ABSTRACT

KERATIN HYDROGELS FOR ANTIBIOTIC DELIVERY IN WOUND HEALING APPLICATIONS: DEVELOPMENT OF HPLC METHODS TO CHARACTERIZE RELEASE

by Rachael Catherine Hall

In the United States, 450,000 burn injuries receive medical treatment per year\(^1\) with approximately 2% experiencing wound infection\(^2\). Current clinical paradigms such as Epicel, Apligraf, and AlloDerm may provide wound coverage and promote wound healing, but do not incorporate antibiotics to fight microbial infection. Thus, dressings or materials that can promote healing while preventing infection are an important unmet clinical need. One possible solution is a biomaterial loaded with antibiotics. Off-the-shelf use of hydrogels made from keratin, collagen, and fibronectin proteins are attractive because of their physiochemical capacity to facilitate skin healing by promoting appropriate cellular responses. This thesis focuses on keratin hydrogels due to their ability to resist proteolytic degradation, promote vascularization, and support cellular ingrowth. The principal hypothesis of these experiments is that antibiotic loaded keratin hydrogels promote sustained release in a manner correlated with their degradation. To investigate this hypothesis, this thesis developed high performance liquid chromatography (HPLC) methods for detection of antibiotics in the presence of keratin proteins. Specifically, the results of this study helped to quantify antibiotic release from keratin hydrogels, and to characterize previous results indicating the sustained release of three specific antibiotics, cephazolin, ciprofloxacin, and neomycin. The results of these studies demonstrate the suitability of keratin hydrogels for antibiotic release and support conducting pre-clinical animal testing as well as possible FDA submissions for future human trials.
KERATIN HYDROGELS FOR ANTIBIOTIC DELIVERY IN WOUND HEALING APPLICATIONS: DEVELOPMENT OF HPLC METHODS TO CHARACTERIZE RELEASE

A Thesis

Submitted to the

Faculty of Miami University

in partial fulfillment of

the requirements for the degree of

Master of Chemical Engineering

Department of Chemical, Paper, and Biomedical Engineering

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2014

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# Table of Contents

Abstract ........................................................................................................................................ i

List of Tables .................................................................................................................................. iv

List of Figures ................................................................................................................................... v

List of Abbreviations .................................................................................................................... vii

Acknowledgements ......................................................................................................................... viii

Chapter I–Introduction to Burn Wound Healing and Keratin Biomaterials .................................... 1

  I.I Natural Healing of the Skin Organ ................................................................................................. 1

  I.II Current Approaches to Burn Wound Healing ............................................................................ 2

  I.III Selection of Biomaterial for Treatment of Burn Wounds ......................................................... 5

    I.III.I Keratin .................................................................................................................................. 7

    I.III.II Keratin Classifications ...................................................................................................... 8

    I.III.III Keratin as a biomaterial ...................................................................................................... 9

    I.III.IV. Use of Keratin for Controlled Release of Antibiotics ....................................................... 10

  I.IV Selection of Antbiotics .............................................................................................................. 12

  I.V High Performance Liquid Chromatography ............................................................................... 14

  I.VI Hypothesis, Objectives, and Approach .................................................................................... 15

  I.VII. Chapter I References ............................................................................................................ 16

Chapter II – HPLC Quantification of Antibiotic from Keratin Hydrogels ........................................ 19

  II.I Introduction ............................................................................................................................... 19

  II.II Materials and Equipment ....................................................................................................... 20

  II.III Methods .................................................................................................................................. 20

    II.III.I. Derivatization of Neomycin ............................................................................................. 21

    II.III.II. Detection of Antibiotics .................................................................................................. 22

    II.III.III. HPLC Method Development ......................................................................................... 22

    II.III.IV. Standard Curve Development ......................................................................................... 25

    II.III.V. Hydrogel Formation and Sample Collection ..................................................................... 26

    II.III.VI. Keratin Extraction ........................................................................................................... 27

    II.III.VII. Protein Assay .................................................................................................................. 28

  II.IV Results .................................................................................................................................... 28

    II.IV.I Chromatographs ................................................................................................................. 29
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>II. IV. II. Standard Curves</td>
<td>31</td>
</tr>
<tr>
<td>II. IV. III. Antibiotic Release from Keratose Hydrogels</td>
<td>33</td>
</tr>
<tr>
<td>II. IV. IV. Protein Assay</td>
<td>37</td>
</tr>
<tr>
<td>II. V. Discussion of Results</td>
<td>40</td>
</tr>
<tr>
<td>II. VI. Chapter II References</td>
<td>44</td>
</tr>
<tr>
<td>Chapter III – Discussion and Future Directions</td>
<td>45</td>
</tr>
<tr>
<td>III. I. Discussion</td>
<td>45</td>
</tr>
<tr>
<td>III. I. I. HPLC Method Development</td>
<td>45</td>
</tr>
<tr>
<td>III. I. II Antibiotic Release Profiles</td>
<td>48</td>
</tr>
<tr>
<td>III. II. III. Role of Sustained Release in Bacterial Inhibition</td>
<td>50</td>
</tr>
<tr>
<td>III. I. IV Broader Impacts</td>
<td>51</td>
</tr>
<tr>
<td>III. II. Future Directions</td>
<td>53</td>
</tr>
<tr>
<td>III. II. I Derivatization Techniques</td>
<td>53</td>
</tr>
<tr>
<td>III. II. II Tunability of Keratin Hydrogels</td>
<td>54</td>
</tr>
<tr>
<td>III. II. III Continuing Research</td>
<td>56</td>
</tr>
<tr>
<td>III. III. Chapter III References</td>
<td>57</td>
</tr>
<tr>
<td>Appendix</td>
<td>58</td>
</tr>
<tr>
<td>A. I. Understanding the Reaction Between OPA and Primary Amines</td>
<td>58</td>
</tr>
<tr>
<td>A. II. “Floating Retention Time” Exhibited by Ciprofloxacin</td>
<td>59</td>
</tr>
<tr>
<td>A. III. Effect of Thiol Group</td>
<td>60</td>
</tr>
<tr>
<td>A. IV. Effect of the Ratio of Volume of OPA to Neomycin</td>
<td>62</td>
</tr>
<tr>
<td>A. V. Tunability of Keratin Hydrogels</td>
<td>62</td>
</tr>
</tbody>
</table>
List of Tables

Chapter I – Introduction to Burn Wound Healing and Keratin Biomaterials

Table 1 - Chemical structures of antibiotics

Chapter II – HPLC Quantification of Antibiotic from Keratin Hydrogels

Table 1 - Antibiotic detection

Table 2 - Instrument parameters
List of Figures

Chapter I – Introduction to Burn Wound Healing and Keratin Biomaterials

Figure 1 - Schematic of a Wool Fiber

Figure 2 - Cumulative release of ciprofloxacin and keratose from keratin as a function of time

Figure 3 - Day of failure for several antibiotics for both gram negative and Gram-positive bacteria

Chapter II – HPLC Quantification of Antibiotic from Keratin Hydrogels

Figure 1 - OPA reaction with a primary amine

Figure 2 - Pump parameters, run time, and mobile phase composition of HPLC methods developed for cephalosporin

Figure 3 - Pump parameters, run time, and mobile phase composition of HPLC methods developed for ciprofloxacin

Figure 4 - Pump parameters, run time, and mobile phase composition of HPLC methods developed for neomycin and combination (of neomycin and cephalosporin)

Figure 5 – HPLC chromatogram for negative control and cephalosporin

Figure 6 - HPLC chromatogram for negative control and ciprofloxacin

Figure 7 - HPLC chromatogram for negative control and neomycin

Figure 8 - Cephalosporin standard curve

Figure 9 - Ciprofloxacin standard curve

Figure 10 - Neomycin standard curve

Figure 11 - Neomycin standard curve for absorbance

Figure 12 - Cephalosporin standard curve for fluorescence

Figure 13 - Cephalosporin cumulative release from 80:20 (α:γ) KOS 15% w/v gels

Figure 14 - Ciprofloxacin cumulative release from 80:20 (α:γ) KOS 15% w/v gels

Figure 15 - Neomycin cumulative release from 80:20 (α:γ) KOS 15% w/v gels

Figure 16 - Combination cumulative release from 80:20 (α:γ) KOS 15% w/v gels
Figure 17 – Keratin degradation for gels with cephazolin and without antibiotic

Figure 18 - Keratin degradation for gels with ciprofloxacin and without antibiotic

Figure 19 - Keratin degradation for gels with neomycin and without antibiotic

Figure 20 - Keratin degradation for gels with combination and without antibiotic

Figure 21 - Cumulative release for cephazolin and keratose 80:20 (α:γ) gels

Figure 22 - Cumulative release for ciprofloxacin and keratose 80:20 (α:γ) gels

Figure 23 - Cumulative release for neomycin and keratose 80:20 (α:γ) gels

Figure 24 - Cumulative release for combination and keratose 80:20 (α:γ) gels

Chapter IV - Appendix

Figure 1 - ESI-MS for streptomycin and OPA

Figure 2 - MALDI for streptomycin and OPA

Figure 3 - “Floating retention time” of HPLC ciprofloxacin trials

Figure 4 - Effect of thiol group (mercaptoethanol v. 3 mercaptopropionic acid) on fluorescence intensity of reaction between neomycin at 0.125 mg/mL and OPA

Figure 5 - Effect of the ratio of volume of OPA to volume of neomycin

Figure 6 - Cumulative release profile for cephazolin from 80:20 (α:γ) KOS and 95:5 (α:γ) KOS gels

Figure 7 - Cumulative release profile for ciprofloxacin from 80:20 (α:γ) KOS and 95:5 (α:γ) KOS gels

Figure 8 - Cumulative release profile for neomycin from 80:20 (α:γ) KOS and 95:5 (α:γ) KOS gels

Figure 9 - Cumulative release profile for combination from 80:20 (α:γ) KOS and 95:5 (α:γ) KOS gels
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC</td>
<td>area under the curve</td>
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<tr>
<td>Cipro</td>
<td>ciprofloxacin</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>HPLC</td>
<td>high-performance liquid chromatography</td>
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<td>KOS</td>
<td>keratose</td>
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<tr>
<td>OPA</td>
<td>o-phthaaldehyde</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>w/v</td>
<td>weight per volume</td>
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<td>w/w</td>
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</table>
Acknowledgements

I would like to give my upmost gratitude to each of the committee members for their patience, guidance, and for challenging me. With each obstacle, new learning and developments changed my view and scope of the project as well as ability to critically think through larger scale issues. Taking these challenges in stride, the project became more encompassing and comprehensive. My committee member’s ability to inspire and provoke stimulating thought throughout the project made my technical and professional skills grow immensely. These committee members were excellent mentors.

Also, I would like to thank my family who has been a source of encouragement, inspiration, and devotion. Without their patience, reminders to trust God’s plan, and unconditional love, I would not be where I am today. Through obstacles and celebrations, no matter where my journey took me, my family was always there to support me.

The author acknowledges funding support from the Department of Defense SBIR program (contract W81XWH-12-C-0004) as pass-through funds from KeraNetics, LLC. The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Defense position, policy or decision unless so designated by other documentation.
Chapter I–Introduction to Burn Wound Healing and Keratin Biomaterials

I.I Natural Healing of the Skin Organ

Skin, as the largest organ in the body, maintains homeostatic balance by helping to regulate internal temperature and to provide protection against pathogens and other external factors while having elasticity necessary to allow motion\textsuperscript{1,2}. The versatility of this organ is a combination of several characteristics provided by the composition of three different layers\textsuperscript{1,3}. Keratinocytes (sometimes referred to as basal cells), make up the outermost layer, the epidermis, which grows outward and flakes off as a function of cornification, or a unique programmed cell death\textsuperscript{4}. The epidermis is the first protection against pathogens and renewal of the cells in this layer can occur every two weeks, facilitating in injury resistance. Below the epidermis, the dermis houses collagen and elastin fibers, which provide strength and elasticity. Also in the dermis, blood vessels, nerves, hair follicles, glands as well as smooth muscle are present. Last, beneath the dermis, a fatty base layer, called the subcutis, provides insulation and padding\textsuperscript{1}. Each of these three layers, acting in combination as a defense mechanism and a regenerative feature, contribute to the functionality and ability of the skin to support the body.

A burn injury to skin is defined as an abrasion resulting from exposure to heat, friction, radiation, chemicals, or electricity, and is categorized according to the severity\textsuperscript{5}. First-degree burns are acute, and damage is only to the epidermis. Second-degree burns have penetrated the epidermis and dermis and take longer to heal than first-degree. Third-degree burns have reached all layers of the skin, while fourth-degree burns involve all layers of the skin as well as surrounding muscle. The seriousness of the burn contributes to the body’s ability to self-renew the cells and promote healing\textsuperscript{5–7}.

