TOPICAL FORMULATIONS FOR DISEASE TREATMENT

by

BRIDG'ETTE BEATRICE ISRAEL

(Under the Direction of Anthony C. Capomacchia)

ABSTRACT

Purpose:
Develop a bioadhesive formulation for the treatment of aquatic life with skin lesions or abrasions. The purpose of the second research project is to develop novel mutual prodrugs (MP) which couple n-acetyl-glucosamine with NSAID, either ketoprofen or ibuprofen. These mutual prodrugs are designed to aid in the treatment of osteoarthritis. Lastly, the transdermal permeability of N-Acetyl-D-Glucosamine is evaluated for the development of the mutual prodrug.

Methods:
For research project one, oleaginous gels were prepared and their viscosity measurements were taken with a rheometer to gain information pertaining to the bioadhesive forces present in each formulation. The mutual prodrugs are synthesized for project two. One mutual prodrug links n-acetyl-glucosamine to ketoprofen and the other links n-acetyl-glucosamine to ibuprofen. N-Acetyl-D-Glucosamine solutions were prepared at different concentrations for the third project. Enhancing agents were also incorporated to evaluate their influence on permeability of N-Acetyl-D-Glucosamine. The Franz cell apparatus and High Performance Liquid Chromatography were utilized to collect and analyze samples, respectively.

Results:
The findings of project one are as follows, gels prepared with safflower oil and wheat germ oil demonstrated the greatest viscosity and perceived bioadhesion when compared to the other gels and all controls. The gel prepared with both safflower oil and wheat germ oil provided the largest inhibition zone. The structure of the oil may be important since both safflower oil and wheat germ oil contain linoleic and linolenic acids, whereas olive oil contains mostly oleic acid. Permeability studies, of project two, show that the ketoprofen mutual prodrug permeates shed snakeskin more than three times greater than either ibuprofen derivative, while ethanol markedly increases the permeation for all three. It was determined, for project three, that the permeability coefficients of the phosphate buffer/ethanol solutions at 5%, 10%, and 25% were about threefold larger in value as those for saturated DMSO solution, whereas the 2% and 50% solution values were lower.

Conclusion:
The bioadhesive gel prepared with safflower oil showed greatest stickiness to chicken breast, greatest viscosity, and best antimicrobial release (for project one). It was concluded, from the results of project two, that the ketoprofen mutual prodrug appears the most likely candidate for

transdermal administration; the ibuprofen mutual prodrugs for oral delivery; all three mutual prodrugs may be candidates for oral delivery or subcutaneous injection. The permeability coefficients calculated, during project three, supports the idea that phosphate buffer/ethanol solutions at 5%, 10%, and 25% are the optimal candidates for formulation while phosphate buffer/ethanol solutions at 2%, 50% contains too little ethanol and saturation has been reached, respectively.

INDEX WORDS: Bioadhesion, Oleaginous, Transdermal Permeation, Mutual Prodrug, NSAID

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by

BRIDG'ETTE BEATRICE ISRAEL

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MS, North Carolina A&T State University, 2003

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BRIDG'ETTE B.J.ISRAEL

Major Professor:	Anthony Capomacchia
Committee:	Casimir Akoh J. Warren Beach Phillip Greenspan Branson Ritchie

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
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DEDICATION

This dissertation is sincerely dedicated to my family and close friends. I would like to leave a few words to express my gratitude in the space provided. Jeremy, you have been a very supportive husband and friend. You never let me quit and I am thankful for your faith in me. JerJuan and JerMeny you all are my favorite lab mates. Thank you for coming to school with me without making a fuss. We have not had a very smooth road, but this is the beginning of the rest of our lives. I love you all so much. I am so proud to be your mother and the queen of the royal Team Israel. My parents, Amos Sr. & Beatrice Johnson, we made it by the grace of the Almighty. I thank God everyday for you all being such wonderful parents to us. A.J., Moni, Jessica, and Adrian; the Johnson 5 has its first doctor. My nephews and nieces have also encouraged me in their own little way. Lil AJ, my first nephew, continue to excel in school and sports. I believe you can do anything your heart desires so never stop dreaming. Ahmad, my science partner, keep in mind you promised me you will major in chemistry. Taneysha, remember to practice the piano every day. I plan to attend your next recital. Tyra, keep perfecting your dancing and singing. Your last performance was outstanding. Deja, you are a great singer and I know you will be a star one day soon. Jaden, since you love electronics, I look forward to using your inventions. Adrian II, you have just arrived and I just pray you are as wonderful as your father. Lastly, I dedicate this document and degree to my maternal grandmother, Beatrice Robinson, who passed away on February 13th, 2010. I am grateful to be your namesake and I will never forget you. Thank you for all the love and support.

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CHAPTER ONE

BIOADHESIVE OLEAGINOUS GEL FORMULATIONS FOR AQUATIC ANIMALS

Purpose:
Develop a bioadhesive formulation for the treatment of aquatic life with skin lesions or abrasions. The formulation must be able to adhere to wounds and mucosa, in a wet environment and release an antimicrobial.

Methods:
Oleaginous gels were prepared by mixing mineral oil, isopropyl myristate, olive oil, safflower oil, and wheat germ oil with lecithin, Carbopol 934 and Tricide antimicrobial. Controls were the oils, oils plus lecithin, and oils plus lecithin and Carbopol. The gels were initially tested for adhesion by applying to wet chicken breast and grading perceived stickiness with a five point Likert Scale. Gel bioadhesion/cohesion measurements were taken by securing strips of chicken breast to a plastic plate, applying gel to the plate, pressing the gel against the chicken, then pulling apart, using a Tensiometer, which resulted in a measurable force. Viscosity measurements on each gel were taken with a rheometer.

Results:
Gels prepared with safflower oil and wheat germ oil demonstrated the greatest viscosity and perceived bioadhesion when compared to the other gels and all controls. Mineral oil and isopropyl myristate showed the least viscosity and perceived bioadhesion; olive oil was intermediate. Tensiometer force measurements were marred by the gels not being cleanly removed from the chicken breast when force was applied. The results were gel cohesion measurements not bioadhesion measurements. Release of the antimicrobial from the gels was measured by plating on agar plates and measuring the zone of microbial inhibition as a measure of drug release from the gels. The gel prepared with both safflower oil and wheat germ oil provided the largest inhibition zone. The structure of the oil may be important since both safflower oil and wheat germ oil contained linoleic and linolenic acids, whereas olive oil contained mostly oleic acid.

Conclusion:
The bioadhesive gel prepared with safflower oil showed greatest stickiness to chicken breast, greatest viscosity, and best antimicrobial release. It remained on the chicken for 12 hours underwater and provided an occlusive function as well as drug release.

INDEX WORDS: Bioadhesion, Oleaginous, Likert Scale

CONCEPT AND SIGNIFICANCE

Many companion fish are exposed, in excess, to antibiotics by producers, fish hobbyists, and retailers. This overexposure can result in the proliferation of multiple drug resistant, "flesh - eating" bacteria (Figure 1). Molecular Therapeutics LLC was determined to develop a product that would address this problem. Tricide^{TM} was developed primarily to distribute potentiated antibiotics for companion fish. Tricide^{TM} has been reported as a safe and effective antimicrobial potentiator that is easy to administer. Molecular Therapeutics has reported that Tricide^{TM} is applicable with a wide variety of antibiotics, increasing their antimicrobial activity against common gram positive and gram negative bacterial pathogens (Figure 2).

The Emerging Diseases Research Group at the University of Georgia, College of Veterinary Medicine, refined and tested Tricide^{TM} for its safety and efficacy when administered to companion fish. Tricide^{TM} potentiated antimicrobial represents the first safe and effective treatment for ulcerative skin, fin, gill, and oral lesions associated with multiple drug resistant bacteria. Tricide^{TM} damages the bacterial cell wall (Figure 3 & 4) destroying the pathogen's barrier, efflux pump and genetic defenses against antibiotics. In addition, Tricide^{TM} potentiator reduces the concentration of antibiotic needed and reduces the potential for a bacteria developing resistance to the potentiated antibiotic.[1]

The marketing potiential of Tricide ^{TM} is high since ornamental fish production is among the leading cash crops of the United States aquaculture economy. The US is recognized for having the largest market for ornamental fish in the world. Ornamental fish production, with koi fish and goldfish being the most popular, has a retail value of nearly 1 billion dollars, and continues to increase.[2]

Ornamental fish commonly are affected by bacterial- associated diseases that the industry has named. Some of these conditions are known as “hole in the side disease”,“gill ill”, “fin root”, and “ulcer disease”. These diseases are most prevelent in pond fish during the spring emergence from hibernation, following shipment, as a result of overcrowding, and poor water quality. A formulation containing Tricide ^{TM} can aid in the prevention of these problems for aquatic life animals.

Tricide ^{TM} potentiated antibiotics have been used in an oil based topical formulation that was administered to a dog. The patient was intentionally set on fire and as a result of this deliberate act, she suffered from some severe burns. This patient had to be treated quickly and carefully, since the damaged tissue covered a large surface area. The injuries suffered also put the patient at risk for infection. Dr. Ritchie and his colleagues discussed treatment options and decided to incorporate an oil based polymer gel containing Tricide ^{TM} , formulated in the Capomacchia laboratory, in the burn victim’s treatment regimen.[1] After a few weeks the patient’s wounds were healing and she showed signs of recovery. Petroleum based ointments are commonly used to treat skin infections , but sometimes petroleum can be toxic and prevent healing. The oil based formulations developed during this research project contain the necessary antimicrobials, but many of the oils contain antioxidants that are known healing agents. This is an added bonus and those formulations seem to render the optimal results.

Gaspar, a beluga whale, had skin abrasions as a result of living in an aquarium, where he constantly came in contact with rocks and other items in his artificial habitat. Inorder to treat Gaspar properly, it was necessary that antimicrobial formulations adhere to Gaspar as he continued his daily routine in the water. Gel formulations were used to treat Gaspar and his

wounds showed signs of recovery. Based on the success of this patient, we were interested in further investigating the potential of these formulations.

INTRODUCTION TO BIOADHESION

The concept of bioadhesion has intrigued the minds of scientists for decades. It really became popular in the scientific community during the early 1980's and from this point until the present it has been exploited for its benefits in the area of pharmaceutics, nanotechnology, tissue engineering, and gene delivery. Bioadhesion can be described as any adhesion phenomenon that occurs with a component of a living organism. The adhesive and substrate must have contact and that is considered the site of action or the adhesive – substrate interface.[3] It is a requirement that at least one of these parts originate from a biological material in order to be classified as a bioadhesive. Water has a mandatory role in bioadhesion since there is a living element present. This is different for conventional adhesion because water usually diminishes the adhesive bonds.

Bioadhesion is the process where natural and man-made macromolecules adhere to mucosal surfaces in the body. The overall goal of utilizing a bioadhesive pharmaceutical formulation is to enhance drug absorption by mucosal cells or increase the time interval of drug released at the target site. For synthetic polymers, like carbomers, the mechanism of bioadhesion is the result of various physicochemical interactions. Bioadhesive drug dosage forms can be administered via the buccal, nasal, ocular, vaginal, and anal cavities, making this a versatile tool. The gastrointestinal tract, however, is a more difficult route of administration for bioadhesive technology because of the rapid turnover of mucus, and relatively constant transit time. [4] Research shows that micro- and nano-particles, coated with either bioadhesive polymers or specific biological bioadhesives maybe another venue in which, bioadhesive polymers can enhance a pharmaceutical formulation.

It is still unclear which current theory is most accurate in its description of the mechanism of bioadhesion. There are some essential elements that must be present in order for bioadhesion to occur. One is that the bioadhesive must spread over the substrate to initiate intimate contact and to increase the surface area of contact. Secondly, the chains of the adhesive should show signs of interdiffusion into the substrate to create a greater area of contact.[5] Lastly, the forces of attraction and repulsion develop and, in the case of successful bioadhesive formulations, the attractive force dominates.

Adhesive properties are influenced by various factors such as molecular weight, concentration of polymer, and chain flexibility. The ideal molecular weight for bioadhesion depends on the type of bioadhesive polymer utilized. It is generally understood that the minimum molecular weight for standard bioadhesion is 100,000 MW. It has been proven that bioadhesiveness improves with increasing molecular weight for linear polymers implies interpenetration is more pertinent for lower molecular weight polymers and entanglement is important for higher molecular weight polymers. An optimum concentration of a bioadhesive polymer does exist and it is the researchers' goal to develop such a formulation that contains that concentration. In systems beyond the optimal level, the adhesive strength drops considerably because the molecules are now removed from the medium and the chains are unable to penetration the substrate. Chain flexibility is critical for interpenetration and entanglement. Polymer cross linking is directly related to the mobility of the individual polymer. [6] As a result, the effective length of the chain that can penetrate into the substrate decreases as the crosslinking decreases. This reduces bioadhesive strength.

There are many theories that attempt to provide an explanation to this phenomenon. Five of them are recognized in the field as possible mechanisms of action. The theories are listed as

follows: electronic, adsorption, wetting, diffusion, and fracture. There are so many parameters that must be considered. I am convinced that one mechanistic scheme will not have the ability to encompass all the possible bioadhesive interactions without being too broad. Combining some principles from the currently recognized theories may give us a better understanding of what drives the system.

The electronic theory of bioadhesion was developed on the assumption that the substrate and the adhesive have different electronic structures. When the two constituents are joined, an electron transfer occurs to balance Fermi levels.[7] This results in a double layer of electrical charge at the adhesive – substrate interface. It is believed that the bioadhesive force can be attributed to the attractive forces across the electrical double layer. Like a capacitor, this system is activated when the adhesive and substrate are in intimate contact and deactivated when they are separated. There is some controversy surrounding this theory. [8]Various scientists think the electrostatic forces are the cause of contact, while others think it is the result.

The adsorption theory states that the bioadhesive bond formed between adhesive and substrate is due to secondary molecular interactions such as hydrogen bonds, van der Waals interactions, ionic bonds and other related forces.[9] It has been proven, that individually these forces are weak. When the sheer number of interactions is combined, they result in the production of a strong adhesive strength. For a bioadhesive polymer with a carboxyl group, hydrogen bonding is considered to be the dominant force at the interface. On the other hand, hydrophobic interactions can explain the fact that a bioadhesive may bind to a hydrophobic substrate more tightly than to a hydrophilic surface. Unlike the electronic theory, the adsorption theory is widely accepted.

The wetting theory was formulated to explain the inner workings of liquid adhesives. The manner in which an adhesive spreads on its substrate is important in the development of the resulting bond strength. Intimate molecular contact is a prerequisite for the development of strong adhesive bonds. Interfacial tensions are responsible for the contact of the adhesive and substrate.[10] They also influence adhesive strength and are used to determine spreading and adhesion. There is a direct relationship between the spreading coefficient and bioadhesive work. The bioadhesive bond is greatly influenced by these parameters. Understanding those variables gives us a better idea of the mechanistic behavior of the system. Young's equation, $g_{tg} = g_{bt} + g_{bg} \cos Q$, is utilized to describe the interfacial tension (g). Subscript b, g, and t are abbreviations for bioadhesive, gastrointestinal contents and tissues, respectively. The formulations designed in this study are topical and therefore, no gastrointestinal contents will be present. Expanded formulas can be derived to accommodate different scenarios.

The diffusion theory supports the idea that the entanglement of polymer chains is responsible for bioadhesion.[11] This theory rests on the primus that interpenetration of polymer chains and substrate may lead to sustained adhesion, by mechanical interlocking between substrate and adhesive. The fundamental nature of this theory is that the adhesive and the substrate infiltrate one another to a sufficient depth to create a semi-permanent adhesive bond. The penetration rate depends on the diffusion coefficients of both interacting polymers, and the diffusion coefficient is dependent upon the molecular weight and crosslinking density. In addition, segment mobility, flexibility of the bioadhesive polymer, and the expanded nature of both networks are important parameters that need to be considered. Interpenetration can be hindered by any crosslinking and this is a major concern when dealing with large molecules. The degree of penetration of polymer into substrate is directly related to the bond strength. Having

similar solubility and structural parameters between the adhesive and substrate will yield a stronger bioadhesive bond.

The fracture theory is quite different from the previous theories, in that it examines the system after adhesion. The forces required to remove the substrate from the adhesive are analyzed to describe and categorize the bond at the adhesive – substrate interface. The physical dimensions of the system must be known to the researcher and the system must be made of one uniform substance in order to evaluate the system utilizing this theory. We also assume that adhesive bond breaks at the adhesive – substrate interface, although separation experiments show that separation rarely occurs at that site. Since the fracture theory only takes into consideration those forces required to separate the adhesive and the substrate, diffusion, entanglement, and interpenetration of polymer chains are not mandatory.

Bioadhesion and adhesion are primarily differentiated by the presence or absence of a biological component, respectively. In many cases, measurements of bioadhesion are in fact measurements of cohesion. Parker and Taylor define adhesion as the use of one material to bond two other materials together and cohesion as the joining together of the same material.[12] It is very difficult to remove all of the bioadhesive material from the testing surface and this decreases the reproducibility of direct measurements of bioadhesion. This obstacle led us to seek an alternative route to obtain this vital information. Adhesion can also be defined as the bond produced by contact between a pressure-sensitive adhesive and a surface. The American Society of Testing and Materials has defined it as the state in which two surfaces are held together by interfacial forces which may consist of valence forces, interlocking action, or both. In biological systems four types of bioadhesion can be distinguished and they are as follows adhesion of a normal cell on another normal cell, adhesion of a cell with a foreign substance, adhesion of a

normal cell to a pathological cell and adhesion of an adhesive to a biological substrate. As it pertains to developments in drug delivery systems, the term bioadhesion implies attachment of a drug carrier system to a specified biological location. The biological surface can be epithelial tissue or it can be the mucus coat on the surface of a tissue. If adhesive attachment is to a mucous coat, the phenomenon is referred to as mucoadhesion. Mucoadhesion can be defined as the interaction between a mucin surface and a synthetic or natural polymer while bioadhesion can be defined as the relationship that occurs when a substance interacts with biological materials and remains in intimate contact with the surface for a prolonged period of time.

Methods Used to Study Bioadhesion

There have been several test methods reported in the literature for studying bioadhesion. These tests are necessary in the screening of a large number of candidate mucoadhesives, but also to study their mechanisms. These tests are important in the design and development of a bioadhesive controlled-release system as they ensure compatibility, physical and mechanical stability, surface analysis, and bioadhesive bond strength. The test methods can be classified in two categories, which are, in vitro and in vivo methods. Most in vitro methods are based on the measurement of either tensile or shear stress.[13] Bioadhesiveness determined by measurement of stress tends to be subjective since there is no standard test method established for bioadhesion.

Tensile Strength Measurement Method

Methods using tensile strength usually measure the force required to break the adhesive bond between a model membrane and the test polymers. The instruments usually employed are modified balances or tensile testers. In this method, the force required to separate the bioadhesive sample from freshly excised rabbit stomach tissue was determined using a modified tensiometer. A section of the tissue, having the mucus side exposed, was secured on a weighed glass vial

placed in a beaker containing USP simulated gastric fluid. Another section of the same tissue was placed over a rubber stopper, again with the mucus side exposed, and secured with a vial cap. Then a small quantity of polymer was placed between the two mucosal tissues. The force used to detach the polymer from the tissue was then recorded. The results of the study provided important information regarding the effects of charge density, hydrophobicity, and experimental conditions such as pH, ionic strength, mucolytic agents, and applied pressure on bioadhesion.[14]

Shear Strength Method

Shear stress measures the force that causes the bioadhesive to slide with respect to the mucus layer in a direction parallel to their plane of contact. The method uses a glass plate suspended from a microbalance which is submerged in a temperature-controlled mucus sample. The force required to pull the plate out of the solution is determined under constant experimental conditions.

Adhesion Weight Method

A test system was developed by Smart and Kellaway that allowed suspensions of ion-exchange resin particles to flow over the inner mucosal surface of a section of guinea pig intestine and the weight of the adherent particles was determined. Although the method was of limited value due to poor data reproducibility resulting from fairly rapid degeneration and biological variation of the tissue, it was possible for them to determine the effect of particle size and charge on the adhesion after 5 min contact with intestine. That study was conducted in an attempt to understand structural requirements for bioadhesion in order to design improved bioadhesive polymers for oral use.

Flow Channel Method

Polymer interaction with the conjunctival epithelial cell membrane was studied by Park and Robinson. They developed a flow channel method that utilized a thin channel made of glass and filled with 2% aqueous solution of bovine submaxillary mucin, at 37°C. Humid air at 37°C was passed through the glass channel. A particle of a bioadhesive polymer was placed on the mucin gel, and its static and dynamic behavior was monitored at various time intervals. The data collected in this study can be used to broaden the current knowledge of the global bioadhesive phenomenon.[15]

Mechanical Spectroscopic Method

Mechanical spectroscopy has been utilized to investigate the interaction between glycoprotein gels and polyacrylic acid, and the effect of pH and polymer chain length.
A similar method was adopted to investigate the effect of Carbopol-934p on the rheological behavior of mucus gel. A Carri- Med CSL 100 rheometer was used and 0.5-mm was the desired gap for that study. The role of mucus glycoproteins and the effect of various factors such as ionic concentration, polymer molecular weight and its concentration, and the introduction of anionic, cationic, and neutral polymers on the mucoadhesive mucus interface were investigated. The use of a Bohlin CS rheometer was investigated to further understand the interactions at the polymer mucin interface. In spite of a number of methods for the determination of bioadhesion, a poor correlation has been found between the bioadhesive strength measured in vitro and the bioadhesive performance in vivo.

It has been found that two formulations exhibiting similar bioadhesive strength determined using the conventional “stress-strain” method in vitro exhibit different adhesion time in vivo. The difference might be due to different erosion resistance of the formulations or to premature

dislodgement of the formulations due to excessive swelling and formation of slippery surface. Hence, there is a need for an effective in vitro method which would sufficiently mimic the in vivo bioadhesive performance of the formulations.

Falling Liquid Film Method

The falling liquid film method was developed by Teng and Ho. Small intestine segments from rats were placed at an inclination of a tygon tube flute. The adhesion of particles to this surface was monitored by passing the particle suspension over the surface. A similar principle was used by Rao et al., to determine the adhesive potentials of various polymers. By comparing the fraction of particles adherent to the tissue, the adhesion strength of different polymers can be determined.[16]

Colloidal Gold Staining Method

Park proposed the colloidal gold staining technique for the study of bioadhesion. The technique employed red colloidal gold particles which were stabilized by the adsorbed mucin-gold conjugates. Upon interaction with mucin-gold conjugates, bioadhesive hydrogels developed a red color on the surface. Thus, the interaction between them could easily be quantified, either by the measurement of the intensity of the red color on the hydrogel surface or by the measurement of the decrease in the concentration of the conjugates from the absorbance changes at 525 nm.[17]

Viscometric Method

A simple viscometric method was used by Hassan and Gallo to quantify mucin-polymer bioadhesive bond strength. Viscosities of 15 % w/v porcine gastric mucin dispersions in 0.1 N HC1 (pH 1) or 0.1 N acetate buffer (pH 5.5) were measured with a Brooksfield viscometer in the

absence or presence of selected neutral, anionic, and cationic polymers. Viscosity components and the forces of bioadhesion were calculated.[18]

Thumb Test

The thumb test is a simple method which can be used to identify mucoadhesives. The adhesiveness is quantitatively measured by the difficulty of pulling the thumb from the adhesive as a function of the pressure and the contact time. It is most likely that any mucoadhesive system is adhesive to fingers, since most mucoadhesives are nonspecific and not mucin specific. Like mucin, the skin has many hydroxyl groups. Although the thumb test may not be conclusive, it provides useful information on mucoadhesive potential. The thumb test is very similar to the perceived stickiness studies conducted in this research project.[19]

Adhesion Number

When a mucoadhesive is present in small particles, the adhesion number can be used as a parameter for mucoadhesion. The determination of adhesion strength for small particles is a difficult task. The adhesion number is typically represented by the following equation:
$N, = (NIN,)$ X 100 where $N,$ is the adhesion number, No is the total number of applied particles, and N is the number of particles attached to the substrate. There is a direct relationship between the adhesion strength and the adhesion number. Therefore, as the adhesion strength increases, the adhesion number also increases.[20]

Electrical Conductance

Bremecker used electrical conductance as a parameter for testing semisolid mucoadhesive ointments. The adhesion of Orabase, Carbopol, Eudispert, guar gum, and methyl cellulose to artificial biomembranes in artificial saliva was studied by using a modified rotational viscometer capable of measuring electrical conductance. This parameter, measured as a function of time,

was found to be influenced by the sample, the artificial saliva, and the artificial biomembrane. In the presence of adhesive material, the conductance was comparatively low.[21] As the adhesive was removed, the value increased to a final value corresponding to the conductance of the saliva, which indicated the absence of adhesion.

In Vivo Methods

In vivo techniques for measuring the bioadhesive strength are relatively few. Some of the reported methods are based on the measurement of the residence time of bioadhesives at the application site. The GI transit times of many bioadhesives have been examined using radioisotopes. In order to investigate the gastrointestinal transit of bioadhesive beads, developed an in vivo method in rats, inserting 55Cr-labeled bioadhesive material in the stomach and measuring the radioactivity in cut segments of the intestine.[22]

Bioadhesive Polymers

To overcome the relatively short gastrointestinal time and improve localization for oral-controlled or sustained release drug delivery systems, bioadhesive polymers which adhere to the mucin/epithelial surface are effective and lead to significant improvement in oral drug delivery. Improvements are also expected for other mucus-covered sites of drug administration.[23] Bioadhesive polymers find application in the eye, nose, and vaginal cavity as well as the gastrointestinal tract, including the buccal cavity and rectum. Polymers that adhere to the mucin-epithelial surface can be conveniently divided into three categories: polymers that become sticky when placed in water and owe their bioadhesion to stickiness; polymers that adhere through nonspecific, noncovalent interactions which are primarily electrostatic in nature (although hydrogen and hydrophobic bonding may be significant); and polymers that bind to specific receptor sites on the cell surface. All three polymer types can be used for drug delivery.

Polymers which can adhere to either hard or soft tissue have been used for many years in surgery and dentistry. Among these "superglues," polymers and monomeric alpha cyanoacrylate esters have been most frequently investigated and used.[24] Other synthetic polymers such as polyurethanes, epoxy resins, polystyrene, acrylates, and cements from natural products were also extensively investigated, as were glues. An ideal polymer for a mucoadhesive drug system should have the following characteristics:

1. The polymer and its degradation products should not be toxic.
2. The polymer should not cause the mucous membrane irritation.
3. The polymer will form a strong noncovalent bond with the mucin-epithelial cell surfaces.
4. The polymer will have the ability to quickly adhere moist tissue.
5. The polymer should be drug compatible and offer little to no hindrance to its release.
6. The polymer should not decompose during the shelf life of the dosage form.
7. The cost of the polymer should not be expensive to ensure that the prepared dosage form remains competitive.

Carbopol/Carbomer

Carbopol/carbomer is a synthetic, high molecular weight, cross-linked polymer of acrylic acid copolymerized with allyl sucrose or allyl pentaerythritol. The carboxyl groups provided by the acrylic acid backbone of the polymer are responsible for many of the product characteristics. The chemical name is carboxy polymethylene. There are many grades of carbopol including: 907, 910, 934, 934P, 940, 941, 971P 974P, 980, and 981. Carbopol 934P, 971P and 974P are the only pharmaceutical grades of the resin intended for internal use.[25] Carbopol 934 P is a high molecular weight polymer of acrylic acid cross-linked with allyl ethers of sucrose and polymerized in benzene. Carbopol 934 P is polymerized in ethyl acetate and is slightly treated

with a potassium base. White, fluffy, and acidic are words that describe the physical appearance of this hygroscopic powder.

Carbopol 934 P is relatively unaffected by temperature variations, not subjected to hydrolysis or oxidation and is resistant to bacterial growth. It is safe and nontoxic. No primary irritation or any evidence of allergic reactions has been observed in human beings following topical application. It is not absorbed in the body and is excreted unchanged. It contributes no off-taste but, contains a slight characteristic odor. In some cases this compound may mask the undesirable taste of the formulation. This effect is observed with phenols, cationic polymers, high concentration of electrolytes, and resorcinol. It is an excellent thickening, emulsifying, suspending, and gelling agent. It is used as a tablet binder in sustained-release formulations affording zero- to near-zero-order release. It is used as the bioadhesive component in muco-adhesive ointments, gels, and tablets.

