$, 350mg of mono-2-(acyrloyloxy)ethyl succinate (1.6 mmol) was added along with EDCI (310mg, 1.6mmol) and DMAP (196 mg, 1.6 mmol) over ice. The solution was then warmed to room temperature and stirred overnight. The product was purified by column chromatography using a 1:1 mixture of hexanes:ethyl acetate to yield 77% lactam 112 as a colorless oil. $^1$H NMR (250 MHz, CDCl$_3$) δ 7.27-7.21 (6H, m), 6.81 (2H, d, J = 8.8 Hz), 6.39 (1H, dd, J = 1.2, 10.8 Hz), 6.18 (1H, d, J = 4.8 Hz), 6.04 (1H, dd, J = 1.2, 10.4 Hz), 5.78 (1H, d, J = 4.8 Hz), 4.32 (4H, q, J = 5.2 Hz), 3.74 (3H, s), 2.42-2.39 (1H, m), 2.33 (2H, t, J = 6.8 Hz), 2.22 -2.18 (1H, m). $^{13}$C NMR (250 MHz, CDCl$_3$) δ 170.8, 169.9, 166.3, 161.8, 157.2, 134.1, 131.7, 130.1, 130.0,
129.6, 129.4, 128.2(2C), 128.1, 127.1, 126.9, 118.9, 114.7, 62.6, 62.3, 60.0, 58.4, 55.7, 28.7, 28.6.

(±)-(3R,4S)-3-Acryloyloxy ethyl succinate-4-(2-chlorophenyl)azetidin-2-one (113): Yellow oil in 86% yield. $^1$H NMR (250 MHz, CDCl$_3$) δ 7.33-7.24 (4H, m), 6.70 (1H, bs), 6.39 (1H, dd, $J = 2.4$, 10.4 Hz), 6.15 (1H, d, $J = 3.2$ Hz), 6.12 (1H, dd, $J = 2.2$, 10.8 Hz), 5.83 (1H, dd, $J = 2.4$, 10.4 Hz), 5.36 (1H, d, $J = 4.8$ Hz), 4.29 (2H, t, $J = 4.4$ Hz), 4.24 (2H, t, $J = 2.8$ Hz), 2.42-2.36 (1H, m), 2.32 (2H, t, $J = 6.4$ Hz), 2.24-2.20 (1H, m). $^{13}$C NMR (250 MHz, CDCl$_3$) δ 170.8, 169.9, 166.3, 161.0, 134.1, 129.4, 127.6, 124.7, 124.6, 123.0, 121.8, 94.9, 80.1, 57.4, 57.1, 57.1, 28.7, 28.6.

(±)-(3R,4S)-3-Acryloyloxy ethyl succinate-4-(2-chlorophenyl)-N-methylthioazetidin-2-one (9): Yellow oil in 72% yield. $^1$H NMR (250 MHz, CDCl$_3$) δ 7.41-7.19 (4H, m), 6.35 (1H, dd, $J = 2.2$, 9.6 Hz), 6.11 (1H, d, $J = 4.4$ Hz), 6.02 (1H, dd, $J = 2.4$, 10.8 Hz), 5.73 (1H, dd, $J = 2.4$, 10.8 Hz), 5.42 (1H, d, $J = 4.9$ Hz), 4.28 (2H, t, $J = 3.6$ Hz), 4.24 (2H, t, $J = 2.8$ Hz), 2.44 (3H, s), 2.36-2.30 (4H, m). $^{13}$C NMR (250 MHz, CDCl$_3$) δ 171.4, 169.9, 168.3, 166.0, 134.6, 131.6, 130.6, 130.3, 130.2, 130.1, 128.9, 128.1, 126.9, 62.6, 62.2, 28.6, 28.5, 22.1.

10.1.3 Modification of Commercially-Available Penicillin Monomers

10.1.3.1 Acrylation of 6-Aminopenicillanic acid (11), Amoxicillin (17), and Cefaclor (19)

Five hundred milligrams of the penicillin (1.0eq) was dissolved in 20mL of dry CH$_2$Cl$_2$, then 0.92mL of N,O-bis(trimethylsilyl)acetamide (1.1eq) was added to the solution and stirred at room temperature for 24 hours. The reaction was then taken directly to the next step by addition of 0.49mL TEA (1.5eq) followed by dropwise addition of 0.19mL of acryloyl chloride (1.0eq). The mixture was stirred for 24 hours at room temperature, followed by washing of the product with water and ammonium chloride. The product was dried over sodium sulfate and the solvent was removed in vacuo. All reactions yielded 100% conversion of reactant to product (via TLC) in a 5:1 system of CH$_2$Cl$_2$:MeOH. 6-Aminopenicillanic acid acrylamide was synthesized in 91% yield. Cefaclor and amoxicillin acrylamide monomers were produced in less than 50% yields due to undesired precipitation during the washing procedure of over half the product. Proton NMR analysis was performed for all of the acylated drug monomers in many deuterated solvents in order to obtain a clear spectrum due to the limited solubility of the acylated products, however, due to solubility issues and issues with solvent purity and interference in the spectral analysis, numerous peaks were observed for the monomers when analyzed in deuterated methanol, acetone, and various mixtures of deuterated chloroform with methanol.
10.1.3.2 Acidification of Penicillin G Potassium Salt (14)

Penicillin G potassium salt (2g) was dissolved in 10mL of d.i. water, followed by addition of dilute HCl dropwise with swirling until a solid precipitate was observed in the solution. The water was decanted from product, which then washed 3x with d.i. water (10mL each) to remove excess acid. Product was dried over night in vacuo. (R<sub>f</sub>=0.09 in 5:1 CH<sub>2</sub>Cl<sub>2</sub>:MeOH)

10.1.3.3 Synthesis of Penicillin G Acrylate (12)

The free acid monomer of penicillin G (14) (1.4g, 4mmol) in 20mL of dichloromethane was treated at 0°C with TEA (0.67ml, 4.4mmol) and ethyl chlorofomate (0.48mL, 5mmol). After stirring at 0°C for 30 minutes, was added a solution of 2-hydroxyethyl acrylate (0.93g, 8mmol) and TEA (0.67ml, 4.4mmol) in 10mL of dichloromethane. The ice bath was removed, and after storage for 3-4 hours, the mixture was washed with cold water, dried over MgSO<sub>4</sub> and column chromatography to give pale yellow oil of penicillin G-acrylate monomer in decent yield (70% crude). <sup>1</sup>H NMR (CDCl<sub>3</sub>, 250MHz): δ 7.35-7.18 (5H, m), 6.38 (1H, d, J = 17.6 Hz), 6.05 (1H, dd, J = 17.6, 7.2 Hz), 5.80 (1H, d, J = 7.2 Hz), 5.50 (1H, dd, J = 7.2, 2.4 Hz), 5.42 (1H, d, J = 2.4 Hz), 4.31 (4H, s), 3.67 (2H, s), 1.35 (6H, s).