The burn itself is differentiated into three concentric zones\textsuperscript{6}. In the outermost zone of hyperemia, both blood circulation and metabolism are still able to occur, and the affected cells are likely to regenerate. As the zones move inward, the zone of stasis loses metabolism and in the zone of coagulation, neither blood circulation nor metabolism are present. For the zone of coagulation, the tissue is necrotic and will require more extensive treatment to return to its normal function. For burns, it is important to note how much of the area is affected by each of these zones to best predict natural healing and a proper method of treatment.
In the United States burns are a prevalent source of injury for civilians, with estimates around 450,000 burn injuries receiving medical treatment per year\textsuperscript{8}. In a 10-year study across 91 U.S. hospitals, it was reported that the majority of burn incidents (61\%) were caused from accidents within the home\textsuperscript{9}. However, burns are also a significant problem for the U.S. military. Since 2003, the United States Army Institute of Surgical Research (USAISR) Burn Center has provided care for more than 800 US military burn casualties and 1250 civilian burn victims\textsuperscript{10}. In recent conflicts including Operations Enduring Freedom/Iraqi Freedom, Kauvar et al. reported that 80\% of combat casualties sustained burns to the hands, and in a similar study by Hedman et al. 77\% of combat casualties were burns sustained to the head\textsuperscript{11,12}.

With destruction of the skin barrier and suppression of cellular response, infection is a common complication that will delay healing of the burn wound\textsuperscript{13}. Risks for infection are not only inherent with severe wounds, but also with delay of wound care, or nutritional challenges. In the 2012 National Burn Repository Study, wound infection was a complication that affected over 3,000 civilian patients (169,998 cases in total)\textsuperscript{9}. While this is a relatively small percentage of overall burn patients, in patients with severe burns (total body surface area >40\%), sepsis from burn wound infections accounts for approximately 75\% of deaths\textsuperscript{13}. With the high tempo of combat operations, infection is more problematic in the theater of battle where military personnel experience infection as frequently as 15-25\% in the current wars in Iraq and Afghanistan\textsuperscript{14–16}.

I.II Current Approaches to Burn Wound Healing

There are current treatments and products on the market for treatment of burns, including autografts and allografts. While these treatments are beneficial to patients, they are not ideal. Several specific problems are discussed below but challenges include lack of availability, infection, and scarring.

Therefore, dressings or materials that can promote healing while preventing infection are an important unmet clinical need. New materials to promote wound healing following burns should meet several criteria, with the optimal scenario leading to scarless healing. Challenges to the use of biomaterials include re-epithelialization and the rate of neovascularization, and development of materials that resist contraction and fibrosis\textsuperscript{17}. First, the material should promote recruitment of endogenous cells (already in the body) that can promote regeneration. This may be accomplished by the controlled release of growth factors to trigger a cell-signaling cascade that would promote recruitment of keratinocytes. Keratinocyte migration is the suggestion that
these cells promote health and wellbeing by allowing other cells to attach and fill in the wound bed. The material should also have minimal inflammatory response that would avoid scarring.

The various approaches to burn healing have considerable variability in success due to differences in the supporting material and immunological response from the body. Human allografts and skin substitutes are approaches to burn healing that are applied to areas of affected tissue that have been cleaned and debrided. Allografts may be seeded with living cells to stimulate growth, or left without the addition of living cells. Either way, the replacement tissue must be properly treated to ensure prevention of disease transmission. Often, a combination of skin substitutes and allograft tissue is utilized, where products such as Epicel, Apligraf, Integra, and Alloderm are in clinical use\textsuperscript{18–21}. These tissue substitutes provide wound coverage, a barrier against infection, and promote wound healing. Each has specific advantages that make consideration of appropriate dressing challenging as each individual injury must be handled on a case-by-case basis. However, the goal of each is to provide a barrier layer of renewable keratinocytes that is securely attached to the underlying dermis, becomes well vascularized, and provides elastic structural support for the skin\textsuperscript{17}.

Epicel is a cultured epidermal sheet best for deep dermal or full thickness wounds, comprising of a total body surface area greater or equal to 30\%\textsuperscript{18}. This product is a xenotransplantation product as it is manufactured with and contains residual amounts of murine cells, with rates of sepsis (related to this product) as high as 3.7\%\textsuperscript{18}. Despite this complication, the Food and Drug Administration has approved this product as a humanitarian use device (device that is intended for treatment of a condition that affects less than 4,000 individuals in the United States per year)\textsuperscript{18}.

A bilayer, bioactive wound healing product, Apligraf, was approved in 1998 by the FDA as a cell-based treatment for diabetic foot ulcers and venous leg ulcers\textsuperscript{22}. The lower dermal layer combines bovine type 1 collagen and human fibroblasts, while the upper epidermal layer contains epidermal cells\textsuperscript{19}. However, rates of infection associated with this product were 5.4\%\textsuperscript{23}. Apligraf is contraindicated for infected wounds, on patients with known allergies to bovine collagen, and for patients with a known hypersensitivity to the agarose shipping medium\textsuperscript{23}.

The FDA has also supported the use of Integra as a bilayer dermal treatment approved for use as a skin substitute\textsuperscript{24}. In this product, a fibrous, collagen, bovine layer is combined with a silicone layer. Two to three weeks later, an epithelial autograft may be used to replace the
silicone layer. This replacement occurs once the tissue has begun to become vascularized, allowing the silicone layer to be extracted. Again, this product is contraindicated for clinically diagnosed infected wounds, third degree burns, and hypersensitivity to bovine collagen or chondroitin materials\textsuperscript{21}.

AlloDerm is a type of allograft used in conjunction with an autograft. This product is an acellular (cadaveric) dermal cover (collagen based) with an autograft overlay, which helps reduce scarring. The FDA has approved this product as human tissue for transplantation and promotes rapid revascularization, leukocyte migration, and cell repopulation\textsuperscript{20}.

For injuries of low enough severity, burn healing occurs through re-epithelialization by the migration of cells to the site of injury for rebuilding the lost tissue. In more severe injuries, this re-epithelialization may be enhanced by the use of skin grafting or skin stretching. Skin stretching has been explored as a method to enhance the area that a graft can cover. It was found that circulating mesenchymal stem cells (MSCs) can be recruited to and migrate into expanded skin, where \textit{in vivo} studies found that 19.42\% of systemically transplanted MSCs migrated into the expanded skin following expander inflation using a computer-controlled bioreactor system\textsuperscript{25}. Both the dermal and epidermal layers must be replaced as the dermis provides the mechanical stability and tensile strength\textsuperscript{26}.

Even though skin stretching may ultimately prove clinically, it is not currently at the clinical level, and will likely not be suitable for severe injuries in which re-epithelialization does not occur. Thus, alternative approaches are required. During autograft surgery, skin is removed from an unburned portion of the body to replace lost tissue from the affected area. This is advantageous for several reasons including minimal immune response from the body as it is the patient’s own tissue. However, current drawbacks and limitations include donor site morbidity, availability of tissue, and current lack of antibiotic incorporation to treat infection. Autograft rate of survival is between 87-97\%, with subsequent wound infection the main risk factor\textsuperscript{27}. The most common bacteria strains found in the study were \textit{Pseudomonas aeruginosa}, \textit{Staphylococci aureus}, and \textit{Acinetobacter baumanii}\textsuperscript{27}. When treated with antibiotics, the rate of autograft survival was 97\%\textsuperscript{27}. With such high risk for infection and the demonstration of the benefit of antibiotic incorporation, alternative approaches are sought for burn healing.

Whereas autografts face possible complications such as donor site morbidity and availability of tissue, allografts face the possibility of an immune response. Consequently, there
exists a need for “off-the-shelf” biomaterial products that have minimal immunological response and low toxicity while also providing anti-microbial properties. Several innovative biomaterial and cell-based products have been improved in an effort to formulate an appropriate and suitable replacement for treatment of burns.

Even in the presence of a system that can promote keratinocyte migration and limit inflammation, it is also necessary to prevent bacterial infection to help achieve scarless wound healing. To prevent infection, methods of debriding the wound, covering the area with sterile dressing, and antibiotic incorporation are a few of the techniques currently used. Because burns and especially those injuries affected by infection may lead to long-term consequences including decreased mobility or activity of the affected person, there is a biomedical need for a solution to decrease rates of infection for burns. According to the 2012 National Burn Repository Report, pneumonia, cellulitis, and urinary tract infections are the most prevalent complications for patients with burns. In a burn wound environment, immediately after injury, both Gram-negative and Gram-positive bacteria may contaminate the injury. Ubiquitous in soil and sand, Acinetobacter baumannii and Pseudomonas aeruginosa are strains of Gram-negative bacteria that often colonize and affect wounds. As the wound progresses into healing, even Gram-positive bacteria such as MRSA may taint rehabilitation. However, at the USAISR, there is no standard protocol regarding the frequency or duration for the use antimicrobial agents. Care providers will apply antibiotics until the skin has healed completely. Given possible problems including antibiotic resistance and systemic toxicity with long-term systemic antibiotic delivery, it is clear that new materials and approaches are necessary for treatment and infection-prevention of burn wounds in clinical practice.

I.III Selection of Biomaterial for Treatment of Burn Wounds

A suitable biomaterial is subject to a number of criteria including mimicking the mechanical strength and function of the original tissue, biodegradability, and cell/tissue integration, among several others. In general, synthetic polymeric materials are readily tunable for degradation and drug delivery but lack the ability to promote cellular attachment and tissue integration. Conversely, natural polymer materials, including polysaccharides and proteins, have several characteristic advantages that make them candidates for biomaterial use. Limited foreign body response, high availability, cost effectiveness, and mechanical properties closely related to
those already in the body are a few of the criteria for the selection of a suitable natural biomaterial.

Hydrogels, a specific class of biomaterials, offer a hydrophilic polymer network with excellent biocompatibility\textsuperscript{29,30}. As these materials are also biodegradable, when the hydrogel breaks down, newly grown tissue has room to develop and assume full functionality. This characteristic is important for the body to heal in the most natural manner. It might be noted that most of the materials described in the preceding section use natural rather than synthetic materials for skin burn treatment.

Polysaccharides such as alginate, chitosan, and agarose mimic certain biological activity properties of the extracellular matrix, but are unable to promote cell adhesion and binding\textsuperscript{7,31,32}. Although relatively inexpensive to obtain, these materials must be chemically modified with appropriate cell binding motifs in order to support cell structure in a manner truly reminiscent of the extracellular matrix. Conversely, proteins (through amino acid sequences) promote integrin binding\textsuperscript{31}. However, some proteins are not as easily tunable (ability to manipulate its physical properties for use in a specific function). As collagen is denatured, its crosslinking changes, and it is unable to be as easily tuned to the parameters required of the biomaterial\textsuperscript{33,34}. 
We have begun to explore the application of keratin biomaterials as a means to satisfy some of the criteria for wound regeneration following burn injury for several reasons. Keratin may promote keratinocyte viability/migration, can achieve sustained release of antibiotics, and has characteristics that may provide a means to chemically control the rates of drug delivery. Unlike collagen, keratin proteins may be formed into biomaterials that meet the criteria of a suitable hydrogel, while providing the ability to degrade in a controlled manner (e.g., more slowly) while promoting cell attachment.

More specifically, keratin hydrogels can be composed of up to 90% (or more) water, providing moisture while also regulating processes of cell recruitment, differentiation, and maturation that are vital for the healing of wounds, especially those that are a result of burns. The ability to facilitate release of growth factors also makes keratin a strong candidate as a material to treat skin burns as the directed delivery of key growth factors may promote more rapid or complete healing.

I.III.I Keratin

Humans and several other animals produce keratin, a fibrous intermediate filament protein that assembles into strong fibrous networks. Examples of keratin include so-called “hard” keratins found in sheep wool, tortoise shells, quills of feathers, cuticle growth, and human hair. In addition to its presence in “hard” form, it is found in “soft” form in epithelial cells that help provide strength and resiliency to the epidermis. In terms of cost, keratins are attractive as they are readily available and are inexpensively purified via extraction. Moreover, its minimal inflammatory and cytotoxic responses as well as its biodegradability make it highly advantageous for use as a biomaterial. Reproducible architectures and porosity are results of keratin’s nature to spontaneously self-assemble at the micron scale. However, reducing the molecular weight of keratin (through hydrolysis) will inhibit keratins from the ability to self-assemble. As fibers, hydrogels, or numerous other forms, keratins promote cell adhesion and migration. Tachibana et al. has shown that cells can live on wool keratin sponge scaffolds for up to 23 to 43 days, making these scaffolds useful for long-term and high-density cell cultivation. Recognizing these specific characteristics has led to extensive research to understand better the process of extraction, processing, and purification. Several chemical and biochemical processes are employed in these techniques to extract proteins suitable for use as a biomaterial, which may be developed into wound dressings or exploited in various tissue engineering applications.
I.III.II Keratin Classifications

Keratins are classified based upon a number of considerations, including molecular structure, physiochemical characteristics, the epithelial cells producing them and the epithelial type containing the keratin-producing cells. With molecular weights in the range of 10 to 90 kDa, keratins are broadly categorized as alpha-, beta-, and gamma-keratins. Low-sulfur proteins in the range of 60-85 kDa are known as α-keratins and form a right-handed, alpha-helical formation in a wool (or hair) fiber (Figure 1). Higher-sulfur content, globular structure, highly crosslinked γ-keratins hold the microfibrils together. Even with its characteristic property of a lower molecular weight (approximately 15 kDa), γ-keratins form the matrix of the fibril (Figure 1). α-keratins, in the native state, are cross-linked by disulfide bonds. Consequently, the intermediate filaments crosslink covalently with matrix proteins. Comparably, γ-keratins are more soluble than α-keratins. Understanding these properties aids in the separation and purification of the proteins. As a result, several chemistry methods have been developed to isolate the different forms of keratins for further use and analysis.