Components of the Studied Bioadhesive Formulations

The bioadhesive formulations used in this study were designed to aid in the treatment of aquatic animals suffering with skin abrasions. It is necessary that the formulation has the ability to adhere to the affected site and remain there while the animal travels in its natural habitat, which in this case is a body of water. Oil and lecithin are combined in three ratios, 1:1, 1:0.5, and 1:0.25. This is referred to as the base because every formulation developed in this study originates from an oil and lecithin mixture. Mineral oil, olive oil, safflower oil, wheat germ oil, and isopropyl myristate are the oils selected for this study. Each experiment was carried out utilizing one kind of oil at a time. Carbopol is used as a thickener and stabilizer. The formulations must have a sticky characteristic that is facilitated by carbopol. The remaining

components consist of antimicrobials and preservatives. They are all listed below with additional information.

Mineral Oil

Paraffin oil, rock oil, and liquid petrolatum are a few synonyms for mineral oil, which has many common names. Mineral oil is a by-product in the distillation of petroleum to produce gasoline and other petroleum based products from crude oil. Mineral oil has no apparent taste or odor. It is also a colorless transparent liquid that is not vulnerable to bacterial decomposition and therefore does not become rancid over time. In the late 1800s, the term mineral oil was first used to describe the petroleum hydrocarbons and associated products that were produced from wells that tapped underground reservoirs. The term was used to differentiate petroleum hydrocarbons produced from underground sources from other oil sources at that time, such as palm oil or whale oil. In today's petroleum exploration and production business, the phrase mineral oil is mainly used in legal documents to define and encompass all of the liquid hydrocarbon and gaseous products produced from wells drilled into underground petroleum-bearing reservoirs.[26]

Olive Oil

The beneficial health effects of olive oil are due to both its high content of monounsaturated fatty acids and its high content of antioxidants. Studies have shown that olive oil offers protection against heart disease by controlling LDL cholesterol levels while raising HDL levels. No other naturally produced oil has as large an amount of monounsaturated as olive oil - mainly oleic acid. Olive oil is very well tolerated by the stomach. Research shows that olive oil's protective function has a beneficial effect on ulcers and gastritis.

Olive oil activates the secretion of bile and pancreatic hormones much more naturally than prescribed drugs. Consequently, it lowers the incidence of gallstone formation. The oil obtained from the first press is named virgin olive oil. Pure olive oil is a mixture of virgin and refined olive oil. Lampante is a highly acidic grade olive oil. Its name is derived from its use as lamp oil. Refined olive oil consists of the lower grade lampante oil from which the color, scent, and acidic nature have been removed through processing. Sulfide olive oil is chemically extracted from the olives via a mechanism that utilizes solvents and is refined many times. Pure olive oil was used in the development of the formulations that contained olive oil.[27]

Safflower Oil

Similar to mineral oil in physical appearance, safflower oil is normally odorless and colorless. The seed of the safflower is pressed to obtain safflower oil. In this study, the safflower oil used to produce the formulations was enhanced with vitamin E. Vitamin E was added by the manufacturer to ensure the freshness of the oil. There are two distinct types of safflower oil, each with very different uses. Monounsaturated safflower oil, which was used in this project, is high in oleic acid and is used as a heat-stable cooking oil. Polyunsaturated oil, high in linoleic acid, is used as a cold oil. Like other products high in oleic acid, monounsaturated safflower oil is not very beneficial to human health. Polyunsaturated oil, on the other hand, has a great deal of nutritional value, making it an excellent choice for dressings, massage oils, and aromatherapy.

Wheat Germ Oil

Wheat germ oil is extracted from the germ of the wheat kernel, which makes up only 2.5% by weight of the kernel. The following fatty acids are present in wheat germ oil: Linoleic acid (55% by weight), Palmitic acid (16% by weight), Oleic acid (14% by weight), and Linolenic acid (7% by weight). Wheat germ oil has been studied to determine if it has physical

performance enhancing properties. Studies indicate that the octacosanol found in wheat germ oil may help to enhance endurance, reaction time, and exercise capacity. Research suggests octacosanol may also have cholesterol-lowering effects, but further testing is necessary to confirm these findings.[28]

Isopropyl Myristate

Isopropyl myristate is the ester of isopropyl alcohol and myristic acid. Isopropyl myristate is a colorless almost odorless liquid. In cosmetic products, isopropyl myristate may be used in the formulation of moisturizers, cleansing products, perfumes, makeup, skin, and hair care products. Isopropyl myristate functions as a binder or a skin conditioning agent. It is also used as a pesticide-free treatment against head lice which works by dissolving the wax that covers the exoskeleton of head lice, killing them by dehydration. Testing showed that undiluted isopropyl myristate irritated the skin and eyes, although products containing it were not irritating. Isopropyl Myristate did not exhibit signs warranting it to be classified as a carcinogenic substance.[29]

Carbopol 934

Carbopol 934 polymer is a cross-linked polyacrylate polymer. It offers excellent stability at high viscosity and produces thick formulations for opaque gels, emulsions, creams and suspensions. Carbopol polymers are polymers of acrylic acid cross-linked with polyalkenyl ethers or divinyl glycol. They are produced from primary polymer particles of about 0.2 to 6.0 micron average diameter. Carbopol polymers are offered as fluffy, white, dry powders. The carboxyl groups of the acrylic acid backbone of the polymer are responsible for many of its attributes. They possess a three dimensional nature which suggests biological inertness, not found in similar linear polymers. The carbopol resins are hydrophilic substances that are not

soluble in water, which adds water sorption characteristics. They swell in water up to 1000 times their original volume and 10 times their original diameter to form a gel when exposed to a pH environment above 4.0 to 6.0. Carbopol 934 was selected for this study because it has been extensively noted for its ability to enhance bioadhesive formulations. The desired formulation in this study should adhere to wet flesh therefore it must have a relatively high viscosity and a sticky nature and carpool enhances these qualities of the final product.

Carbopols are very versatile polymers and can be used to formulate a variety of drug dosage forms and there is a number assigned to them to differentiate between the various molecular weights. Because of this characteristic, they have been an excellent component of different types of controlled release solid dosage forms, like gels, ointments, tablets, and etcetera. Recently, carbomers are becoming a very popular component in the manufacturing of controlled release tablets. These polymers are effective at low concentrations, which are recognized as weight percentages less than 10%. At these concentrations, carbopol polymers continue to show extremely rapid and efficient swelling characteristics. Carbomers show larger dissolution times at lower concentrations than other excipients.

Carbopol polymers possess many characteristics that make them excellent components of topical dosage forms.[30] They are outstanding thickening, emulsifying, and suspending agents. Common in the case of many water insoluble compounds, carbopol swells in the presence of water. The network formed is attributed to the covalent and ionic bonds that stem from the crosslinking agent. This allows carbopol to greatly increase the viscosity of the formulation it is added to. Carbopols have been extensively documented as safe and effective polymers that can be used in topical gels, creams, lotions, and ointments. They have also shown signs of having low irritancy properties and are non-sensitizing with repeat usage. Carbopol polymers provide an

excellent vehicle for drug delivery. Due to their extremely high molecular weight, they cannot penetrate the skin or affect the activity of the drug. Carbopol polymers are used to permanently suspend the active ingredients in transdermal reservoirs as well as in topical gels and creams.

Methyl Paraben

Methyl paraben is an antifungal agent that is commonly used as a preservative in the food, drug, and cosmetic industries. It can be found in many of the leading skin, hair, and facial products. This compound is often found in carpules of local anaesthetic, acting as a bacteriostatic agent and preservative. Methyl paraben is extracted benzoic acid which originates from benzoin tree gum. Methyl paraben is readily absorbed from the gastrointestinal tract and through the skin. Since methyl paraben is an ester of *p*-hydroxybenzoic acid, when hydrolyzed methyl paraben converts back to *p*-hydroxybenzoic acid and is excreted without accumulation in the body. Extensive studies have shown that methylparaben is not toxic following oral or parenteral administration. When examining patients with normal skin, the use of methyl paraben does not show signs of casing skin irritations. There have been reports of allergic reactions occurring due to ingesting parabens.[31]

Propyl Paraben

Propyl paraben is a well documented fungicide and microbiocide. It is the propyl ester of *p*-hydroxybenzoic acid that occurs as a natural substance found in many plants and some insects. Propyl paraben is synthetically manufactured for its use in cosmetics, pharmaceutical preparations and food items. Since propyl paraben is water soluble, it is commonly used as a preservative in many water-based cosmetics, such as creams, lotions, shampoos and bath products. Although the United States Food and Drug Administration has approved the use of

propyl paraben in food, cosmetic, and pharmaceutical products there have been reports of mild skin irritations from products that contain parabens.[32]

Sorbic Acid

Sorbic acid is a natural organic compound used in the food industry as a preservative to prevent the growth of mold, yeast and fungi. It has the chemical formula $C_6H_8O_2$. Sorbic acid and its mineral salts, such as sodium sorbate and calcium sorbate, are antimicrobial agents. In general the salts are preferred over the acid form because they are more soluble in water. The optimal pH for the antimicrobial activity is below pH 6.5. Sorbic acid should not be confused with other similarly named food additives sorbitol, polysorbate, and ascorbic acid. Some molds and yeasts are able to detoxify sorbates by decarboxylation, producing trans-1,3-pentadiene. The pentadiene manifests as a typical odor of kerosene or petroleum. Other detoxification reactions include reduction to 4-hexenol and 4-hexenoic acid.[33]

Neomycin

Neomycin was first isolated by American microbiologist Selman Waksman in 1949 from a strain of the bacterial species Streptomyces fradiae. Neomycin is a broad spectrum antibiotic that has been effective against both gram positive and gram negative bacteria. It is used in the prevention or treatment of skin infections caused by bacterial invasion. It is not a successful agent in the treatment fungal or viral infections. Neomycin is commonly administered as a topical preparation. Neosporin is the most popular brand that incorporates neomycin in its formulation. Neomycin can also be given orally, where it is usually combined with other antibiotics.[34] Another use of neomycin includes its role as a preservative in some vaccines - typically 0.025 mg per dose.

Fluconazole

Fluconazole is a triazole antifungal drug that is noted for its effective treatment and prevention of superficial and systemic fungal infections. The powder form of fluconazole is a white crystalline powder that is barely soluble in water but, is soluble in alcohol. It is commonly marketed under Diflucan, Trican, and Loitin.[35] Fluconazole is widely recognized as the best form of treatment for patients suffering from yeast infections. It is active against many microorganisms such as *Blastomyces dermatitidis, epidermophyton, and Histoplasma capsulatum*. Research shows that fluconazole inhibits human cytochrome P450, particularly the isozymes CYP2C9 and CYP3A4.

Theoretically, fluconazole decreases the metabolism and increases the concentration of any drug metabolized by these enzymes. Fluconazole is most commonly dispensed in tablet form. Fluconazole can be used to treat yeast infections of the mouth, throat and esophagus. It has also been proven to treat vaginal yeast infections, fungal urinary tract infections, pneumonia caused by yeast, and fungal infections throughout the whole body and in the blood. Fluconazole is also used to prevent fungal infections from occurring in people with suppressed immune systems such as cancer chemotherapy, organ transplant, and AIDS patients. The role of fluconazole, in the bioadhesive formulations developed during this research project, was to protect the products from fungal growth and the powder form of fluconazole was utilized to achieve this goal.

Tricide^{TM}

The focus of this project is to develop a bioadhesive formulation for the treatment of aquatic life with skin ulcers. The ideal formulation must possess the ability to adhere to wounds, mucosa, wet surfaces and release the drug (an antimicrobial). This is a novel research endeavor,

which makes it interesting and challenging. Developing such a product may expand our options for wound treatments for animals. Figure 1 and 2 illustrates the affect of Tricide™ potentiated antibiotic on koi fish. This medicament is very useful in the treatment of lesions present on companion fish. Figures 3 and 4 shows the difference in a normal cell wall and a cell treated with Tricide. These figures support the idea and current studies that suggest that formulations containing the therapeutic dose of Tricide™ potentiated antibiotics will be useful in the treatment regimen of aquatic animals.[36] With further testing, the formulations developed may be useful for humans to deliver potentiated antimicrobials.

MATERIALS AND METHODS

Olive oil, 100 % pure, was purchased from Wal- Mart Incorporated, Bentonville, AR, USA. Safflower oil, high oleic 100 % expeller pressed, was acquired from Hain Celestial Group Incorporated, Melville, NY, USA. Isopropyl myristate and soy lecithin were purchased from Gallipot, Saint Paul, MN, USA. Wheat germ oil, expeller pressed unrefined, was acquired from Spectrum Essentials, Melville, NY, USA. Light mineral oil and neomycin were obtained from Fischer Scientific Incorporated, Pittsburg, PA, USA. Carbopol 934 was acquired by Noveon Incorporated, Cleveland, OH, USA. Fluconazole was purchased from Spectrum Chemical MFG Corporation, Gardena, CA, USA. Dr. Branson Ritchie and Molecular Therapeutics LLC donated the Tricide used in this research project. The experimental oils can be obtained from Molecular Therapeutics LLC.Sodium hydroxide was obtained from J. T. Baker Incorporated, Phillipsburg, NJ, USA. Sorbic acid, methyl paraben, and propyl paraben were obtained from Pharmacy Compounding Chemicals, Houston, TX, USA.

Hypothesis

A bioadhesive gel formulation can be prepared, that adheres to wounds, mucosa and wet surfaces, such that it releases neomycin for 24 hours by zero order kinetics.

Specific Aim 1

Prepare bioadhesive formulations using various oils, polymers, neutralizing agents, water, neomycin (or alternative antimicrobial drugs), and adjuvants; test the formulations for bioadhesiveness on various tissues/surfaces; a formulation must be developed that sticks to and remains on wet tissue for at least 24 hours.

Specific Aim 2

Screen/evaluate neomycin release rate from formulations using Ninhydrin assay, microbiologic assays, modified dissolution apparatus, or modified Franz cell apparatus; a formulation must be developed that releases neomycin over 8-24 hours.

Methodology to Achieve Specific Aim 1

Sample Preparation

Bioadhesive formulations were prepared with a mixture of lecithin and one of nine oils. The oils that will be utilized for this research project are as follows: experimental oil 1(EO1), experimental oil 2 (EO2), experimental oil 3 (EO3), experimental oil 4 (EO4), safflower oil, olive oil, mineral oil, wheat germ oil, and isopropyl myristate. The specific identification of the experiment oils is proprietary. The oil and lecithin were combined in ratios of 1:1, 1:0.5, and 1:0.25 (oil: lecithin; OL). A batch of each OL is prepared and utilized as needed. It was very difficult to incorporate lecithin in the viscous oils. In order to overcome this challenge the OL mixture was placed in the Weksler incubator at 40° C for 48 – 72 hours and stirred periodically.

All OL mixtures were examined by a Rheometric Scientific SR – 5000 rheometer and the viscosity of each sample was calculated from the data obtained.

Each formulation was prepared from fifty grams of the OL mixture to which was added 12.5 grams of carbopol 934. Carbopol 934, a cross-linked polyacrylate polymer, was incorporated into the OL mixture as a source of adhesiveness and is denoted as OLC. The relationship between carbopol 934 and the formulation's bioadhesiveness was tested. Adding different quantities of carbopol 934 to OL mixtures was performed to assess the best quantity to achieve adhesiveness. Sodium hydroxide was used as the neutralizing agent, and after the addition of carbopol 934, 0.75 grams of NaOH (50.5% aqueous solution, about 12.6 molar in hydroxide ions; where the 0.75 g of solution is equivalent to 0.38g NaOH solid) was incorporated along with the other agents below to prepare a new formulation denoted as OLCA.

Each of the following dry components was sieved prior to its addition to the formulation. The preservatives methyl paraben (0.125 grams), propyl paraben (0.075 grams), sorbic acid (0.125 grams), were dissolved in distilled water (6.0 grams) prior to adding to the formulation; after which fluconazole (1.0 gram), neomycin (0.45 grams), and Tricide^{TM} (1.0 gram) were sprinkled over the formulation after all other components were added to aid in the release of these medicaments. The final OLCA products were placed in the ointment mill two times to ensure that the formulations were homogeneous. The effects of these components on formulation viscosity were evaluated by a rheometer and compared to OL and OLC. The viscosities of nine different oils with three different concentrations of lecithin were measured in triplicate. The experiments were repeated in triplicate in the presence of homogenized chicken breast to assess viscosity change and thus bioadhesion force as a function of adhesion to the chicken breast tissue.

Stickiness

Non-biased individuals (students) were randomly selected to record the physical appearance of all the formulations (UGA IRB approved). Perceived stickiness, color, texture, and smell were determined by the subjects independently of the researcher. Physical properties of formulation samples were evaluated at room temperature. This study was designed to determine if there is a relationship between the degree of bioadhesion of the formulation and its perceived stickiness.

Rheometric Data

Initially we attempted to utilize the Brookfield viscometer to determine the viscosity of the samples, but many of the samples were too viscous. This made it difficult to obtain accurate data and decreased reproducibility of viscosity measurements. The rheometer used during this research project was the Rheometric Scientific SR – 5000. Dynamic strain sweep test was performed utilizing the cone and plate apparatus. The diameter of the plate was measured at 40 mm and the cone angle was 0.0385 radians. The tool serial number was 3262 and the gap was set at 0.50 mm.

The plate and cone of the rheometer was cleaned and polished prior to each test or run. One gram of sample was carefully placed on the plate and the lock was disarmed. The parameters were set as follows: strain: 1%, temperature: 20 °C, sweep mode: log, initial frequency: 0.1 Hz, and final frequency: 79 Hz. The rate of shear (σ): 0.973/s.

Methodology to Achieve Specific Aim 2

Ninhydrin Assay

The release of neomycin and Tricide^{TM} was evaluated via an amino acid assay, which utilizes ninhydrin colorimetric method. The samples were taken from time zero to 24 hours, at

various time intervals. The ninhydrin assay works on the premise that a reaction occurs between alpha-amino acids and ninhydrin that results in color development.

After the initial screening process was complete, there was not enough evidence to support the idea that neomycin and Tricide^{TM} are released. Even after the amount of neomycin and Tricide^{TM} were increased in the formulation, there were no positive signs of drug release. Further studies were abandoned on drug release.

RESULTS AND DISCUSSION

Assessing Bioadhesion

Viscosity measurements of the oil mixtures oil/lecithin (OL), oil/lecithin/carbomer (OLC), and oil/lecithin/carbomer/adjuvants (OLCA) were conducted and treated as *per* Hassan and Gallo in order to assess bioadhesiveness. This approach was used in place of a tensiometer or modified torsion balance since pilot experiments with the latter resulted in ointment sticking to both plates as force was applied to separate the plates. The sticking to both plates occurred, when metal was used, plastic tape, and tissues like chicken breast or flank steak to mimic flesh. Therefore, the measurements recorded indicated cohesion forces in the formulation and not adhesion to either inanimate material or bioadhesion to tissue as shown for two representative formulations presented in Tables 1 and 2.

In order to assess formulation bioadhesion rather than cohesion, the method of Hassan and Gallo were used. Their equation which relates total system viscosity to viscosity caused by bioadhesion, $\eta_t = \eta_{m+} \eta_p + \eta_b$ (η_t = viscosity coefficient of the system; η_m, η_p, and η_b are the individual viscosity coefficients of mucin, polymer and bioadhesion, respectively) may be used to calculate the Force of Bioadhesion by using $F = \eta_b\sigma$, ($\eta_b = (\eta_t - \eta_m - \eta_{p;}$ and σ = rate of shear/s)(Figures 3 & 4). For our system mucin was replaced by homogenized chicken breast, and

the polymer was Carbopol 934 (cross-linked polyacrylic acid). This system displayed Newtonian characteristics similar to those described by Hassan and Gallo since viscosity was independent of time and rate of shear.

Bioadhesion as a Function of Formulation Variables

Tables 5-10 and Figures 5-10 show the results of experiments conducted on nine different oil/lecithin mixtures at 3 different levels, and other ingredients to determine optimum conditions for a bioadhesive gel, in the presence and absence of chicken breast tissue. The viscosity measurements were conducted under the same conditions reported by Hassan and Gallo with homogenized chicken breast replacing the mucin.

The viscous and elastic gel-like properties found in mucin are due to its glycoprotein structure with molecular weights ranging from 0.5 to 20 MDa. Its viscosity is caused by flow resistance caused by chain segments and entanglement, van der Waals forces and hydrophobic bonding[3]. These forces are the same as those present in mucin-polymer interactions, and most likely the same present in polymer-protein interactions reported here. Therefore, polymer-protein interactions may be determined through polymer viscosity measurements in the absence and presence of homogenized proteins like chicken breast. The effect of additives such as surfactants and other adjuvants on formulation bioadhesion may also be assessed.

The data in Tables 5-7 and Figures 5-7 show a near exponential increase in viscosity and thereby apparent bioadhesive force for OL, OLC and OLCA regardless of lecithin levels. This is more clearly apparent in Figure 8. Carbopol in the OLC formulation only doubled the viscosity seen with OL, whereas addition of adjuvants in the OLCA formulation increased viscosity almost 10 fold. The adjuvants used in OLCA were water, NaOH, neomycin, fluconazole, methyl paraben, propyl paraben, and potassium sorbate. Pilot experiments conducted in our laboratory

indicated that the parabens, potassium sorbate, neomycin and fluconazole were a factor in actual stickiness to animal flesh discussed but had a small effect on viscosity. Water at the concentration employed, about 8%, demonstrated only a small effect on viscosity but it did affect the ease of applying the formulation to sliced tissue. NaOH had the greatest effect on viscosity, ease of application and stickiness. NaOH and other organic bases may be used to deprotonate the carboxcylic acid groups present in the crosslinked polyacrylic acid that composes Carbopol 934. Deprotonation causes the crosslinked polymer to unwind resulting in an increase in polymer viscosity and thereby formulation viscosity (R). This is seen upon comparing Figures 5 - 7 where the effect of carbopol in Figure 6 almost doubles viscosity compared to Figure 5, but upon addition of adjuvants and particularly NaOH viscosity increases almost ten-fold.

The data in Tables 9-11 and Figures 9-11 shows an almost linear relationship upon going from OL to OLC to OLCA in regards to viscosity and thereby bioadhesive force. Each formulation adheres to homogenized chicken breast, whole chicken breast and thinly sliced flank steak. Viscosity measurements with solid tissue were not possible therefore only homogenized chicken breast was examined. Adhesion to whole chicken breast was observed to be more difficult than to sliced flank steak owing to a smoother tissue surface. So the more difficult conditions for assessing tissue adhesion to homogenized chicken breast were chosen. Figure 9 shows that OL-T adheres to homogenized chicken breast; and demonstrates an almost 10-fold increase in viscosity when compared to the results in Figure 5. Addition of carbopol 934 almost doubles this viscosity (OLC-T, Figure 10) and the addition of carbopol plus adjuvants more than doubles it again (OLCA-T, Figure 11). Lecithin seems to impart some degree of adhesion possibly owing to its amphoteric properties as seen in Table 1 when compared to Table 4 which contains viscosity values for the oils used. It is interesting to note that the viscosity values for the

four experimental oils EO1 – EO4 plus lecithin (OL) did not decrease as rapidly as the other oils as lecithin levels were reduced from 1:1 to 1:0.25. This may be a factor in their greater bioadhesion to tissue.

Oleaginous Gel

Two formulations reported here. OLC and OLCA we define as oleaginous gels. They are novel organogel-like gels which are non-thermoreversible. Organogels are defined as non-crystalline, non-glassy thermoreversible (thermoplastic) solid material composed of a liquid organic phase entrapped in a three-dimensionally cross-linked network[16]. The liquid can be, for example, an organic solvent, mineral oil, or vegetable oil. The solubility and particle dimensions of the structurant (polymer) are important characteristics for the elastic properties and firmness of the organogel. Often, these systems are based on self-assembly of the structurant molecules[17].

The phenomenological definition of a gel by Amdal[18] states that a gel is a soft solid or solid like substance consisting of no less than two materials, one of which is a liquid in abundance. The elastic and resilient nature should be recognized with no magnification. The gel should not flow under the influence of its own weight on a second timescale.

In our system the abundant liquid is oil, not water although water was most likely the liquid intended in the phenomenological definition. The gels we report stick to wet flesh and are formed by mixing oil, lecithin and carbopol, and adjuvants to achieve maximum adhesion. OL (which is not a gel but simply a mixture of oil and lecithin) and OLC do not contain water, only OLCA contains water (8%), NaOH and other adjuvants. As a comparison, organogels usually contain water at about 40% water by weight. A literature search did not reveal reports on oleaginous gels, but several patent applications concerning oil formulations containing polymers. There was one report on nonaqueous, hydrophilic gels used for minocycline HCl delivery[19].

The concept we present of an oleaginous gel is not only novel but very useful for the treatment of marine animals both warm and cold-blooded and those animals that thrive in wet environments like some turtles and snakes. The formulation has potential for human use.

Assessing Perceived and Actual Stickiness

Table 11 and Figures 13 and 14 show the results of a study that assessed the perceived stickiness of OL *versus* OLCA. OLC was omitted since we sought only the relative perceived stickiness of starting *versus* final formulations. The study and the participant consent document were sanctioned by the UGA-IRB. The data presented in Table 11 was plotted to clarify the role of the amphoteric surfactant lecithin in formulation adhesion to tissue (Figures 9 and 11). Figures 13 and 14, when compared to Figures 8 and 12 show some differences in perceived stickiness *versus* viscosity that may indicate viscosity (or bioadhesive force calculated from viscosity measurements) may not decisively correlate with apparent adhesion. In Figure 8 and 12 the viscosity of the 1:1, oil: lecithin ratio demonstrates the greatest viscosity for most oils, especially those that show the greatest viscosity excepting EO1 in Figure 8. In Figures 9 and 11 perceived stickiness measured for the ratio of oil: lecithin at 1: 0.5 is nearly equal to that of the 1: 1 ratio for most oils. This corroborates application of the formulations in field work when applying it to ulcers on the fins of marine mammals. Subjectively, formulations that are less viscous seem to possess mobility that is difficult to define; they apply easily, stick best to wet flesh, and stay on longer underwater. In fact they describe it (OLCA) as honey-like. The above experiments and field applications lead us to suspect that lecithin plays an important role in the adhesion process. This observation is under further study. Moreover, the observation in Figures 5 and 7 that reducing lecithin levels diminishes but not eliminates perceived stickiness shows that carbopol also plays an important role in the adhesion process.

CONCLUSIONS

The question of why the oleaginous formulations OLC but especially OLCA stick to wet flesh underwater is provocative and remains elusive, however we are making strides in understanding the adhesion process from an empirical perspective. Both carbopol and lecithin appear to be important in the adhesion process. The addition of salts like Tricide^{TM} appears to diminish adhesion. This suggests that an electrostatic interaction exists between the amphoteric lecithin and the surface of damaged tissue. The fact that carbopol exerts a larger adhesive effect after treatment with NaOH suggests that negatively charged carboxylic groups interact with positively charged proteins on the tissue surface. Owing to the lipophilic nature of the oleaginous gel, hydrophobic interactions are not only expected but most likely dominate the adhesive interactions. OLCA containing oil, lecithin, carbopol, and adjuvants at an oil: lecithin ratio of about 1: 0.5 was the stickiest and adhered the best to wet flesh underwater. It also adhered to ulcers on marine mammals for at least 24 hours as the gel absorbed water and was gently washed away.

Figure 1: Companion koi fish with ulcerative lesion prior to Tricide - Neo™ treatment.

Figure 2: Companion koi fish with ulcerative lesion after Tricide - Neo^{TM} treatment (three, five minute treatments).