10.1.3.4 Synthesis of Penicillin G Acrylamide (13)

Penicillin G free acid monomer (14) was dissolved in 40mL of dry dichloromethane (1.00g, 3.37mmol) and 0.92mL of N,O-bis(trimethylsilyl)acetamide (3.71mmol) was added to the solution and was subsequently stirred at room temperature for 24 hours. Reaction was then placed over ice, and 202mg of sodium hydride (60% oil immersion, 5.06mmol) was added to the reaction. After bubbling ceased, the ice bath was removed and the solution was stirred for 10 minutes, followed by addition of acryloyl chloride (0.27mL, 3.37mmol) dropwise and stirring for 24 hours at room temperature. Product was washed with water followed by ammonium chloride and brine, then dried with sodium sulfate and solvent removal in vacuo to yield 95% yellow crystalline solid product after washing with hexanes to remove any non-polar impurities. TLC analysis showed spot-to-spot conversion of reactant in 5:1 CH<sub>2</sub>Cl<sub>2</sub>:MeOH (R<sub>f</sub> prod=0.6857).

10.1.3.5 Acrylation of Ampicillin Sodium Salt (16)

To a solution of ampicillin sodium salt (500mg, 1.35mmol) dissolved in 15mL of water, add 15mL CH<sub>2</sub>Cl<sub>2</sub> and stir at room temperature. Next, tetrabutylammonium bromide (435mg, 1.35mmol) was added and the solution was stirred until no separation of solvents was observed. N,O-bis(trimethylsilyl)acetamide was then added (0.37mL,
1.50mmol) and the reaction was stirred for 24 hours at room temperature. The silyl-protected ampicillin was then extracted with 20mL of CH₂Cl₂ and dried in vacuo to yield 700mg of the yellow silyl-protected solid intermediate, which was then re-dissolved in 20mL of CH₂Cl₂ and subsequently deprotonated using triethylamine (TEA) (0.28mL, 2.03mmol) and stirring over 10 minutes at room temperature. Acryloyl chloride (0.11mL, 1.35mmol) was then added dropwise to the reaction mixture and the reaction was then stirred at room temperature for 24 hours. The mixture was washed with brine solution then dried over MgSO₄ to give a dark yellow solid in 52-70% crude yield. TLC in 5:1 mixture of CH₂Cl₂:MeOH showed spot to spot conversion of product with no by products observed.

10.1.3.6 Diacrylation of Amoxicillin

Amoxicillin (100mg, 0.24mmol) was dissolved in 5mL of d.i. water, then treated with 0.48mL of 2M NaOH (0.60mmol) and stirred over ice for 10 minutes. Acryloyl chloride (38.8µL, 0.96mmol) was added dropwise to the reaction mixture, which was then stirred at 0°C for an additional 4 hours. The reaction was then terminated with the addition of 0.5mL of concentrated HCl, and the precipitated diacrylate free acid amoxicillin was filtered from the solution. Product was a light yellow solid.

10.1.3.7 Acrylation of Ciprofloxacin

The commercially available ciprofloxacin hydrochloride monomer (500mg, 1.5mmol) was dissolved in 15mL of dry dichloromethane and 0.63mL of TEA (4.50mmol) was added to the solution then stirred at room temperature for ten minutes. Acryloyl chloride (0.197mL, 3.0mmol) or methacryloyl chloride (0.29mL, 3.0mmol) was subsequently added to the reaction mixture in a dropwise fashion, and the solution was then stirred at room temperature for 24 hours. The resulting acrylamide product was immediately recovered in vacuo after aqueous work up in order to prevent polymerization. A yellow solid was produced in 75-90% yield, depending on the acryloyl chloride reagent used.

**Ciprofloxacin acrylamide (7):** Yellow solid, 74% yield. ¹H NMR (250 MHz, CDCl₃): δ 7.33-7.29 (2H, m), 6.5 (1H, m), 6.33 (1H, d) 6.26 (2H, d), 5.74 (1H, s), 3.87-3.69 (8H, d), 1.38-1.33 (1H, m), 1.30-1.18 (4H, s). ¹³C NMR (63 MHz, CDCl₃) δ 187.0, 170.6, 164.7, 116.5, 141.2, 138.7, 135.6, 130.8, 129.1, 118.4, 111.6, 108.9, 99.0, 57.4, 49.0, 35.2.

**Ciprofloxacin methacrylamide (8):** Yellow solid, 90% yield.
10.2 Antibacterial Assays

Media was purchased from Becton–Dickinson Laboratories, Cockeysville, MD, and supplies were purchased from Fisher Scientific Company. Bacteria were purchased from Hardy Diagnostics.

10.2.1 Culture Preparation

From a freezer stock in tryptic soy broth (Difco Laboratories, Detroit, MI) and 20% glycerol, a culture of each microorganism was transferred with a sterile Dacron swab to Trypticase® Soy Agar (TSA) plates (Becton-Dickinson Laboratories, Cockeysville, MD), streaked for isolation, and incubated at 37°C for 24 h. A 10⁸ standardized cell count suspension was then made in sterile phosphate buffered saline (PBS, pH 7.2) and swabbed across fresh TSA plates.