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Numerous methods exist for extraction of keratin, depending on the desired application of the product. Soluble keratins may be extracted by oxidation or reduction, during which the amino acid cystine is converted to either cysteic acid or cysteine. An oxidative treatment will yield keratins in which the cysteine residues form a cysteic acid derivative, and these oxidatively extracted keratins are commonly called keratose. Reductive extraction will yield a keratin
protein with cysteine residues that can form disulfide crosslinks, and these reductively extracted keratins are referred to as **kerateine**.

The reductively extracted kerateine can be obtained by reduction with thioglycolic acid at a concentration of 1.0 M or 0.5 M, pH at 10.2, temperature of 37°C, reduction time of 12 hours, and liquid to solid ratio of 20:1. Reduced keratins are not as hydrophilic as those that are oxidized and will not swell and split open the hair cuticle, resulting in a relatively low yield. Kerateines display properties that show they are less polar than their counterpart (keratose, described below), more stable at extremes of pH and can be re-crosslinked through oxidative coupling of cysteine groups.

Oxidative extraction of keratose can be achieved in 2% w/v peracetic acid for 10 hours at 37°C and liquid to solid ratio of 20:1. The resulting keratose product is unstable at pH extremes due to polarization of the backbone caused by the electron withdrawing properties of the cysteic acid. In comparison to kerateines, keratoses are generally hygroscopic and water-soluble. Also, they are non-disulfide crosslinkable due to sulfonic acid residues on the cysteic acids preventing formation of disulfide bonds. For this project, only keratose formulations were used. This is because a burn wound environment in inherently hydrolytic as part of the natural healing response, a water-soluble keratose (instead of hydrophobic kerateines) is best for wound healing management.

### I.III.3 Keratin as a biomaterial

Keratin biomaterials are used in a variety of applications such as in local drug delivery of antibiotics and other bioactive materials, for cosmetic use as hair growth and epidermis substitutes, and electron spun membranes for wound healing, as they exhibit excellent physiochemical properties. In tissue engineering, they may be used as scaffolds to support cell growth or proliferation in de novo tissue formation. In one example, keratose biomaterials were used as a conduit to aid in neuronal regeneration across a critically-sized mouse tibial nerve defect. Similarly, studies have shown excellent cyto-compatibility of keratin in biological systems, by promoting NIH 3T3 fibroblast and Schwann cell proliferation and adhesion. Keratins have been demonstrated to be conducive to cell infiltration, and do not form adhesions or “abnormal” scar tissue. Also, keratin biomaterial extracts did not adversely affect the viability of MC3T3 at low concentrations of 0.1 mg/mL or less. Considering the combination
of cell viability, adhesion, proliferation, and tissue response, a strong case can be made for the use of keratins as biomaterials.43

Mechanical and chemical properties of the keratin derivatives directly influence their use in biomaterials. The percentage of the components of a hydrogel may affect properties such as viscosity, film elasticity, and hydrolytic resistance. Decreased hydrolytic susceptibility is exhibited when higher amounts of the α- composition of a keratose are present.38 Keratin resists proteolytic degradation, as human keratinases are not known to exist. By introducing exogenous crosslinking, the degradation of the keratose is decreased, and the compound with a high binding affinity for keratose can be released over a longer time.38 Each of these properties may be modeled and manipulated for use as a specific biomedical application. The ability to facilitate release of growth factors also makes keratin a strong candidate for the hydrogel as regulating cell processes, cell differentiation, and cell maturation are vital for the healing of wounds, especially those that are a result of burns.

I.III.IV. Use of Keratin for Controlled Release of Antibiotics

Previous studies by our group have shown that keratin hydrogels can support sustained release of the antibiotic ciprofloxacin.36,46 In this previous report, release profiles of ciprofloxacin and keratose from keratin hydrogels were studied, and their relationship correlated (as a clear overlap between cumulative release profiles).36 In other words, as the keratose gel degraded, ciprofloxacin was released at the same rate as the degradation of the gel. The rate of the gel degradation may impact the ability of the loaded antibiotic to be released from the hydrogel, which is crucial for wound healing applications. As the (burn) affected tissue begins to regenerate, the hydrogel should degrade in a manner that is proportional to the healing and proportional to the rate of antibiotic delivery. The results reported in Saul et al.36 (see Figure 2 below) show a clear correlation between the hydrogel degradation and antibiotic release, as well as support the idea of using keratin hydrogels for applications in wound healing with infection control via antibiotic release.
In the same way, ciprofloxacin must be shown to be bioactive over its release from the hydrogel. In another finding by Saul et al, results indicate that the bioactivity of ciprofloxacin is maintained over the course of 23 days, by remaining above the minimum inhibitory concentration (MIC), a measure to determine an antimicrobial’s ability to inhibit growth of microorganisms. Again, these results were encouraging for the use of keratin hydrogels for infection control and suggested that further studies (including those in this thesis) are warranted.

Further, it has been demonstrated that flowable keratin biomaterials loaded with antibiotics (KeraStat-ICG) supported keratinocyte and fibroblast proliferation while preventing colony formation of Gram positive, Gram negative and anaerobic bacteria. Previous in vitro studies show that ciprofloxacin or combination of neomycin and cephazolin provide long-term inhibition of pseudomonas aeruginosa (Gram negative bacteria) and staphylococcus aureus (Gram positive). These results of these previous studies are shown in Figure 3 below and provide validation for the concept of the studies that will be described in this thesis, as keratin has been shown to be a suitable scaffold material and the antibiotics have been shown to be bioactive.
In summary, keratin hydrogels have been shown to release antibiotic with respect to the rate of the keratin scaffold degradation, promote tissue regeneration, elicit minimal foreign body response and may be adjusted to mimic certain mechanical and material properties similar to those present in the body. Given that these are the key criteria for a successful burn treatment, there is a need for development of methods to assess the release of antibiotics within keratin gels. While the detection of various antibiotics has been previously reported by using fluorescence (for ciprofloxacin), ELISAs (for neomycin), and absorbance (for cephazolin), a more accurate and repeatable measure is necessary. As noted below (Section I.V), HPLC is a method widely accepted by regulatory agencies and is considered the standard to which other methods are compared. Considering the high sensitivity and ability to use multiple detectors in series, HPLC may be used for multiple antibiotic formulations. However, specific consideration must be given to the selection of antibiotics according to the biomedical application, as well as the chemical properties of the compound that allow for HPLC detection.

### I.IV Selection of Antibiotics

To address the need for antibiotic incorporation into biomaterials used for skin healing with infection control, three antibiotic drugs (ciprofloxacin, cephazolin, and neomycin sulphate), each with different physiochemical properties, different mechanism of actions, and from different drug classes that are commonly used in medical practice, were investigated for incorporation into and release from keratin hydrogels. For prevention against Gram-negative infections, neomycin sulphate (an aminoglycoside with molecular weight ~ 614 Da) demonstrates interference with bacterial protein synthesis. Similarly, cephazolin (~ 454 Da, 1st
generation cephalosporin) fights against Gram-positive infections by disrupting the synthesis of the bacterial cell wall, adversely affecting cell wall integrity. In yet another class of antibiotics (a quinolone), ciprofloxacin (molecular weight ~331 Da) is effective against both Gram-positive and Gram-negative bacteria. Ciprofloxacin’s effectiveness comes from its ability to slow or stop DNA and protein synthesis, but is a 2<sup>nd</sup>/3<sup>rd</sup> line drug. These specific antibiotics were selected because they exhibit bacterial inhibition against specific types of bacteria, which may be useful to promote improved wound healing following burn.

While each individual antibiotic was formulated into keratin individually, we recognize the neomycin (Gram negative) and cephazolin (Gram positive) are more selective to certain types of bacteria. We note again that ciprofloxacin is a broad spectrum antibiotic that is likely to be effective against both Gram positive and Gram negative bacteria. We therefore also investigated a combinatorial approach of in which neomycin and cephazolin were formulated with keratin hydrogels together as an alternative to ciprofloxacin. This combination of 1<sup>st</sup> line treatment drugs (cephazolin and neomycin) would provide a means treat both Gram-positive and Gram-negative bacteria from a single formulation. The combination of neomycin and cephazolin together would provide more bacterial inhibition against different strains of bacteria, in a manner that may be more effective than second/third-line drugs such as ciprofloxacin. This combinatorial approach is discussed further in Chapter II. The structures of the antibiotics are shown in Table 1 below.

<table>
<thead>
<tr>
<th>Table 1. Chemical structures of antibiotics</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1" alt="Cephazolin" /> <img src="image2" alt="Ciprofloxacin" /> <img src="image3" alt="Neomycin" /></td>
</tr>
</tbody>
</table>

With the incorporation of antibiotics in this design, it would be the goal that wound management would become less burdensome as dressings would need to be changed less frequently due to the sustained release achieved with keratin hydrogels. Ideally, patient care would be more favorable as a result of the success of this model.
I.V High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) detection methods offer high accuracy, precision, and reliability through processes that separate, identify, and quantify components of a mixture. HPLC has been previously used in pharmaceutical manufacturing, and in medical research (for separation of components within a biological sample) among numerous other applications\(^{48}\), due to the controlled experimental environment, which consistently provides reliable data. For a wide range of applications, on industrial or small scales, HPLC is the “gold standard” to which other methods are compared.

HPLC techniques are developed based upon the interaction between a specific compound and the HPLC column’s adsorbent material. Pressurized liquid (containing the dissolved compound of interest within a mobile solvent phase of defined composition) is passed through a series of capillaries, instrument column, and eventually through detectors such as mass spectrometers, UV-VIS, fluorescence, or charged aerosol, among many others. During this time, temperature, mobile phase composition and flow rate, as well as the material of the column itself influence the interaction of the mixture components with the column. Most importantly, special consideration must be given to the column material as it may be the most crucial factor affecting separation.

To quantify the release of these antibiotics from a keratin hydrogel, a C18 column was selected in consideration of the suggested hydrophobicity of the antibiotic, where 18 carbon chains (hydrocarbon) is attached to a silica bead. The antibiotics each interact with the column to separate them from the mixture. Hydrophilic (polar) molecules in the mobile phase will pass through the column first since they do not interact with the hydrophobic stationary phase. As the hydrophobic antibiotics adsorb to the column, they can be subsequently eluted from the column by reducing the hydrophobic interactions by using a non-polar solvent mobile phase.

By using methods specifically developed for detection of the selected antibiotics, more accurate detection and less measurement error may be obtained. In this thesis, the results of HPLC analysis allow for determination of release profiles of these antibiotics from keratin hydrogels.
I.VI Hypothesis, Objectives, and Approach

The overarching hypothesis guiding this work is that keratin hydrogels are useful in the treatment of burn injury because they promote cell interaction/migration, can be tuned for the rate drug delivery, and can achieve sustained release of antibiotics or antimicrobial agents. The specific objective of this thesis proposal was to better characterize previous results from our group indicating the sustained release of several types of antibiotics from keratin hydrogels. More specifically, methods were developed to allow the use of high performance liquid chromatography (HPLC) as a means to characterize antibiotic release from keratin hydrogels.

The goal for this work was to better characterize keratin hydrogels as a material, where consideration was given to ensure repeatable formulation of the antibiotic-loaded keratin hydrogels. More specifically, reliability of formulation would be required for prospective FDA submissions. HPLC provides a means to ensure that the drug loading meets the specifications of the product formulation before further use. Ultimately, the completion of this project may be useful for justification of future animal testing or human clinical trials, and should aid in demonstrating whether keratin hydrogels can play a role in the treatment of burn wounds by demonstrating (confirming) previous drug release profiles.
I.VII. Chapter I References

38. Van Dyke ME, Saul JM, Smith TL, de Guzman R. Controlled Delivery System. 2011;1(61).
Chapter II – HPLC Quantification of Antibiotic from Keratin Hydrogels

II.I Introduction

As noted in the previous chapter, current burn wound dressing products on the market (both auto- and allografts) have important technical drawbacks to wound treatment including availability of tissue (auto- and allograft), donor site morbidity (autograft), and potential for disease transmission (allograft). The susceptibility to infection in some cases is also an important aspect of wound treatment, management, and healing that must be addressed. We are investigating the use of keratin biomaterial hydrogels as an alternative material to promote wound healing due to their biocompatibility\(^1\), ability to facilitate an appropriate healing response\(^2\), and the ability to achieve sustained release of antibiotics\(^3\). The last of these is the primary focus of this thesis.

While we have previously demonstrated antibiotic release from keratin hydrogels, in this thesis work, release of several antibiotics from keratin hydrogels was investigated by HPLC. While the emphasis here in on antibiotic release, in the future, it is foreseeable that these hydrogels could also have cells incorporated as a tissue engineering approach to address the need for cell-mediated repair or that they could be further formulated to achieve desirable rates of antibiotic release.