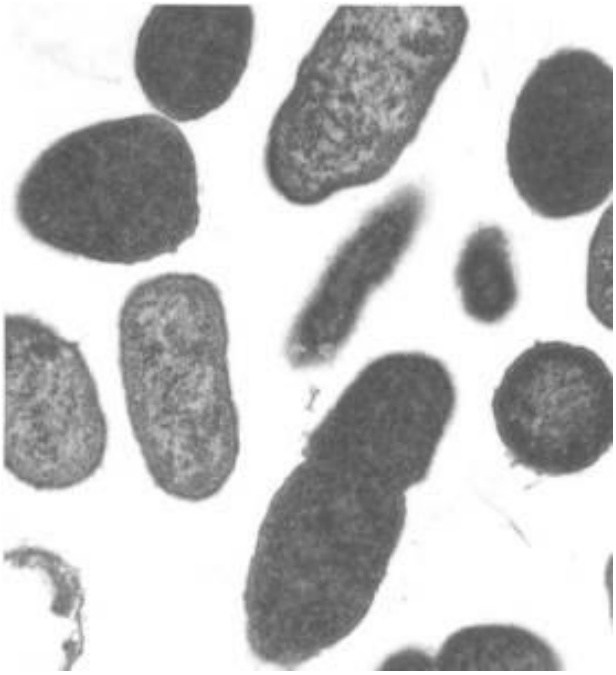

Figure 3: *Pseudomonas* sp. before exposure to Tricide - Neo^{TM}. Normal, Intact Cell Wall.

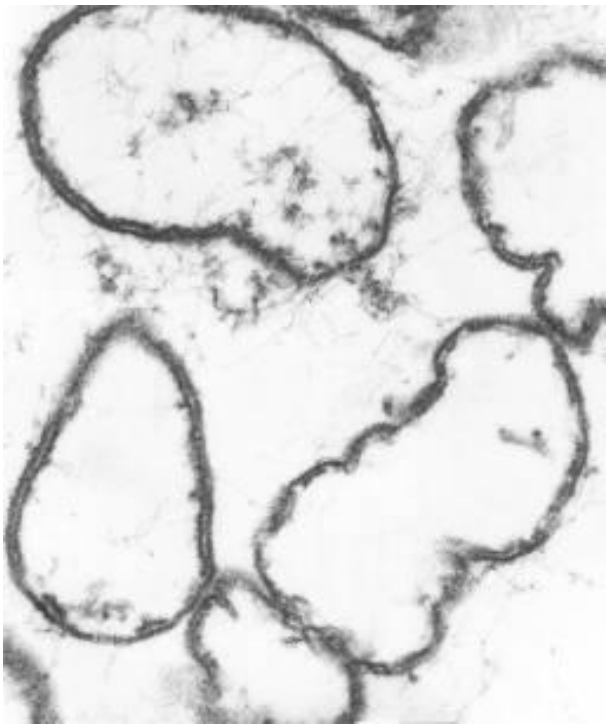

Figure 4: *Pseudomonas* sp. after exposure to Tricide - Neo^{TM}. Tricide ^{TM} Induced Cytoplasmic Leakage

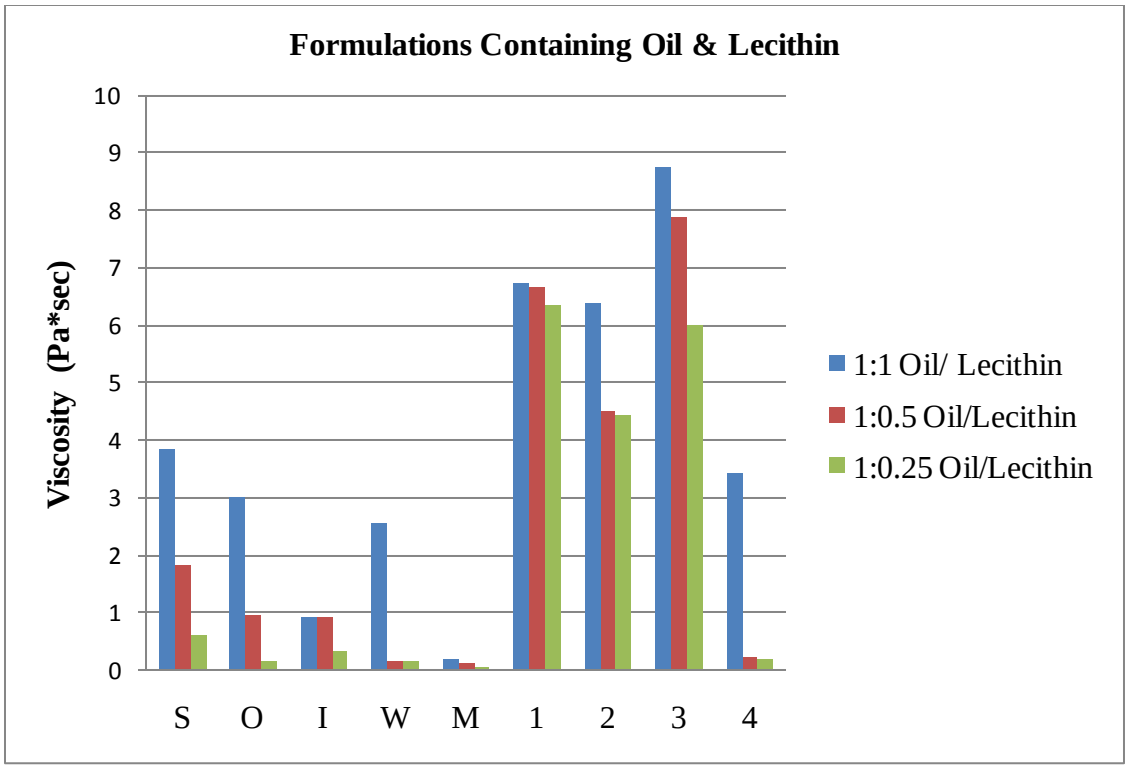

Figure 5: Viscometric data of formulations containing only oil and lecithin. The oil and lecithin ratios are as follows:1:1, 1:0.5, and 1:0.25. The sample composed of EO3 and lecithin at a 1:1 ratio had the highest viscosity. The oils are abbreviated as follows safflower oil (S), olive (O), isopropyl myristate (I), wheat germ oil (W), mineral oil (M), experimental oil 1 (1), experimental oil 2 (2), experimental oil 3 (3), experimental oil 4 (4).

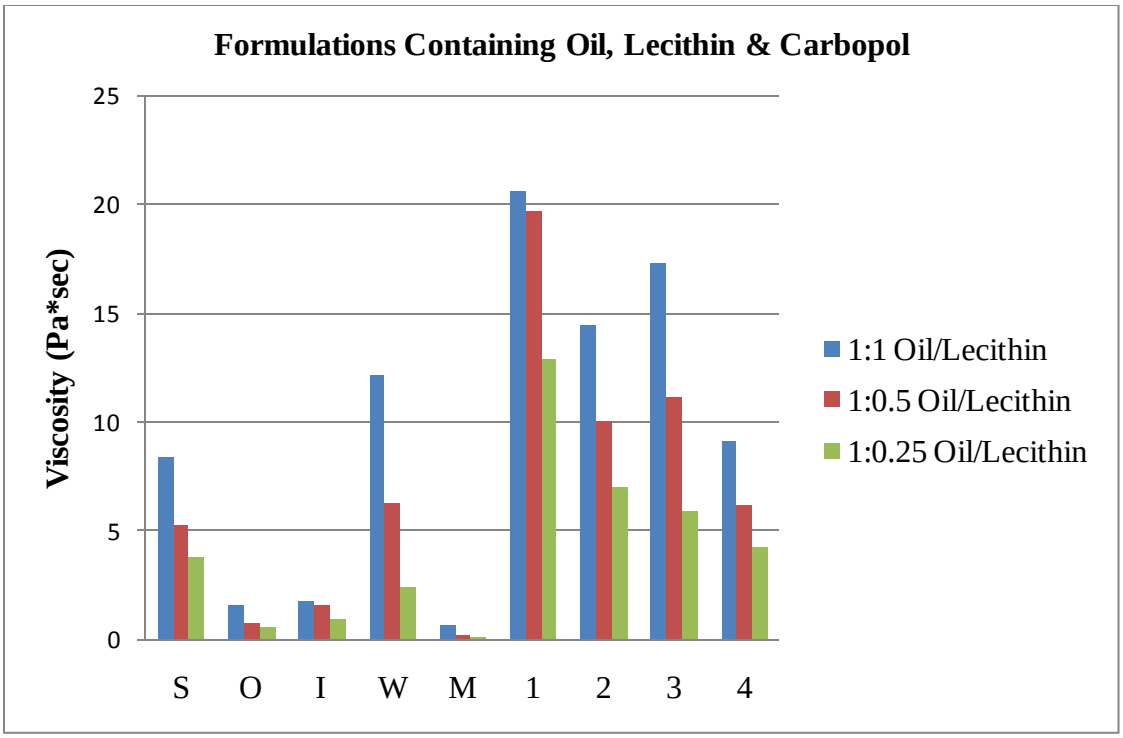

Figure 6: Viscometric data of formulations containing only oil, lecithin, and carbopol. The oil and lecithin ratios are as follows:1:1, 1:0.5, and 1:0.25. The sample composed of experimental oil 1 and lecithin at a 1:1 ratio had the highest viscosity.

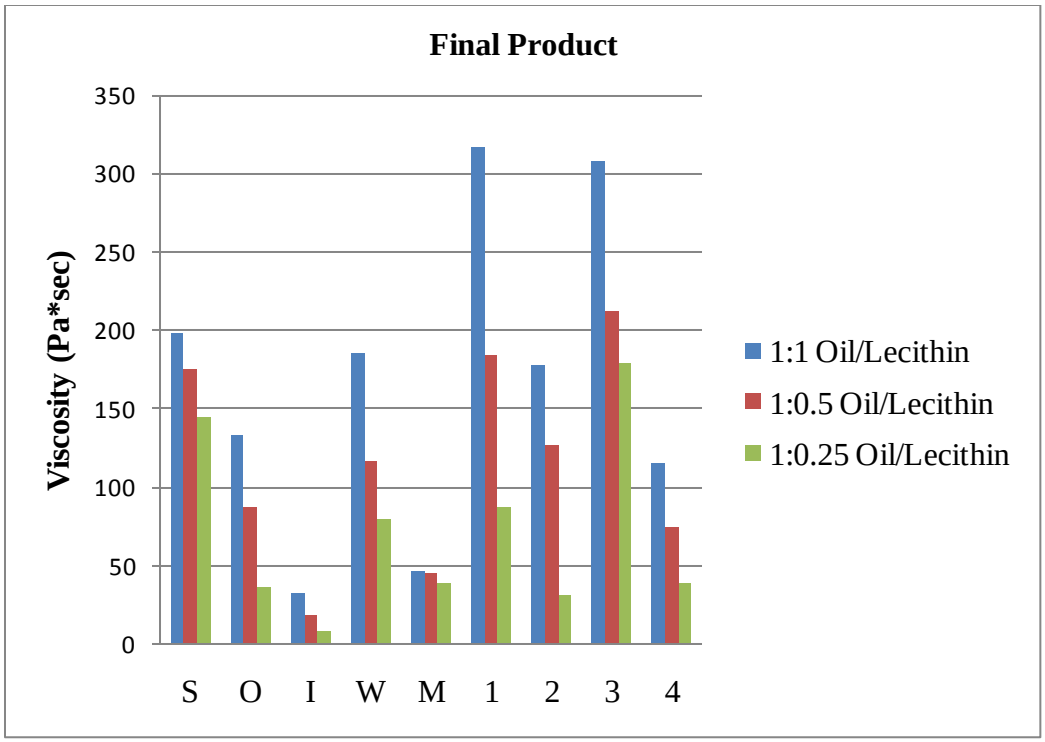

Figure 7: Viscometric data of formulations containing only oil, lecithin, carbopol, and adjuvants. The oil and lecithin ratios are as follows:1:1, 1:0.5, and 1:0.25. The samples composed of experimental oil and lecithin at a 1:1 ratio had the highest viscosity.

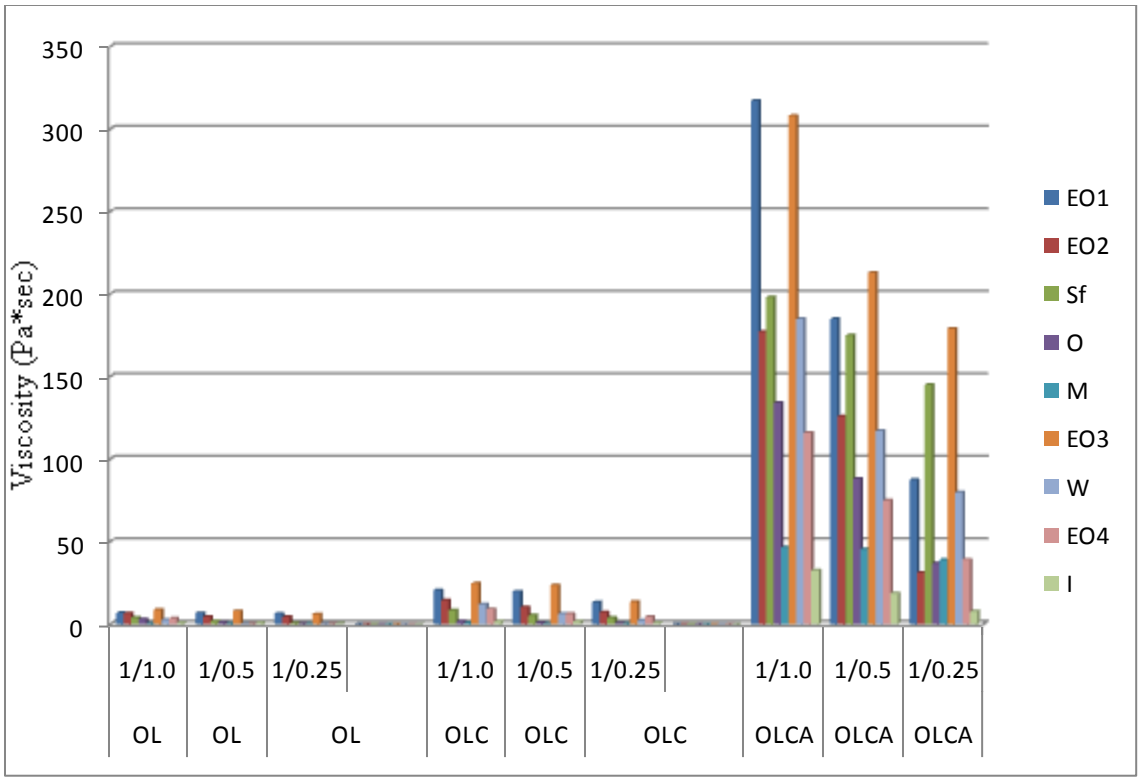

Figure 8: Comparison of viscometric data from figures 5, 6, and 7.

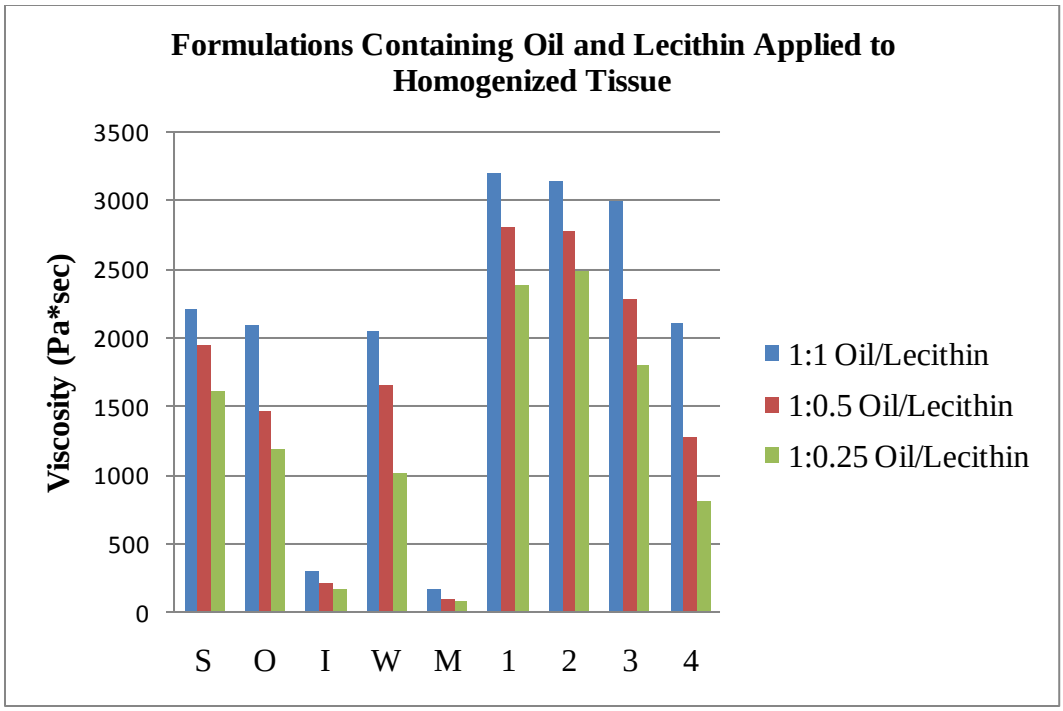

Figure 9: Viscometric data of formulations containing only oil and lecithin applied to homogenized tissue. One gram of sample containing oil and lecithin was incorporated with one gram of homogenized tissue. Viscosity was measured for these samples to gain insight on the bioadhesive properties of these systems.

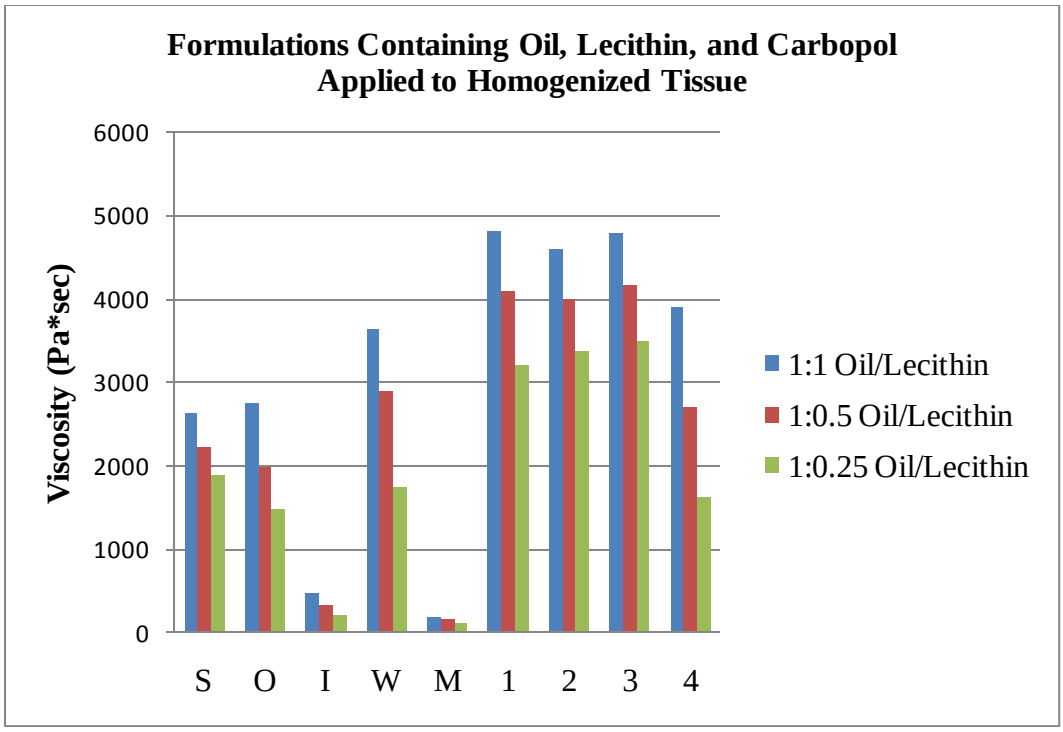

Figure 10: Viscometric data of formulations containing only oil, lecithin, and carbopol applied to homogenized tissue. One gram of sample containing oil, lecithin, and carbopol was incorporated with one gram of homogenized tissue. Viscosity was measured for these samples to gain insight on the bioadhesive properties of these systems.

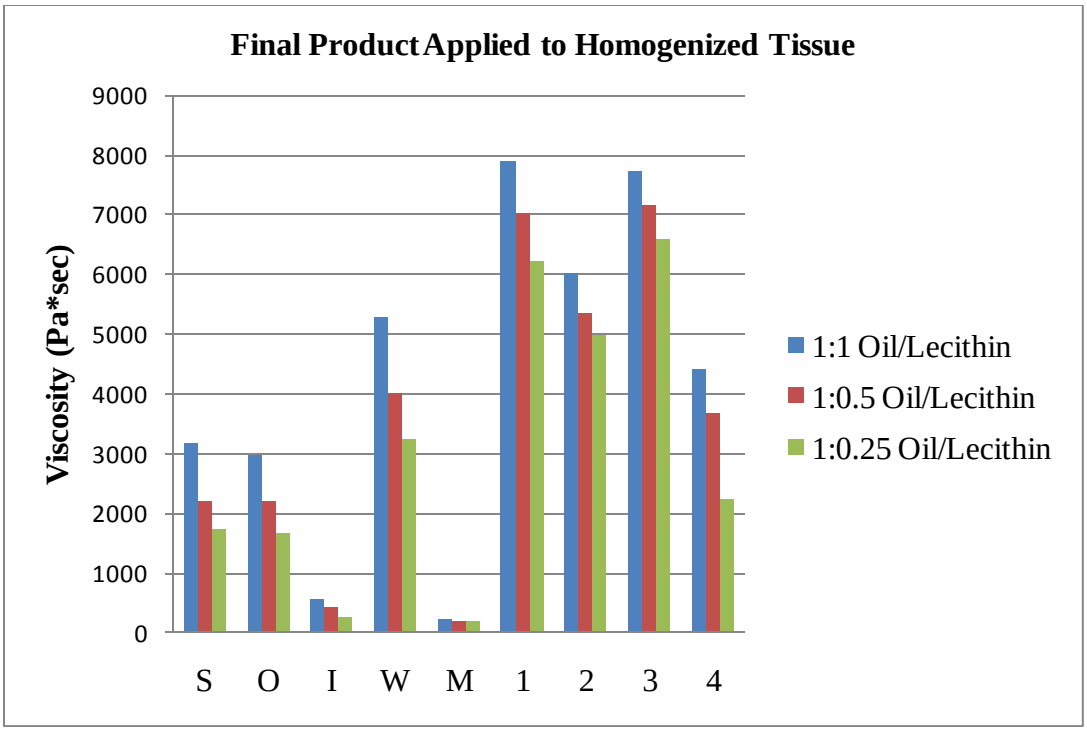

Figure 11: Viscometric data of formulations containing only oil, lecithin, carbopol, and adjuvants applied to homogenized tissue. One gram of sample containing oil, lecithin, carbopol, and adjuvants was incorporated with one gram of homogenized tissue. Viscosity was measured for these samples to gain insight on the bioadhesive properties of these systems.

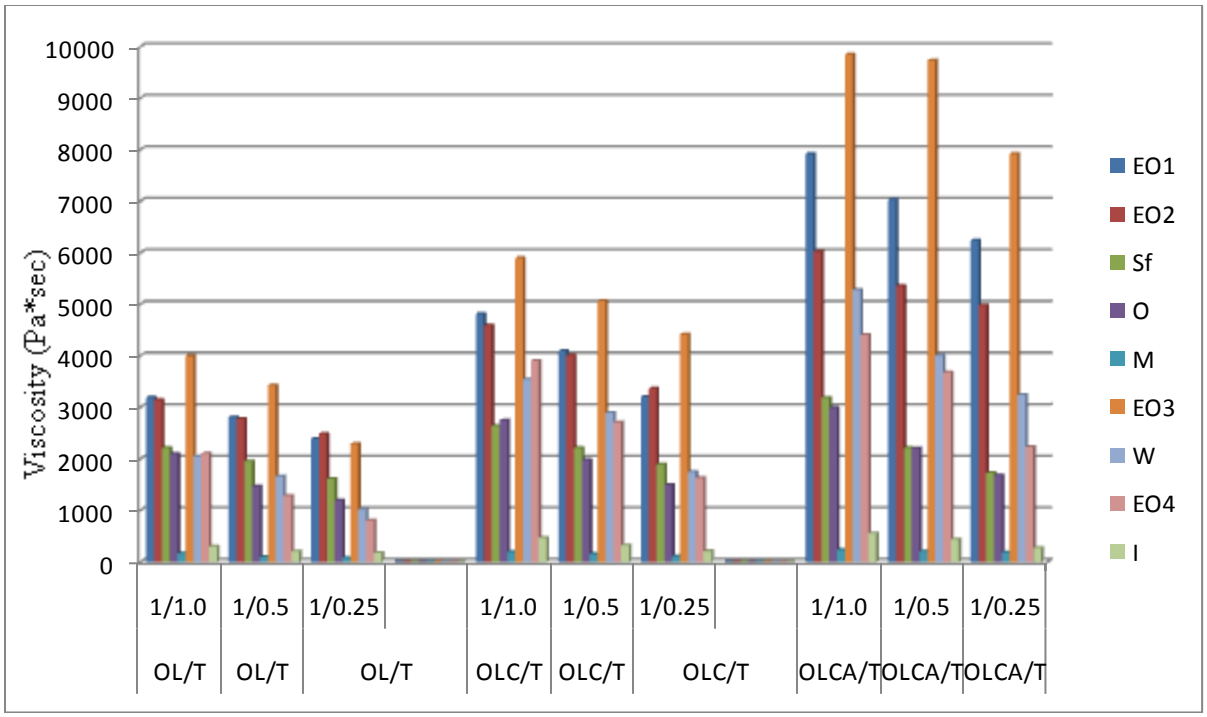

Figure 12: Comparison of viscometric data of formulations applied to homogenized tissue.

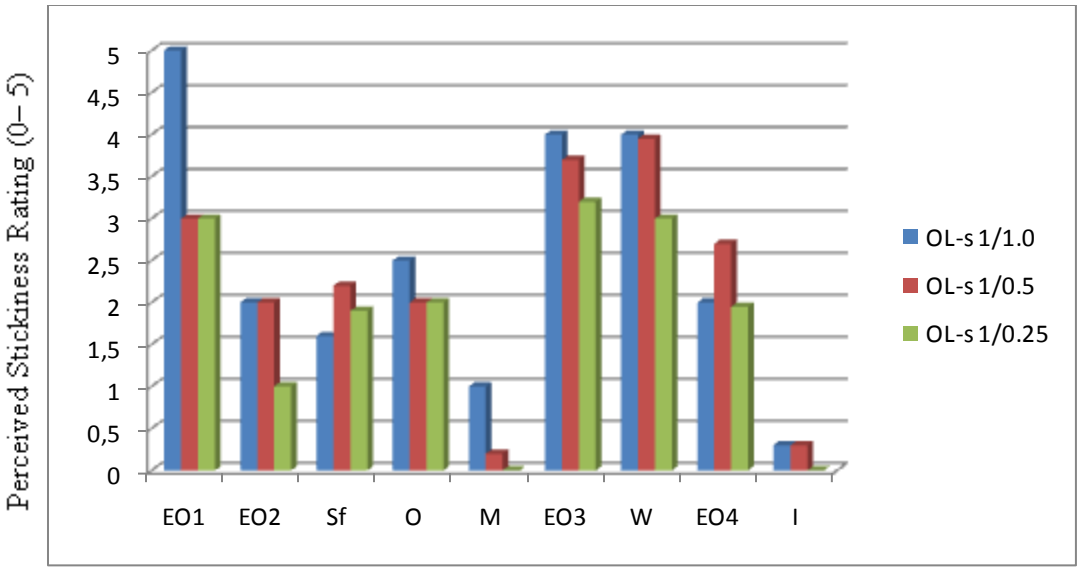

Figure 13: Perceived Stickiness of formulations containing only oil and lecithin. EO1 – EO4 are experimental proprietary oils; Sf = safflower oil; O = olive oil; M = mineral oil; W = wheat germ oil; I =isopropyl myristate.