10.2.2 Kirby-Bauer Assay

Five milliliters of sterile phosphate buffered saline (PBS) was inoculated with the desired bacteria and the concentration was adjusted to 0.5 McFarland Standard. The bacteria-containing solution was then streaked evenly onto a Mueller-Hinton agar plate and wells were drilled into the agar using sterile 1-30uL pipette tips. The wells were filled with 20uL of antimicrobial solution or nanoparticle emulsion which were at a 1mg/mL concentration, and the plates were incubated for 24 hours at 37°C. Zones of inhibition were determined for each well using a millimeter scale and measuring the zone through the center of the well.

For antibacterial analysis of the polyacrylate films, instead of drilling wells in the agar for addition of the emulsion, the concentrated (20% solid content) emulsion was placed on top of an already inoculated Mueller-Hinton agar plate, where the volume of emulsion added was dictated by the surface area of the agar plate used. The plates were then stored uncovered in a biosafety class 2A cabinet to promote film formation for 30 minutes, then were incubated at 37°C overnight. Zones of inhibition were determined for each film using a millimeter scale and measuring the zone through the center of the film, subtracting the film diameter from the measurement.

10.2.3 Agar Dilution MIC Assay

Antimicrobial concentrations were determined by the standard NCCLS protocol M7-A2. The concentrations of the emulsions analyzed were based on the drug content incorporated in the emulsions, and these concentrations were placed in a well of a 24 well plate (Costar 3524, Cambridge, MA). Mueller Hinton agar was added to each well in
liquid form to produce a total well volume of 1.5mL. The contents of each well were thoroughly stirred to evenly distribute the antimicrobial within the agar. Using a sterile inoculating loop, a small amount of each standardized bacterial strain cultured on TSA plates for 24 hours was transferred into a sterile test tube containing 5mL TSB and incubated at 37°C for 24 h. One microliter of each culture was then applied to the appropriate well of solidified Mueller Hinton agar and incubated for 24 hours at 37°C. Bacterial growth was assessed by visual observation and subsequent analysis on a TSA plate if necessary.

10.2.4 Broth Dilution MIC Assay

MIC values were determined by broth serial dilution assay, according to NCCLS protocols. The reported MIC values for emulsions analyzed were based on the concentration of the drug incorporated in the emulsions, were the initial concentration of drug was 512µg/mL emulsion, which was serially diluted in Mueller-Hinton broth, which is subsequently inoculated with 5µL of bacterial culture in TSB. Bacterial growth/inhibition was determined by optical density measurement at either 540nm (Staphylococcal species) or 550nm (Bacillus species).

10.3 Nanoparticle Emulsion Polymerization and Analysis

10.3.1 Emulsion Polymerization Process

For the drug-containing emulsion polymerizations, a water insoluble drug monomer was dissolved in a 7:3 (w/w) ratio of butyl acrylate and styrene, respectively, at 70°C. Nanopurified water was then added to the solution, followed by addition of sodium dodecyl sulfate (SDS) as surfactant (5%, 3%, or 1% w/w). If no drug is added, the two comonomers, at their respective ratios, were heated to 70°C with stirring prior to addition of surfactant and nanopurified water. The resulting mixture was stirred rapidly at 70°C under a nitrogen atmosphere until homogenous micelle formation occurs (between 10 and 15 minutes). The radical initiator, potassium persulfate (1% or 0.5% w/w), was then added to the emulsified solution and the solution was allowed to stir at 70°C for 6-8 hours under a nitrogen atmosphere. The resulting emulsions are milky white to a white iridescent solution, depending on amount of surfactant and the size of the drug monomer incorporated.

10.3.2 Process for the Purification of Nanoparticle Emulsion NP7

A sample of the nanoparticle emulsion NP7 (20% solid content) was diluted to 10% solid content concentration using nano-pure water. The emulsion (10mL) was then placed in a continuous extractor and extracted for 3 days with refluxing cyclohexane. The emulsion
was then removed from the apparatus and separated from the organic layer using a separatory funnel. The extracted and non-extracted samples were then diluted to 1mg drug content in 1mL of emulsion (the drug content for the washed emulsion was assumed to be the same as the original unwashed emulsion) and the antibacterial activity was analyzed by Kirby Bauer diffusion assay as described in Chapter 2.

10.3.3 Analysis of Particle Size Distribution by Dynamic Light Scattering

Dynamic light scattering data was acquired on a UPA Honeywell MicroTrac at the University of Florida’s Particle Engineering Research Center (PERC). One drop of concentrated emulsion solution (20% solid content) was placed in a 10 mL well filled with nanopure water. Data was collected per sample over 3 runs of 180 seconds per run.

10.3.4 Zeta Potential Analysis of the Nanoparticle Emulsions

The zeta potential of each emulsion solution was obtained using a Brookhaven ZetaPALS at PERC. The emulsion solution was diluted to 1.5% solid content (~ pH = 7) using nanopure water for analysis. Two runs were performed per sample where each run recorded 10 data points. The dispersant viscosity and dielectric constant of the poly(butyl acrylate-styrene) emulsion solution used for analysis was 0.8872 cP and 78.55 respectively.

10.3.5 Film Formation and Characterization

An aliquot (~0.5-1.0mL) of concentrated emulsion solution was pipetted onto a glass or Teflon surface and was allowed to sit at room temperature overnight. Film formation was not solely dependant on evaporation of the water constituent of the emulsion, so film formation was also performed under humid or moist atmospheres and at increased or decreased temperatures. Body temperature (37°C) appeared to be the optimal temperature for film formation however. Upon film formation, the emulsions turned from a cloudy white liquid to a clear, translucent pliable film. Films were removed from glass surfaces using a straight edge razor blade or peeled off manually from Teflon surfaces and further manipulated.

Nuclear magnetic resonance (NMR) analysis was performed on some of the polymer films synthesized by placing a small piece of the film in a slender tube containing deuterated chloroform. While the chloroform did not dissolve the film, it caused the film to swell and become translucent enough for NMR analysis. Fourier transform infrared spectroscopy (FTIR) analysis was also performed on a Perkin Elmer Spectrum. Polymer film samples were cut into 1 x 1cm² pieces and analyzed with this instrument for sample identification.
10.4 Microscopy Studies of Nanoparticles

All chemicals were purchased from Sigma-Aldrich Chemical Company, and the copper grids for TEM imaging were purchased from Electron Microscopy Sciences.