In previous studies, different methods were used to characterize release of four different types of antibiotic drugs\(^3,4\). Ciprofloxacin release from keratin hydrogels was characterized by using a fluorescence reading at 340/460 nm excitation/emission wavelengths, UV absorbance (270nm) was used to detect cephazolin release, and neomycin was detected by a competitive enzyme linked immunosorbant assay (ELISA)\(^4\). These previous studies have indicated that each of these drugs can be delivered in sustained fashion from keratin hydrogels and are bioactive against several model strains of bacteria including \textit{S. aureus} and \textit{P. aeruginosa}\(^3,4\).

While these approaches provided information on the release characteristics of the antibiotics, several of these (UV and ELISA methods) are inherently “noisy” due to high background readings from keratin. Therefore, it is necessary to explore alternative methods for characterizing the release of antibiotics from the hydrogels as an application for wound dressings. More specifically, we were interested in investigating the ability of HPLC to provide more accurate and repeatable measurements of the antibiotics than our previous techniques. We also improved on our previous approach by achieving separation of keratin from the antibiotic
drugs with an extraction approach that removes keratin from the samples in an effort to improve sensitivity and repeatability in antibiotic quantification. Ultimately, this thesis work may prove useful in the characterization of keratin hydrogels for applications to regulatory agencies as these formulations have similarities to a product being commercially developed by KeraNetics, LLC.

II.II Materials and Equipment

Sterile, lyophilized keratose powder, including α-keratose and γ-keratose powder, was obtained from KeraNetics, LLC (Winston-Salem, NC) with no further modifications. Ciprofloxacin (catalog number 17850-5G-F) was obtained from Sigma-Aldrich Corp (St. Louis, MO). Cephazolin (catalog number C2242) was from TCI (Tokyo, Japan) and neomycin sulphate (catalog number 1405-10-3) was from Indofine Chemical Company (Hillsborough, NJ). Phosphate-buffered saline (PBS) was obtained from Thermo Scientific (Rockford, IL).

The Ultimate 3000 High Performance Liquid Chromatography unit with Chromelone 7 analysis software (Thermo Scientific Dionex, West Palm Beach, FL) was used for the detection of the antibiotics. The HPLC unit is equipped with a diode array detector (DAD) for UV detection and a fluorescence detector. For all experiments, a Syncronis aQ C18 column with a diameter of 3 mm length of 100 mm, and particle size of 3.0 μm was used.

HPLC grade solvents were used for all experiments. Water, methanol, chloroform, and acetonitrile were obtained from Fisher (Fair Lawn, NJ). Acetic acid was from Sigma-Aldrich Corp (St. Louis, MO). O-Phthaldealdiahyde (OPA) (A13299) for neomycin derivatization was obtained from Alfa Aesar (Heysham, England). Boric acid (10043-35-3) and potassium hydroxide (1310-58-3) were from Fisher (Fair Lawn, NJ). A DC protein assay kit was obtained from Bio-Rad (Hercules, CA).

II.III Methods

The purpose of the following experiments was to characterize the antibiotic release profile over time from the keratin hydrogels. Characterizing the antibiotics with respect to their release profiles from keratin hydrogels helps validate previous antibiotic release studies with a more widely accepted technique (HPLC), and demonstrates the suitability of keratin hydrogels for antibiotic release. In order to determine the antibiotic release kinetics, we first developed an extraction technique to remove keratin from samples, we developed HPLC methods for each antibiotic (or combination of antibiotics) described in Chapter 1, and we developed a standard curve as a means to quantify release from keratin hydrogels.
II.III.I. Derivatization of Neomycin

Successful quantification of the antibiotics by HPLC techniques is contingent upon ability to identify each compound, using inherent physiochemical properties or chemically modifying the compound for detection. While ciprofloxacin (fluorescence) and cephazolin (UV peak) have chromophores that can be detected without modification, neomycin does not contain such a group that could be detected by the diode array or fluorescence detectors of our HPLC system. We therefore used a pre-column derivatization technique for neomycin.

O-phthalaldehyde has been used in numerous applications, including the fluorimetric detection of aminoglycosides and amino acids\textsuperscript{5-7}. The reaction of a primary amine with o-phthalaldehyde has been shown to proceed by the reaction shown in Figure 1.

\[
\text{CHO} + \text{R-NH}_2 + \text{R'}-\text{SH} \rightarrow \text{fluorophore}
\]

---

Potassium borate buffer was prepared by dissolving 426 mg of boric acid and 494 mg of potassium hydroxide in 20 mL of water. Then, the OPA solution was prepared by dissolving 10 mg of o-phthalaldehyde in 100 μL of methanol. Then 24.8 μL of 3-mercaptopropionic acid was added. This solution was brought to a final volume of 2 mL by adding potassium borate buffer. This solution was vortexed thoroughly to ensure proper mixing. The final concentration of OPA in solution was 5 mg/mL.

Neomycin was prepared in PBS (see Section II.III.V for antibiotic preparation) at concentrations ranging from 0 – 2.0 mg/mL. Since neomycin contains six primary amines, OPA has plenty of opportunity to attach to and derivatize this antibody. Using 150 μL of neomycin sample and 50 μL of OPA reagent (a 3:1 volume ratio of OPA at 5mg/mL to neomycin at the concentrations noted above) and allowing a 120 second reaction time upon addition of the OPA. At the neomycin concentrations tested, the signal to noise ratio was found to be greater than 3.
This range was suitable for the detection of neomycin in the time point samples (see standard curves in Section II.IV.II).

**II.III.II. Detection of Antibiotics**

The mode of detection for each antibiotic is shown in Table 1, using a diode array or fluorescence detector.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Detection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>Fluorescence at 340/450 nm excitation/emission⁴</td>
</tr>
<tr>
<td>Cephazolin</td>
<td>Absorbance at 254 nm⁴</td>
</tr>
<tr>
<td>Neomycin</td>
<td>Fluorescence at 340/450 nm excitation/emission (by OPA derivatization)⁷</td>
</tr>
<tr>
<td>Combination (Cephazolin and Neomycin)</td>
<td>Cephazolin: Absorbance at 254 nm Neomycin: Fluorescence at 340/450 nm excitation/emission (by OPA derivatization)⁷</td>
</tr>
</tbody>
</table>

To ensure consistent addition of a derivatization agent, as well as uniform reaction time, a user-defined program was written in the Chromeleon software. In this program, the needle drew 50 μL of OPA reagent from a 1.5 mL vial, deposited it into the desired sample well, drew up and then deposited 100 μL from the well (this served as mixing), and was then allowed to react for 120 seconds. After this time, the needle drew 10 μL from the well and injected the reaction product to begin the analysis. The fluorescent products were then detected at 340/450 nm excitation/emission. Since the methods and procedures were well documented, the measure of antibiotic loading was found to be repeatable and reliable by HPLC methods. Using the fluorescence and absorption properties of the antibiotics, the concentration and, therefore, the respective drug loading into the keratin hydrogel was quantified.

**II.III.III. HPLC Method Development**

The instrument parameters were as shown Table 2 (below). For a reverse phase HPLC column, selection of the mobile phase includes a hydrophobic (polar) solvent, where acetonitrile is commonly used. Run time length is based upon how the antibiotic interacted with the column, the column diameter and height, as well as the flow rate. For each antibiotic, the run time was variable since they each interacted with the column differently, and therefore each required a specific method (e.g., change in mobile phase, run time, etc.). For trials that included a step
change, the column was equilibrated back to starting conditions (86% of 2% acetic acid and 14% acetonitrile), after the antibiotic eluted from the column. Each run was extended for at least 10 minutes to allow equilibrium to occur before starting a new sample injection.

<table>
<thead>
<tr>
<th>Table 2. Instrument Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow Rate</td>
</tr>
<tr>
<td>Injection Volume</td>
</tr>
<tr>
<td>Column Temperature</td>
</tr>
<tr>
<td>Fluorescence Detector Temperature</td>
</tr>
<tr>
<td>Detection</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

Figures 2 through 4 below show the method and HPLC parameters used for the run time, pump flow, and mobile phase for cephazolin, ciprofloxacin, and neomycin, respectively. For cephazolin, an isocratic method of 86% of acetic acid (2%) in water and 14% acetonitrile was used throughout the duration of the run (Figure 2).

![Figure 2](image)

Figure 2. Pump parameters, run time, and mobile phase composition of HPLC methods developed for cephazolin

For ciprofloxacin, the same isocratic method used for cephazolin could not be used. Ciprofloxacin has limited solubility in certain aqueous solvents and is susceptible to precipitation, especially if the pH is above 5.2. Further, ciprofloxacin was not completely eluted from the C18 column within a suitable timeframe when using 86% of 2% acetic acid and 14% acetonitrile, and exhibited a “floating retention time” (discussion in Appendix, A.II.). Using a
step change to increase the hydrophobic (acetonitrile) composition of the mobile phase helped to elute ciprofloxacin from the column. For ciprofloxacin, the acetonitrile composition was increased from 14% to 30% at 4 minutes and then allowed to return to 14% acetonitrile at 20 minutes, where the balance was water with 2% acetic acid. From 20 to 30 minutes, the column was allowed to re-equilibrate at 14% and prepare for the start of the next injection. Figure 3 represents the pump parameters and mobile phase composition.

Figure 3. Pump parameters, run time, and mobile phase composition of HPLC methods developed for ciprofloxacin

After pre-column derivatization (see above), a different HPLC method than those described above for cephazolin and ciprofloxacin was required to detect neomycin and resolve it from the unreacted OPA reagent. To clearly resolve fluorescent products, it was necessary to again use a step change in the mobile phase composition. At 6 minutes the acetonitrile was increased to 100% to essentially wash the column from the reaction product of the derivatization method. At 17 minutes, the mobile phase was brought back to 86% of 2% acetic acid and 14% acetonitrile and then allowed to equilibrate for 10 minutes (Figure 4). The same pump parameters, run time, and mobile phase composition were used for the combinatorial approach, where neomycin and cephazolin were prepared in the same formulation (Table 4, below). As noted in Chapter I, the combination of neomycin and cephazolin is of interest due to their ability to inhibit a variety of bacterial strains in a way that is more effective than second/third-line drugs (ciprofloxacin).
II.III.IV. Standard Curve Development

Integration of the area under the curve (AUC) from the HPLC chromatogram, by comparison to a standard curve, was used to quantify the release of antibiotics from the keratin hydrogel (see Section II.III.V below). Each antibiotic was prepared following Table 4 (Section II.III.V below). Then serial dilutions were prepared in PBS by using a 1:2 ratio to prepare a 7-point standard curve. A blank (PBS only) was also prepared. The standards were prepared in triplicate. All (known) concentrations of antibiotic were submitted to the extraction process mentioned below (in Section II.III.VI), and then run over the HPLC column with the methods described for each antibiotic in the previous section (Section II.III.III). Table 3 describes the integration time for each antibiotic, which is based on the elution peak of the antibiotic as determined by UV (cephazolin) or fluorescence (ciprofloxacin and neomycin).

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cephazolin</td>
<td>4-6</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>6.5-12</td>
</tr>
<tr>
<td>Neomycin</td>
<td>8-10</td>
</tr>
</tbody>
</table>
| Combination  | Fluorescence: 8-10 (for detection of neomycin)  
UV: 6.0-6.75 (for detection of cephazolin) |

For each antibiotic, the peak found between in the specified time range was found to be concentration dependent. The integration time was selected to best characterize and represent the
area under the curve by integrating from valley to valley of the peak of interest. The area under the integrated curve is proportional to the concentration of the antibiotic in the sample. Since the concentration of these samples was known, the slope of the curve could subsequently be used to determine the unknown concentration of the samples (Section II.III.V).