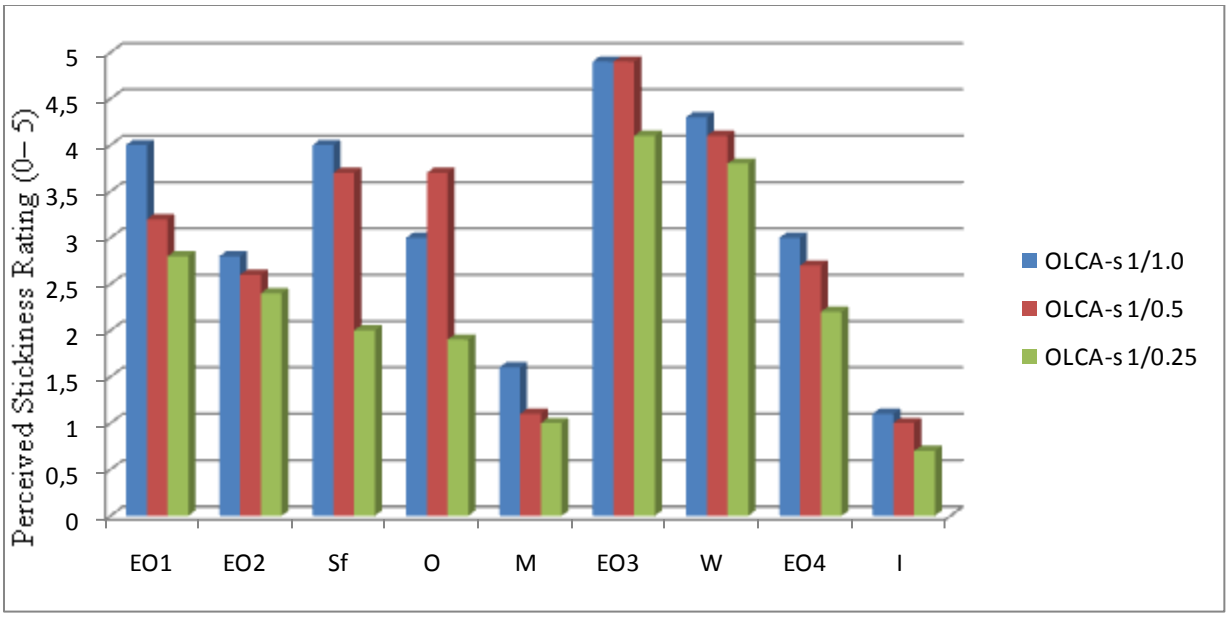

Figure 14: Perceived Stickiness of formulations containing oil, lecithin, carbopol and adjuvants (OLCA-s). EO1 – EO4 are experimental proprietary oils; Sf = safflower oil; O = olive oil; M = mineral oil; W = wheat germ oil; I =isopropyl myristate

Table 1:
Average ointment cohesive forces when gel spread between two layers of cellophane tape and separated[15]

GEL # BA9:	Mass Applied	Average Force Applied
Trial 1:	314 grams	average force: 1853 Newtons/m^2
	123	
	260	
	205	
GE: # BA7:		
Trial 1:	464 grams	average force: 3755 Newtons/m^2
	450	
	464	
Trial 2:	444 grams	average force: 3735 Newtons/m^2
	369	
	437	
	382	

Table 2:
Average ointment cohesive forces when gel spread between two layers of tissue (flank steak) and separated[15]

GEL # BA9:	Mass Applied	Average Force Applied
Trial 1:	400 grams	average force: 1,912.92 Newtons/m^2
	320	
Trial 2:	290	average force: 1,433.35 Newtons/m^2
	320	
	294	
GEL # BA7:		
Trial 1:	400	average force: 3,037.5 Newtons/m^2
	560	
Trial 2:	533	average force: 2,673.22 Newtons/m^2
	453	
	373	
Trial 3:	453	average force: 2,893.9 Newtons/m^2
	507	

Table 3: Bioadhesive Force Measurements[16]

$F_{bioadhesive}$ = (W x G)/A, where,
F = bioadhesive force, Newtons (N)/m^2, where (Newtons are *per* area (A), in meters squared)
W = detachment weight (the weight, in kilograms required to overcome gel adhesive force; N has units of {kg x m)/s^2})
G = acceleration due to gravity, m/s^2
A = area covered by gel that is subjected to adhesion test, in units of m^2 (the area measured had a constant value of 0.0012 m^2 determined from, $A = \pi r^2$ since the tested surface was a circle of diameter 3.9 cm or 0.039 m)
Calculations: F = W x G/A; F = W x (9.81$m/^2$)/0.0012 m^2

Table 4: Reported Viscosity for the oils used in study formulations.

Oil	Viscosity (Pa*sec)
EO 1	2.12
EO 2	1.0
Safflower	0.0178
Olive	0.084
Mineral	0.0024
EO 3	2.9
Wheat Germ	0.092
EO 4	0.110
Isopropyl Myristate	0.006

Table 5: Rheometric measurements of formulations containing oil and lecithin. Oil and lecithin are present in ratios of 1:1, 1:0.5, and 1:0.25. The sample composed of oil EO3 and lecithin at a 1:1 ratio had the highest viscosity.

Formulation	Viscosity (Pa*sec)	Rate of Shear (1/sec)	Bioadhesive Force (N/m^2)
1:1 EO1/Lecithin	6.7505	0.973	6.57
1:0.5 EO1/Lecithin	6.6795	0.973	6.50
1:0.25 EO1/Lecithin	6.3648	0.973	6.19
1:1EO2/Lecithin	6.4033	0.973	6.23
1:0.5EO2/Lecithin	4.5053	0.973	4.38
1:0.25EO2/Lecithin	4.4310	0.973	4.31
1:1 Safflower Oil / Lecithin	3.8572	0.973	3.75
1:0.5 Safflower Oil/ Lecithin	1.8136	0.973	1.76
1:0.25 Safflower Oil / Lecithin	0.6059	0.973	0.57
1:1 Olive Oil/ Lecithin	3.0218	0.973	2.94
1:0.5 Olive Oil / Lecithin	0.9651	0.973	0.94
1:0.25 Olive Oil / Lecithin	0.1427	0.973	0.14
1:1 Mineral Oil/ Lecithin	0.1770	0.973	0.17
1:0.5 Mineral Oil / Lecithin	0.1285	0.973	0.13
1:0.25 Mineral Oil / Lecithin	0.0528	0.973	0.05
1:1 EO3/ Lecithin	8.7463	0.973	8.51
1:0.5 EO3 / Lecithin	7.8916	0.973	7.68
1:0.25 EO3 / Lecithin	6.0154	0.973	5.85
1:1 Wheat Germ Oil/ Lecithin	2.5624	0.973	2.49
1:0.5 Wheat Germ Oil / Lecithin	0.1581	0.973	0.15
1:0.25 Wheat Germ Oil / Lecithin	0.1450	0.973	0.14
1:1 EO4/ Lecithin	3.4243	0.973	3.33
1:0.5 EO4 / Lecithin	0.2199	0.973	0.21
1:0.25 EO4 / Lecithin	0.1864	0.973	0.18
1:1 Isopropyl Myristate/ Lecithin	0.9380	0.973	0.91
1:0.5 Isopropyl Myristate / Lecithin	0.9279	0.973	0.90
1:0.25 Isopropyl Myristate / Lecithin	0.3457	0.973	0.34

Table 6: Rheometric measurements of formulations containing oil and lecithin. and carbopol (at ratios 1:1, 1:0.5, and 1:0.25). The sample composed of oil EO3 and lecithin at a 1:1 ratio had the highest viscosity. The sample composed of experimental oil 1 and lecithin at a 1:1 ratio had the highest viscosity.

Formulation	Viscosity (Pa*sec)	Rate of Shear (1/sec)	Bioadhesive Force (N/m^2)
1:1 EO1/Lecithin /Carbopol	20.5802	0.973	20.02
1:0.5 EO1/Lecithin /Carbopol	19.6994	0.973	19.17
1:0.25 EO1/Lecithin /Carbopol	12.8997	0.973	12.55
1:1EO2/Lecithin /Carbopol	14.4957	0.973	14.10
1:0.5EO2/Lecithin /Carbopol	10.0239	0.973	9.75
1:0.25EO2/Lecithin /Carbopol	6.9645	0.973	6.78
1:1 Safflower Oil / Lecithin /Carbopol	8.3928	0.973	8.17
1:0.5 Safflower Oil/ Lecithin /Carbopol	5.2308	0.973	5.09
1:0.25 Safflower Oil / Lecithin /Carbopol	3.8296	0.973	3.73
1:1 Olive Oil/ Lecithin /Carbopol	1.5507	0.973	1.51
1:0.5 Olive Oil / Lecithin /Carbopol	0.7541	0.973	0.73
1:0.25 Olive Oil / Lecithin /Carbopol	0.5441	0.973	0.53
1:1 Mineral Oil/ Lecithin /Carbopol	0.6405	0.973	0.62
1:0.5 Mineral Oil / Lecithin /Carbopol	0.2141	0.973	0.21
1:0.25 Mineral Oil / Lecithin /Carbopol	0.1463	0.973	0.14
1:1 EO3 / Lecithin /Carbopol	17.2678	0.973	16.80
1:0.5 EO3 / Lecithin /Carbopol	11.1823	0.973	10.88
1:0.25 EO3 / Lecithin /Carbopol	5.8919	0.973	5.73
1:1 Wheat Germ Oil/ Lecithin /Carbopol	12.1646	0.973	11.84
1:0.5 Wheat Germ Oil / Lecithin /Carbopol	6.2585	0.973	6.09
1:0.25 Wheat Germ Oil / Lecithin /Carbopol	2.3783	0.973	2.31
1:1 EO4/ Lecithin /Carbopol	9.1132	0.973	8.87
1:0.5 EO4 / Lecithin /Carbopol	6.2165	0.973	6.05
1:0.25 EO4 / Lecithin /Carbopol	4.2853	0.973	4.17
1:1 Isopropyl Myristate/ Lecithin /Carbopol	1.7269	0.973	1.68
1:0.5 Isopropyl Myristate / Lecithin /Carbopol	1.5679	0.973	1.53
1:0.25 Isopropyl Myristate / Lecithin /Carbopol	0.9267	0.973	0.90

Table 7: Rheometric measurements of final formulations (containing oil, lecithin. carbopol, and suitable adjuvants for use on aquatic animals). The oil to lecithin ratios are as follows: 1:1, 1:0.5, and 1:0.25. The sample composed of experimental oil 1 and lecithin at a 1:1 ratio had the highest viscosity.

Formulation	Viscosity (Pa*sec)	Rate of Shear (1/sec)	Bioad. Force (N/m^2)
1:1 EO1/Lecithin /Carbopol/Adjuvants	316.742	0.973	308.19
1:0.5 EO1/Lecithin /Carbopol/Adjuvants	184.867	0.973	179.88
1:0.25 EO1/Lecithin /Carbopol/Adjuvants	87.3052	0.973	84.95
1:1EO2/Lecithin /Carbopol/Adjuvants	177.405	0.973	172.62
1:0.5EO2/Lecithin /Carbopol/Adjuvants	126.444	0.973	123.03
1:0.25EO2/Lecithin /Carbopol/Adjuvants	31.2179	0.973	30.38
1:1 Safflower Oil / Lecithin /Carbopol/Adjuvants	198.0840	0.973	192.74
1:0.5 Safflower Oil/ Lecithin /Carbopol/Adjuvants	174.9220	0.973	170.20
1:0.25 Safflower Oil / Lecithin /Carbopol/Adjuvants	145.3630	0.973	141.44
1:1 Olive Oil/ Lecithin /Carbopol/Adjuvants	133.773	0.973	130.16
1:0.5 Olive Oil / Lecithin /Carbopol/Adjuvants	87.5471	0.973	85.18
1:0.25 Olive Oil / Lecithin /Carbopol/Adjuvants	36.7377	0.973	35.75
1:1 Mineral Oil/ Lecithin /Carbopol/Adjuvants	46.4024	0.973	45.15
1:0.5 Mineral Oil / Lecithin /Carbopol/Adjuvants	45.2829	0.973	44.06
1:0.25 Mineral Oil / Lecithin /Carbopol/Adjuvants	38.8559	0.973	37.81
1:1 EO3 / Lecithin /Carbopol/Adjuvants	307.8191	0.973	299.51
1:0.5 EO3 / Lecithin /Carbopol/Adjuvants	212.5144	0.973	206.78
1:0.25 EO3 / Lecithin /Carbopol/Adjuvants	178.9468	0.973	174.12
1:1 Wheat Germ Oil/ Lecithin /Carbopol/Adjuvants	185.638	0.973	180.63
1:0.5 Wheat Germ Oil / Lecithin /Carbopol/Adjuvants	117.271	0.973	114.10
1:0.25 Wheat Germ Oil / Lecithin /Carbopol/Adjuvants	79.9265	0.973	77.77
1:1 EO4/ Lecithin /Carbopol/Adjuvants	115.733	0.973	112.61
1:0.5 EO4/ Lecithin /Carbopol/Adjuvants	74.9571	0.973	72.93
1:0.25 EO4 / Lecithin /Carbopol/Adjuvants	39.1186	0.973	38.06
1:1 Isopropyl Myristate/ Lecithin /Carbopol/Adjuvants	32.728	0.973	31.84
1:0.5 Isopropyl Myristate / Lecithin /Carbopol/Adjuvants	18.5504	0.973	18.05
1:0.25 Isopropyl Myristate / Lecithin /Carbopol/Adjuvants	7.64096	0.973	7.43

Table 8: Rheometric measurements of formulations containing oil and lecithin applied to homogenized tissue. One gram of sample (oil and lecithin) was incorporated with one gram of homogenized tissue. Viscosity was measured for these samples to gain insight on the bioadhesive properties of these systems.

Formulation	Viscosity (Pa*sec)	Rate of Shear (1/sec)	Bioadhesive Force (N/m^2)
Tenderloin	2.3405		
1:1 EO1/Lecithin	3197.47	0.973	3111.14
1:0.5 EO1/Lecithin	2812.19	0.973	2736.26
1:0.25 EO1/Lecithin	2391.72	0.973	2327.14
1:1EO2/Lecithin	3150.71	0.973	3065.64
1:0.5EO2/Lecithin	2778.14	0.973	2703.13
1:0.25EO2/Lecithin	2490.67	0.973	2423.42
1:1 Safflower Oil / Lecithin	2217.83	0.973	2157.95
1:0.5 Safflower Oil/ Lecithin	1953.69	0.973	1900.94
1:0.25 Safflower Oil / Lecithin	1613.97	0.973	1570.39
1:1 Olive Oil/ Lecithin	2097.17	0.973	2040.55
1:0.5 Olive Oil / Lecithin	1469.08	0.973	1429.41
1:0.25 Olive Oil / Lecithin	1194.46	0.973	1162.21
1:1 Mineral Oil/ Lecithin	173.32	0.973	168.64
1:0.5 Mineral Oil / Lecithin	102.85	0.973	100.07
1:0.25 Mineral Oil / Lecithin	79.33	0.973	77.19
1:1 EO3/ Lecithin	3005.19	0.973	2924.05
1:0.5 EO3 / Lecithin	2287.63	0.973	2225.86
1:0.25 EO3 / Lecithin	1801.22	0.973	1752.59
1:1 Wheat Germ Oil/ Lecithin	2049.31	0.973	1993.98
1:0.5 Wheat Germ Oil / Lecithin	1663.12	0.973	1618.22
1:0.25 Wheat Germ Oil / Lecithin	1021.78	0.973	994.19
1:1 EO4/ Lecithin	2108.54	0.973	2051.61
1:0.5 EO4 / Lecithin	1283.19	0.973	1248.54
1:0.25 EO4 / Lecithin	811.92	0.973	790.00
1:1 Isopropyl Myristate/ Lecithin	302.10	0.973	293.94
1:0.5 Isopropyl Myristate / Lecithin	211.08	0.973	205.38
1:0.25 Isopropyl Myristate / Lecithin	176.84	0.973	172.07

Table 9: Rheometric measurements of formulations containing oil, lecithin, and carbopol applied to homogenized tissue. One gram of sample (oil, lecithin, and carbopol) was incorporated with one gram of homogenized tissue. Viscosity was measured for these samples to gain insight on the bioadhesive properties of these systems.

Formulation	Viscosity (Pa*sec)	Rate of Shear (1/sec)	Bioadhesive Force (N/m^2)
Tenderloin	2.3405		
1:1 EO1/Lecithin /Carbopol	4815.21	0.973	4685.20
1:0.5 EO1/Lecithin /Carbopol	4097.67	0.973	3987.03
1:0.25 EO1/Lecithin /Carbopol	3204.75	0.973	3118.22
1:1EO2/Lecithin /Carbopol	4596.36	0.973	4472.26
1:0.5EO2/Lecithin /Carbopol	4012.18	0.973	3903.85
1:0.25EO2/Lecithin /Carbopol	3369.79	0.973	3278.81
1:1 Safflower Oil / Lecithin /Carbopol	2643.09	0.973	2571.73
1:0.5 Safflower Oil/ Lecithin /Carbopol	2217.91	0.973	2158.03
1:0.25 Safflower Oil / Lecithin /Carbopol	1896.45	0.973	1845.25
1:1 Olive Oil/ Lecithin /Carbopol	2746.18	0.973	2672.03
1:0.5 Olive Oil / Lecithin /Carbopol	1979.56	0.973	1926.11
1:0.25 Olive Oil / Lecithin /Carbopol	1493.47	0.973	1453.15
1:1 Mineral Oil/ Lecithin /Carbopol	197.76	0.973	192.42
1:0.5 Mineral Oil / Lecithin /Carbopol	154.39	0.973	150.22
1:0.25 Mineral Oil / Lecithin /Carbopol	111.68	0.973	108.66
1:1 EO3 / Lecithin /Carbopol	4780.23	0.973	4651.16
1:0.5 EO3 / Lecithin /Carbopol	4163.46	0.973	4051.05
1:0.25 EO3 / Lecithin /Carbopol	3501.29	0.973	3406.76
1:1 Wheat Germ Oil/ Lecithin /Carbopol	3648.57	0.973	3550.06
1:0.5 Wheat Germ Oil / Lecithin /Carbopol	2894.01	0.973	2815.87
1:0.25 Wheat Germ Oil / Lecithin /Carbopol	1752.34	0.973	1705.03
1:1 EO4/ Lecithin /Carbopol	3906.11	0.973	3800.65
1:0.5 EO4 / Lecithin /Carbopol	2714.53	0.973	2641.24
1:0.25 EO4 / Lecithin /Carbopol	1637.48	0.973	1593.27
1:1 Isopropyl Myristate/ Lecithin /Carbopol	469.48	0.973	456.80
1:0.5 Isopropyl Myristate / Lecithin /Carbopol	326.24	0.973	317.43
1:0.25 Isopropyl Myristate / Lecithin /Carbopol	212.63	0.973	206.89

Table 10: Rheometric measurements of final formulations applied to homogenized tissue. One gram of sample (final formulation) was incorporated with one gram of homogenized tissue. Viscosity was measured for these samples to gain insight on the bioadhesive properties of these systems.

Formulation	Viscosity (Pa*sec)	Rate of Shear (1/sec)	Bioadhesive Force (N/m^2)
Tenderloin	2.3405		
1:1 EO1/Lecithin /Carbopol/Adjuvants	7916.25	0.973	7702.51
1:0.5 EO1/Lecithin /Carbopol/Adjuvants	7036.59	0.973	6846.60
1:0.25 EO1/Lecithin /Carbopol/Adjuvants	6241.38	0.973	6072.86
1:1EO2/Lecithin /Carbopol/Adjuvants	6024.17	0.973	5861.52
1:0.5EO2/Lecithin /Carbopol/Adjuvants	5361.75	0.973	5216.98
1:0.25EO2/Lecithin /Carbopol/Adjuvants	4981.34	0.973	4846.84
1:1 Safflower Oil / Lecithin /Carbopol/Adjuvants	3190.02	0.973	3103.89
1:0.5 Safflower Oil/ Lecithin /Carbopol/Adjuvants	2217.68	0.973	2157.80
1:0.25 Safflower Oil / Lecithin /Carbopol/Adjuvants	1733.41	0.973	1686.61
1:1 Olive Oil/ Lecithin /Carbopol/Adjuvants	2989.57	0.973	2908.85
1:0.5 Olive Oil / Lecithin /Carbopol/Adjuvants	2204.83	0.973	2145.30
1:0.25 Olive Oil / Lecithin /Carbopol/Adjuvants	1684.35	0.973	1638.87
1:1 Mineral Oil/ Lecithin /Carbopol/Adjuvants	240.38	0.973	233.89
1:0.5 Mineral Oil / Lecithin /Carbopol/Adjuvants	211.82	0.973	206.10
1:0.25 Mineral Oil / Lecithin /Carbopol/Adjuvants	188.58	0.973	183.49
1:1 EO3 / Lecithin /Carbopol/Adjuvants	7731.78	0.973	7523.02
1:0.5 EO3 / Lecithin /Carbopol/Adjuvants	7169.36	0.973	6975.79
1:0.25 EO3 / Lecithin /Carbopol/Adjuvants	6591.23	0.973	6413.27
1:1 Wheat Germ Oil/ Lecithin /Carbopol/Adjuvants	5282.73	0.973	5140.10
1:0.5 Wheat Germ Oil / Lecithin /Carbopol/Adjuvants	4013.88	0.973	3905.51
1:0.25 Wheat Germ Oil / Lecithin /Carbopol/Adjuvants	3245.62	0.973	3157.99
1:1 Solgar/ Lecithin /Carbopol/Adjuvants	4410.17	0.973	4291.10
1:0.5 Solgar / Lecithin /Carbopol/Adjuvants	3679.49	0.973	3580.14
1:0.25 Solgar / Lecithin /Carbopol/Adjuvants	2232.10	0.973	2171.83
1:1 Isopropyl Myristate/ Lecithin /Carbopol/Adjuvants	553.62	0.973	538.67
1:0.5 Isopropyl Myristate / Lecithin /Carbopol/Adjuvants	439.42	0.973	427.56
1:0.25 Isop. Myristate / Lecithin /Carbopol/Adjuvants	272.21	0.973	264.86

Table 11. Perceived Stickiness of nine oils with differing concentrations of lecithin (OL-s), and the same nine oils formulated with oil, lecithin, carbopol and adjuvants (OLCA-s). EO1 – EO4 are experimental oils with proprietary value; Sf = safflower oil; O = olive oil; M = mineral oil; W = wheat germ oil; I = isopropyl myristate. The Table values are averages from a five point Likert scale utilizing 24 participants. The scale was calibrated such that 5 was most sticky and 1 least sticky. The study and informed consent document were approved by the UGA IRB (IRB Study 1, and IRB Consent 1.)

	OL-s	OL-s	OL-s		OLCA-s	OLCA-s	OLCA-s
	1/1.0	1/0.5	1/0.25		1/1.0	1/0.5	1/0.25
EO1	5	3	3	EO1	4	3.2	2.8
EO2	2	2	1	EO2	2.8	2.6	2.4
Sf	1.6	2.2	1.9	Sf	4	3.7	2
O	2.5	2	2	O	3	3.7	1.9
M	1	0.2	0	M	1.6	1.1	1
EO3	4	3.7	3.2	EO3	4.9	4.9	4.1
W	4	3.95	3	W	4.3	4.1	3.8
EO4	2	2.7	1.95	EO4	3	2.7	2.2
I	0.3	0.3	0	I	1.1	1	0.7

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CHAPTER TWO

TRANSDERMAL DRUG DELIVERY

Abstract

The current investigation reports the synthesis of three novel mutual prodrugs (MP) which couple n-acetyl-glucosamine with an NSAID, either ketoprofen or ibuprofen. They were evaluated for transdermal permeation using shed snakeskin, and to our knowledge represent the first MPs synthesized for this purpose, although they could be used for oral or subcutaneous delivery as well. MPs are defined as two active drug compounds usually connected by an ester linkage. Glucosamine administration has been linked to damaged cartilage repair, and pain relief in joints afflicted with osteoarthritis. NSAIDs are commonly used orally and in transdermal creams or gels for joint pain relief. The two novel compounds we report (MP1 and MP 2) covalently link ibuprofen and ketoprofen directly to the amide nitrogen of n-acetyl-glucosamine (NAG); the other compound (MP3) covalently links ibuprofen to the amide nitrogen, using a short chain acetyl linker. Permeability studies show that the ketoprofen mutual prodrug (MP2) permeates shed snakeskin more than three times greater than either ibuprofen derivative, while ethanol markedly increases the permeation for all three. The ketoprofen mutual prodrug appears the most likely candidate for transdermal administration; the ibuprofen mutual prodrugs for oral delivery; all three mutual prodrugs may be candidates for oral delivery or subcutaneous injection.

Keywords: transdermal permeation, mutual prodrug, NSAID, osteoarthritis, N-Acetyl-Glucosamine, glucosamine

INTRODUCTION TO TRANSDERMAL DRUG DELIVERY

Transdermal drug delivery systems offer an alternative to delivering drugs orally and may provide an alternative to hypodermic injections. For many years, therapeutic agents have been applied to the skin. Overtime, a variety of topical formulations have been developed to treat local conditions. Transdermal delivery is favored over oral delivery when oral delivery results in low bioavailability, multi-dose delivery is required or steady delivery is necessary. The first transdermal, systemic delivery was approved in the United States in 1979. This three-day patch delivered scopolamine to treat motion sickness. Ten years later, the development of the nicotine patch increased interest in transdermal delivery. Transdermal delivery systems currently transport analgesic agents, contraceptives and hormones used in hormone replacement therapy. From 1979 until 2002, new transdermal patches were approved on average of one every 2 years. During the years 2003–2007, that rate tripled to a new transdermal delivery system every 7.5 months. This rate is expected to continue to rise exponentially.[22]

There are many advantages to utilizing transdermal delivery compared to other delivery systems. In particular, it is used when there is a significant first-pass effect of the liver that can prematurely metabolize drugs. Transdermal delivery is preferable to hypodermic injections, which are painful and can potentially generate dangerous medical waste and pose the risk of disease transmission by needle re-use, especially in developing countries. In addition, transdermal systems are non-invasive and can be self-administered. They can provide release drug for several hours to several days. They also increase patient compliance and are generally inexpensive compared to other delivery methods. The main challenge for transdermal delivery is the requirements of drug candidates. Transdermal drugs have molecular masses that are only up to a few hundred Daltons. They also exhibit octanol-water partition coefficients that heavily

favor lipids and require doses of milligrams per day or less. It has been difficult to exploit the transdermal route to deliver hydrophilic drugs; the transdermal delivery of peptides and macromolecules, including new genetic treatment employing DNA or small-interfering RNA (siRNA), has posed particular challenges.[26]

Another area of great interest is the delivery of vaccines. In addition to avoiding hypodermic needles, transdermal vaccine delivery could improve immune responses by targeting delivery to immunogenic Langerhans cells in the skin. Given the external placement and patient control over patches, it might also be possible to develop modulated delivery, which could involve feedback control. Recently, an analgesic patch was approved in the United States that uses patient-regulated delivery of fentanyl modulated by electricity to control pain (iontophoresis). This product has also been researched in European laboratories.

Drug delivery is not the only manner in which transdermal technology can render itself useful to the scientific community and the populations we serve. There is a possibility that molecules can be extracted through the skin. This has already been achieved for glucose monitoring by extracting interstitial fluid using electrical means and other approaches, such as ultrasound. Transdermal delivery systems can be categorized as undergoing three generations of development from the first generation of systems that produced many of today's patches by cautious selection of drugs that can cross the skin at therapeutic rates with little or no enhancement; through the second generation that has yielded additional advances for small molecule delivery by increasing skin permeability and driving forces for transdermal transport; to the third generation that will enable transdermal delivery of small molecule drugs, macromolecules (including proteins and DNA) and vaccines.[8]

First generation transdermal formulations consist of most transdermal patches that are currently in clinical use. Significant advances in patch technology and public acceptance is responsible for the recent rise in first-generation transdermal patches on the market. Since it is necessary for viable first-generation delivery candidates to be low-molecular weight, lipophilic and efficacious at low doses, there is a limit on the number of formulations that can be developed. The stratum corneum is the outermost layer of the skin and provides the body with a physical barrier. Since first-generation transdermal formulations don't utilize enhancing agents, any compound has to cross the 10 to 20 μm thick layer without enhancers in order to be considered a viable medicament. Underneath this layer is the epidermis, which measures 50 to 100 μm and is avascular. Deeper still is the dermis, which is 1–2 mm thick and contains a rich capillary bed for systemic drug absorption just below the dermal–epidermal junction.