Scanning electron microscopy (SEM) analysis of the nanoparticle emulsions was done in the College of Engineering at the University of South Florida. The samples were prepared by diluting the initial emulsion with nanopure water (3,000-fold), then evaporating the aqueous content under a N₂ stream prior to coating by gold sputter under high vacuum. The gold-coated nanoparticles were then observed by SEM (Hitach S 800). Transmission electron microscopy (TEM) analysis was performed on a FEI Morgagni 268D electron microscope in the biology department at the University of South Florida. The initial emulsion solution was diluted to a $10^{-10}$ concentration using nanopure water then the solution was drop cast onto a formvar-coated copper grid (Electron Microscopy Sciences). The water content was evaporated by applying a cool stream of air to the drop, and the grid was subsequently viewed on the microscope. Atomic force microscopy (AFM) analysis was performed with the aid of August Heim in the Department of Physics at the University of South Florida. Glass slides were plasma cleaned to establish a molecularly flat surface prior to emulsion deposition. A 5µL aliquot from the original concentrated emulsion is placed in 5mL of nanopure water, then a 15µl aliquot is placed on a round glass coverslip and the particles are allowed to sit uncovered for 1-2 minutes in order for the particles to attach to the glass coverslip. Thereafter, the excess water was shaken off the coverslip and any residual water was evaporated by applying a cool stream of air to the drop. For liquid imaging, the coverslip containing the adhered nanoparticles is submerged in DI water and then imaged. AFM imaging was performed using the Asylum Research MFP-3D and µMasch cantilevers (nominal spring constants between 1–2 nN/nm) using the tapping mode of the instrument under ambient air at room temperature and <45% humidity.

10.5 TEM Imaging of Bacterial Cell Interactions with Drug Monomers and Nanoparticles

10.5.1 Whole Cell Imaging

*S. aureus* (ATCC 25923) was grown up on tryptic soy agar (TSA) plates overnight at 37°C. The bacteria were then carried through a series of dilutions in order to obtain a concentration of $10^2$ CFU/mL in trypticase soy broth (TSB). The cells were then exposed to a $10^{-10}$ concentration of control nanoparticles for 30 minutes. Thereafter, the broth was treated with 5mL of 2.5% gluteraldehyde for 4 hours at 4°C. A solution of $10^2$CFU/mL *S. aureus* in TSB was also treated with 5mL of 2.5% gluteraldehyde for 4 hours at 4°C and served as a control for the experiment. The broth suspension was then drop cast onto a formvar-coated copper grid and was allowed to dry at room temperature under a cool
stream of air. The control cells and cells treated with control nanoparticles were then observed on the FEI Morgagni 268D TEM.

10.5.2 Sectioned Cell Imaging

*S. aureus* (ATCC 25923), *B. anthracis*-Sterne, or *Candida albicans* was grown up on TSA plates overnight at 37°C. The bacterial culture was then carried through a series of dilutions in order to obtain a concentration of 10^7 CFU/mL in TSB. The diluted samples were then exposed to a concentration of 10^5 control nanoparticles or lactam monomer 6 dissolved in DMSO for 30 minutes (bacteria) or up to 4 hours (Candida) and then centrifuged at 10,000g for 5 minutes. The pellet was washed twice with PBS, then resuspended in 5mL of PBS and fixed with 5mL of 2.5% glutaraldehyde for 4 hours at 4°C. The fixed cells were centrifuged at 10,000g for 5 minutes and then resuspended in PBS supplemented with 0.1 M sucrose. The cells were then centrifuged and resuspended twice in PBS at 10,000g for 5 minutes. The cells were then centrifuged again at 10,000g for 5 minutes then the pellet was embedded in agar for easier handling. The agar blocks were then washed with PBS and postfixed in 2% osmium tetroxide for 1 hour at room temperature. The cells were then washed twice with PBS then once with 0.9% saline and stained with 1.5% uranyl acetate. The cell-containing agar blocks were then carried through a series of dehydrations using graded ethanol. The cells were then infiltrated and embedded in Spurr’s Plastic and ultrathin sections were cut using a Sorvall MT-2B ultramicrotome and placed on copper mesh grids. The grids were then examined on the FEI Morgagni 268D TEM. No post-embedding staining was performed on the sections in order to minimize any unnecessary metal precipitation that could be mistaken as nanoparticles.

10.6 Mechanical Testing

All mechanical studies were performed at the Shriners Hospital for Children, Tampa, FL. Each film was placed on a flat glass surface and cut into a rectangular shape of set dimensions, which were then calculated with a dial caliper to the nearest 0.01mm in order to calculate the cross-sectional area of the film. For cyclic, uni-axial analysis, the films were analyzed as is. However, for measurement of tensile strength, a 3mm biopsy punch tool was used to convert the film into a dumbbell shape by punching semi-circle holes on either side of the center of the film. The width and thickness of the film was measured at the center of the dumbbell at the thinnest point with the dial caliper in order to calculate the cross-sectional area of the film at the most likely spot of failure.

This protocol was used for tensile strength measurements because, when the film is a solid rectangular shape, failure occurred at variable locations, thereby decreasing the accuracy of the measurement since the film’s width and thickness slightly varies throughout the film. Actual clamp-to-clamp distance was measured after the film was
loaded to the nearest 0.01mm with a dial caliper. The clamps were mounted in recessed holes in aluminum jigs produced from 1/4 in stock. Uniaxial tensile tests were performed on a tensiometer fabricated in the laboratory. The polymer film was stretched to 100mm in length at a strain rate of 1mm/s, creating 1000% deformation under uniaxial tensile load. Distance was continually measured with LVDT (Lucas Control Systems Products, Schaevitz Sensors, Hampton, VA) mounted on the piston. Load was measured with an LVDT force transducer (Lucas Control Systems Products, Schaevitz Sensors, Hampton, VA).