**II.III.V. Hydrogel Formation and Sample Collection**

Table 4, below, shows the keratin hydrogel antibiotic formulations (right hand column). The antibiotic loading concentrations selected were based on previous work in our group, which demonstrated sustained release of bioactive antibiotics suitable to achieve bacterial inhibition for acute infections over the course of 7 – 21 days when these concentrations were used. These concentrations are also non-toxic to mammalian cells, and provide a therapeutic window when released from the hydrogel.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC</th>
<th>Hydrogel formulations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>0.4 ~ 4mg/L for <em>S. aureus</em>&lt;sup&gt;9,10&lt;/sup&gt;</td>
<td>2 mg/mL in 15% (w/v) gel *PBS at pH of 5.2</td>
</tr>
<tr>
<td></td>
<td>0.25mg/L for <em>P. aeruginosa</em>&lt;sup&gt;11&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Cephazolin</td>
<td>0.5mg/L for <em>S. aureus</em>&lt;sup&gt;12&lt;/sup&gt;</td>
<td>20 mg/mL in 15% (w/v) gel</td>
</tr>
<tr>
<td>Neomycin</td>
<td>8~16mg/L for <em>P. aeruginosa</em>&lt;sup&gt;13&lt;/sup&gt;</td>
<td>20 mg/mL in 15% (w/v) gel</td>
</tr>
<tr>
<td></td>
<td>2~256mg/L for <em>S. aureus</em>&lt;sup&gt;14&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Cephazolin and Neomycin</td>
<td>0.5mg/L for <em>S. aureus</em>&lt;sup&gt;12&lt;/sup&gt; (Cephazolin) AND</td>
<td>20 mg/mL of cephazolin AND 20 mg/mL of neomycin in 15% (w/v) gel</td>
</tr>
<tr>
<td></td>
<td>8~16mg/L for <em>P. aeruginosa</em>&lt;sup&gt;13&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2~256mg/L for <em>S. aureus</em>&lt;sup&gt;14&lt;/sup&gt; (Neomycin)</td>
<td></td>
</tr>
</tbody>
</table>

All antibiotic formulations were prepared in PBS at the concentrations indicated in Table 4. For example, for neomycin or cephazolin preparation, 200 mg of respective antibiotic was weighed and then dissolved in 10 mL of PBS (pH ~7.4) by vortexing the solution. For ciprofloxacin formulations, 20 mg of antibiotic was weighed, and then dissolved in 1M of HCl. 8 mL of PBS was then added, and the pH brought to 5.2 by addition of 0.1 M NaOH. The final volume was brought to 10 mL in PBS. We note that for ciprofloxacin at 2 mg/mL, the PBS was at a pH of 5.2. We have found that this is necessary as this pH maintains solubility of ciprofloxacin (precipitates at higher pH) and keratin (precipitates at pH < ~5).
For each antibiotic-keratin hydrogel formulation, a consistent method was followed. First, keratin hydrogels were formed by methods previously described by our group\(^4\). Briefly, the hydrogel was formulated by using a mixture of 80% w/w α-keratose powder and 20% w/w γ-keratose powder. The rheological and degradation profiles of this hydrogel formulation have been shown to be suitable in skin infection models\(^8\). For a typical 600 mg mixture of keratose, 480 mg of α-keratose and 120 mg of γ-keratose was mixed with 4 mL of PBS or 4 mL of specific antibiotic formulation to give a 15% weight per volume (w/v) formulation (see Table 4). The resulting formulations were vortexed, mixed manually with a spatula, vortexed, and then centrifuged at 1500 RPM for 3 minutes to ensure thorough mixing and homogeneity. After centrifugation, known masses of the keratose mixtures were loaded into a syringe and ~ 400μL was injected into 1.5 mL tubes. The tubes were kept overnight in a 37°C incubator to complete gelation\(^1\). The keratose-only formulation (PBS with no antibiotic) was used as the (negative) control group. All 400 μL gels were made in quadruplicate.

After 24 hours in the incubator, 150 μL of PBS was placed on top of each gel. This was then taken to be time zero for the release experiments. At pre-specified time points (1.5, 3, 6, 12, 24 hours, daily for one week, and weekly for one month), the 150μL of PBS was removed and placed in 1.5mL labeled tube for further analysis. Then 150μL of fresh PBS was placed on top of each gel sample. The collected PBS samples were stored at -20°C until ready for HPLC analysis.

**II.III.VI. Keratin Extraction**

As the antibiotic release proceeds, soluble (and some insoluble pieces of keratin hydrogel) also elute into the PBS due to degradation (or erosion) of the hydrogel. The HPLC methods established above are not compatible with keratin passing over the HPLC C18 column due to solubility issues with keratin in the mobile phase as well as particulate matter. Therefore, it was necessary to remove all keratin from the samples at each time point. A method for extracting protein was developed by KeraNetics and was used here. Briefly, 100 μL of sample (whether from the negative control group or the antibiotic loaded group) was added to a clean 1.5 mL conical tube. 400 μL of methanol was added, then 100 μL of chloroform, and finally 300 μL of water. Between additions of each solvent, the tube was vortexed to ensure good mixing. After addition of the water and a final vortex, the solution was centrifuged at 3600 rpm for 6 minutes at 25 °C. In this extraction method, the hydrophobic chloroform denatures the keratose, exposing the hydrophobic residues in the keratose. As a result of the addition of water and centrifugation,
keratin deposited as a layer at the phase interface (between the organic and aqueous layers) to minimize free energy. Only the top aqueous layer (purified of keratin), which contained the water-soluble antibiotic, was removed for analysis. 200 μL from this aqueous layer was placed into a 96 well plate for HPLC analysis.

II.III.VII. Protein Assay

To determine the keratin protein content in the samples (due to gel degradation), a colorimetric modified Lowry’s protein assay was performed. We used the DC Protein Assay from Bio-Rad according to the manufacturer’s instructions. Briefly, 5 μL of each sample or standard were placed into individual wells of a 96-well microplate. Then, 25 μL of reagent A (an alkaline copper tartrate solution) was added followed by 200 μL of reagent B (a dilute Folin Reagent), in sequential order. After 20 minutes, the absorbance was read on a plate reader at 750 nm.

II.IV Results

We anticipated that the results obtained by HPLC would be more accurate than those by other techniques, potentially providing a better explanation to previous results related to the timeframe of bacterial inhibition in a broth assay in the presence of antibiotic-loaded keratin hydrogels. Using consistent and repeatable HPLC methods helps to remove background noise and interference, therefore increasing the sensitivity. This helps to eliminate ambiguity from the experiments that previously used fluorescence, UV, and ELISA methods for cephazolin, and neomycin.

To accomplish this task, a method of fabricating the hydrogels, introducing the three antibiotics into the gels, removing a sample of released antibiotic, performing an extraction, and then running the sample over HPLC was developed.
II.IV.I Chromatographs

The chromatograms displayed below represent the Chromeleon 7 software outputs. For Figure 5, the y-axis is absorbance (mAU), and the x-axis is time (min). For cephazolin, integration from 4 through 6 minutes was performed and the area under the curve, with the baseline manually set at 0, was evaluated. Figure 5 represents the integration peak for cephazolin at concentration of 4 mg/mL.

![Figure 5. Chromatograms for negative control (left) and cephazolin (right) at 4 mg/mL, where the circled peak indicates the integrated peak. In both figures, the y-axis is absorbance at 254 nm (mAU), and the x-axis is time (min).](image)

For ciprofloxacin, fluorescence at 340/450 nm (ex/em) was used. The y-axis represents the fluorescence (in counts), and the x-axis is time (min).

Figure 6 below depicts the chromatograms for the control as well as the peak of interest ciprofloxacin at concentration of 0.125 mg/mL. Again, for detection of ciprofloxacin, fluorescence at 340/450 nm (ex/em) was used. The y-axis represents the fluorescence (in counts), and the x-axis is time (min).

![Figure 6. Chromatograms for negative control (left) and ciprofloxacin (right), where the circled peak indicates the integrated peak. In both figures, the y-axis is fluorescence (counts) at 340/450 nm (ex/em), and the x-axis is time (min).](image)
Because it was known that neomycin does not have a fluorophore or chemical group that gives an absorbance peak at a certain wavelength, typical UV and fluorescent detection methods would not work for this antibiotic. Therefore, derivatization was required for this compound, where a fluorophore is covalently coupled to amino groups on neomycin to achieve fluorescence. For pre-column derivatization, the reaction stoichiometry, and resulting product structure should be known to predict the elution peak and optimize instrument conditions as well as a suitable mobile phase. Similarly, the rate of the reaction should be rapid, and an HPLC method should be developed that allows the resulting reaction product to be separated from the reactants. However, it is not always advantageous to complete a pre-column reaction, due to concern for loss of antibiotics resulting from pre-column derivatization (mass balance on the column), instability of reaction products, or small reaction volume. Nonetheless, due to lack of required equipment (i.e. heaters and reaction coils) for the HPLC instrument, pre-column derivatization was determined to be the best suited for this method development based on available resources.

Figure 7 represents the chromatogram for the negative control and neomycin at 0.25 mg/mL. Again, the y-axis is fluorescence (counts), and the x-axis is time (min).

Figure 7. Chromatograms for negative control (left) and neomycin (right), where the circled peak indicates the integrated peak. In both figures, the y-axis is fluorescence (counts) at 340/450 nm (ex/em), and the x-axis is time (min).
II.IV.II. Standard Curves

From the integrated area under the curve (AUC), a standard curve was produced for each antibiotic (Figures 8-10). Standards for each antibiotic were assessed in triplicate and the error bars indicate standard deviation. For cephazolin, the standard curve was linear from 0 to 4 mg/mL, which provided a sufficient range for detection of unknown concentrations of cephazolin within the samples from keratin hydrogel release experiments (see below).

The same method of determining the standard curve was performed for ciprofloxacin. However, the range of linearity was limited between 0 to 0.25 mg/mL, due to “quenching,” where the detector was overloaded at higher concentrations. Nonetheless, this range was sufficient for the detection of the unknown concentration of ciprofloxacin in the samples (Figure 9).

![Cephazolin Standard Curve](image8.png)

Figure 8. Cephazolin standard curve. For n=3, the range of linearity was between 0-4 mg/mL, and bars indicate standard deviation.

![Ciprofloxacin Standard Curve](image9.png)

Figure 9. Ciprofloxacin standard curve. For n = 3, the range of linearity was between 0-0.25 mg/mL, and bars indicate standard deviation.
Similarly, neomycin exhibited “quenching,” and was confined to a linear detection range from 0 to 1 mg/mL (Figure 10). Dilution of time point samples (1:2 in PBS, before extraction) would be necessary to bring the concentration within the linear detectable range on the HPLC instrument.

![Figure 10. Neomycin standard curve. For n = 3, the range of linearity was between 0 and 1 mg/mL, and bars indicate standard deviation.](image)

To determine the concentration of neomycin and cephazolin in the combination formulations, it was necessary to ensure the contribution of fluorescence from cephazolin and absorbance from neomycin were both minimal. Figures 11 and 12 below describe the standards. Since the slope of both curves was very small and because the values of the AUCs were less than ~ 10% or less of the signal from the other antibiotic of interest in that detection model (e.g., cephazolin at 254 nm), absorbance from neomycin and fluorescence from cephazolin were neglected. It is noted that the error bars for Figures 11 and 12 appear large since the scales of the y-axis are considerably smaller, but the error (noise) is similar to that shown in Figures 8 and 10.
II.IV.III. Antibiotic Release from Keratose Hydrogels

To determine antibiotic release, keratose hydrogels were loaded with each antibiotic or combination of antibiotics and allowed to release their contents into PBS over the course of 1 month. The mass of antibiotic in each sample was determined at each time point by comparison to the standard curves shown above. The analysis was performed in quadruplicate (1 HPLC analysis on release from 4 separate keratin hydrogels; experimental quadruplicate) for each time
point. The data is presented as cumulative release over 4 weeks for each antibiotic formulation. Figures 14 through 17 show the cumulative release profiles, and the error bars indicate standard deviation. For all antibiotic cumulative release profiles, the trend follows a first order release profile.

![Figure 13. Cephazolin cumulative release from 15% w/v KOS gels loaded at 20 mg/mL (cephazolin). Detection for cephazolin was at UV 254 nm, n=4, and bars indicate standard deviation.](image)

For cephazolin, UV detection at 254 nm was used. From Figure 13, it can be shown that for gels made of 80:20 (α:γ) keratose, the release of cephazolin is nearly complete by day 7. However, release was still detectable through 4 weeks and this may have significant biological implications (i.e., values higher than minimum inhibitory concentration for certain types of bacteria), as will be discussed below. Four weeks of data was collected to show that the profile plateaus over time and no more release occurs.

Similarly, the ciprofloxacin cumulative release profile was measured through four weeks by using fluorescence detection with the HPLC method described above. As shown in Figure 14 through four weeks ciprofloxacin release reached nearly 95%. The other 5% could be accounted for in experimental error, left in the part of the gel that did not degrade, or a small amount of ciprofloxacin may be separating to the chloroform layer while non-covalently bound to keratose left behind in the extraction process. Nonetheless, from the data it is clear that nearly all of the loaded drug is released by 7 days and reaches a plateau at approximately 14 days. Again, ciprofloxacin was detectable through all four weeks.
Based on preliminary studies, it was apparent that neomycin concentrations in the time point samples might exceed the linear range of the standard curve. Therefore, the neomycin and neomycin in the combination samples (neomycin with cephazolin) up through 6 days were diluted 1:2 before being subjected to HPLC analysis in order to bring their concentrations into the linear range.

Figure 15 demonstrates the cumulative release of neomycin over 4 weeks as less than 20%, which is much lower release than observed above for cipro and cephazolin from keratose hydrogels. Visual observation suggested that the reduced release of neomycin was because these gels remained intact longer than the other antibiotic gels. Therefore, the rate of protein degradation was explored to see if this visual observation could be confirmed quantitatively. As with cephazolin and ciprofloxacin, neomycin was detectable in the time point samples through all four weeks.