Closer examination of the stratum corneum barrier reveals a brick and mortar structure, where the bricks represent non-living corneocyte cells composed primarily of cross-linked keratin and the intercellular mortar is a mixture of lipids organized largely in bilayers. Drug transport across the stratum corneum typically involves diffusion through the intercellular lipids via a path that winds tortuously around corneocytes, where hydrophilic molecules travel through the lipid head group regions and lipophilic molecules travel through the lipid tails. This transport pathway is highly constrained by the structural and solubility requirements for solution and diffusion within stratum corneum lipid bilayers.[23] A variation on the traditional transdermal patch of first-generation delivery systems involves no patch at all, but applies a metered liquid spray, gel or other topical formulation to the skin that, upon evaporation or absorption, can drive small lipophilic drugs into the stratum corneum, which in turn serves as the drug reservoir for extended release into the viable epidermis over hours. For example, testosterone gels have been

in use for several years and a transdermal spray has been recently approved for estradiol delivery.

The second generation transdermal delivery systems take into consideration that permeation is difficult for large molecules and polar compounds. Many of the second generation drugs are formulated with chemical excipients. Second generation transdermal chemical enhancers generally disrupt the bilayer of the intracellular lipids in the stratum corneum by inserting amphiphilic molecules into the bilayers, which cause molecular packing to become less uniform or by extracting lipids using solvents and surfactants to create lipid packing defects of nanometer dimensions. Permeability enhancement is necessary to increase the development of novel transdermal drugs. The optimal enhancing agent should increase skin permeability by reversibly disrupting stratum corneum structure. It is also necessary that this agent provide an additional driving force for drug transport into the skin without causing injury to deeper tissues.

However, enhancement methods developed in this generation, such as conventional chemical enhancers, iontophoresis and non-cavitational ultrasound, have struggled with the balance between achieving increased delivery across stratum corneum, while protecting deeper tissues from damage. Iontophoresis uses a low voltage current as its driving force. This application is ideal for small charged molecules and some macromolecules that are less than 3,000 Daltons. The pressure gradient and oscillation associated with ultrasound act as a driving force to transport drugs across the stratum corneum when non-cavitational ultrasound technology is used. Non-cavitational ultrasound temporarily disrupts the lipid structure of the stratum corneum and thereby increases drug permeation. As a result, this second generation of delivery systems has advanced clinical practice primarily by improving small molecule delivery for

localized, dermatological, cosmetic and some systemic applications, but has made little impact on delivery of macromolecules.[16]

The third generation transdermal delivery systems are designed to improve stratum corneum penetration. It is important when incorporating any enhancing agents that there is little to no damage inflicted on tissue in the other layers of the skin. Isolating the treatment to the stratum corneum increases the damage applied to this barrier and in turn increases effective transdermal delivery. Chemical enhancers, microneedles, electroporation, cavitational ultrasound, thermal ablation and microdermabrasion have been shown to deliver macromolecules, including therapeutic proteins and vaccines, across the skin in human clinical trials. Transdermal drug delivery systems have evolved into an excellent choice for drug administration. The following manuscript has been submitted but notification of its acceptance has not been received.

Historical objectives of prodrug synthesis and development were to improve drug stability and to target drug delivery, for drugs administered orally and IV. [34]Stability is the key to drug activity; and for water and enzyme labile drugs stability is achieved by protecting the drug from chemical hydrolysis and enzyme degradation subsequent to drug administration. Targeted delivery for prodrugs is based on enhancing drug lipid solubility and permeability and is especially important in lipid membranes in order to penetrate hydrophobic regions. The most common form of prodrug utilizes an ester linkage formed synthetically through reaction of a carboxylic acid with an alcohol to modify the parent drug's in vivo metabolic fate. The ester prodrug may also possess other advantages like reduced side effects. For example gastric distress should be reduced if the prodrug were formulated with a nonsteroidal anti-inflammatory drug (NSAID) as compared to the NSAID alone. [9]

In contrast a typical mutual prodrug (MP) is composed of two active drug compounds also linked by an ester as for a prodrug. [40] The concept arises from the practice of clinically co-administering two drugs in order to enhance pharmacological activity or prevent clinical side effects. [43] The MP concept aims to produce a more efficacious product; based on the same principles as for prodrug synthesis; such that the ester linkage is easily degraded by mammalian esterases or hydrolysis. The linking of the two drugs imparts a protective effect that decreases degradation/toxicity. MPs also exhibit different aqueous/lipid solubility profiles, which usually aids in formulation and/or delivery, just as for prodrugs. Initially, they were named chimera prodrugs, since composed of two parts; however mutual prodrug is a better description since both entities are active.[16]

The current research uses this MP model; with the initial goal to develop a transdermal compound for local delivery of N-acetyl-glucosamine (NAG), to osteoarthritic joints, with the knowledge that a subcutaneous or oral formulation would also be a viable delivery method.[10, 11] The three compounds synthesized are mutual prodrugs of NAG and an NSAID; and are potentially active anti-osteoarthritis therapeutic agents since they release both NAG and an NSAID following cleavage of the amide bond by either chemical hydrolysis or an amide esterase. Two compounds directly link ibuprofen or ketoprofen, MP1 and MP2, respectively to the amide nitrogen on glucosamine. [19] The other novel compound, MP3, covalently links ibuprofen to the amide nitrogen of glucosamine using a short chain acetyl linker. To the best of our knowledge these compounds represent the first MPs synthesized for and shown to be useful for transdermal delivery.[17]

MATERIALS AND METHODS

All reagents and solvents utilized were purchased from Fisher Scientific. TLC and preparative TLC chromatographs were performed on Analtech Co. Uniplates. Melting points were determined on a Fisher-Johns apparatus and are uncorrected. Nuclear magnetic resonance spectra were recorded on Varian Inova 500 MHz spectrometer for 1H NMR and 13C NMR with tetramethysilane as an internal stand. Chemical shifts are reported in parts per million (ppm) and signals are reported as s (singlet), d (doublet), t (triplet), m (multiplet), or br (broad singlet). A Beckman DU-650 and a Thermo Electron Corp. Aquamate were used to record the UV spectra. Staff at the University of Georgia's Chemical and Biological Sciences Mass Spectrometry Facility completed ESI (electrospray ionization) mass spectra. Column chromatographs were performed using silica gel >440 mesh.

In-vitro Membrane Permeation and Analysis of MP

Shed snakeskins were used as a model membrane for all permeation studies using MP in phosphate buffer (pH 7.4, 0.1M) solutions (PBS) and in PBS containing 10% ethanol. [18] The skins were hydrated in distilled water at room temperature for 30 minutes before use to allow for complete hydration. Franz-cell diffusion experiments were carried out; receptor cells were filled with pH 7.4, 0.1 M PBS and the donor cell filled with MP in 0.1 M PBS/10% ethanol solution. Receptor solutions were maintained at 37°C and stirred with a magnetic stirrer. The snakeskins were mounted between the receptor and donor cells. The surface exposed to diffusion was 2.54 cm2 (diameter 1.8 cm) and the receptor cell volume was 6 cm3; MP1 – 3, were added to their respective donor cells as saturated PBS or PBS-10% ethanol solution [18]; donor cells were covered with plastic film to prevent evaporation. The system was allowed to equilibrate at 37°C for two hours before each experiment. Triplicate samples were taken over a 24 hour period; 200

µl samples of receptor solution were removed and replaced with fresh buffer. The amounts of NAG permeating through the snakeskin were determined by HPAE-PAD.[5,21]

MP analysis was carried out using high-performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD); Dionex, Sunnyvale, CA USA): Dionex DX-500 HPLC system consisting of a GP40 gradient pump, ED40 Electrochemical detector, AS3500 autosampler and PeakNet Chromatography Workstation. [12-15] The HPAE-PAD was equipped with CarboPac™ PA20 (3 x 150 mm), analytical anion-exchange column for the rapid, high resolution separation of monosaccharides and disaccharides, using pulsed amperometric detection, [12-15], and a CarboPac PA20 analytical guard column (3 x 30 mm) and a carbonate trap column (25 x 15 mm). Mobile phase (A) was degassed and deionized water. Mobile phase (B) consisted of 0.02 N NaOH prepared with deionized water and filtered with 0.45 µm filters in a solvent filtration apparatus (Waters-Millipore, Milford, MA, USA) that was degassed under vacuum. The mobile phase system was run at a gradient concentration of 16 mM NaOH at a flow rate of 0.5 ml/min. NAG was used as the standard since the NSAIDs used are not detected by HPAE-PAD. A standard calibration curve of NAG was obtained with linear regression and value of $R_2 = 0.9934$ in 0.1 M phosphate buffer at pH 7.4. Each sample set was run with external standards. The sample concentration values were obtained via the Peak Net software. These values were compared with to those obtained by calculations of the peak area and peak height observed as functions of the standard curve linear regression equation. The instrument LOD (limit of detection) was 0.05 ng/ml.

Permeation was determined from the increasing amount of NAG in the receptor medium, and cumulative steady state permeation from plots of cumulative amount of NAG, per unit area

(μg/cm^2) that permeated through the snakeskin *versus* time (Figures 15 -17; Table 12); where the slope of the linear portion of the plot was used to determine steady state flux, Jss (μg/cm^2/hr) for each MP. The latter calculated using Fick's first law from plots of M (t) = Jss x t where M(t) is the cumulative amount of MP that has permeated through the snakeskin per unit time (t), and the slope (Jss) is the steady state flux [10, 18].Permeation was rapid with almost no lag time observed for permeation of the ethanol laced phosphate buffer solutions of NAG after examination of the intersection of the linear portion of the plots with the x-axis.

In vitro hydrolysis studies

Hydrolysis rates of MP1 – 3 were studies, as described below, at 37˚C in 0.1M PBS solution at pH 7.4; hydrolysis half-life is reported in Table 12. [19, 33] MP1 – 3 samples were weighed and placed in 1L of buffer and stirred continuously. Aliquots of 5ml were withdrawn at various time intervals from 0 minutes to 12 hours and measured at the uv spectral maxima of either ibuprofen (220 nm; log € 351) or ketoprofen (260 nm; log € 335). Over the time interval the initial spectrum of the individual MP increased in magnitude with time as either ibuprofen or ketoprofen appeared; the half-life of the appearance was calculated from: lnC = lnCo- kt.

In vitro solubility studies

The aqueous solubility of finely divided MP1 - 3 was determined by equilibrating excess amounts in PBS at pH7.4 at 37˚C for 1.0 hour. After equilibration the samples were filtered (0.45μm pore size; Whatman Inc. Haverhill, MA); and immediately assayed by ultraviolet spectrometry, in triplicate. [6]

Synthetic Procedures

Procedure for Scheme 1 (ibuprofen directly linked to NAG, MP 1)(Figure 18):

Ibuprofen (compound 1 in scheme 1) (2.1g, 10 mmoles) was dissolved in 50 mL of dichloromethane. To this stirred solution was added, one drop of DMF as catalyst and two equivalents of oxalyl chloride (1.7 ml). The mixture was allowed to stir at room temperature until the evolution of carbon dioxide ceased. The solvent was then removed under reduced pressure and the residue (Ibuprofen acid chloride, compound 2) was dissolved in 5 ml of dichloromethane and used in the next reaction without further purification. Glucosamine hydrochloride (compound 3; 2.16 grams, 10 mmoles) was suspended in 200 ml of methanol.[39]

Triethylamine (3 mL, ~3 equivalents) was added and the mixture allowed to stirred at room temperature until the glucosamine had dissolved. To this solution was added the ibuprofen acid chloride from above and mixture was stirred at room temperature, during which the product began to crystallize from the reaction mixture. Thin Layer Chromatography (15 % methanol in chloroform) showed complete conversion to the amide (~1.5 hrs). The solid was filtered and recrystallized from methanol to give 1.03 grams of the mutual prodrug MP 1 (compound 4). The average melting point range for this compound was 227 – 230 °C. FW 352; 1H NMR (DMSOd6) δ 0.83 (d, J = 6.0 Hz, 6 H), 1.27 (d, J = 7.5 Hz, 3 H), 1.77 (dt, J = 6.0, 6.5 and 7.0 Hz, 1 H), 2.48 (s, OH), 2.37 (d, J = 7.0 Hz, 2 H), 3.33 – 3.71 (m, 6 H), 4.51(t, J = 5.5 Hz, 1 H), 4.79 (pseudo t, J = 5.0 and 3.5 Hz, OH), 4.90 (dd, J = 4.5 and 5.0 Hz, 2 OH), 6.34 (d, J = 4.0 Hz, 1 H), 7.03 (m, 4 H, ar).

Procedure for Scheme 2 (ketoprofen directly linked to NAG, MP 2)(Figure 19):

Ketoprofen (compound 1 in scheme 2; 2.8 g, 11 mmoles) was dissolved in 50 mL of dichloromethane. To this solution were added one drop of DMF, as catalyst, and two equivalents

of oxalyl chloride (1.88 ml). The mixture was allowed to stir at room temperature until the evolution of carbon dioxide ceased. The solvent was then removed under reduced pressure and the residue (compound 2; Ketoprofen acid chloride) was dissolved in 5 mL of dichloromethane and used in the next reaction without further purification. Glucosamine HCL (compound 3; 2.16 grams 10 mmoles) was suspended in 200 ml methanol. Triethylamine (3 mL, ~3 equivalents) was added and the mixture stirred at room temperature until the glucosamine dissolved. To this solution was added the ketoprofen acid chloride from above and the reaction mixture was allowed to stir at room temperature for approximately two hours at which time Thin Layer Chromatography (15 % methanol in chloroform) indicated that the reaction was complete. The reaction mixture was evaporated under reduced pressure and the residue was taken up into hot isopropanol and allowed to cool slowly to give the product, MP 2, as a crystalline solid (1.26 grams). The average melting point range for this compound was 173 – 176 °C. FW 400; 1H NMR (DMSO-d6) δ 1.32 (d, J = 7.5 Hz, 3 H), 2.48 (s, OH), 3.39 – 3.70 (m, 5 H), 3.86 (q, 1 H), 4.52 (pseudo t, J = 5.0 and 5.5 Hz, 1 H), 4.75 – 4.83 (m, 3 OH), 7.15 (d, 4.5 Hz, 2 H), 7.55 (pseudo t, J = 7.0 and 8.0 Hz, 2 H), 7.73 (m, 5 H, ar).

Procedure for Scheme 3 (ibuprofen coupled to NAG with a short acetyl linker, MP 3)(Figure 20):

Preparation of 1,3,4,6-tetra-O-acetyl-2-deoxy-2-{[{[2- (4-isobutylphenyl) propanoyl] oxy} (phenyl) acetyl] amino}-ß-D-glucopyranose (MP3). Starting compounds 1-3 were synthesized from adapted procedures.[31,39]

Preparation of 2-deoxy-2-amino-1, 3, 4, 6-tetra-O-acetyl-β-D-glucopyranosyl (5): compound 4 (100 g, 0.26 mole) was titrated with triethylamine to yield a white precipitate. The precipitate was filtered and washed with CH_2C_{l2} (2x150 ml). The filtrate was dried under vacuum for 24 hrs.

The organic layer was washed with brine (2x100) dried with MgSO4. The solvent was removed via reduced pressure rotary evaporation and product dried for 24 hrs under vacuum. Compound 5 was afforded as a white solid (87.9 g, 97% yield). Mp 134º C; FW 669. 1H NMR (d-acetone) 9.21 s, 1H), 6.15 (d, 1H), 5.38 (t, 1H), 5.08 (t, 1H), 4.31 (dd, 1H), 4.11-4.03 (m, 2H), 3.56 (t, 1H), 3.03-2.05 (dd, 6H), 2.25 (d, 3H), 2.10-2.09 (m, 3H). 13C NMR (d-acetone) 205.7, 170.1, 169.87, 169.39, 169.0, 95.20, 74.85, 72.28, 68.61, 61.90, 55.46, 19.98, 19.85, 19.82, 19.77. ES1 for C14H21NO9: FW 347 found m/z 348 [M + H+]. Preparation of 2-deoxy-2-(2-chloro-2-phenyl) acetylamino-1, 3, 4, 6-tetra-O-acetyl-β-Dglucopyranosyl (6): α-Chlorophenylacetyl chloride (20g, 0.105 mol) was added drop-wise to a stirred solution of compound 4 (29.88g, 0.105 mol), triethylamine (12.4 ml, 0.90 ml) in 50 ml CH2Cl2 at –10º to room temperature for 24 hours. The reaction mixture was washed with HCl (1.5 N, 2 x 7 ml), H_2O (1 x 100 ml) and brine (1 x 100 ml). The organic phase was dried with Mg2SO4 and solvent removed via reduced pressure rotary evaporation. The resultant syrup was crystallized with ice-cold acetonitrile and dried under vacuum for 24 hrs. Compound 6 (27.4g, 93.5%) was obtained as a white solid. 1H NMR (d-acetone) 7.80 (s, 1H), 7.37(s, 2H), 7.25 (s, 2H), 5.79 (s, 1H), 5.33 (d, 2H), 4.90 (s, 1H), 4.09 (d, 2H), 3.95 (s, 1H), 3.84 (s, 1H), 3.17 (s, 1H) 1.87-1.64 (m, 12H). 13C NMR (d-acetone) 205.55, 16.88, 169.69, 169.18, 168.51, 167.69, 128.85, 128.61 (2C), 127.81 (2C) 91.03, 68.54, 61.70, 60.60 53.13, 19.73, 19.70 (2C), 19.62. ES1 for C14H21NO9: FW [M + H+] 499 found m/z 500 440 [M + H+]. Mp >200(238)º C Preparation of 1,3,4,6-tetra-O-acetyl-2-deoxy-2-{[{[2-(4-isobutylphenyl) propanoyl]oxy} (phenyl)acetyl] amino}-ß-D-glucopyranose (7): Compound 6 (653 mg, 1.45 mmol) and α-methyl-4-[isobutyl] phenylacetic acid-Na salt in anhydrous 10 ml CH_2C_{l2} were stirred at room temperature for 16 hours. The solution was washed with brine (2 x 10ml) and reduced via rotary

evaporation to give 6 (763 mg, 93.5%) as a white powder, compound 7, MP3. 1H NMR (d11 acetone) 7.73 (s, 1H), 7.41-7.13 (m, 9H), 5.81 (d, 2H), 5.38 (m, 1H), 5.04 (m, 1H), 4.26 (s, 2H), 4.10 (s,1H), 3.96 (s, 2H), 2.48 (s, 2H), 2.01 (s, 6H), 1.84 (s, 6H), 1.65 (s, 1H), 1.53 (s, 3H), 0.90 (s, 6H) . 13C NMR (d-acetone) 205.69, 173.37, 169.61, 168.73, 168.70, 140.38, 138.43, 138.03, 129.25, 128.36, 127.31, 126.84, 91.96, 75.91, 72.54, 71.66, 68.31, 52.07, 44.61, 21.72. 19.61, 17.96: UV 203λ nm. ES1 for C35H43NO12 FW 669 found; MP 238.

RESULTS AND DISCUSSION

Membrane permeability

Permeability investigations were carried out using shed snakeskin as a model membrane to human skin; a widely recognized model for preliminary studies due to its similarity in composition to the human stratum corneum. [22,24,29,30] Steady state flux values for the mutual prodrugs MP 1, MP 2, and MP3, collected in Table 12, were obtained from graphs in Figures 15 – 17. The profile of these graphs resemble those we reported earlier for NAG in PBS ethanol solutions; and exhibit an initial linear region from time zero to 2.67 hours. [7] Permeation was determined from the increasing amount of MP in the receptor medium, and cumulative steady state permeation from the plots of cumulative amount of MP, per unit area ($\mu g/cm^2$) that permeated through the snakeskin *versus* time (Figures 15 - 17); where the slope of the initial linear portion of the plot was used to calculate steady state flux, Jss $\mu g/cm^2/h$. Permeation was rapid with almost no lag time observed for MP1, MP2, and MP3 in the ethanol laced phosphate buffer solutions after examination of the intersection of the linear portion of the plots with the x-axis.[31] Steady state flux values for MP2, the ketoprofen analog, are significantly greater than those for either MP1 - 3, the ibuprofen analogs, in both PBS and PBS-10% ethanol, under

identical experimental conditions; and ethanol at 10% concentration increases the permeation rate for each mutual prodrug compared to the rate in PBS (Table 12).

Permeation of NSAID prodrugs through skin is sparsely documented; however, the synthesis and human skin permeation of glucoside and mannoside esters of various NSAIDs, including ketoprofen and ibuprofen, have been reported. [42] Polyoxyethelene esters as dermal prodrugs of ketoprofen, naproxen and diclofenac have been reported (Bonina, et al.) In addition, the esters, amide esters and piperazinylalkyl esters of ketorolac have also been examined. [25] These five studies focused on comparing NSAID permeation to NSAID-ester permeation. Our work takes the opposite tack by comparing the current study, NSAID-NAG (MP) permeation, to previously reported NAG permeation.[18] Moreover, MP1 – 3 are mutual prodrugs with both entities able to serve as active agents either topically, orally, or through injection.

Compared with our earlier work on NAG permeation through snakeskin, flux values for MP1 in PBS-10% ethanol show little change (27.4 ($\mu g/cm^2 hr$) reported here for MP1 versus 29.5 ($\mu g/cm^2 hr$) for NAG) even though molecular weight has increased with synthesis of the mutual prodrug. [18,33] In this connection for MP3 in PBS – 10%, ethanol, even though molecular weight increased even more owing to a linker molecule rather than direct attachment, flux values are virtually the same as those reported for NAG (5.6 ($\mu g/cm^2 hr$) reported here for MP3 versus 6.8 ($\mu g/cm^2 hr$) for NAG). It seems that even though MP1 and MP3, the two ibuprofen analogs, present with greater molecular weight their comparative fluxes through the model skin remains almost the same in 10% ethanolic solution.

Apparently, esterification with a known skin permeant (NSAID) may overcome the reduced permeation expected with greater molecular size, in each case.[26] In contrast, MP2 presents with an almost threefold increase in flux compared to MP1 and MP3. This verifies, in

part, those flux values reported by Swart, et al.[42]; ketoprofen-glycoside prodrug flux was reported to be 3.8 times greater than that for the ibuprofen-glycoside prodrug; but the opposite was reported for the same mannoside prodrugs; ibuprofen analog flux being 2.5 times greater than for the ketoprofen analog.

Mutual prodrug hydrolysis

Solubility of MP1 – 3 was examined (Table 12) and found to be of the same order of magnitude as reported for ibuprofen and ketoprofen glycoside and mannoside ester prodrugs.[42] Aqueous hydrolysis of MP1 – 3 was measured to assure that the solubility values reported here were not affected by chemical hydrolysis rate (Table 12). [33] The half-life values we report for the ibuprofen mutual prodrugs, MP1 and MP3, are similar in magnitude to a literature report for ibuprofen. [19]

MP1 – 3 were designed to locally treat osteoarthritis through local delivery of both an NSAID and glucosamine moiety, NAG. MP 1 and MP 2 are ibuprofen and ketoprofen covalently bound directly to the amide of glucosamine, respectively; and MP 3 is ibuprofen molecule linked to glucosamine with a short acetyl linker. Physiological enzymatic and hydrolysis reactions are expected to affect both ester and imido-ester linkage of each compound, because of differences in the linkage. [13] The importance of the ester chain link owes to how glucosamine is metabolized. Orally administered glucosamine promotes glycosaminoglycan synthesis and the production of proteoglycans that compose the lubricating fluids and support joint tissues *i.e.* cartilage. [27] Glucose and glucosamine are substrates of glucokinase; and phosphorylated glucosamine, glucosamine-6-phosphate inhibits glucokinase and alters both glucose and subsequent glucosamine metabolism; glucokinase has a low affinity for NAG [28] NAG kinase mediates the phosphorylation of NAG to produce NAG-6-phosphate which does not affect

glucokinase activity; because of this glucose and glucosamine may proceed through metabolism to a glycosaminglycan unrestricted. The point is that biosynthesis of glycosaminoglycans would be better promoted with the use of NAG or some other rate-limiting glucosamine analogue rather than by parent glucosamine.[20] Glucosamine and analogues thereof such as NAG as well as glucosamine with varying N-linkage-chains have shown degrees of human chondrocyte cell culture growth in matrix gene expression in vitro. [32] Evidently by protecting glucosamine amide, the half-life is increased which affects its activity. [2-4] Chain linkage effects have been reported in literature studies such as coupling a polymer to a molecule through an ester bond to increase its half-life in order to modify chemical dissolution properties and/or biopharmaceutical properties. [13] Glucosamine bioavailability (approximately 12-13 %) is the problem when administered orally. This perhaps could be solved if a NAG entity were administered. Despite the lack of bioavailability data, studies indicate potency in mild to moderate cases of OA. [1,35-38, 14]

CONCLUSIONS

The objectives of the study were complete with the synthesis of the three MP candidates that improve the pharmaceutical properties in regard to permeability and stability relative to the parent glucosamine and NSAIDs. The ketoprofen and ibuprofen analogs, MP1 and MP2, are projected primarily for transdermal delivery with subcutaneous depot, or oral formulation as alternative delivery routes; the ibuprofen analog, MP3, is projected for either oral or depot formulation delivery. All three MP derivatives provide a means to deliver both a glucosamine analog and an NSAID to an afflicted joint if used either transdermally or subcutaneously for osteoarthritis. If used for oral administration the glucosamine moiety prevents NSAID gastric irritation, and the NSAID moiety may improve glucosamine bioavailability. To the best of our

knowledge these compounds represent the first MPs synthesized specifically for and shown to be potential agents for transdermal delivery.

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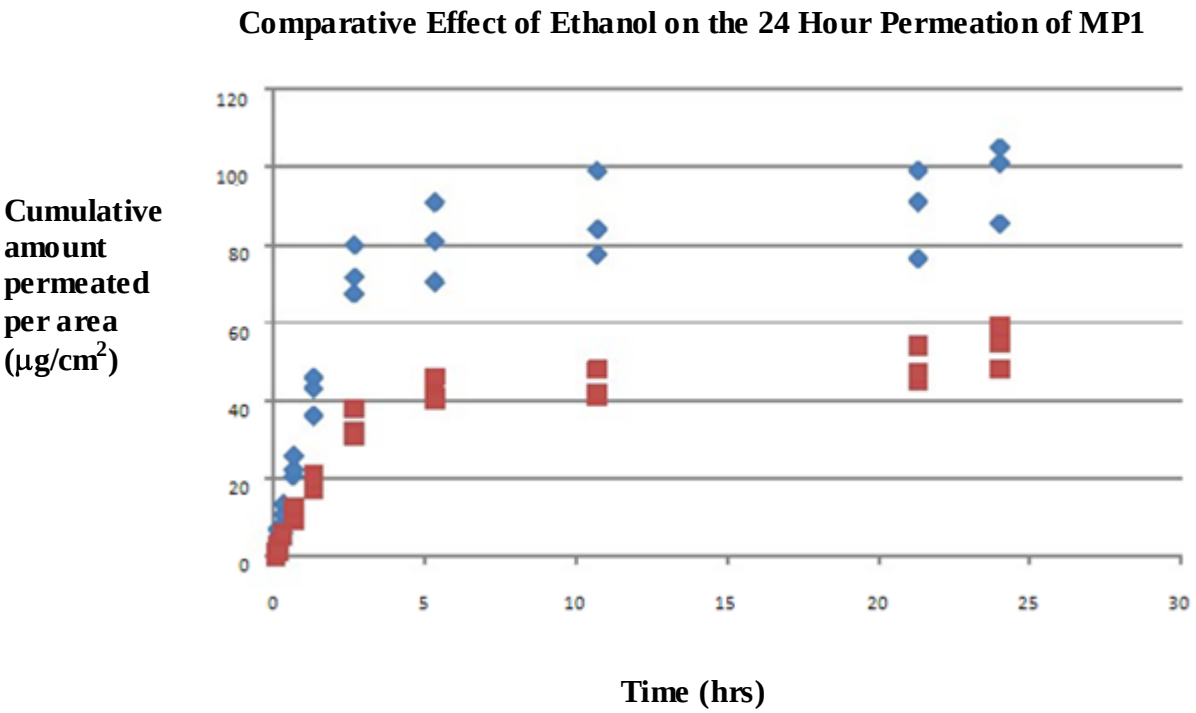

Figure 15: Comparative effect of ethanol on cumulative 24 hr NAG release from the ibuprofen-NAG analog MP1 (initial linear portion, 0 – 2.67 hrs; ♦ PBS with 10% ethanol, R_2= 0.977; ■ , PBS, R_2= 0.9712).