To account for displacement of the force transducer core, an LVDT was placed in line with the core and the distance was recorded. The mVolt analogue outputs were digitized with a Quatec A-D PCMCIA card and imported directly into Microsoft Excel. Tensile strength was taken as the highest load attained before failure normalized to the cross-sectional area. The linear portion of the stress/strain curve was used to calculate the elastic modulus. Due to the high number of measurements obtained, a moving average was employed for each data set analyzed in order to visualize the data in a more seamless fashion.

10.7 Bacterial Permeability Assays

The nutrient-rich agar used for this assay, 0.5% Bacto Acetate Differential Agar, was purchased from Fisher Scientific, along with M9 minimal salts agar and tryptic soy broth (TSB).

10.7.1 Multi-Layered Agar Assay

All agars were prepared following the manufacturer’s recommended procedure prior to addition to the test tube or Petri dish. For each assay, each layer of agar or emulsion was added to the test tube or plate and allowed to solidify in the biosafety cabinet until completely hardened. Upon addition of the nutrient-free agar layer and subsequent solidification, the agar was inoculated by dipping a thin metal rod into a saline solution containing $10^5$CFU/mL of multiple microbes prior to the next agar layer addition. Once all layers solidified and the nutrient-free agar was inoculated, the assay was incubated at 37°C for 7 days, while monitoring the progress of the assay each day.

A positive control was used that represented a substance that does not permit bacterial migration into the bottom agar layer, but does allow pH indicator and nutrients migration into the nutrient-free agar layer. A 2% agar solution is prepared, autoclaved, and then a 1mL aliquot of the cooled solution was placed on top of the solidified bottom agar layer and allowed to solidify overnight. Once solidified, the top agar layer was applied to the tubes. Also, a negative control was used to represent a substance that did not permit bacterial migration but allowed the flow of nutrients and pH indicator into the nutrient-
free agar layer. A 3x3cm square of double ply hospital-grade gauze was placed between the nutrient-rich and nutrient-free agar layers at the same stage of assay preparation as when the emulsions were added.

10.7.2 Broth-Based Diffusion Assay

A solution of TSB was prepared following the manufacturer’s recommended procedure, then placed in the bottom chamber of either the diffusion chamber or the diffusion cell used. A pre-formed polymer film sample (as described in Section 10.7.3) was placed over the opening(s) of the diffusion apparatus so that no air bubbles were present between the broth and the film, then was clamped in place by applying the top chamber to the apparatus. The top chamber was then filled with a saline solution containing $10^5$ CFU/mL of multiple microbes, and the chamber was then sealed either by a lid or with parafilm and incubated at 37°C for 7 days. For the diffusion chamber, at day 7, the apparatus was dismantled, making sure not to contaminate the TSB in the bottom chamber, and a sample of the TSB was then streaked onto an agar plate containing the Bacto acetate differential agar. The inoculated plate was then incubated overnight at 37°C and any observance of bacterial growth was recorded.

10.7.3 Carbon Source Assay

Small agar plates were formed using M9 minimal salts agar (1.5%). Once the agar solidified, a 1.00mL aliquot of the emulsion sample was added to the surface of the agar and the plates were left open overnight in a biosafety class 2A cabinet to permit film formation. Once the film solidified, a microbial suspension containing $10^5$ CFU/mL in saline was added to the film surface by placing 5-6 drops (approximately 50µL each) over the film. The plates were then incubated at 37°C for 7 days, checking the progress of bacterial growth each day.

10.7.4 Molecular Weight Cutoff Analysis

In order to determine if small molecules, such as water, oxygen, and various nutrients, are able to penetrate the polymer films to provide a wound bed, for example, with the elements it needs to heal, a study was designed using dyes of various molecular weights and microdialyzer chambers. These chambers allowed the polymer films to be exposed to various dyes at once, and the subsequent percentage of each dye that penetrated the film was analyzed by capillary zone electrophoresis (CZE).

Chambers used to determine the permeability of a few select polymer films samples was similar to the chambers used by Ussing to test the permeability of egg capsules of marine animals. (Ussing et al., ’52; Lombardi and Files, ’93). The top of the chamber has ten
circular openings that, when placed on top of the bottom portion, create ten separate wells, allowing for ten polymer film samples to be analyzed at once. The bottom portion of the chamber has one large well containing phosphate buffered saline (PBS) solution for the dyes that are able to penetrate the film samples to flow into. Dialysis tubing was used as a positive control material, and a hard plastic material was used as a negative control, with each sample being analyzed in duplicate. An N of 6 was used for the polymer film samples analyzed. Once the bottom chamber was filled with PBS, the materials were placed over each well so that there were no air bubbles between the material and the PBS, then the top of the chamber was clamped into place over these materials. Once secured, the material being analyzed separated the bottom well from the top wells, and the top wells were subsequently filled a solution different molecular weight dyes. Samples were taken from the top wells after 24 hours to determine whether the different molecular weight materials were able to diffuse across the material.

PBS containing molecular weight materials ranging from 404 to 844g/mol was used to determine the permeability of polymer film samples to low molecular weight materials. The solution contained 1mM concentrations of each of the following materials: cresol red (404g/mol), thymol blue (488g/mol), bromothymol blue (646g/mol), bromocresol green (720g/mol), and methylthymol blue (845g/mol). Eighty microliter aliquots of the solution were introduced into the top wells of the chamber so that the solution is in direct contact with the film sample covering each well and the PBS solution in the bottom chamber was stirred continuously for 24 hours. The amount of each dye was measured by removing an aliquot of the solution from the top chambers after 24 hours using a capillary zone electrophoresis system (Dionex) monitoring at an absorbance of 280nm.