Figure 14. Ciprofloxacin cumulative release from 15% w/v KOS (80:20 α:γ) gels loaded at 2 mg/mL (ciprofloxacin). Detection for ciprofloxacin was fluorescence at 340/450 nm (ex/em), n=4, and bars indicate standard deviation.
Similar to the methods used for quantifying neomycin individually, the release of neomycin in the combination gels (with cephazolin) was determined through OPA derivatization by using fluorescence at 340/450 nm (ex/em) (for neomycin) and UV at 245 nm (for cephazolin). However, the signal to noise ratio was low (<2.5) after 7 days, and neither cephazolin nor neomycin was detectable after this time. At 7 days, the release of neomycin reached only 30% and cephazolin was approximately 55% (Figure 16). For cephazolin, this is half of the release the drug exhibits when loaded individually (Figure 13). However, for neomycin, the release increased slightly. This may be due to the interaction between cephazolin and neomycin within the keratose hydrogel. Again, a protein assay was used to quantify the rate of gel degradation, and therefore help explain the difference in release between combination (of neomycin and cephazolin) gel formulations and those of the individual drugs (see below).
As noted above, the release profiles of neomycin and combination were lower than ciprofloxacin and cephazolin. Visually, the latter gels seemed to be nearly fully degraded by 2 weeks, whereas the gels containing neomycin remained largely intact. Protein assays were performed on all of the time point samples to quantitatively describe the correlation between gel degradation and antibiotic release. For both cephazolin and ciprofloxacin, the gel degradation profiles for antibiotic loaded gels overlaps the degradation for unloaded (control) (Figures 17 and 18). A t-test was performed for each time point, and it was found that there was no significant difference (p>0.05) for any time up to 4 weeks between degradation of gels containing cephazolin and those that did not contain an antibiotic (control). This indicates that the incorporation of cephazolin does not have a significant impact on the rate of gel degradation. However, it should be mentioned that the protein assay only detects soluble protein. In Figures 17 and 18, the rate of gel degradation may be underestimated due to the inability of the assay to quantify insoluble proteins (pieces of undissolved keratin gels).

**Figure 16.** Combination cumulative release from 15% w/v KOS gels (80:20 α:γ) loaded at 20 mg/mL neomycin and 20 mg/mL cephazolin. Detection for neomycin was possible through OPA derivatization using fluorescence at 340/450 nm (ex/em) and cephazolin detection was UV at 254 nm. n=4, and bars indicate standard deviation.
Similar to cephazolin, the rate of degradation of ciprofloxacin gels was not significantly different for any time point up to 4 weeks, as determined by a t-test (p>0.05), than those that did not contain an antibiotic (unloaded). The profiles almost completely overlap over the entire 4 weeks (Figure 18), with the ciprofloxacin curve directly underneath the unloaded, again indicating that the presence of ciprofloxacin had no impact on rate of gel degradation. As with cephazolin, however, the protein degradation may be underestimated due to insoluble protein.

Figure 17. Keratin degradation for gels with cephazolin (at 20 mg/mL) and without antibiotic (control) from 15% w/v KOS (80:20 α:γ) gels. Detection was through DC protein assay with absorbance at 750 nm, n=4, bars indicate standard deviation.

Figure 18. Keratin degradation for gels with ciprofloxacin (at 2 mg/mL) and without antibiotic (unloaded) from 15% w/v KOS (80:20 α:γ) gels. Detection was through DC protein assay with absorbance at 750 nm, n=4, bars indicate standard deviation.
Unlike ciprofloxacin and cephazolin, neomycin gels degraded more slowly than the negative control (gel only, no antibiotic). As determined by a t-test, by day 4 the neomycin samples were significantly different (p<0.05) than unloaded samples (Figure 19). This relationship is interesting to note and may help explain the cumulative release of neomycin only reaching 20%. Since the gels did not fall apart, neomycin may have remained in the KOS gel, and not eluted into the PBS solution (withdrawn for each time point).

Figure 19. Keratin degradation for gels with neomycin (at 20 mg/mL) and without antibiotic (unloaded) from 15% w/v KOS (80:20 α:γ) gels. Detection through DC protein assay with absorbance at 750 nm, n=4, bars indicate standard deviation.

Similar to neomycin alone, the combination gels degraded more slowly than the control (Figure 20). By 6 hours, the gels were significantly different (determined by a t-test with p<0.026 at 6 hours) for combination than the control samples. Again, this relationship explains the lower release of combination gels (as compared with cephazolin and ciprofloxacin) as the neomycin must have some interaction with the KOS, which helps the gel to remain intact.
II.V. Discussion of Results

As noted before, the ability of the keratose gel to degrade as wound tissue begins to regenerate and heal is potentially important to wound healing applications. The antibiotics will exhibit sustained release from the keratin hydrogel so as to inhibit bacterial growth for as long as possible. As the skin begins to heal, the natural infection barrier will take over and infection will be less likely to occur. As the keratose gel degrades, simultaneous release of antibiotic from the hydrogel would help to fight bacterial infection, thereby promoting wound healing. Previously the results of the release of each antibiotic (Figures 13-16) as well as the rate of gel degradation (Figures 17-20, as determined by protein assay) were shown (Sections II.IV.III and II.IV.IV). Figures 21 through 24 represent this data together on one figure (for each individual antibiotic or the combination formulation) to further demonstrate and explain these results.

Figure 21 shows that the relationship between cephazolin cumulative release and keratose 80:20 gel degradation is correlated in that as the gel degrades over time, more drug is released.

Figure 20. Keratin degradation for gels with combination (neomycin and cephazolin each at 20 mg/mL) and without antibiotic (control) from 15% w/v KOS (80:20 α:γ) gels. Detection was through DC protein assay with absorbance at 750 nm, n=4, bars indicate standard deviation.
Similarly, ciprofloxacin also shows a correlation between the release of the antibiotic and the rate of gel degradation (Figure 22).
In Figure 23, the correlation between neomycin release and gel degradation was less than ciprofloxacin and cephazolin. In this case, the correlation (as determined by Excel function) was 0.95 (compared with 0.99 for the other antibiotics). However, the most interesting note, as shown previously, is that the neomycin gel degrades more slowly (plateauing at ~35%) than its counterparts (plateau ~40%), and also releases considerably less (max release ~20%). The gel remaining intact longer may explain the lower release (Figure 23). To better characterize and further understand this relationship, more experiments would be necessary (discussion in section III.1.II).

![Figure 23. Cumulative release of neomycin and keratose from 15% w/v KOS (80:20 α:γ) gels over 4 weeks. Neomycin detection was through using HPLC methods, previously determined, and fluorescence at 340/450 nm (ex/em). Keratose gel degradation is reported as cumulative (%) release, and was determined through protein assays. Bars indicate standard deviation, and for all tests, n = 4.](image)

Similar to neomycin, the combination (of neomycin and cephazolin) release increased as the keratose gel degraded (Figure 25). Again, the rate of gel degradation plateaued at ~35%, and further experiments would be necessary to specifically determine the effect the interaction between neomycin and the gel has upon the degradation (discussion in section III.1.II).
Figure 24. Cumulative release of combination (neomycin and cephazolin) and keratose from 15% w/v KOS (80:20 α:γ) gels over 4 weeks. Detection of each antibiotic was through using HPLC methods, previously determined, and fluorescence at 340/450 nm (ex/em) (for neomycin) and UV at 254 nm (for cephazolin). Keratose gel degradation is reported as cumulative (%) release, and was determined through protein assays. Bars indicate standard deviation, and for all tests, n = 4.
II. VI. Chapter II References


Chapter III – Discussion and Future Directions

III.I. Discussion

Keratin hydrogels demonstrate several attributes that make them desirable for burn wound applications, including ability to promote vascularization\(^1\), elicit minimal foreign body response\(^2\), and support cellular ingrowth\(^3\). The experiments in this thesis have confirmed previous reports that antibiotic-loaded keratin hydrogels promote sustained release in a manner correlated with their degradation. Through the development of HPLC methods for the detection of antibiotics in the presence of keratin proteins, antibiotic release from keratin hydrogels was quantified for three specific antibiotics (cephazolin, ciprofloxacin, and neomycin) as well as a combination of two of these (cephazolin and neomycin). The results of these studies helped to validate previous antibiotic release studies by more standardized and widely accepted techniques. Along with pre-clinical animal testing, these data may be acceptable for regulatory approval processes aimed at future human clinical application of these materials.

III.I.I HPLC Method Development

The specific objective of this thesis was to assess the release of antibiotics from keratin hydrogels. To quantify the concentration at each time point (therefore quantifying the release) by the widely accepted practice of HPLC, it was necessary to develop the HPLC techniques for detection of each antibiotic either individually or in combination. As described in Chapter II, it is important to note that for all samples, an extraction was performed to remove keratin from the sample, therefore reducing potential interference and background signal from the keratin. Below we discuss the method development for each antibiotic formulation.

Cephazolin

HPLC methods developed for cephazolin were relatively simple and did not require much optimization in order to achieve a chromatograph with sufficient resolution and signal intensity. The peak of interest for cephazolin was repeatable for all concentrations, the standard curve was linear over the range of 0 to 4 mg/mL, and the release profile exhibited error bars within 10% of the average. Overall, the methods (described in Chapter II) were found to be suitable for the detection of cephazolin from keratin hydrogels.

Ciprofloxacin

The method for ciprofloxacin was originally developed from literature review, where a similar apparatus, and column were used\(^4\). From literature, a mobile phase of 86% of 2% acetic
acid with 14% acetonitrile was used isocratically for the duration of a 15-minute run. However, HPLC trials utilizing this method exhibited an issue of “floating retention time” (see Appendix, Figure 3). In these isocratic trials, the elution time of ciprofloxacin from the HPLC column varied from 5 to 8 minutes with different concentrations of ciprofloxacin. Similarly, the peak shape was very broad and did not allow for a good way to integrate the area under the curve that could be standardized. When a standard curve was produced by using this method, the correlation coefficient was only 0.95. We were unable to determine a clear reason for the cause of this change in retention time. In an effort to overcome this unexpected and unexplained phenomenon of the floating retention time and to increase the sharpness and resolution of the peak curve, an increase in acetonitrile was considered as a step function.

At 4 minutes post-injection, the acetonitrile content in the mobile phase was increased from 14% to 30%, which increased the hydrophobicity of the mobile phase by increasing the wettability of the C18 column and reducing the aqueous content. By increasing the acetonitrile content, ciprofloxacin was better eluted from the column and exhibited sharper, more reproducible peaks. Production of a standard curve this step change produced a correlation coefficient of 0.99. Also, the “floating retention” issue was reduced from 3 minutes to less than 30 seconds, although the exact reason for the “floating retention” time was still not fully elucidated. The results of the chromatographs were more repeatable and reproducible with the step function method developed and shown in Chapter 2. Thus, the use of the step function was beneficial to help more accurately quantify the release of ciprofloxacin in keratin hydrogels.

**Neomycin and Derivatization Methods**

Neomycin has no inherent chromophores that could be detected with our DAD or fluorescence detectors. We therefore needed to develop a derivatization technique and preferred to use a pre-column derivatization. Again, it is important to note for all time samples, a chloroform/methanol extraction was performed to remove keratin. Particularly important for detection of neomycin, the selected functional groups to be derivatized were the primary amines (there are 6 such primary amines neomycin’s structure). Keratin as a protein contains numerous amine groups and would, consequently, also be derivatized if not removed by extraction. Despite the removal of keratin, the low signal-to-noise ratio of the OPA and neomycin reaction product on the HPLC (~2) was identified as a concern for the OPA derivatization technique. It would be ideal to improve the ratio (to at least 3), and one approach would be to consider other derivatizing agents. To achieve the attachment of a fluorophore to neomycin, several other pre-
column derivatization techniques were explored. Our first attempt to derivatize neomycin was through the use of ninhydrin. However, ninhydrin coupling requires a very high reaction temperature, which was not supported by our HPLC instrument. The reaction would not proceed even using a plate reader heated to 40°C. Consequently, another derivatization method using fluorescamine was investigated. The reaction of fluorescamine with the primary amine on neomycin yielded a fluorescent product, but the signal to noise ratio was very poor even at high concentrations of neomycin, which thus provided lower limits of detection (poor sensitivity). For this reason, the fluorescamine method was not considered further during the completion of this thesis work. However, future work could be focused on further optimizing these methods in an effort to increase the signal-to-noise ratio. In consideration of the timeline of this thesis project, method development for OPA was pursued as the HPLC equipment was sufficient for the reaction temperature, and the ratio of background to signal was acceptable.

In initial attempts to develop the derivatization of neomycin with OPA, mercaptoethanol was used as the thiol group in the coupling reaction, but resulted in a loss of signal over time (the fluorescence intensity decreased as a function of time). We therefore elected to use 3-mercaptopropionic acid, which has been suggested to add stability of fluorescent derivatization products compared to mercaptoethanol. An equimolar amount of neomycin and 3-mercaptopropionic acid were used. Under the same reaction conditions, our results confirmed that 3-mercaptopropionic acid provided stability to the reaction product, increased the signal-to-noise ratio, and increased the detectable range of neomycin (Appendix, Figure 4) compared to use of mercaptoethanol at a 1:1 ratio (with neomycin). As a result, 3-mercaptopropionic acid was used in all subsequent methods to better enhance detection of neomycin for all time samples.