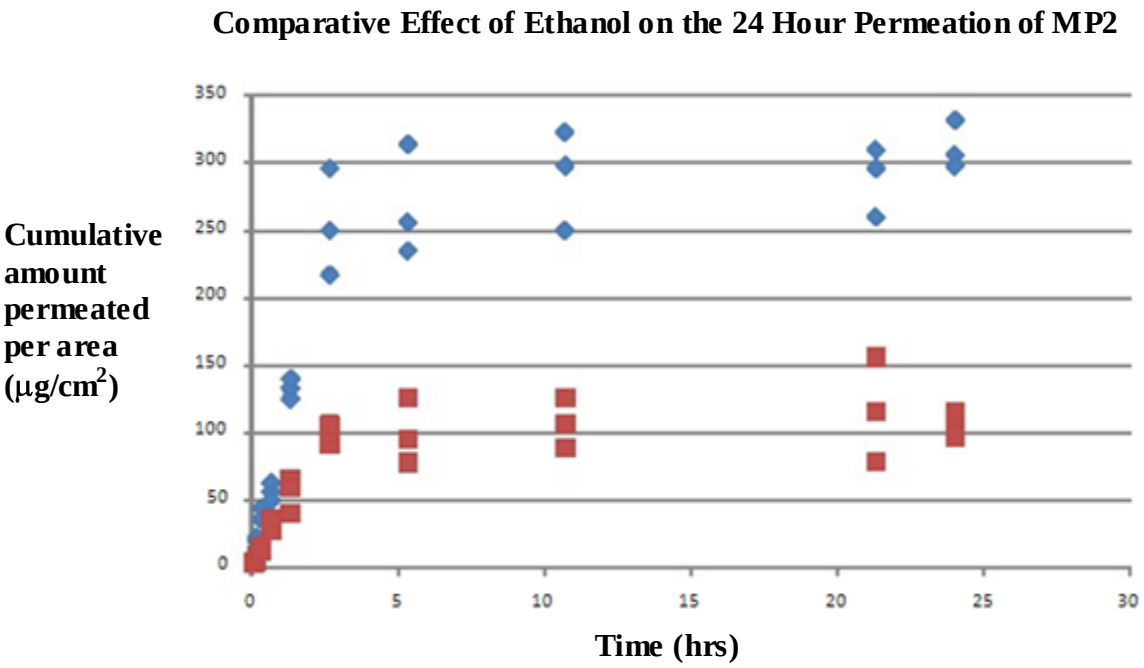

Figure 16: Comparative effect of ethanol on cumulative 24 hr NAG release from the ketoprofen-NAG analog MP2 (initial linear portion, 0 – 2.67 hrs;♦ PBS with 10% ethanol, R_2= 0.9701;■ , PBS, R_2= 0.9442).

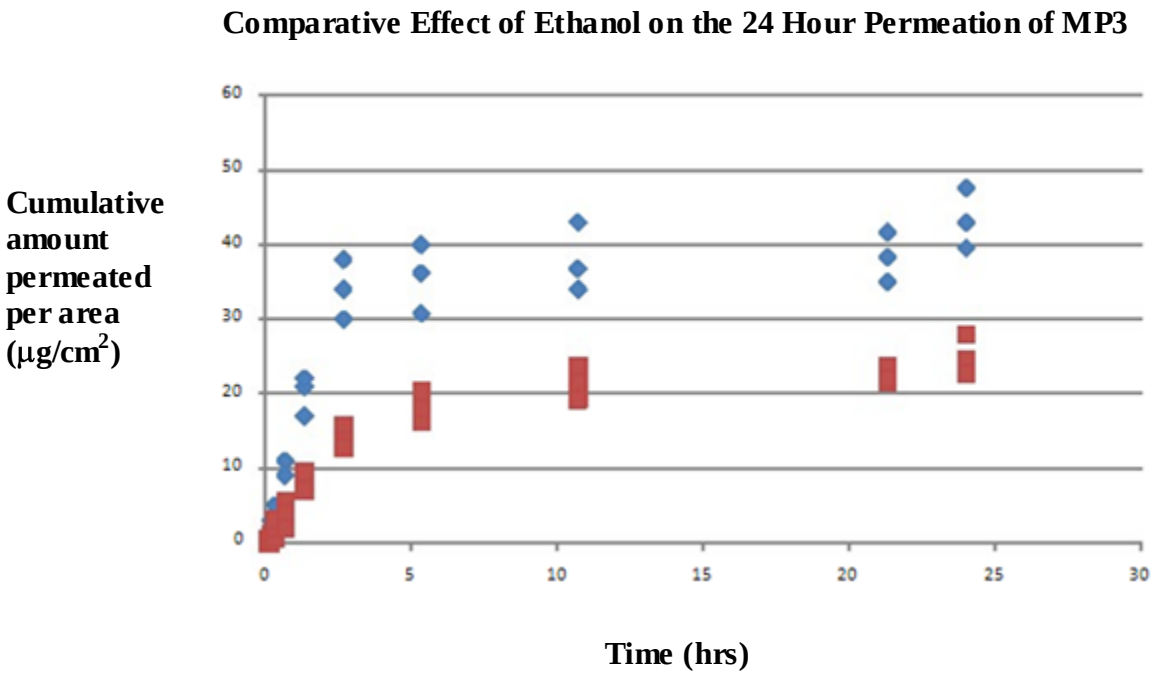

Figure 17: Comparative effect of ethanol on cumulative 24 hr NAG release from the ibuprofen-acetyl-NAG analog MP3 (initial linear portion, 0 – 2.67 hrs; ♦ PBS with 10% ethanol, R_2= 0.9675; ■ , PBS, R_2= 0.9528).

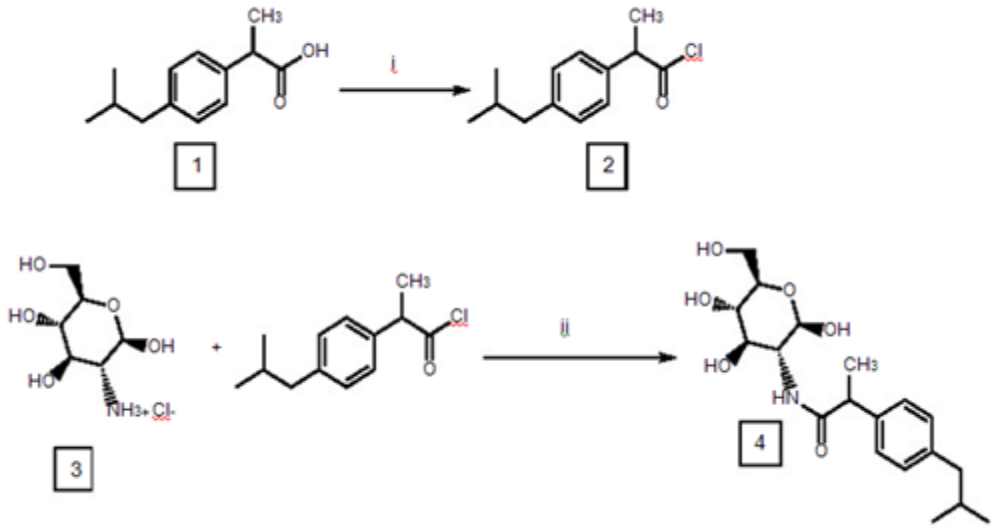

Figure 18: Synthesis schematic for MP1

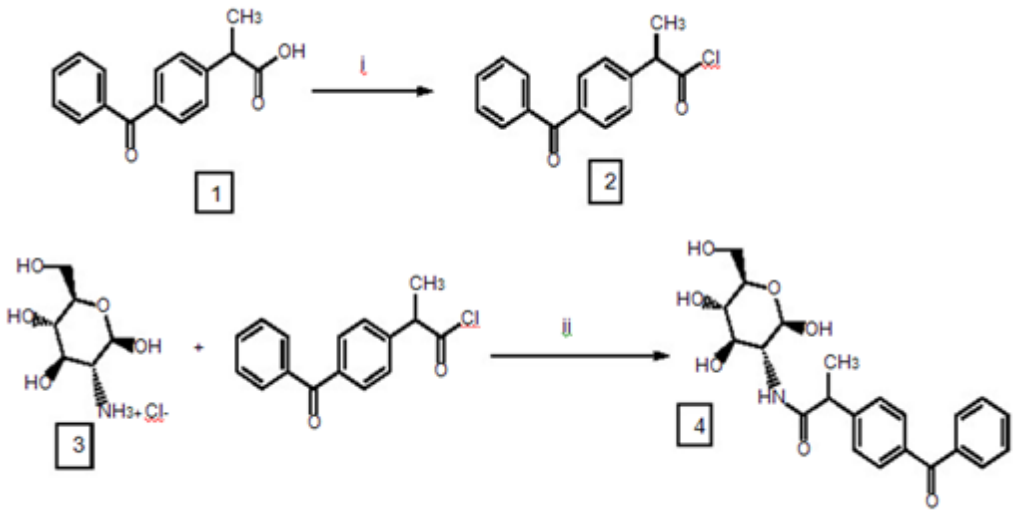

Figure 19: Synthesis schematic for MP2

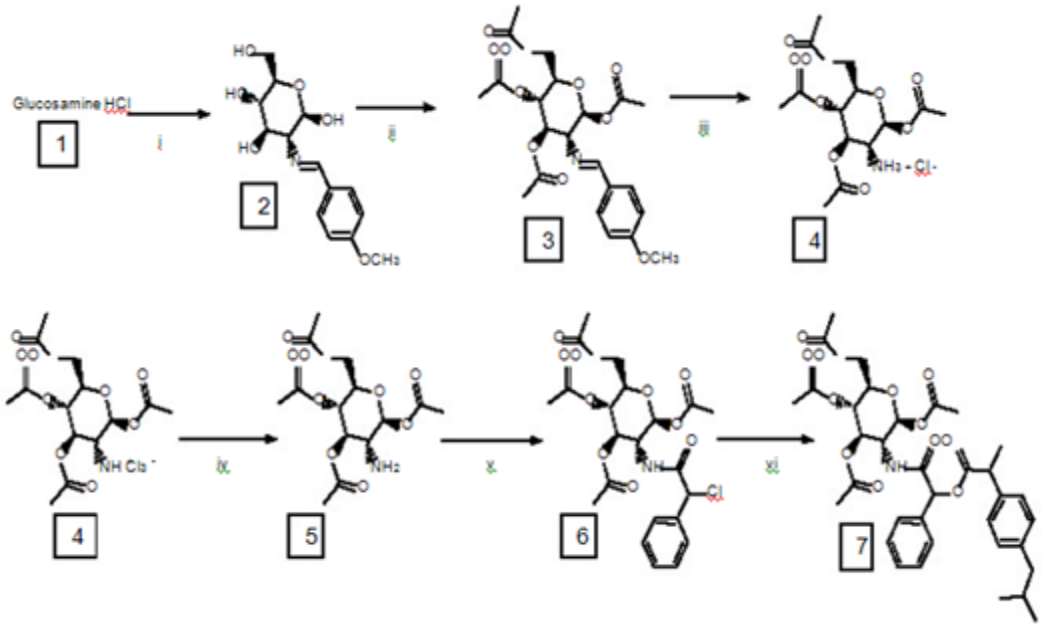

Figure 20: Synthesis schematic for MP3

Table 12: Steady state flux values, Jss for the permeation of mutual prodrugs MP 1-3 through shed snakeskin in phosphate buffer (PBS) and phosphate buffer (PBS)- 10% ethanol, from linear portions (0-2.67 hrs) of figures 15,16,17; and the experimentally determined physicochemical parameters, solubility (Molar); hydrolysis half-life (hours); NSAID solubility: ibuprofen – relatively insoluble in water; ketoprofen – slightly soluble in water (Merck Index,12th Edition).

Mutual Prodrug	**Jss($\mu g/cm^2 hr$) in PBS**	**Jss($\mu g/cm^2 hr$) in PBS – 10% Ethanol**	**Solubility, M PBS 7.4**	**Solubility, M PBS-10% EtOH**	**Hydrolysis Half – life (hrs)**
MP1	12.7	27.4	0.0019	0.00094	5.1
MP2	37.2	85.1	0.0037	0.0010	5.9
MP3	5.6	13.1	0.00072	0.00034	6.5

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CHAPTER THREE

TRANSDERMAL PERMEABILITY OF N-ACETYL-D-GLUCOSAMINE

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Garner, S., Israel, B., Ahmed, H., Abney, T., Azadi, P., and Capomacchia, A. 2007. Trandermal Permeability of N- acetyl D- glucosamine, Pharmaceutical Development and Technology, 12, 169.

Transdermal permeation of N-acetyl-D-glucosamine (NAG), a metabolite of glucosamine was examined. Glucosamine salts are nutraceuticals used in the oral treatment of osteoarthritis. Sparse information is available regarding glucosamine and NAG trans- dermal or percutaneous transport and absorption. Permeability of NAG in various enhancer suspensions was evaluated by using shed snakeskin as a model membrane via Franz-type cell diffusion studies. Negligible permeability was observed for NAG in neat solutions of known membrane permeation enhancers, ethanol, oleic acid, isopropyl myristate, and isopropyl palmitate, as well as from saturated solutions of NAG in water or phosphate buffer. Permeability measurements obtained from saturated solutions of NAG in DMSO and phosphate buffer solutions containing ethanol at 2%, 5%, 10%, 25%, and 50% demonstrated excellent permeation. Permeability coefficients of the phosphate buffer/ethanol solutions at 5%, 10%, and 25% were about threefold larger in value as those for saturated DMSO solution, whereas the 2% and 50% solution values were lower.

Background

The oral use of glucosamine supplements became popular after being featured in the book, The Arthritis Cure by Jason Theodasakis, MD.[1] Currently, glucosamine and its metabolites are not classified as drugs but as nutraceutical/dietary supplements under United States Food and

Drug Administration's Dietary Supplement Health and Education Act of 1994 (DSHEA). Oral dosage formulations of N-Acetyl-D-glucosamine (NAG) and its parent compound glucosamine in salt form (sulfate, hydro- chloride, etc.) are commercially available nutraceuticals and are commonly administered in conjunction with chondroitin sulfate, also a readily available nutraceutical. Glucosamine and chondroitin have been reported effective in the oral treatment of osteoarthritis but have not undergone the rigorous studies needed for FDA approval as pharmaceuticals.[1,2] The National Institutes of Health (NCCAM) (Bethesda, MD, USA) has an ongoing multicenter study— GAIT (Glucosamine/Chondroitin Arthritis Intervention Trial), which has evaluated the efficacy of orally administered glucosamine and chondroitin oral supplements. Published study results indicate that a combination of glucosamine and chondroitin might be most effective in patients with osteoarthritis with moderate to severe pain and in many cases obviates the need for NSAID use.[3]

Glucosamine and chondroitin salts are charged, highly polar, aqueous soluble, and apparently poor candidates for transdermal absorption. Currently, there are topical products containing these salts, and other ingredients, marketed as nutraceuticals for the treatment of osteoarthritis. NAG, an acetylated glucosamine metabolite, is less polar, uncharged, and appears to be a more likely candidate for transdermal or percutaneous absorption.

Glucosamine and its orally delivered salt forms are metabolized to NAG via the hexosamine pathway; glucosamine or galactosamine, plus a uronic acid, is incorporated as a disaccharide unit into all macromolecules requiring amino sugars, such as keratan sulfates, dermatan sulfates, chondroitin 4- and 6-sulfates, hyluronates, and heparin and heparan sulfates, to produce glycosaminoglycans (GAGs). GAGs are highly negatively charged molecules, with an extended conformation, that demonstrate high viscosity and low

compressibility—ideal as a lubricating fluid for anatomical joints. The majority of GAGs in the body are linked to core proteins to form proteoglycans or mucopolysaccarides, which are basic components of skin, tissue, and cartilage.[4,5]

INTRODUCTION

The study objectives were to evaluate stratum corneum permeability of NAG with use of shed snakeskin and to assess the feasibility of pursuing a percutaneous formulation for local therapy to osteoarthritic joints. In this connection, a provisional patent application, followed by patent application 235.00560201 was filed with the U.S. Patent and Trademark Office.[6] Permeability was evaluated by using NAG solutions of various known membrane transport-enhancing reagents, ethanol, oleic acid, isopropyl myristate, and isopropyl palmitate; NAG solutions of water, phosphate buffer, phosphate buffer, and aqueous ethanol; and NAG saturated DMSO solution.

Oral administration of glucosamine, its salts, and NAG are affected by the liver's first-pass metabolism.[7] However, a more recent report indicates that these agents may be metabolized mostly in the gut rather than solely by the liver.[8] Few pharmacokinetic literature reports exist on the disposition of these agents in articular cartilage. Setnikar et al. reported on the pharmacokinetic properties of glucosamine in dogs and man.[9,10] It is estimated that approximately 87% of the original glucosamine oral dose is absorbed and excreted; <13% is widely distributed in the body; and <<1% reaches osteoarthritic joints. Chondroitin is known to degrade into its basic disaccharide components within the gut prior to further metabolism.[11] Although only a small fraction of glucosamine reaches the articular cartilage target site, it is reported to exhibit a high potency; and together glucosamine and chondroitin therapy demonstrate therapeutic efficacies over time.[2] NAG was selected because it is an active

metabolite and prodrug of glucosamine and, owing to its commercial availability, relatively low cost and stability. It possesses the following physical and chemical characteristics, making it a reasonable candidate for transdermal delivery and percutaneous absorption: 1) high potency, 2) reasonably lipid soluble, 3) low molecular weight, 4) unique biochemical pathway with active transport from blood into articular cartilage.[5]

MATERIALS AND METHODS

Chemicals

NAG, 99.9% purity was purchased from MP Biomedical (Aurora, OH). All enhancer reagents purchased for this study were 99.9+% pure. All other reagents were of analytical grade and used without further purification.

Analysis

NAG analysis was carried out by using high-performance anion exchange chromatography with pulsed amperometric detection (HPAE-PAD); Dionex, Sunnyvale, CA, USA): Dionex DX-500 HPLC system consisting of a GP40 gradient pump, ED40 Electro- chemical detector, AS3500 autosampler and PeakNet Chromatography Workstation.[12-15] The HPAE-PAD was equipped with CarboPac™ PA20 (3 ×150 mm), analytical anion-exchange column for the rapid, high- resolution separation of monosaccharides and disaccharides, using pulsed amperometric detection,[12–15] a CarboPac PA20 analytical guard column (3 ×30 mm), and a carbonate trap column (25 ×15 mm). Mobile phase (A) was degassed and deionized water. Mobile phase (B) consisted of 0.02 N NaOH prepared with deionized water and filtered with 0.45-µm filters in a solvent filtration apparatus (Waters-Millipore, Milford, MA, USA) that was degassed under vacuum. The mobile phase system was run at a gradient concentration of 16 mM NaOH at a flow rate of 0.5 mL/min. A standard calibration curve of NAG (Figure 21) was obtained with linear

regression and value of R2 = 0.9934 in 0.1 M phosphate buffer at pH 7.4. Each sample set was run with external standards. The sample concentration values were obtained via the Peak Net software. These values were compared with to those obtained by calculations of the peak area and peak height observed as functions of the standard curve linear regression equation. The instrument LOD (limit of detection) was 0.05 ng/mL.

Shed snakeskins were used as a model membrane for all permeation studies using the NAG solutions in known membrane permeation enhancers; ethanol, oleic acid, isopropyl myristate, and isopropyl palmitate; saturated solutions of NAG in water and in phosphate buffer; as well as in DMSO solution (Table 13; Figure 22) and phosphate buffer containing ethanol at 2%, 5%, 10%, 25%, and 50% solutions (Table 14; Figure 23). The skins were stored at 20°C before use; a piece of the dorsal section was trimmed to fit the Franz cell and hydrated at 37°C for 30 min.[16] Franz-cell diffusion experiments were carried out.

For experiments using ethanol, oleic acid, isopropyl myristate, isopropyl palmitate, DMSO, and phosphate buffer containing ethanol at 2%, 5%, 10%, 25%, and 50% solutions in the donor phase, receptor cells were filled with 7.4 pH 0.1 M phosphate buffer, and the donor cell was filled with the corresponding NAG solution. Receptor solutions were maintained at 37°C and stirred with a magnetic stirrer. The snakeskins were mounted between the receptor and donor cells. The surface exposed to diffusion was 2.54 cm2 (diameter 1.8 cm) and the receptor cell volume was 6 cm3. The donor cell was covered with plastic film. The system was allowed to equilibrate at 37°C for 2 hr before each experiment. To the donor cells, 5 mL of the NAG-enhancer solution (100 mg/mL) maintained at 37°C were added. Samples were taken at intervals over a 24-hr period, 200-μL samples of receptor solutions were taken and replaced with fresh buffer; experiments were conducted in triplicate. The amounts of NAG that permeated

through the snakeskin were determined by HPAE-PAD.

Data Treatment

Permeation was determined from the increasing amount of NAG in the receptor medium, and cumulative steady-state permeation from plots of cumulative amount of NAG, per unit area ($\mu g/cm^2$) that permeated through the snakeskin versus time (Figures 22 and 23); where the slope of the linear portion of the plot was used to calculate the steady-state flux,[16–18] NAG permeability coefficients (kp) were calculated from the expression $kp = Jss \times \Delta C$ where Jss and ΔC are the flux and concentration change, respectively (Tables 13 and 14). Permeation was rapid with almost no lag time observed for NAG in DMSO (Figure 22; Table13)[20]; and also for permeation of the ethanol-laced phosphate buffer solutions of NAG after examination of the intersection of the linear portion of the plots with the x-axis (Figure 23; Table 14).

Determination of Partition Coefficients

The oil/water partition coefficient for NAG was determined by using n-octanol/phosphate buffer (pH 5.5, 6.5, and 7.4 at 0.1 M) and n-octanol/water (Table 15).[19] In each case 5 mL of n-octanol was mixed with 5 mL of aqueous solutions containing NAG and shaken at 37°C for 24 hr. The mixture was centrifuged, and the organic and aqueous phases were separated. NAG concentration in the filtrates was determined by HPAE-PAD after appropriate dilution.

RESULTS AND DISCUSSION

Initial permeability investigations were carried out by using shed snakeskin as a model membrane to human skin, a widely recognized model for preliminary studies due to its similarity in composition to the human stratum corneum.[20,21] Negligible NAG transport was observed from neat saturated solutions of the membrane permeability enhancers; pure ethanol, oleic acid, isopropyl myristate, and isopropyl palmitate. No permeation was observed from the aqueous

saturated solutions of NAG in water or phosphate buffer solutions (pH 5.5, 6.5, and 7.4; 0.1 M). This indicates that permeation of NAG probably does not involve interactions with intercellular lipids as presented in Barry's lipid-protein partitioning theory.[18,22] Partition coefficients are contained in Table 15; the NAG oil-water partition coefficient is shown to increase concomitantly with the increase in buffer solution pH, no doubt owing to the greater concentration of unionized species at pH values greater than the pKa of 6.73 (Table 3). The theoretically calculated permeation coefficient (kp) of 1.910 is similar in magnitude to those we report in Tables 13 and 14.

DMSO was chosen for evaluation as a benchmark permeation enhancer due to its physical properties and well-documented enhancement properties.[22] Enhancers in the category of DMSO (also ethanol) are reported to disrupt intercellular lipids of the stratum corneum by increasing a drug's partitioning into the stratum corneum with a concomitant increase in drug permeation through the intercellular junctions.[23–25] From the plot containing cumulative NAG concentration per unit area ($\mu g/cm^2$) versus time, NAG's in vitro flux was 8.282 (Figure 22; Table 13). The assumption is that NAG's high polarity and low partition coefficient (Table 15) contributes to its permeation in DMSO and poor permeability in purely aqueous solution owing to solubility considerations. The study shows that DMSO allows NAG to be transported immediately and continuously as seen in Figure 22 with a linear concentration increase over time.

NAG was also incorporated in ethanol/buffer solution at various ethanol concentrations. Ethanol is known to promote transdermal penetration and percutaneous absorption of many drugs.[26] NAG transdermal transport was not observed from phosphate buffer or pure ethanol where it is highly soluble and insignificantly soluble, respectively. NAG permeation was

observed in sink conditions from phosphate buffer containing ethanol at 2%, 5%, 10%, 25%, and 50% (Figure 22). The cumulative concentration of the solutions containing 5% and 10% ethanol are very similar after 24 hr; the 25% solution slightly lower; and the 2% and 50% ethanol in buffer solutions deliver comparatively less NAG. Beyond 50% ethanol concentration in buffer, the NAG precipitated. The flux values for 5%, 10%, and 25% ethanol concentrations are also close in value, whereas the 50% and 2% values are significantly lower.

The results indicate that thermodynamic and solubility effects may control NAG permeation in DMSO at 100%, and in the different concentrations of ethanol buffer solutions.[27] NAG in vitro flux and cumulative permeations from 5%, 10%, and 25% ethanol solution were larger in magnitude than that observed in DMSO and are also larger than those recently reported for glucosamine sulfate in a transdermal permeation study.[28] The results indicate that a solution concentration of 5–25% ethanol as an enhancer in delivery vehicles appears to be an excellent starting point toward a percutaneous formulation of NAG.

CONCLUSIONS

In conclusion, DMSO is an excellent skin penetration enhancer for NAG. DMSO is generally used in veterinary drug delivery, and the use of DMSO in NAG formulations may be useful for localized osteoarthritis treatment in animals because DMSO is not an FDA-approved excipient for human use in topical or transdermal pharmaceutical products. Results also show that ethanol enhances the permeation of NAG in the concentration range of 5–25%, in shed snakeskin. This finding indicates that formulations containing 5–25% ethanol may enhance NAG permeation in humans.

Acknowledgements

This work was supported in part by NIH grant GM066321–03; the Alfred P. Sloan Foundation Minority Ph.D. Program; the Department of Pharmaceutical and Biomedical Sciences; and a private grant from AC Capomacchia.

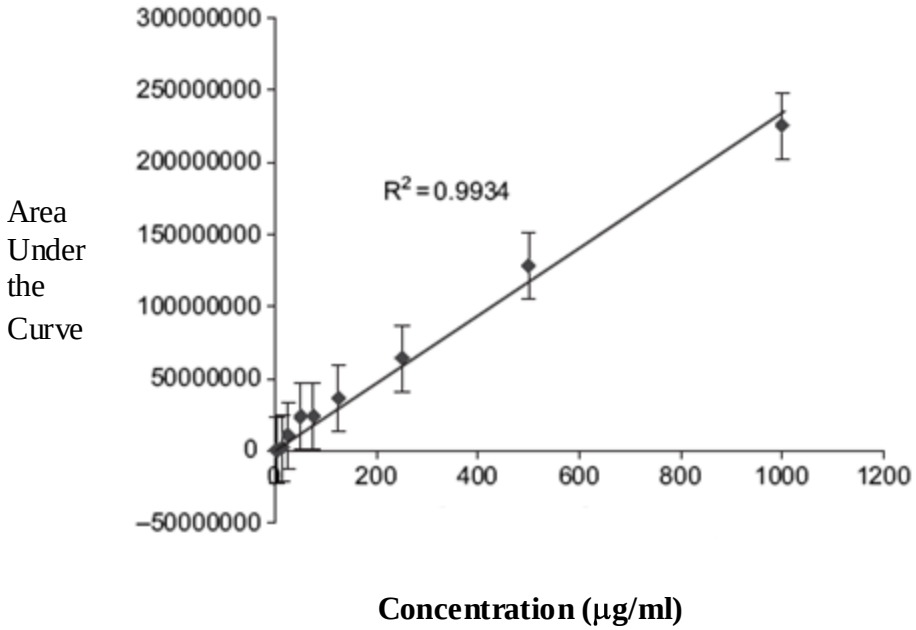

Figure 21: Standard calibration curve of NAG (R^2 = 0.9934) obtained in 0.1M phosphate buffer at pH 7.4.