10.8 Biocomposite Fiber Formation and Analysis

10.8.1 General Mechanical Analysis

Mechanical properties were established using a tensiometer fabricated in the laboratory at Shriners Hospital for Children, Tampa, FL. The fibers were stretched to 10mm in length at a strain rate of 1mm/s. Distance was continually measured with LVDT (Lucas Control Systems Products, Schaevitz Sensors, Hampton, VA) mounted on the piston. Load was measured with an LVDT force transducer (Lucas Control Systems Products, Schaevitz Sensors, Hampton, VA). Displacement of the force transducer core was accounted for by placing the LVDT in line with the core. The mVolt analogue outputs were digitized with a Quatec A-D PCMCIA card and imported directly into Microsoft Excel. Tensile strength was taken as the highest load attained before failure normalized to the cross-sectional area. The linear portion of the stress/strain curve was used to calculate the elastic modulus, and the maximum strain was obtained by the highest displacement before failure normalized to the length of the fiber between the clamps of the instrument.
10.8.2 Emulsion Dilution Biocomposite Fibers

Collagen fibrils were extracted from the echinoderms *Cucumaria frondosa* obtained from Frenchman Bay in the Gulf of Maine. The extraction process was performed and reported by Thomas Koob. Five-10mL aliquots of a sea cucumber collagen fibril suspension were placed into 15mL centrifuge tubes, and either 0, 25, 50, 100, or 150µL of CNP3 nanoparticle emulsion was added to the tube and the solution was gently mixed to avoid fibril tangling. The mixture was then gently transferred to 10mm wide dialysis tubing, placed in fiber-forming buffer (3% acetic acid), and stirred overnight at room temperature. The fibers were then removed from the dialysis tubing and slowly lifted from the fiber forming buffer over 4 hours to dry the fibers.

10.8.3 Collagen Dilution Biocomposite Fibers

A suspension of collagen fibrils in water was obtained as described in Section 6.8.2 and 10mL was transferred into one test tube, then 5mL, 4mL, 2mL, and 1mL into other individual test tubes. The tubes containing 5mL or less of collagen was diluted with deionized water to give dilutions of 1:1, 1:2, 1:4, and 1:10 collagen:water, which corresponded to 50%, 33%, 20% and 9% collagen in the fiber formulation. If the dilution volume was greater than 10mL, a 10mL aliquot was removed and placed in a fresh test tube. A 100µL aliquot of CNP3 was pipetted into each test tube and gently mixed for even distribution of emulsion. The mixture was then gently transferred to 10mm wide dialysis tubing and placed in fiber-forming buffer (3% acetic acid) and stirred overnight at room temperature. The fibers were then removed from the dialysis tubing and slowly lifted from the fiber forming buffer over 4 hours to dry the fibers.

10.8.4 Fiber Analysis

Dried fibers were cut into 15mm long pieces and then ends of each piece were taped and marked for identification. Half of the prepared fibers from a single formulation (3) were hydrated with a drop of deionized water in the center of the exposed fiber for one hour, and the other half were kept dry. The diameter of each fiber was then measured in duplicate using a Nikon Eclipse E800M microscope and incorporated software. The percent of fiber swelling was obtained for the hydrated fibers based on the measurements in Equation 5.1. The fibers were then clamped in place on the tensiometer described in Chapter 5, the length between the clamps was measured, and the fibers were tested under uniaxial tension as described in Section 10.6.
10.8.5 Calcium Doping Study

Biocomposite fibers containing 100mL of CNP3 and 10mL of sea cucumber collagen fibril suspension were formed as described in section 6.8.1. The dry fiber was subsequently cut into 8-10mm long segments and tape was placed on both ends of the segments and labeled. Half of the fiber segments cut (5) were fully submerged in a prepared solution containing 10mM calcium chloride (CaCl$_2$) overnight, and the other half were submerged in ddi H$_2$O. The diameter of the fibers was then measured using the Nikon Eclipse E800M camera/microscope in two locations towards the center of each fiber segment, and the fibers were then analyzed under uniaxial tension by the MTS instrument as described in Section 10.6.

10.8.6 TEM Analysis of Fibers

The TEM analysis procedure used for the collagen and biocomposite fibers was based on numerous publications of imaging of biological tissues.$^{37-39}$ A control, collagen fiber and a biocomposite fiber formulated with 100µL of CNP3 nanoparticle emulsion and 10mL of sea cucumber collagen fibril suspension were cut to 3mm in length and rinsed twice with distilled water. The fibers were then rinsed with 0.1M sodium cacodylate buffer then submerged in a 0.1M cacodylate buffer containing 3% gluteraldehyde and 0.5% tannic acid overnight. The fibers were then rinsed again two times with 0.1M cacodylate buffer then post-fixed using 1% osmium tetraoxide for one hour at room temperature. Thereafter, the fibers were rinsed three times for 15 minutes each in double distilled water (ddi H$_2$O), then submerged in 1% uranyl acetate in ddi H$_2$O for two hours at room temperature. The fibers were then rinsed twice with ddi H$_2$O, followed by total dehydration of the fibers through an increasing gradient of pure ethanol, where the fibers are first subjected to 30% ethanol for 5 minutes, followed by 50% ethanol for 5 minutes, 70% ethanol for 10 minutes, 95% ethanol for 10 minutes, and finally 100% ethanol twice for 20 minutes each. The fibers were then submerged in propylene oxide twice for 10 minutes each to completely dehydrate the fibers. The dehydrated fibers were then embedded in a specialized embedding plastic (Spurr’s Low Viscosity Embedding Media) through a series of increasing concentrations of the plastic in propylene oxide, 1:2, 1:1, 2:1, and finally only Spurr’s plastic. The fibers sat in the different concentrations of Spurr’s plastic: propylene oxide for 30 minutes each. The fibers in Spurr’s plastic were then place into a rectangular mold containing a small volume of hardened Spurr’s plastic so that the fiber would be in the center of the plastic, and the two plastics were allowed to gel together sitting at room temperature for 4-6 hours. The fiber-embedded plastic was then cured at 70°C for 8 hours. Once hardened, the plastic was removed from the mold and sectioned into nano-thin cross sections with the fiber centralized in the plastic using an ultramicrotome and a diamond-tip knife. The sections are then carefully transferred to a TEM grid and subsequently viewed on a TEM.
10.9 In vitro Cytotoxicity Assay using Human Dermal Fibroblast Cells

Human dermal fibroblast cells were a gift from the University of Cincinnati, College of Medicine. All assays were performed with permission from the University of South Florida’s Internal Review Board (IRB) for the use of human cell lines. All supplies were purchased from Fisher Scientific Company, fetal bovine serum (FBS) was purchased from Fisher Scientific (Hyclone), and the cellular media, contents and the CyQUANTA Cell Proliferation Assay Kit and the MTT Cell Proliferation Assay Kit were purchased from Invitrogen. Fibroblast cells were incubated at 37°C with 5% CO₂ for optimal growth and proliferation.