**Combination**

The same method that was used for neomycin was also used for quantification of neomycin and cephazolin (within the combination formulation). Again, to reiterate, the structure of cephazolin does not have any primary amines, which allowed for successful pre-column derivatization of neomycin without interference or cross reactivity (from cephazolin). Although the signal-to-noise ratio of OPA derivatization was low, detection of cephazolin and neomycin from the same sample was achieved by using fluorescence (for neomycin) and UV (for cephazolin) where the diode array and fluorescence detectors were run in series. Overall, the method developed for quantifying these antibiotics was effective and suitable for the work established in this thesis.
III.I.II Antibiotic Release Profiles

As described in Chapter II, keratose hydrogels were shown to support sustained release of cephazolin, ciprofloxacin, neomycin, and the combination (of neomycin and cephazolin) up to 4 weeks. Continued release (specifically of these antibiotics from the keratose hydrogels) is important as this would help achieve less burdensome wound management for burn victims by reducing the number of bandage changes.

Again, as tissue begins to regenerate, it is important for the antibiotic loaded gel to help fight infection as well as degrade at a proportional rate. For all antibiotic formulations, the antibiotic release profile is correlated with the rate of gel degradation (Chapter II, Figures 21 through 24), with a correlation of 0.94 or better. Specifically for cephazolin and ciprofloxacin, the antibiotic release reaches approximately 100%, and the gel degrades nearly 40%. Again, it should be noted that the protein degradation is most likely underestimated, since only soluble protein is quantified, and visual inspection would suggest degradation closer to 80%.

Nonetheless, this result would suggest that diffusion must play some role in the mechanism of release to account for the difference between gel degradation, and ciprofloxacin or cephazolin release. The contribution of diffusion (rather than interaction between the antibiotic and keratin, discussed in the next paragraph) would seem to be significant given the differences in release (~40% for the keratose gel vs. ~100% for ciprofloxacin and cephazolin). It is possible, however, that the diffusion does not play as large a role as it appears. By visual observation, over the course of 4 weeks, we observed keratose gels (unloaded or loaded with cephazolin or ciprofloxacin) to be nearly fully degraded. The protein assay does not indicate this, and the reason for the difference in our quantitative result versus visual observation could be that the protein assay detects only soluble protein. We have observed larger pieces of gel come off during our experiments due to mechanical effects of adding/removing PBS for the release experiments. These pieces of gel would not be quantified by the modified-Lowry protein assay, and could account for the difference from what we observed. Thus, while diffusion may play a role in the release, it is not clear to what extent this contributes based on this current study. This HPLC approach with extraction, however, is useful in showing that nearly 100% of cephazolin and ciprofloxacin release can be detected. This is an improvement over our previous reports in which only ~60% release was observed\textsuperscript{6,7}, although we cannot discount the possibility of
differences in the keratin compositions due to differences in keratin extraction techniques between these studies.

In regards to the mechanism of action it should also be noted that, in an unpublished thesis work completed by our group, ciprofloxacin exhibited a different release profile from keratose than from kerateine gels. This may be due to the differences in their rates of gel (keratin) degradation (determined by protein assay), though this could also be attributed to differences in the way that ciprofloxacin interacts with keratose compared to kerateine. In either case, this suggests the possibility of some binding affinity between the antibiotic and keratins (keratose or kerateine) that leads to control over the release by manipulation of the gel rate of degradation. As such, we are unable to draw a definitive conclusion regarding the exact mechanism of release at this time. Nonetheless, despite the limited window of understanding about the mechanism, the results from this thesis did confirm previous release studies showing sustained release of cephazolin and also ciprofloxacin from keratose hydrogels.

Unlike the other antibiotics, the neomycin cumulative release profile exhibited a slower release profile over 7 days. Using OPA derivatization methods to attach a fluorophore to the primary amines in neomycin, the product of the reaction was detected by fluorescence. However, even after 7 days, the gel only released less than 20% of the initial neomycin mass. Visual inspection of the negative control and neomycin gels during sample collection demonstrated that the neomycin gels remained intact longer. Over time, the unloaded gels degraded and broke into small fragments. However, at the same time, neomycin gels did not appear to degrade as quickly as unloaded, ciprofloxacin-loaded, or cephazolin-loaded gels. We have identified two possible explanations for this observed behavior. First, the neomycin could interact via electrostatic interactions with keratin (e.g., between positively charged amines on neomycin and negatively charged sulfonic acid residues on keratose). It is also possible that neomycin may facilitate increased levels of hydrophobic interactions in the keratin gels by affecting keratin’s conformation within the gel. The change in conformation may increase gel stability, thereby slowing the rate of degradation as well as the release from the gel. Again, this trend is demonstrated in Figure 15.

To further understand the mechanism of release, several experiments could be conducted. First, a solid-phase assay would provide more insight into the possibility of interaction between neomycin (or cephazolin or ciprofloxacin) and keratose (or kerateine). Additionally, changing
the salt concentrations or the pH of aqueous buffers (in this thesis, PBS) used for release studies could be used to explore the role of electrostatic interactions. Lastly, urea could be used to disrupt hydrophobic interactions to determine the effects of these interactions on gel stability and antibiotic (especially neomycin) release. Each of these proposed experiments would provide insight for the interaction between the antibiotic and keratin, therefore helping to explain the gel degradation and antibiotic cumulative release (from the gel).

Similarly, in the combination (neomycin and cephazolin together) gels, neomycin exhibited nearly two times more release than when delivered alone (with no cephazolin present) and cephazolin exhibited half the release than when present alone (with no neomycin present). It is possible that the presence of cephazolin reduces the binding affinity between neomycin and the gel due to changes in hydrophobicity of the gel or that cephazolin affects the strength of any electrostatic interactions between neomycin and keratose that to gel stability (as discussed in the previous paragraph). Regardless of the mechanism, the cumulative release (Figure 15) and gel degradation (Figure 21) for neomycin and keratose gels loaded with neomycin were both considerably lower than expected based upon results from cephazolin and ciprofloxacin. It seems apparent that the physiochemical properties of the antibiotics themselves have significant effects on the gel stability and release rates and that these effects become more manifest in the presence of relatively high concentrations of these antibiotics.

III.II.III. Role of Sustained Release in Bacterial Inhibition

Over time, the sustained release may be sufficient to reduce the likelihood of infection, with consideration given to the antibiotic’s bioactivity over time, as a measure to determine its effectiveness against infection. Previously, other detection techniques (i.e., fluorescence determined by platereader for ciprofloxacin, ELISA for neomycin, and absorbance determined by platereader for cephazolin) seemed to indicate the point at which these drugs would fail in a broth inhibition assay based upon the published MICs for each antibiotic compared to several common bacterial strains including S. aureus and P. aeruginosa. One advantage of using HPLC methods (Chapter II) would be to determine if any additional information could be provided concerning the amount of drug present and how this correlates to the level of bioactivity over time. For example, this thesis work has shown that nearly 100% of cephazolin and ciprofloxacin were released from keratose hydrogels, which is greater than we have previously quantified. This may be due to underestimation in our previous studies that has now been improved by
extraction of the antibiotic from the keratin and the use of HPLC as the detection technique. We note that there were several differences from this thesis work compared with our previous studies, including use of bleached keratose (a proprietary formulation with potentially different properties than unbleached keratose, which was used in previous studies) as well as different gel volumes/geometries (400 µL compared with 1 mL). Despite these differences, from the concentration of antibiotic (for each timepoint as determined by HPLC methods), a simple calculation was performed to directly compare the time at which the release was previously shown to drop below the MIC and the time this thesis study would drop below. Extrapolation of the data to a larger gel (1 mL in broth inhibition assays compared 400 µL used in this thesis) with more volume (10 mL compared with the 0.6 mL used in the release study) yielded the concentration of each antibiotic for every timepoint. For cephazolin, ciprofloxacin, and combination (of neomycin and cephazolin), the concentration remained above the MIC for the entire 4 weeks. Previous studies reported by our group showed that the antibiotics would fail sooner than at 4 weeks. The differences noted above could be one reason for these differences, but another possibility is that the delivered antibiotics are not fully bioactive or bioavailable. To fully explain the results, it may be necessary to conduct broth inhibition assays under the same conditions (e.g., keratin formulation and, more importantly, gel mass and geometry) to directly quantify at what time the antibiotic release sample would cross below the MIC (Chapter II, Table 4 for each antibiotic). Regardless, for acute bacterial treatment, up to 7 days can be critical to ensure wound healing is without infection. This study suggests keratin hydrogels can sustain release of antibiotics over time, while remaining above their individual MIC’s.

III.I.IV Broader Impacts

Ciprofloxacin, is a 2nd/3rd line broad spectrum antibiotic that is effective against Gram-positive and Gram-negative bacteria. Using this drug as the first approach to fight infection may leave fewer alternatives in the event this quinolone is ineffective or that antibiotic resistance develops. This thesis work assessed release profiles in order to establish the suitability of using this combinatorial antibiotic approach (i.e. cephazolin and neomycin together, both 1st line drugs) as an alternative to ciprofloxacin. In this case, this combination of antibiotics should be effective against Gram-positive (cephazolin) and Gram-negative (neomycin) bacteria, such as Staphylococcus aureus and Pseudomonas aeruginosa, but may do so as efficiently or successfully as ciprofloxacin. The advantage of the combinatorial approach is the ability to use
more 1st line drugs, thus providing opportunity to use other antibiotics such as amoxicillin (moderate-spectrum aminopenicillin with ability to treat both Gram-positive or Gram-negative strains) or metronidazole (a nitroimidazole used to fight anaerobic bacteria and protozoa) as a secondary approach to fight infection. A benefit of the HPLC methods developed in this study is the ability to apply the general techniques (if not the exact HPLC methods) to quantification of other antibiotics (beyond those studied in this thesis work). As new classes of antibiotics are discovered or used in biomedical applications, the same general methods shown in this study could be utilized depending on the physiochemical properties of the drugs. Overall, the techniques and methods used in this study showed the ability to detect multiple types of drugs used in combinations that may ultimately be used to fight multiple bacterial strains/types.

Relevance of this work may be used to address other biomedical needs besides wound care for skin burns. Since the keratin hydrogel is both “flowable” and relatively viscous, the antibiotic loaded keratin hydrogel may be applied topically for skin injury dressings, which is the primary focus of this research project. However, the findings of this work may be applicable to other areas of tissue engineering and regenerative medicine. For example high-energy fractures such as those in automobile accidents or from warfare may lead to multi-tissue injuries that affect not only skin but also, muscle, soft tissue, and bone. In these scenarios, tissue-engineering strategies seek to promote tissue regeneration. However, infections are also common in these injuries and can affect all injured tissues, not just skin. In one treatment method, osteomyelitis (or bone infection) in a bone fracture is treated with gentamycin loaded into polymethylmethacrylate (pMMA) beads, or so-called “beads on a string”\textsuperscript{11}. While this method prevented bone infection, the drawback is that these beads later need to be surgically removed and pMMA itself does not promote bone regeneration. In skin applications, as discussed in Chapter I, auto- and allografts promote healing, but do not have the technology for antibiotic incorporation. Keratin, on the other hand, has been used to promote bone regeneration through delivery of BMP-2 and, in an unpublished study by our group, has been used for cell delivery strategies that may ultimately be used to treat volumetric muscle loss injuries or other injuries that require cell-mediated approaches \textsuperscript{8}. In summary, keratin can be used to bridge the gap by providing favorable cell response for tissue regeneration while promoting bacterial resistance. Even further, in using a flowable keratin biomaterial that could be directly applied to wounds, local delivery of antibiotic (as opposed to systemic) could decrease the incidence of antibiotic
resistance while lowering the dosage of the antibiotic or antibiotics. Therefore, it is foreseeable that these types of tissue-engineering strategies (growth factor and cell delivery, as well as local delivery) using keratin biomaterials could be supplemented with sustained antibiotic release for new or existing antibiotics as an additional design component.

The work established in this study developed HPLC methods to characterize the release of three antibiotics from keratin biomaterials, specifically those made with a specific ratio α:γ-keratose (80:20). Again, the α- component of KOS provides the structural, helical component of the formulation, where γ- fractions of KOS are short, globular structures that act as a glue (holding together the gel). The results from this thesis work, using HPLC techniques, helped to validate previous (“non-standard”) methods, providing a more complete picture of antibiotic release from these keratin hydrogels. Also, the use of HPLC was justified as the outcome of this study showed that the methods produced more repeatable and reliable results as a consequence of using this approach. While this work was successful in many ways, there is still potential for optimization of methods or future development (discussed below).

III.II. Future Directions

III.II.1 Derivatization Techniques

The OPA derivatization method for aminoglycosides (neomycin and also streptomycin, which was not previously discussed in this thesis) was not without complications. Detection of streptomycin (an aminoglycoside with MW ~581 Da, and no inherent fluorophore to allow for detection) was unsuccessful, as the reaction with OPA did not yield a favorable signal-to-noise ratio for this antibiotic. To overcome the issues with OPA-derivatization of aminoglycosides, several possibilities could be explored. One potential approach would be use of another derivatization agent, which may increase the signal-to-noise ratio. Another possibility would be further development of the extraction technique, used to remove antibiotics from the solutions containing keratin, in order to enhance fractional recovery of the antibiotic. Even further, other opportunities would be present in optimization of the OPA reaction with neomycin (or other aminoglycosides or amine-containing antibiotics) by installation of more HPLC instrument equipment for a post-column derivatization method.