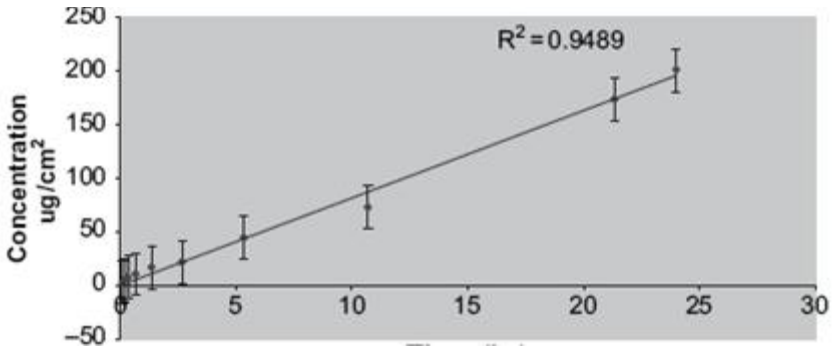

Figure 22: N-acetylglucosamine (NAG) permeation through shed snakeskin at 37.5°C (cumulative concentration vs. time)in DMSO solution (100 mg/ml). Each point represents the mean ± D, n = 3.

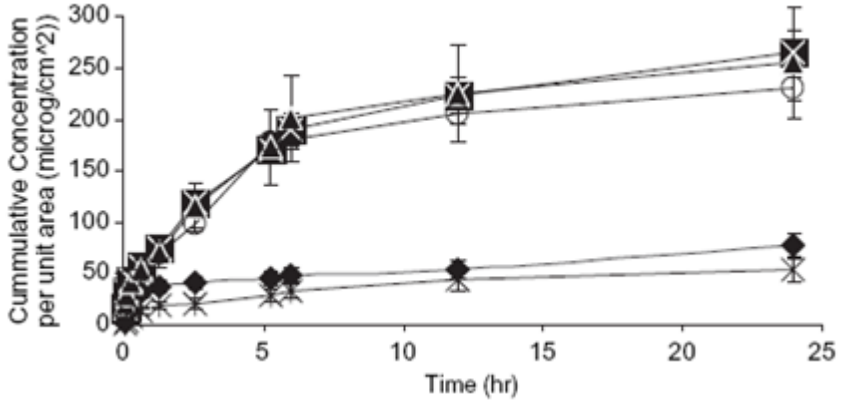

Figure 23: The effect of ethanol concentration on N-acetyl glucosamine (NAG; 100 mg/ml) permeation at 37.5°C through shed snakeskin (cumulative concentration, $\mu g/cm^2$, vs. time). Each point represents the mean ± D, n= 3.2% ethanol:♦ ; 5% ethanol: ∎, 10% ethanol:▲, 25% ethanol:○ ,50% ethanol:×.

Table 13: Physiochemical data obtained for the permeation of N-acetylglucosamine (NAG) through shed snake skin using dimethyl sulfoxide (DMSO) solution in the donor phase and pH 7.4 phosphate buffer in receptor phase (from Figure 22).

Parameter	
Jss ($\mu g/cm^2/hr$)	8.28
Cumulative 24 hr steady state permeation (μg)	510.5
Permeation coefficient, k_p (cm/hr) 10^3	0.425
% NAG in receptor phase after 24 hr	2.57

Table 14: Physicochemical data obtained for the permeation of N-acetylglucosamine (NAG) through shed snake skin via phosphate buffer containing ethanol at 2%, 5%, 10%, 25%, and 50% solutions in the donor phase and pH 7.4 phosphate buffer in the receptor phase.

% Ethanol, enhancing agent	2	5	10	25	50
Jss (μg/cm^2/hr)	6.88	26.8	29.5	25.5	5.14
Cumulative 24hr permeation (μg)	196	572.8	572.1	482.6	54.0
Permeation coefficient, kp (cm/hr) 10^3	0.347	1.38	1.52	1.31	0.264
% NAG in receptor phase after 24 hrs	0.98	2.89	2.88	2.44	0.273

Table 15: Experimentally Determined Partition Coefficients for N-Acetyl-D- Glucosamine (NAG) [pKa 6.73] in pH solutions 5.5, 6.0, 6.5, 7.4, and water; and permeation coefficient theoretically calculated according to Potts-Guy equation using our experimentally derived value for NAG Octanol/water ratio of 0.116.

Octanol/pH 5.5 buffer	Octanol/pH 6.5 buffer	Octanol/pH 7.4 buffer	Octanol/water	k_p(cm/hr)
0.085	0.089	0.110	0.116	$1.9\ 10^{-4}$

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APPENDIX A

ACCELERATED DISSOLUTION TESTING FOR CONTROLLED RELEASE MICROSPHERES USING THE FLOW – THROUGH DISSOLUTION APPARATUS

ACCELERATED DISSOLUTION TESTING FOR CONTROLLED RELEASE MICROSPHERES USING THE FLOW – THROUGH DISSOLUTION APPARATUS

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Collier, J., Thakare, M., Garner, S., Israel, B., Ahmed, H., Granade, S., Strong, D., Price, J., Capomacchia, A. 2008. Pharmaceutical Development and Technology 1 – 9.

Theophylline controlled release capsules (THEO-24 CR) were used as a model system to evaluate accelerated dissolution tests for process and quality control and formulation development of controlled release formulations. Dissolution test acceleration was provided by increasing temperature, pH, flow rate, or adding surfactant. Electron microscope studies on the theophylline microspheres subsequent to each experiment showed that at pH values of 6.6 and 7.6 the microspheres remained intact, but at pH 8.6 they showed deterioration. As temperature was increased from 37–57°C, no change in microsphere integrity was noted. Increased flow rate also showed no detrimental effect on integrity. The effect of increased temperature was determined to be the statistically significant variable.

INTRODUCTION

The objective was to develop an accelerated dissolution test for controlled release formulations for quality and process control, and formulation development. Reproducible dissolution testing has long been recognized as extremely important for quality control. In this study, theophylline controlled release capsules (THEO-24 CR) were used as a model drug system. The variables of temperature, pH, and flow rate for aqueous dissolution medium were

examined; the effect of adding surfactant (sodium lauryl sulfate) to the medium was also evaluated using the flow-through cell method (USP Apparatus IV). The test criteria were that it be substantially shorter than the USP eight-hour test, and that it correlated with USP specifications for products labeled for dosing every 24 h as described in USP Monograph test 6 for products labeled for dosing every 24 h.[1] The specific goal was to attain USP acceptable theophylline release in less than three hours in order to make the accelerated test a viable alternative for process and quality control, and formulation development. THEO-24 CR was chosen as the model drug since as an extended release product it is a suitable dosage form for flow through dissolution testing and therefore, to evaluate accelerated flow through dissolution.[2–9] Moreover, the flow through test, initially developed in FDA laboratories 40 years ago, has been reported to offer the best discrimination due to manufacture or product composition changes.[2–4]

Two studies report on accelerated dissolution testing for process and quality control and formulation development of controlled release formulations. The goals were to substantially reduce testing time compared to USP requirements. The first used increased temperature to accelerate dissolution from a controlled release Roxiam formulation in a USP IV flow through apparatus. Analytical times were reported to be less than 30 min for one assay.[10] The second study used increased temperature, solvent and stirring to accelerate dissolution of USP salicylic acid calibrator tablets in a USP II apparatus. Dissolution times could be accelerated by a factor of 5 which meant that a single analysis could be performed in less than one hour.[11] Both of these ground-breaking studies showed that substantial time could be saved using the ‘Accelerated Dissolution Rate Analysis’ (ACDRA) method with no loss in correlation compared to the standard USP test. Further discussions on the possibility of using increased temperature to

accelerate the dissolution process, and the use of surfactants and hydroalcoholic media have also been reported.[12,13]

MATERIALS AND METHODS

The following materials were obtained from the commercial suppliers below and used without further purification. Theophylline (mw 180.16) as THEO-24 Controlled Release capsules (100 mg) from Wal-Mart Pharmacy, Athens, GA, USA; acetonitrile, ammonium hydroxide, sodium acetate trihydrate, from Sigma-Aldrich Chemicals, St Louis, MO, USA; glacial acetic acid, potassium phosphate monobasic, hydrochloric acid, and sodium hydroxide from Aldrich Chemicals, Milwaukee, WI, USA; methanol and sodium lauryl sulfate (SLS; powder/NF/FCC) from Fisher Chemicals, Fairlawn, NJ, USA; Theophylline RS Anhydrous (USP) from Spectrum Chemical Manufacturing Corporation, New Brunswick, NJ, USA.

Instruments

Spectronic 2000 Spectrophotometer, Bausch and Lomb, NJ; Thermo Spectronic Helios Aquamate, Rochester, NY; Haake E-52 Water Bath, Haake Instruments, Inc., Saddle Brook, NJ; Sigma Motor Peristaltic Pump Model, 100 T8, Middleport, NY, USA.

UV Spectrophotometric Studies

UV Spectrophotometric analyses for all samples were conducted at 271 nm, the uv maximum for Theophylline using a 10 mm quartz at 25°C. Absorbance standard curves were constructed using solutions of reference standard Theophylline RS Anhydrous (USP) in either phosphate buffer dissolution medium (0.05M) at the indicated pH values; 0.05 M phosphate buffer at pH 1.2 and 7.6 with SLS at 25°C; theophylline samples were prepared at percent concentrations from 5–100%. The absorbance versus percentage concentration plots for Theophylline RS were linear over this concentration range and were used to determine the

percent drug released in all dissolution samples at 25°C.

Dissolution Method

In vitro dissolution studies of THEO-24 CR capsules were conducted in triplicate; a sample was withdrawn at each time point in three separate experiments using the flow-through cell method (USP Apparatus IV). Samples of 4 mL were withdrawn every 30 min for 3 h; the 4 mL sample removed was immediately replaced with 4 mL of fresh dissolution medium (either phosphate buffer, or phosphate buffer with SLS). Dissolution profiles of Percent Released versus Time (n = 3) were constructed as shown in Figures 24 – 27. Medium temperature, pH and flow rate were changed to evaluate the effect on release rate.

One hundred milligrams of THEO-24 CR microspheres were introduced into the flow cell. Dissolution media (1000 mL) pH was altered depending on experiment requirements. Phosphate buffer 0.05 M was adjusted to the desired pH by adding small increments of 1M NaOH. Samples were withdrawn from the media reservoir at the predetermined time intervals stated above. The percent concentration of dissolved Theophylline in each sample was measured spectrophotometrically at 271 nm against a reference cell containing only the dissolution medium.

Flow – Through Dissolution

The flow-through cell, of transparent and inert material, was mounted vertically containing a filter system that prevented escape of undissolved particles from the top of the cell; standard cell diameter was 12 mm; the bottom cone was filled with small glass beads of about 1 mm diameter with one bead of about 5 mm positioned at the apex to protect the fluid entry tube.[1] The flow-through cell used had three parts: the lower cone, the middle cylindrical portion, and the filter head on top. The cone is separated from the cylindrical portion by a #40 mesh screen

and a microfiber filter; the filter head also holds a glass microfiber filter. The entire cell is immersed in a water bath with the temperature maintained at 37, 47, or 57 ± 0.5ºC as per experiment requirements; and the dissolution medium is kept at the corresponding bath temperature for each experiment. All experiments were conducted in a closed loop setup. Flow rates of the dissolution medium through the cells were within USP specifications of 4, 8, and 16 mL/min.

Electron Microscope Study

Microspheres were dried for 24 hours at 50°C then placed in a dessicator for 48 hours at room temperature before being submitted for electron microscopy imaging at the UGA Electron Microscopy Laboratory as shown in Figures 28–34.

Process Variables

To determine the influence of the process variables pH (A), temperature (B), and flow rate (C), a two – level 3 factor (2_3) factorial design was selected. Standard runs were generated for times 0.5, 1.0, and 1.5 h, as shown in Table 1 for 0.5 h, but not for times 2.0–3.0 h since Theophylline release ended at about 1.5 h at higher temperatures. Pareto charts were constructed for experimental runs; the chart for 0.5 h is shown in Figure 35. The factorial design and analyses were generated using Stat-Ease Design-Expert Version 7.0.3 (Stat-Ease Inc., Minneapolis, MN, USA).

RESULTS AND DISCUSSION

Dissolution Profiles

The process variables associated with flow through dissolution testing: temperature, pH, and flow rate were manipulated to assess the potential for accelerated dissolution testing. In this study, the drug release of commercially available theophylline microspheres (THEO-24 CR) was

determined using a flow-through cell apparatus (USP Apparatus IV) at three different flow rates, 4, 8, and 16 mL/min; three different pH values, 6.6, 7.6, and 8.6; and three temperatures, 37, 47, and 57°C. Figures 24–27 show the linear dissolution profiles and regression coefficients upon changing these variables, which closely resemble those published by Macheras et al. for Theo-Dur under comparable conditions.[6,14] The most benign parameters for the current study were pH 7.6, 37°C, and flow rate 8 mL/min showing 55% linear drug release in 3 h (Figure 25). The addition of 0.1% SLS to the same solution increased drug release from 55–65% in 3 h (Figure 27). Dissolution under these conditions correlates with USP test 6 which provides for 55–75% release of theophylline within 8 h for products labeled for dosing every 24 h. Fastest drug release occurred at 57°C at flow rates of 8 and 16 mL/min at which point 85% of the theophylline was linearly released in 1.5 h from solutions at pH 7.6 and 8.6, also meeting the USP requirements (Figures 25 and 26). These results compare with those of Zackrisson et al., where a flow through apparatus was employed using increased medium temperature to accelerate dissolution rate of remoxipride microcapsules.[10] Their results indicate that the time needed for one analysis of Roxiam takes less than 5% of the time required for the USP method; estimated to be about one hour. They report a direct correspondence between their data recorded at 85°C with USP data recorded at 37°C with no mention of microsphere integrity.

However, the reported correspondence with USP data strongly indicates little or no loss in integrity. The data we present here convincingly show that increased drug release at 57°C, causes no apparent change in microsphere integrity as shown in the electron micrograph, Figure 33, at pH 7.6, or observed as change in linearity of release in Figures 25 and 26. Based on these results, the results of Zackrisson et al., and those of Quist and Ostling, increasing temperature should be considered first as a method for accelerating drugs release from microspheres constructed with

polymers responsive to temperature.[10,11,15,16] Further, the Pareto chart in Figure 35 indicates temperature as the significant variable for theophylline release in the current study. The Pareto Charts were generated from data obtained from three standard 23 factorial runs at 0.5, 1.0, and 1.5 hours; Figure 35 is for the 0.5 h time point. They were statistically analyzed to determine the significance of the process variables pH (A), temperature (B), and flow rate (C) on theophylline release.[17] The chart in Figure 35 presents as a vertical bar graph in which the height of the bars are proportional to the value of the estimated effects of the three variables, temperature, pH or flow rate, as well the effects of interacting variables AB, AC, or ABC. Bars above the first dotted line (T-Value Limit) are most likely significant. Bars below it are not likely to be significant. Bars above the Bonferroni line are almost certainly significant but must be confirmed by ANOVA. For timepoints 0.5, 1.0, and 1.5, ANOVA calculates P-values of 0.0057, 0.169, and 0.0045, respectively, indicating the significance of the variable temperature on theophylline released from the microspheres. The effects of variables pH and flow rate ranked 2, and 3, respectively, were not statistically significant as were the effects of the interactive variables, even though flow rate and pH appear to increase drug release as seen in Figures 24 -27.

Electron Micrographs

Figures 28 - 34 show representative electron micrographs of Theo-24 CR microspheres after dissolution testing and how their physical appearance is altered after changing the variables, pH, temperature, and surfactant. Figure 28 shows intact microspheres before dissolution which are similar to microspheres after dissolution at pH 6.6, 37°C and 8 mL/min flow rate (Figure 29). Microspheres at pH 8.6 appear degraded compared to those at pH 6.6 and 7.6 (Figures 6, 7, 8). However, the apparent degradation did not adversely affect drug release or cause a dose dumping effect since theophylline release remained linear and zero order (Figures 24 -26). The effect of

pH on increasing drug release is also shown in Figures 24 -26 ; even though not statistically significant the increase may reflect changes in theophylline ionization upon changing the medium from pH 6.6–8.6. In aqueous media of pH 8.6 theophylline (pKa 8.77) is almost 50% ionized to the conjugate base suggesting that a relatively minor pH related solubility effect may be causing increased drug release. This view supported by the results shown in the Pareto charts where pH is ranked second in effects but is not significant. It is clear that increased temperature does not alter microsphere integrity upon comparing Figures 30, 32, and 33 as previously discussed.

Surfactant

Adding sodium lauryl sulfate (0.1%) to the buffer medium at 37°C, pH 7.6, and flow rate 8 mL/min increased drug release by roughly 20% at the three hour mark as shown in Figure 27 with no loss of microsphere integrity as seen in Figure 34. Moreover, when compared to data in Figure 4, addition of surfactant results in even greater drug release than elevating medium pH to 8.6. A recent study showed that 1% sodium lauryl sulfate increased carbamazepine from controlled release tablets fourteen fold.[18]

Upon review of this article it was noted that potassium salts (from the potassium phosphate buffer) precipitate SLS by forming the relatively insoluble potassium lauryl sulfate (PLS; solubility at room temperature about 0.02%).[19,20] This is a dynamic equilibrium and the effect of PLS formation is nullified by increasing concentrations of SLS.[21] PLS solubility has been reported to be temperature dependent such that at 37°C its solubility is about 0.11%.[20] The current study used SLS at 0.1% concentration and potassium ion concentration was about 0.6% from the buffer solution. Therefore PLS could only form to the extent of its solubility at 37°C, and for this reason PLS solubility or precipitation was not a factor in the results presented in

Figure 27 of the current study.

CONCLUSIONS

Accelerated dissolution testing of theophylline–24 CR capsules using USP Apparatus IV may be attained by increasing medium temperature, pH, increasing flow rate, and adding a surfactant; however only the effect of temperature is significant. Increasing dissolution medium above pH 7.6–8.6 caused a loss of microsphere integrity. The most benign parameters for the current study were pH 7.6, 37°C, and flow rate 8 mL/min showing 55% linear drug release in 3 h. Addition of the surfactant sodium lauryl sulfate increased the maximum drug released in 3 h to 65%. Dissolution under both of these conditions correlates with USP test 6 which provides for 55–75% release of theophylline in 8 h for products labeled for dosing every 24 h. Fastest drug release (85%/1.5 h) occurred at 57°C, pH 7.6 or 8.6, and flow rate either 8 or 16 mL/min. A combination of increased medium temperature and added surfactant may prove to be the optimal conditions for accelerated drug release of controlled release formulations while maintaining microsphere integrity.

Acknowledgements

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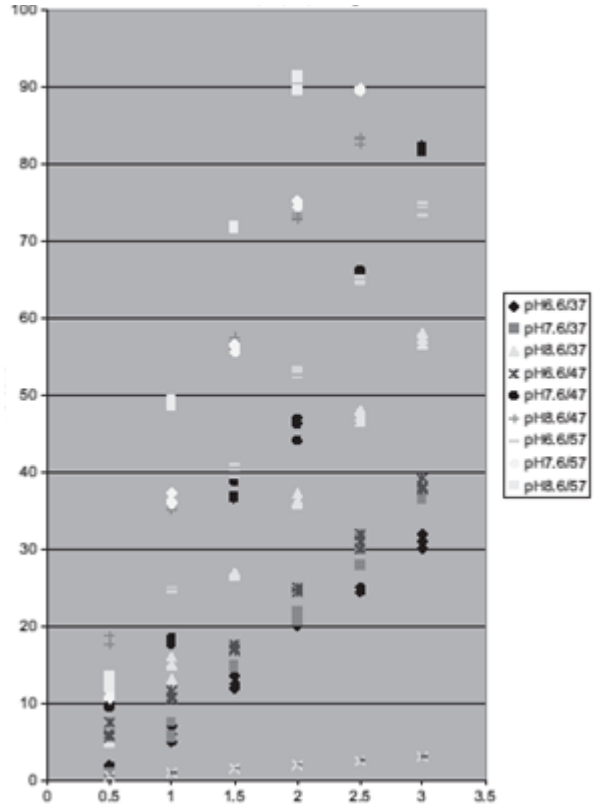
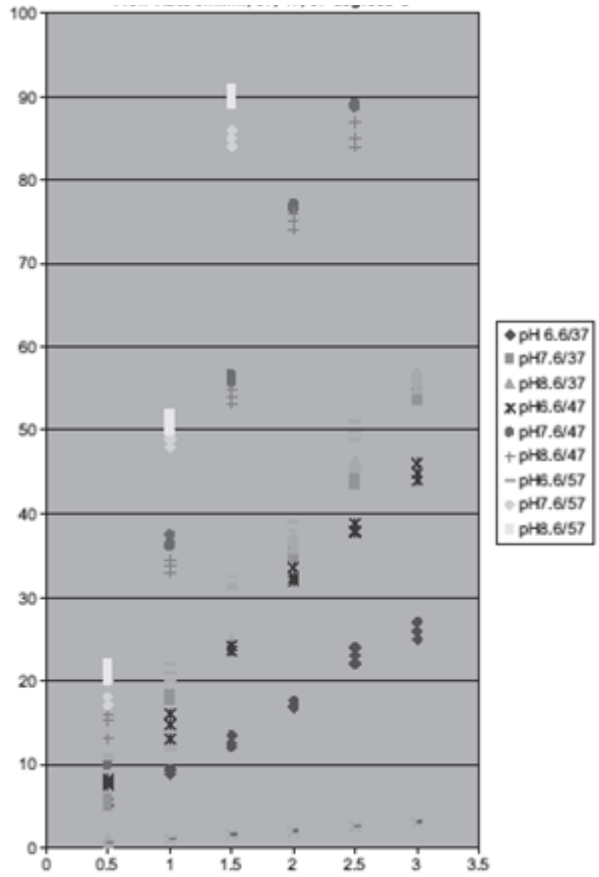
Accelerated Dissolution Testing for Controlled Release Microspheres
Flow Rate 4ml/min, 37, 47, 57 degrees C



Time, hrs

Figure 24: Dissolution profiles of Theo 24-CR in phosphate buffer 0.05M; flow rate 4ml/min. Temperature (pH/r^2): 37°C (6.6/0.9776, 7.6/0.9932, 8.6/0.9977); 47°C (6.6/0.9928, 7.6/0.9958, 8.6/0.9856); 57°C (6.6/0.9944, 7.6/0.9902, 8.6/0.9761); n=3.

Accelerated Dissolution Testing for Controlled Release Microspheres
Flow Rate 8ml/min, 37,47,57 degrees C



Time, hrs

Figure 25: Dissolution profiles of Theo 24-CR in phosphate buffer 0.05M; flow rate 8ml/min. Temperature (pH/r^2): 37°C (6.6/0.9871, 7.6/0.9971, 8.6/0.9948); 47°C (6.6/0.9847, 7.6/0.9901, 8.6/0.9811); 57°C (6.6/0.9921, 7.6/0.9935, 8.6/0.9835); n=3.

Accelerated Dissolution Testing for Controlled Release Microspheres
Flow Rate 16ml/min, 37,47,57 degrees C

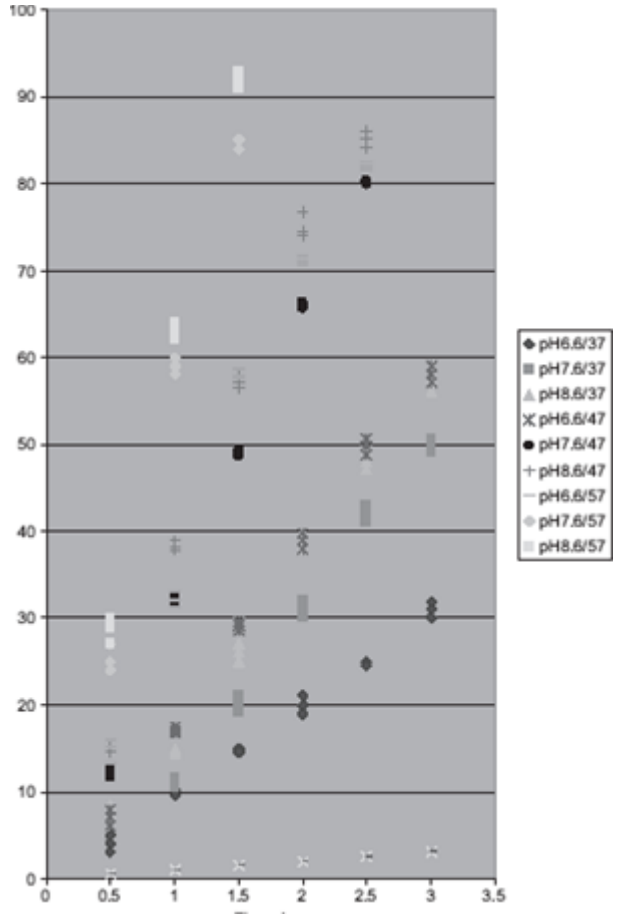


Figure 26: Dissolution profiles of Theo 24-CR in phosphate buffer 0.05M; flow rate 16 ml/min. Temperature(pH/r^2): 37°C (6.6/0.9938, 7.6/0.9851, 8.6/0.9943); 47°C (6.6/0.9808, 7.6/0.9966, 8.6/0.9965); 57°C (6.6/0.9755, 7.6/0.9926, 8.6/0.9962); n=3.

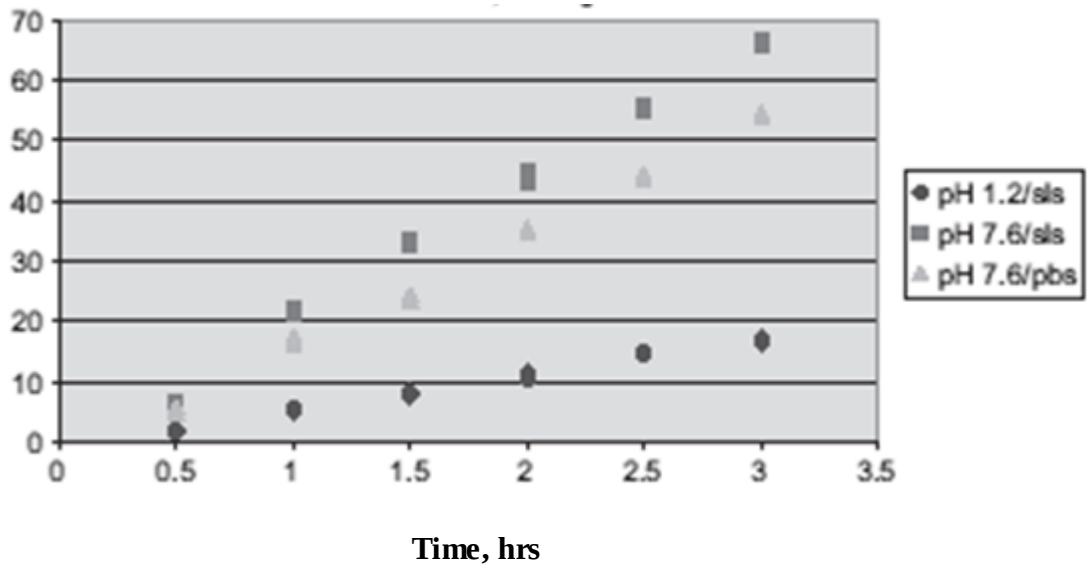


Figure 27: Dissolution profiles of Theo 24-CR at 37°C, in phosphate buffer at pH 1.2 (r^2: 0.9924[j] ; and 7.6 (r^2: 0.9973) with SLS (0.1 %) compared to pH 7.6 (r^2: 0.9965), in buffer alone, n=3.

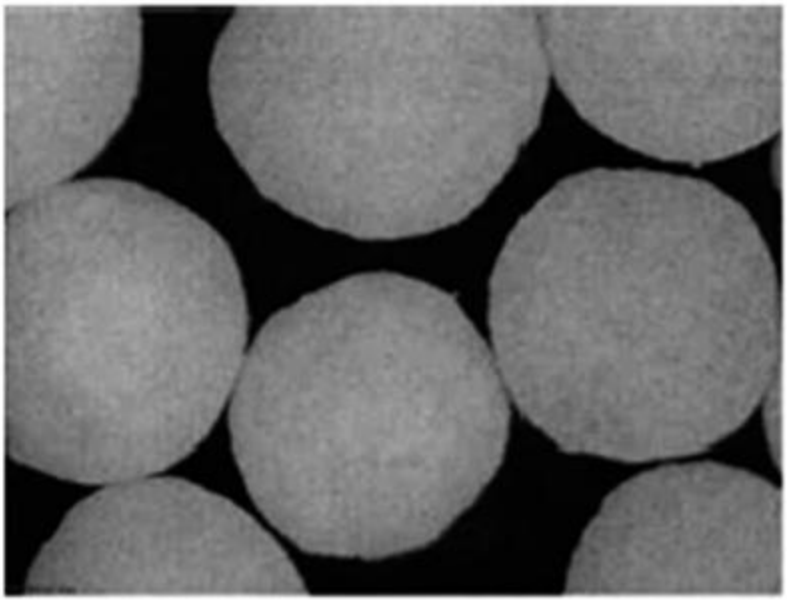


Figure 28: Electron micrograph of intact theophylline microspheres before dissolution.