10.9.1 Cell Culture Preparation

A vial of cryofixed or frozen yet still viable human dermal fibroblast cells in 10% glycerol-containing media were plated out into 5 sterile flasks using Dulbeco’s Modified Eagle Medium (DMEM) and incubated for 2-4 hours, then the media in each flask was replaced with 20mL of DMEM containing 10% FBS and the cells were incubated for 3-4 days or until they reached confluence. The media was then removed and the cells were treated with 10mL of 1% trypsin in phosphate buffered saline (PBS) for 10 minutes to detach the cells from the flasks. The cell suspension was then centrifuged at 20,273 x g for 15 minutes and the cellular pellet was re-suspended in 15mL DMEM containing 10% FBS. The total number of cells in the suspension was then determined using a hemocytometer (a cell counting instrument), and the number of cells per milliliter was calculated in order to determine the number of cells that will be in each well for the toxicity assay. The cell suspension was then diluted in DMEM in accordance with the calculations in order to obtain the correct volume of cell suspension needed for the assay.

10.9.2 CyQUANTA Cytotoxicity Assay

A cellular suspension in DMEM, as prepared in 10.9.1, was added in 2mL aliquots to each well of a 24 well microplate and the cells were incubated for 24 hours in order for the cells to adhere to the wells prior to sample exposure. Acrylated drug monomers dissolved in DMSO or nanoparticle emulsions were added to the wells in 20uL aliquots, or 80mg of disinfected polymer film samples were added to the wells and the cells were incubated for 24 hours. The media was then removed, the cells were washed three times with 2mL of PBS, then the cells were lysed using 0.5mL of 1% lysis buffer provided in the CyQUANTA kit followed by a series of freezing and defrosting at -108°C. A 100uL aliquot from each well containing the lysed cells was placed in a 96 well plate and 100uL of lysis buffer containing 1% CyQUANTA DNA dye was added to each well. The amount of fluorescence emission was then recorded for each well using the Victor Wallace 1420 spectrophotometer at 485nm excitation and 535nm emission.
10.9.3 MTT Cytotoxicity Assay

A suspension of human dermal fibroblast cells in DMEM, prepared as described in 10.9.1, was added in 100µL aliquots to each well of a 96 well microplate, and the cells were incubated for 24 hours in order for the cells to adhere to the wells prior to sample exposure. Drug monomers dissolved in DMSO or nanoparticle emulsions were added to the wells in 1µL or 2µL aliquots and the cells were incubated for 24 hours. The MTT dye solution was then added to each well in 15µL volumes and the cells were incubated for 4 hours. The cells were then treated with 100µL of solubilization/stop solution provided in the MTT kit and the formazan product was allowed to form for one hour. The amount of fluorescence absorbance was then recorded for each well using the Victor Wallace 1420 spectrophotometer at 570nm.

10.10 In vivo Nanoparticle Toxicity Studies using a Mouse Model

10.10.1 Nanoparticle Synthesis

Synthesis of the penicillin G acrylamide monomer (13) is described in Section 10.1.3, as well as synthesis of all the acrylated drug monomers that constituted MDNP1. The emulsion polymerization process used to form CNP16, NP13, and MDNP1 is described in Section 10.3.1.

10.10.2 Systemic Model for Toxicity Analysis of Nanoparticles

All animal procedures were conducted under the approval of the University of South Florida Institutional Animal Care and Use Committee (IACUC). Female CF1 mice weighing 25-27g (Harlan, Indianapolis, Ind., or Charles River Laboratories, Inc., Wilmington, Mass.) were acclimated upon arrival at the College of Medicine Vivarium at the University of South Florida for 7 days prior to use.

Emulsions CNP16 and NP13 were diluted to solid content concentrations of 5%, 3%, 2%, 1.5%, 0.75%, 0.50%, or 0.25% using nano-purified water. The emulsions were then either purified by low speed centrifugation using an Eppendorf desktop centrifuge 5415D at 13.2K rpm (16.1K rcf) for 30 minutes in 2.0 ml Eppendorf Safe-Lock centrifugation tubes or were used “as is” for the remainder of the study. A 0.1mL volume of either an emulsion solution or a saline solution was administered to the mouse by intraperitoneal injection twice a day for 8 days. Mice were also administered the analgesic ketoprofen at the mouse dose rate of 10mg/kg body weight subcutaneously every 12 hours for the first 72 hours, then as needed per veterinary advice. The animals were monitored for 6 additional days after final sample injection for signs of pain, inflammation, toxicity, irritation, discomfort, and distress. Blood samples were collected via submandibular vein bleeds on the final day of treatment (day 8), and via cardiac puncture post-euthanasia
(day 14). Samples were immediately centrifuged, and serum was stored at -20°C until measurement of cytokine concentrations could be performed.

10.10.3 Tape-Stripping Abrasion Model for Toxicity Analysis of Nanoparticles

Mice were anesthetized using 3-5% isoflurane at a flow rate of 1L of O₂ per minute and received analgesic treatment via ketoprofen at the mouse dose rate of 10mg/kg body weight subcutaneously every 12 hours for the first 72 hours, then as needed per veterinary advice. Mice were kept on an isothermal warming blanket while under anesthesia and during recovery to maintain normal body temperature. Mice were removed from the blanket only after consciousness was regained. A 2cm² area on the dorsal surface was shaved and cleansed followed by administration of a skin abrasion through the use of a recently popular tape stripping technique. This technique has been histologically proven to create an abrasion by removal of most of the epidermal layers of the skin, exposing the basal layer of epidermis, and in some instances, the upper dermis. Nanoparticle-containing emulsions were then administered to the exposed area at 9%, 7%, 5%, or 2% solid content concentrations in 0.10mL doses two or three times a day for 8 days. The animals were monitored for 6 additional days after final sample administrations for signs of pain, inflammation, toxicity, irritation, discomfort, and distress. Blood samples were collected from each mouse via submandibular vein bleeds on the final day of treatment (day 8), and via cardiac puncture post-euthanasia (day 14). Samples were immediately centrifuged, and serum was stored at -20°C until measurement of cytokine concentrations could be performed.