Post-column derivatization techniques offer several advantages in developing methods suitable for the quantification of antibiotics from keratin hydrogels. In a post-column method, the stability of the reaction product is less of a concern, as the amount of time the sample takes to
travel from the column to the detector is minimal (important to maintain strong fluorescent intensity). Specifically since we are only interested in quantifying the concentration of antibiotic in the sample, post-column derivatization would provide a response characteristic of all the OPA reaction products (as opposed to pre-column, where separate peaks are supposed to be different degradation products). In the methods established in Chapter II, an extraction (methanol, water, and chloroform) was necessary for OPA-derivation since OPA reacts with primary amines and keratin (a protein) has numerous reaction sites. A post-column derivatization may open the possibility of removing the extraction step by putting samples directly onto the column. The column (C18 or another type such as a size exclusion or ion exchange column) could be used to separate keratin from antibiotic, which would then be compatible with OPA derivation. Also, using post-column equipment would provide additional heating components (to increase temperature beyond 50°C), potentially providing opportunity to revisit the fluorescamine or ninhydrin derivatizations. In either case of using another derivatization agent or post-column techniques, there is opportunity to develop better methods for quantification of neomycin and other drugs that do not contain a fluorophore (i.e. streptomycin).

III.II.11 Tunability of Keratin Hydrogels

Traumatic injuries that create volumetric loss of tissue (skin, muscle, bone) require considerable time to heal, and thus may require the release of antibiotics to last the duration of the healing period. In a hydrolytic environment, characteristic of wound healing, the hydrogel should exhibit controlled degradation at a rate inversely proportional to the rate of tissue regeneration. To control degradation, gel formulations may be “tuned” for different degradation profiles to meet different healing time requirements depending on the tissue, size, and severity of the injury. For example, using different formulations of α (alpha) and γ (gamma) keratins will alter the physiochemical (and rheological) properties of the gel. γ- keratins are short, less structurally sound keratins that have a globular structure. Conversely, α-keratins form in an alpha-helical formation and provide more stability in the gel than γ- keratins. By using these physical characteristics, different gel degradations and release profiles may be achieved with different α:γ keratin ratios.

As an initial example of this approach, we have conducted studies with 95:5 (α:γ) KOS and assessed antibiotic release profiles. These 95:5 formulations KOS consistently showed lower cumulative release for cephazolin, ciprofloxacin, neomycin, and combination (of
neomycin and cephazolin) than similar formulations of 80:20 (α:γ) KOS. This is most likely due to the increased alpha content providing additional hydrogel stability (decreased degradation rate). For example, the cumulative release of cephazolin from 95:5 (α:γ) KOS gels reached a plateau at only 70% (compared with 100% for 80:20 (α:γ) KOS) (Appendix, Figure 6). Similarly, for ciprofloxacin, the release from the 95:5 (α:γ) KOS was nearly 10% less (85% for 95:5 compared with 95% for 80:20) (Appendix, Figure 7). A t-test performed at each time point on for each antibiotic revealed that the cumulative release from 95:5 (α:γ) KOS was statistically lower than release from 80:20 (α:γ) KOS (p<0.05) (Appendix, Figures 6-9). Protein assays have not yet been performed on 95:5 (α:γ) KOS to determine if the gel degradation is different, but visual observation indicated that the 95:5 gels seemed to remain intact longer than 80:20 formulations. Nonetheless, based upon the cumulative release profiles the higher content of α-KOS (in 95:5) in the gels seems to slow the release of the antibiotics tested. Using different ratios of α to γ KOS would potentially provide tunability for long term wound healing by controlling the release from the hydrogel.

Outside of tuning gels based solely upon the α to γ KOS ratio, other keratin extracts also provide opportunity to change the physiochemical properties of the gel. As previously noted (Section I.III.II), keratoses are generally hygroscopic and water-soluble, making them advantageous for use in burn wounds. However, the rate of degradation of keratose is relatively rapid when compared with kerateines (reductively extracted keratins) due to the presence of disulfide crosslinking (in kerateines). Our group has explored alkylation of kerateine hydrogels as a means to tune the gel degradation profile, which may be valuable for slower healing wounds (i.e. slower hydrogel degradation for extensive tissue damage that may require extended time to heal). In this alkylation approach, free thiol groups on cysteine residues present in kerateine (as opposed to sulfonic acid groups present on cysteine residues of keratose) are chemically modified by using iodoacetamide, which have been shown to affect the rate of gel degradation. Since keratin contains a cysteine residue content (more than other proteins such as collagen), it can be tuned to a specific degradation rate by modification of increasing numbers of cysteine residues. Gels made from various ratios of modified kerateine exhibited slower degradation than those made from keratose alone. Clearly, changes in the physiochemical properties of the keratin (by alkylation) will have effects on the gels themselves as well as their interaction with antibiotics or other drugs (see discussion above regarding mechanism of drug
release). Again, a slower rate of degradation may be advantageous for extensive wound healing, where more time is required for tissue regeneration. Regardless of the method (alkylation or changing α:γ ratios), tunability of keratin hydrogels by manipulation of the inherent properties of keratin (namely, cysteine content) provides a unique advantage in providing flexibility in material properties and behavior compared to other naturally derived biomaterials such as collagen.

**III.III. Continuing Research**

This thesis supports other work that has shown sustained release and bioactivity\(^7\) as well as effect in rodent (mouse) models\(^6\). As noted above, these studies play a role in assessing whether keratin hydrogels may be useful for clinical applications. In order to reach clinical translation, definitive pre-clinical studies must be performed. This thesis work should provide support to identify proper keratin-antibiotic formulations for large (pig) animal studies that involve excisional or burn wounds. The results shown in this study demonstrate the important role that quantification of release kinetics and methods to determine them play in properly formulating these materials for antibiotic delivery and, more generally, to the delivery of other therapeutic agents.
III.III. Chapter III References


Appendix

A. I. Understanding the Reaction Between OPA and Primary Amines

The reaction between OPA and neomycin was neither straightforward nor easy to predict as the presence of several amine groups (in the structure of neomycin) provided a possibility of multiple reaction products, and consequently resulted in multiple HPLC peaks. Trying to isolate one peak on the HPLC was difficult, and so the area under a collective three peaks was used for integration and in determination of the concentration present in each sample. To help understand the reaction between OPA and primary amines, especially with an aminoglycoside, electrospray ionization mass-spectrometry (ESI-MS) tests were conducted. Specifically, the structure of streptomycin (an aminoglycoside with MW ~581 Da) only has four primary amines (compared with five in neomycin), but has a similar molecular weight, and assumed mechanism (as neomycin) when reacted with OPA. In theory, ESI-MS would help determine the number of OPA’s bound to streptomycin.

In these tests, streptomycin was prepared at 0.01 M by mixing 29 mg of streptomycin with 5 mL of PBS. OPA was prepared in methods previously established (Section II.III.I) and allowed to react with streptomycin for 120 seconds by adding 150 μL of streptomycin and 50 μL of OPA. This reaction solution was then submitted to the ESI-MS. Figure 1 below describes the results.

![Streptomycin ESI-MS](image1)

![OPA ESI-MS](image2)

![Streptomycin and OPA ESI-MS](image3)

Figure 1. ESI-MS for streptomycin (top), OPA (middle), and the reaction product of streptomycin and OPA (bottom).
Unfortunately, no peaks at the expected addition of 1, 2, or 3 OPA’s to streptomycin were detected. This could be due to lack of optimization of ESI-MS parameters, or to the low ratio of reaction product. Consequently, ESI-MS tests were inconclusive and further experiments would be necessary to identify the reaction chemistry.

Similarly, MALDI tests were conducted on the streptomycin and OPA reaction products and results were published in negative mode (Figure 2). Again, the test was inconclusive. Initial results showed peaks at the suggested masses for addition of 1, 2, and 3 OPA’s. However, it was suggested that the machine was not clean and these were actually residual peaks from previous trials. Again, further experiments would be necessary to determine any specifics about the interaction between streptomycin and OPA.

![Streptomycin and OPA MALDI](image)

Figure 2. MALDI spectrum (in negative mode) for the reaction between streptomycin and OPA. The largest peak at 581 is attributed to the signal from streptomycin. Unfortunately, no detectable peaks are present where the addition of 1, 2, or 3 OPA’s would be expected.

**A. II. “Floating Retention Time” Exhibited by Ciprofloxacin**

As noted in Section III.I.I, ciprofloxacin exhibited a “floating retention time” when analyzed using an isocratic mobile phase (86% of 2% acetic acid and 14% acetonitrile). Figure 3
below shows the broad peaks, which span over 15 minutes. The retention time for each trial varied from 5.1 minutes (for 0.125 mg/mL cipro) to 7.3 minutes (for 0.007 mg/mL cipro).

![Graph showing retention time for different concentrations of ciprofloxacin.](image)

Figure 3. “Floating retention time” exhibited for ciprofloxacin at mobile phase composition of 86% of 2% acetic acid and 14% acetonitrile (isocratic). The y-axis is fluorescence (counts) and the x-axis is time (min) for all concentrations of ciprofloxacin. For all samples, injection volume was 10 µL, the mobile phase flow rate was 0.5 mL/min, and concentrations ranged from 0.007-0.125 mg/mL. The retention time varied from 5.1 minutes (for 0.125 mg/mL cipro) to 7.3 minutes (for 0.007 mg/mL cipro).

The issue of “floating retention time” was not previously published in literature, and no exact conclusion as to the cause has been determined. However, as noted in Section II.III.III, a step change in the mobile phase composition from 14% to 30% acetonitrile at 4 minutes helped sharpen the ciprofloxacin peak as well as reduce the variability in retention time.

**A. III. Effect of Thiol Group**

Exchanging the mercaptoethanol thiol group for 3-mercaptopropionic in the OPA reagent increased the fluorescence intensity for products of the reaction between neomycin (at 0.125 mg/mL) and OPA. Over 30 minutes the fluorescence intensity was higher and more stable for OPA prepared with 3-mercaptopropionic acid than for OPA with mercaptoethanol (Figure 4). Results from this experiment were encouraging for the use of 3-mercaptopropionic acid in the
derivatization technique described above (Section II.III.II) to increase the signal-to-noise ratio, and therefore increase the detection limits for neomycin.

Figure 4. Effect of thiol group (mercaptoethanol v. 3 mercaptopropionic acid) on fluorescence intensity of reaction between neomycin at 0.125 mg/mL and OPA. For this experiment, n=3, and 100 uL of neomycin was reacted with 100 uL of OPA. The y-axis is fluorescence (RFU) at 340/450 nm(ex/em), as determined by the plate reader at sensitivity = 50, and the x-axis is time (min).
A. IV. Effect of the Ratio of Volume of OPA to Neomycin

Similarly, it was necessary to determine the volume of neomycin that would yield the best signal-to-noise ratio when reacted with OPA. Figure 5 (below) shows that for a reaction of 150 uL of neomycin and 50 uL of OPA (3:1 molar ratio of neomycin to OPA), the fluorescence intensity was higher than 100 uL of neo and 100 uL of OPA (1:1 ratio). Again, these results were encouraging and supported use of a 3:1 molar ratio (of neomycin to OPA) in all OPA derivatization techniques.

![Figure 5](image)

Figure 5. Effect of the ratio of volume of OPA to volume of neomycin for concentrations from 0 to 2 mg/mL. For this trial, n=1, the y-axis is fluorescence (RFU) at 340/450 nm (ex/em) as determined by the plate reader at sensitivity = 50 and the x-axis is time (min). For all cases, the reaction was allowed to proceed for 2 minutes.

A. V. Tunability of Keratin Hydrogels

Similar to the methods established in Chapter 2 (for hydrogel formation and antibiotic quantification), hydrogels were formed using 95:5 (α:γ) KOS. The data and results in Figures 6 through 9 show the relationship between gel formulations (15% w/v KOS) for 80:20 (α:γ) and 95:5 (α:γ) KOS. For all antibiotics, the release was consistently lower for 95:5 (α:γ) KOS than for 80:20 (α:γ) KOS.
Figure 6. Cumulative release profile for cephazolin from both 80:20 (α:γ) and 95:5 (α:γ) 15% w/v KOS gels. Detection for cephazolin was through HPLC methods, established previously, and absorbance at 254 nm. In both studies, n=4, and bars indicate standard deviation.

Figure 7. Cumulative release profile for ciprofloxacin from both 80:20 (α:γ) and 95:5 (α:γ) 15% w/v KOS gels. Detection for ciprofloxacin was through HPLC methods, established previously, and fluorescence at 340/450 nm (ex/em). In both studies, n=4, and bars indicate standard deviation.
Figure 8. Cumulative release profile for neomycin from both 80:20 (α:γ) and 95:5 (α:γ) 15% w/v KOS gels. Detection for neomycin was through HPLC methods, established previously, and fluorescence at 340/450 nm (ex/em). In both studies, n=4, and bars indicate standard deviation.

Figure 9. Cumulative release profile for combination (of neomycin and cephazolin) from both 80:20 (α:γ) and 95:5 (α:γ) 15% w/v KOS gels. Detection was through HPLC methods, established previously, and fluorescence at 340/450 nm (ex/em) (for neomycin) as well as absorbance at 254 nm (for cephazolin). In both studies, n=4, and bars indicate standard deviation.