10.10.4 Biochemical Analysis of Mouse Blood Samples

Concentrations of TNF-α and IL-6 in serum were determined for all blood samples obtained using an enzyme-linked immunosorbent assay (ELISA) kit (Endogen; Pierce, Rockford, IL) following the manufacturer’s recommended procedure. Cytokine concentrations were determined using three wells per serum sample, where a 10uL serum aliquot was used per well.
Appendix A: $^1$H and $^{13}$C NMR Spectra
Spectrum 11.1: $^1$H NMR (250MHz, CDCl$_3$) of β-lactam 103.

Spectrum 11.2: $^1$H NMR (250MHz, CDCl$_3$) of β-lactam 105a.
Spectrum 11.3: $^1$H NMR (250MHz, CDCl$_3$) and $^{13}$C (63MHz, CDCl$_3$) of $\beta$-lactam 105b.
Spectrum 11.4: $^1$H NMR (250MHz, CDCl$_3$) of β-lactam 106a.

Spectrum 11.5: $^1$H NMR (250MHz, CDCl$_3$) of β-lactam 2.
Spectrum 11.6: $^1$H NMR (250 MHz, CDCl$_3$) and $^{13}$C NMR (63 MHz, CDCl$_3$) of $\beta$-lactam 106b.
Spectrum 11.7: $^1$H NMR (400MHz, CDCl$_3$) of β-lactam 107.

Spectrum 11.8: $^1$H NMR (400MHz, CDCl$_3$) of β-lactam 10.
Spectrum 11.9: $^1$H NMR (250MHz, CDCl$_3$) and $^{13}$C (63MHz, CDCl$_3$) of β-lactam 6.
Spectrum 11.10: $^1$H NMR (250MHz, CDCl$_3$) and 13C (63MHz, DMSO) of $\beta$-lactam 109.
Spectrum 11.11: $^1$H NMR (250MHz, CDCl$_3$) of $\beta$-lactam 108.

Spectrum 11.12: $^1$H NMR (250MHz, CDCl$_3$) of $\beta$-lactam 4.
Spectrum 11.13: $^1$H NMR (250MHz, CDCl$_3$) and $^{13}$C (63MHz, CDCl$_3$) of β-lactam 110.
Spectrum 11.14: $^1$H NMR (250MHz, CDCl$_3$) of β-lactam 111.

Spectrum 11.15: $^1$H NMR (250MHz, CDCl$_3$) of β-lactam 3.
Spectrum 11.16: $^1$H NMR (400MHz, CDCl$_3$) of β-lactam 1.
Spectrum 11.17: $^1$H NMR (250MHz, CDCl$_3$) and $^{13}$C (63MHz, CDCl$_3$) of β-lactam 118.
Spectrum 11.18: $^1$H NMR (250MHz, CDCl$_3$) and $^{13}$C (63MHz, CDCl$_3$) of β-lactam 119.
Spectrum 11.19: $^1$H NMR (250MHz, CDCl$_3$) and $^{13}$C (63MHz, CDCl$_3$) of β-lactam 5.
Spectrum 11.20: $^1$H NMR (250MHz, CDCl$_3$) and $^{13}$C (63MHz, CDCl$_3$) of ciprofloxacin acrylamide 7.
Spectrum 11.21: $^1$H NMR (250MHz, CDCl$_3$) of penicillinac acid acrylamide 11 and polymer NP11.
Spectrum 11.22: $^1$H NMR (250MHz, CD$_2$OD) of ampicillin acrylamide 16.

Spectrum 11.23: $^1$H NMR (250MHz, DMSO-d$_6$) of amoxicillin.
**Spectrum 11.24:** $^1$H NMR (250MHz, CD$_3$OD) of amoxicillin diacrylate 18.

**Spectrum 11.25:** $^1$H NMR (250MHz, DMSO-d$_6$) of cefaclor.
Spectrum 11.26: $^1$H NMR (250MHz, Acetone-d$_6$) of cefaclor acrylamide 19.

Spectrum 11.27: $^1$H NMR (250MHz, CDCl$_3$) of poly(butyl acrylate) film CNP2.
Spectrum 11.28: $^1$H NMR (400MHz, CDCl$_3$) of poly(butyl acrylate:styrene) film CNP5.

Spectrum 11.29: $^1$H NMR (400MHz, CDCl$_3$) of poly(butyl acrylate:styrene) film CNP7.
Spectrum 11.30: $^1$H NMR (250MHz, CDCl$_3$) of β-lactam:ethyl acrylate polymer NP1a.

Spectrum 11.31: $^1$H NMR (250MHz, CDCl$_3$) of β-lactam encapsulated polymer NP6.
Spectrum 11.32: $^1$H NMR (250MHz, CDCl$_3$) of $\beta$-lactam encapsulated polymer NP2.

Spectrum 11.33: $^1$H NMR (250MHz, CDCl$_3$) of ciprofloxacin:butyl acrylate:styrene polymer NP7.
Spectrum 11.34: $^1$H NMR (250MHz, CDCl$_3$) of doxycycline encapsulated polymer NP24.
ABOUT THE AUTHOR

Kerriann Greenhalgh received her bachelor’s degree at the University of South Florida, Tampa, FL, majoring in Chemistry with an emphasis in Biochemistry. During her junior year, she began research in the lab of Edward Turos, contributing to the synthesis of numerous β-lactam analogues and biochemical analysis of the analogues. Kerriann has enjoyed performing highly interdisciplinary research and following the development of the antimicrobial therapy from the preliminary synthetic stages all the way to in vivo toxicity analysis. She hopes to further develop this multi-drug conjugated nanoparticle treatment for burn wound infections and ultimately establish a commercial product from this research.