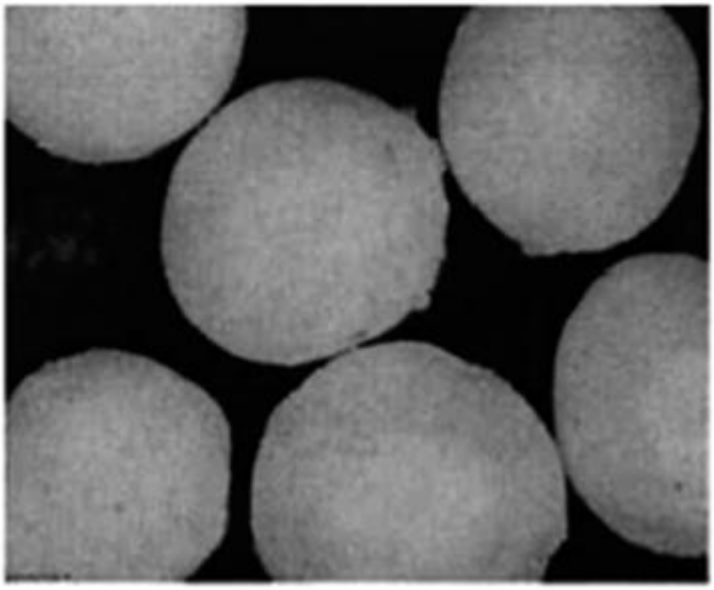


Figure 29: Electron micrograph of microspheres after dissolution at 37°C, pH 6.6, 8 ml/min flow rate.

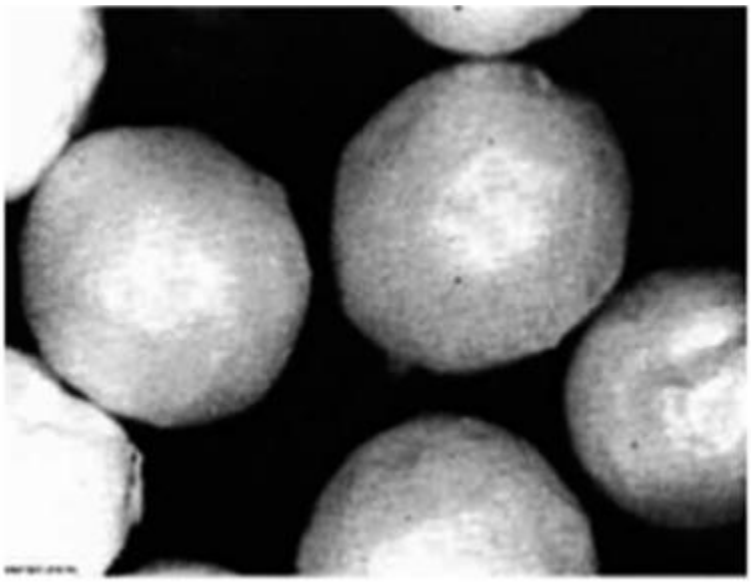


Figure 30: Electron micrograph of microspheres after dissolution at 37°C, pH 7.6, 8 ml/min flow rate.

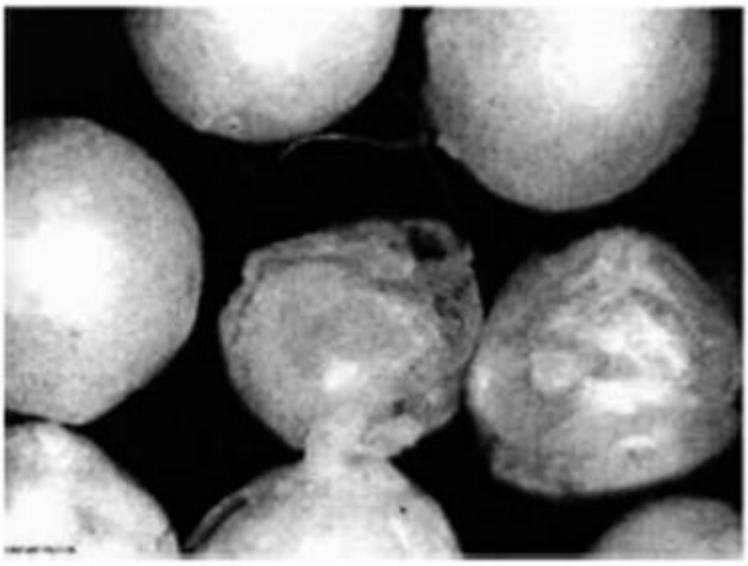


Figure 31: Electron micrograph of microspheres after dissolution at 37°C, pH 8.6, 8 ml/min flow rate.

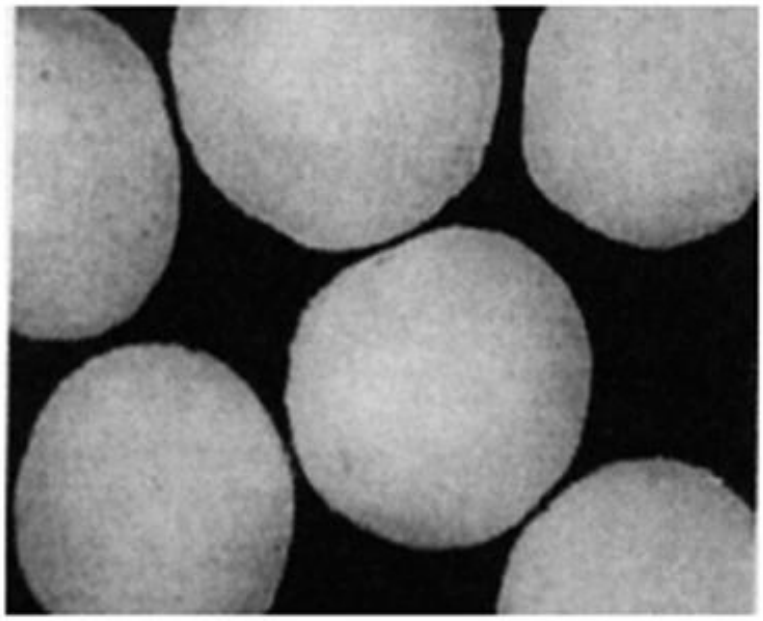


Figure 32: Electron micrograph of microspheres after dissolution at 47°C, pH 6.6, 8 ml/min flow rate.

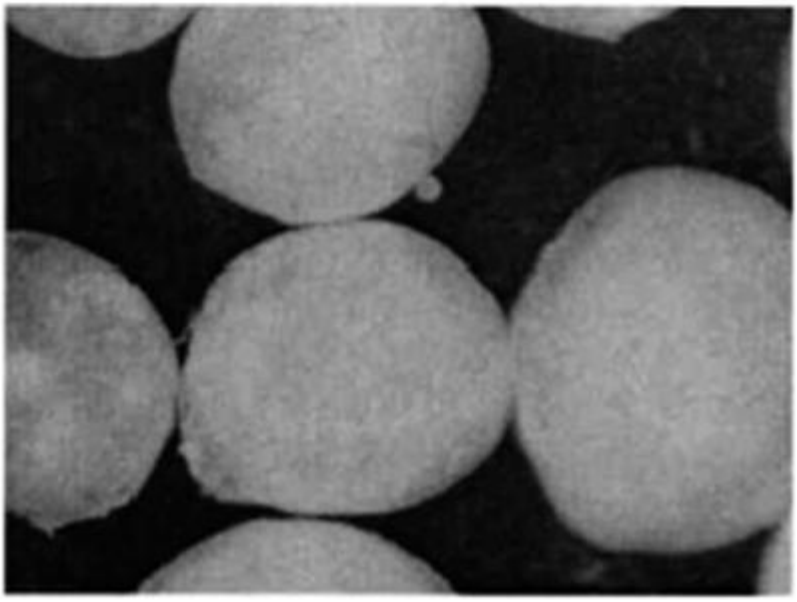


Figure 33: Electron micrograph of microspheres after dissolution at 57°C, pH 7.6, 8 ml/min flow rate.

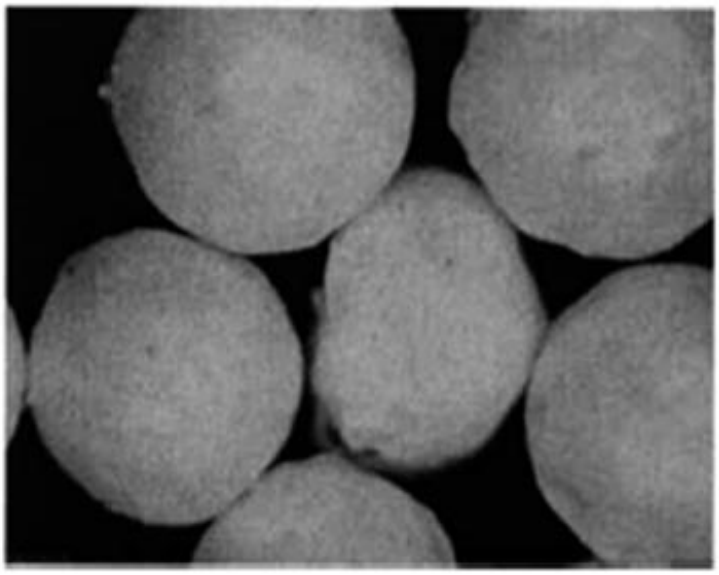


Figure 34: Electron micrograph of microspheres after dissolution at 37°C, pH 7.6, 8 ml/min flow rate and containing 0.1% sodium lauryl sulfate.

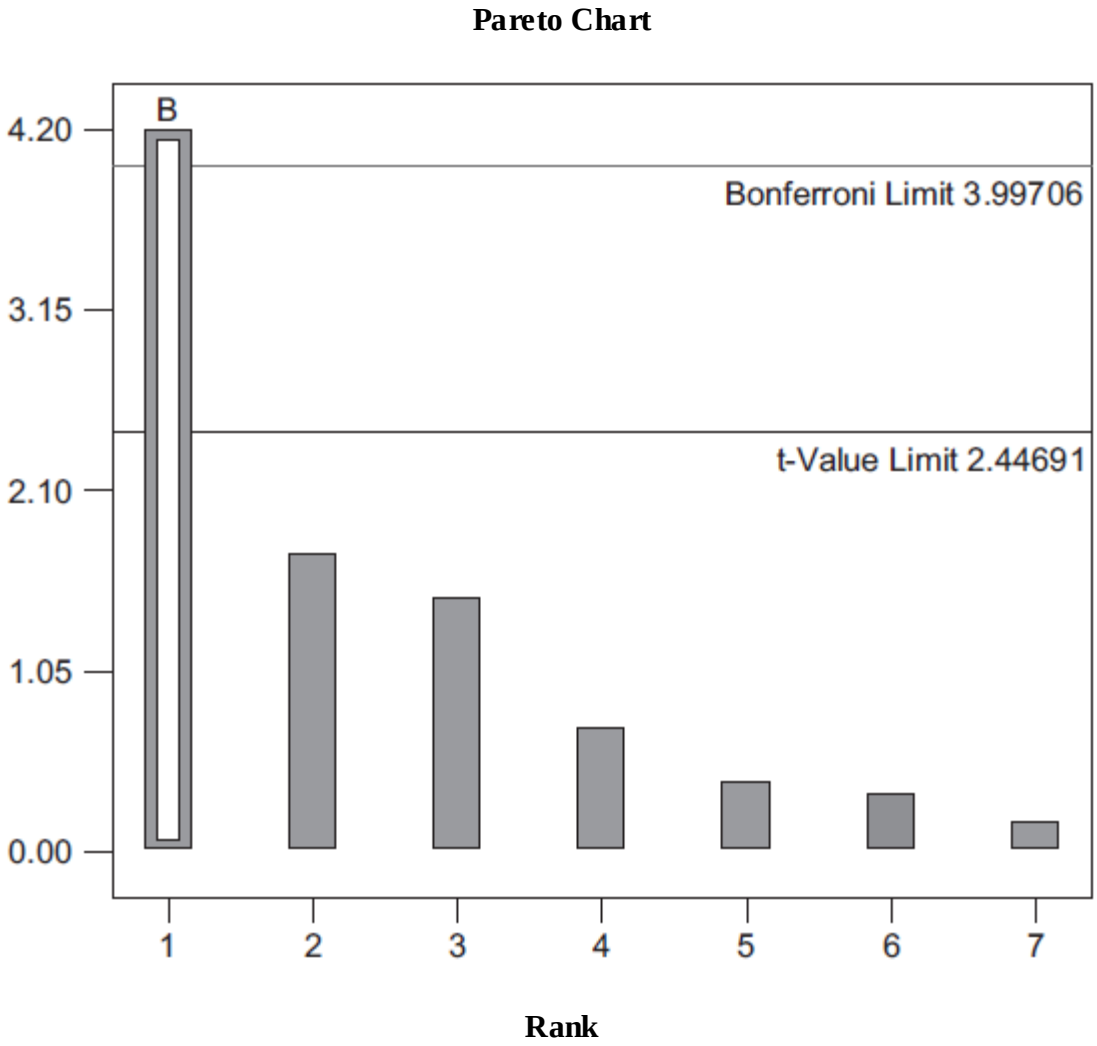


Figure 35: Pareto chart: t-Value of [Effect] versus Rank, at time point 0.5 hours for percentage release of theophylline for the effect of variables ranked according to significance: B, Temperature (rank 1); pH, A (rank 2); Flow Rate C, (rank 3); and interactions AB (rank 4); AC (rank 5); BC (rank 6); ABC (rank 7).

Table 16: Standard 2^3 factorial run at time 0.5 hours for the three factors pH, temperature, and flow rate at two levels

Std	Run	Factor 1 A: pH	Factor 2 B: Temp	Factor 3 Flow rate	Response % Theo released
3	1	6.6	57°C	4	12
5	2	6.6	37°C	16	4
1	3	6.6	37°C	4	3
4	4	8.6	57°C	4	13
6	5	8.6	37°C	16	8
2	6	8.6	37°C	4	5
7	7	6.6	57°C	16	14
8	8	8.6	57°C	16	29

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APPENDIX B

CONSENT FORM FOR PARTICIPANTS IN PERCEIVED STICKINESS STUDY

CONSENT FORM FOR PARTICIPANTS IN PERCEIVED STICKINESS STUDY

I agree to take part in a research study entitled:

Topical Formulations for Disease Treatment which is taking place at the University of Georgia in the College of Pharmacy. This study is being conducted by Bridg'ette Israel under the direction of Dr. Anthony Capomacchia, principal investigator.

The purpose of this research project is to develop a bioadhesive formulation for the treatment of aquatic life with skin lesions or abrasions. The formulation must be able to adhere to wounds and mucosa, in a wet environment and release an antimicrobial. The stickiness of the formulation is important for this experiment because the formulation must adhere to wet flesh. The human subjects are evaluating the physical properties of the formulations prior to the addition of any drug. My participation is voluntary; I can refuse to participate or stop taking part at any time without penalty or loss of benefits to which I am otherwise entitled. All participants MUST be 18 years of age or older.

If I volunteer to take part in this study, I will be asked to do the following:

I. Wear gloves while handling formulations

II. Evaluate physical properties of formulations

III. Record evaluation on the provided form

The formulations contain lecithin and one of the following oils: olive oil, mineral oil, wheat germ oil, safflower oil, and isopropyl myristate. These are natural products and they do not normally present a health risk when used as directed. Latex gloves will be provided for all participants as a safety precaution. Wheat germ allergy is a known condition. Those who possess

such a condition will be asked not to participate if they do not feel comfortable wearing gloves and a mask as a safety precaution. There should not be any topical exposure to the formulations, if all participants use the gloves. If the integrity of the gloves degrades there will be plenty of gloves available. The evaluations should not exceed three hours but breaks can be taken during this time. The researchers will exercise all reasonable care to protect me from harm as a result of my participation. In the event of an injury as an immediate and direct result of my participation, the researchers' sole responsibility is to transport me to an appropriate facility if additional care is needed. The researchers will not provide any compensation or payment for medical care. As a participant, I do not give up or waive any of my legal rights.

Please list ALL known allergies below:

_____

_____

_____

_____

This research will be conducted in room 337 in Wilson Pharmacy at the University of Georgia. The evaluations will be taken anonymously. This consent form will not be attached to the evaluation form. The research participants will be given an opportunity to gain a basic understanding of designing a research project. Their input will aid in the design of the final formulation. This project has been designed to determine if there is a relationship between bioadhesion and viscosity. This is a relationship that has not been explained in great detail and the scientific community may greatly benefit from this information.

Pertinent questions about this research project should be directed to the researchers whose contact information is provided below. Additional questions or problems regarding your rights as a research participant should be addressed to The Chairperson, Institutional Review Board, University of Georgia, 612 Boyd Graduate Studies Research Center, Athens, Georgia 30602-7411; Telephone (706) 542-3199; E-Mail Address IRB@uga.edu

I understand my involvement in this project and my questions have been answered to my satisfaction. I agree to participate in this study. I have been given a copy of this form.

A.C. Capomacchia:_____
Signature & Date

B.B. Israel:_____
Signature & Date

Participant:_____
Signature & Date

Please sign two copies. Keep one and return one to the researcher. Thank you for your contribution to science.

Contact information:

Dr. Capomacchia- 706-542-5339 or 706-340-6400 tcapomac@rx.uga.edu

Bridg'ette Israel- 706-542-3868 or 706-461-3771 johnsonb@rx.uga.edu

APPENDIX C

IRB FOR PERCEIVED STICKINESS STUDY

(INSTITUTIONAL REVIEW BOARD: IRB)

The University of Georgia

Human Subjects Office (HSO)
612 Boyd GSRC ▪ Athens, GA 30602-7411
Phone: 706-542-3199 ▪ Fax: 706-542-3360 ▪ irb@uga.edu
DHHS Assurance No.: FWA00003901

Institutional Review Board (IRB)

HUMAN RESEARCH APPLICATION

To submit: http://www.ovpr.uga.edu/hso/how/application

IMPORTANT: Please respond to all the questions. Do not leave items blank; if not applicable, mark N/A. Please note that incomplete applications may result in delayed review. Click on the hyperlinks (text underlined in blue) to obtain additional information.	*For Human Subjects Office Use Only* Project #: Date Received: Type of Review: ☐ Exempt ☐ Expedited ☐ Full Board

Section A: PROJECT INFORMATION

1. **Study Title:** TOPICAL FORMULATIONS FOR DISEASE TREATMENT
2. **Application Type:** ☒ New Project ☐ Response to Initial Review (*All revisions must be in italics or different font color.*)
   ☐ 5-Year Renewal; Previous IRB number:
3. **Principal Investigator:** (*Must be UGA faculty or senior staff. See Eligibility to Serve as PI.*)
   Name: Anthony Capomacchia **Title:** Dr.
   Department Name: Pharmaceutical and Biomedical Sciences
   Mailing Address: 233 Wilson Pharmacy
   Phone: 706-542-5339 **UGA E-mail** (Required): tcapomac@rx.uga.edu
4. **Co-Principal Investigator:** (*Required only if for thesis/dissertation or other student project.*)
   Name: Bridgette Israel **Title:** Ms.
   Department: Pharmaceutical and Biomedical Sciences
   Mailing address: 204 Wilson Pharmacy
   Phone: 706-542-3868 **UGA E-mail** (Required): johnsonb@rx.uga.edu
5. **Anticipated Start Date:** (*Must be at least 4 weeks after application is received.*) March 17th, 2010

Section B: PROJECT FUNDING

1. **Funding Status:** ☒ Funded ☐ Pending ☒ No Funding
2. **Funding Source:** ☐ Internal Account #:
   ☐ External Funding Source: OSP Proposal or Award #:
3. **Name of Proposal or Award PI** (if different from PI of IRB protocol):
4. **Proposal or Award Title** (if different from title of IRB protocol):

Section C: STUDY PERSONNEL / RESEARCH TEAM

Including the PI, identify all personnel who will be engaged in the conduct of human research. ***Important Note:*** *All researchers listed below are required to complete the CITI IRB Training prior to submission of this application. This application will be returned to PI for resubmission if training requirement has not been satisfied. To add more names, bring cursor to outside of last row, and press "enter" key.*

Name	E-mail	*Institution
Anthony Capomacchia	tcapomac@rx.uga.edu	University of Georgia
Bridgette Israel	johnsonb@rx.uga.edu	University of Georgia

** Submit an Individual Investigator Agreement for all study personnel affiliated with an institution that does not have an assurance with the Office for Human Research Protections or OHRP (typically, local schools, private doctors' clinics).*

Section D: PRINCIPAL INVESTIGATOR'S ASSURANCE

As the Principal Investigator, I have the ultimate responsibility for the conduct of the study and the protection of the rights and welfare of human participants. *By affixing my signature below,*

- I assure that all the information contained in this Human Research Application is true and all the activities described for this study accurately summarize the nature and extent of the proposed participation of human participants.
- If funded, I assure that this proposal accurately reflects all procedures involving human participants described in the grant application to the funding agency.
- I agree to comply with all UGA policies and procedures, as well as with all applicable federal, state, and local laws on the protection of human participants in research.
- I assure that all personnel listed on this project are qualified, appropriately trained, and will adhere to the provisions of the approved protocol.
- I will notify the IRB regarding any adverse events, unexpected problems or incidents that involve risks to participants or others, and any complaints.
- I am aware that no change(s) to the final approved protocol will be initiated without prior review and written approval from the IRB (except in an emergency, if necessary to safeguard the well-being of human participants and then notify the IRB as soon as possible afterwards).
- I understand that I am responsible for monitoring the expiration of this study, and complying with the requirements for an annual continuing review for expedited and full board studies.
- If human research activities will continue five years after the original IRB approval, I will submit a new IRB Application Form. (*Exceptions:* If the research is permanently closed to the enrollment of new participants, all participants have completed all research-related interventions, and the research will remain active only for long-term follow-up of participants; or if the remaining research activities are limited to analysis of individually-identifiable private information.)
- I understand that the IRB reserves the right to audit an ongoing study at any time.
- I understand that I am responsible for maintaining copies of all records related to this study in accordance with the IRB and sponsor guidelines.
- I assure that research will only begin after I have received notification of final IRB approval.

Signature of Principal Investigator _____ **Date** (mm/dd/yyyy): 2162010

Section E: CONFLICT OF INTEREST (COI)

1. **Is there any real, potential, or perceived conflict of interest on the part of any study personnel** *(e.g., financial or business interest, stock or stock options, proprietary interest, inventorship, consultant to sponsor)*? ☐ **Yes** ☒ **No**
2. **If yes, please identify personnel and explain.** ***Important Note:*** *Please review the UGA Conflict of Interest Policy. Final IRB approval cannot be granted until all potential conflict matters are addressed.*

Section F: LAY PROJECT SUMMARY

Briefly describe in simple, non-technical language a summary of the study, its specific aim(s)/objective(s), and its significance or importance. *Response should be limited to 250 words and easily understood by a layperson.* **This project is designed to develop a bioadhesive formulation for the treatment of aquatic life with skin lesions or abrasions. The formulation must be able to adhere to wounds and mucosa, in a wet environment and release an antimicrobial. The human subjects will evaluate the formulation prior to the addtion of experimental drug. The physical properties will be evaluated along with the degree of stickiness of each formulation.**

Section G: HUMAN RESEARCH PARTICIPANTS

1. **Provide a general description of the targeted participants** *(e.g., healthy adults from the general population, children enrolled in an after-school program, adolescent females with scoliosis)*, **and indicate the estimated total number, targeted gender, and age.** *To add a row, bring cursor to outside of last row, and press "enter" key.*

Targeted Population	Total Number	Targeted Gender	Specify age or age range
Healthy adults from the general population	50	Male and Female	18-100

2. **Identify the inclusion and exclusion criteria.** If two or more targeted populations, identify criteria for each.
   - **a. List inclusion criteria.**
   - **b. List exclusion criteria.**
3. If the research will exclude a particular gender or minority group, please provide justification.
4. Will participants receive any incentives for their participation (e.g., payments, gifts, compensation, reimbursement, services without charge, extra class credit)? ☐ **Yes** ☒ **No**
   - **a. If yes, please describe.** For multiple sessions, include scheme to pro-rate incentives.
   - **b. If offering extra class credit, describe a comparable non-research alternative for receiving incentive.**

Section H: RECRUITMENT AND ELIGIBILITY OF PARTICIPANTS

1. Describe how potential participants will be initially identified (e.g., public records, private records, etc.). They will be assigned a number.
2. **Describe when, where, and how participants will be initially contacted.** Those how attend the information session will be selected and they will complete the project that same day.
3. Advertisements, flyers, and any other materials that will be used to recruit participants must be reviewed and approved before their use. Check all that apply below and submit the applicable recruitment material/s.
   ☐ **No Advertising** ☒ **Bulletin boards** ☒ **Electronic media (e.g., listserv, emails)** ☒ **Letters**
   ☐ **Print ads/flyers (e.g., newspaper)** ☐ **Radio/TV** ☐ **Phone call** ☐ **Other** *(please describe)*
4. **Describe any follow-up recruitment procedures.** No follow up recruitment

5. **Describe how eligibility based on the above inclusion/exclusion criteria will be determined** (e.g., self-report via a screening questionnaire, hospital records, school records, additional tests/exams, etc.). screening questionnaire

Section I: RESEARCH, DESIGN, METHODS AND PROCEDURES

1. Describe the research design and methods of data collection. **The formulations are topical and are made from a mixture of an oil and lecithin. The ratio of oil: lecithin (1:1,1:0.5,1:0.25) is chanced for each set of samples. This set of formulations will give us an data that will help us determine if there is a significant relationship between the ratio of oil and lecithin and bioadhesion. Isopropyl myristate, mineral, olive, wheat germ, and safflower oils will be used in this reseach project to determine if there is a relationship between the degree of bioadhesion and the oil. There will be 100 samples and their physical properties need to be evaluated by atleast 50 subjects.**

2. If applicable, identify specific factors or variables and treatment conditions or groups (include control groups).
3. Indicate the number of research participants that will be assigned to each condition or group, if applicable.
4. Describe in detail, and in sequence, all study procedures, tests, and any treatments/research interventions. Include any follow-up(s). ***Important Note:*** *If procedures are long and complicated, use a table, flowchart or diagram to outline the study procedures from beginning to end.* The formulations will be developed by the research team. The subjects will wear gloves and evaluate the physical properties of the topical formulations on the form provided.
5. Describe the proposed data analysis plan and, if applicable, any statistical methods for the study.
6. Anticipated duration of participation.
   a. Number of visits or contacts: 1
   b. Length of each visit: 4 hours
   c. Total duration of participation: 4 hours

Section J: DATA COLLECTION INSTRUMENTS

List and describe all the instruments (interview guides, questionnaires, surveys, etc.) to be used for this study. **Attach a copy of all instruments that are properly identified and with corresponding numbers written on them.** *To add a row, bring cursor to outside of last row, and press "enter" key.*

Number	Instrument	Brief Description	Identify group(s) that will complete
001	Screening questionnaire	Potential subjects will be asked questions and the researchers will decide if the individuals are suitable for this study.	All subjects
002	Consent Form	Subjects will agree to the terms of the project.	All subjects
003	Formulation Evaluation	Subjects will complete this form about each formulation they evaluate and turn this information into the researchers.	All subjects

Section K: RISKS AND BENEFITS

1. Risks and/or discomforts

   Describe any reasonably foreseeable psychological, social, legal, economic or physical risks and/or discomforts from all research procedures, and the corresponding measures to minimize these. ***Important Note:*** *If there is more than one study procedure, please identify the procedure followed by the responses for both (a) and (b).*

   a. **Risks and/or discomforts.** The subjects are not expected to experience any discomfort and are not at risk of injury.

   b. **Measures to minimize the risks and discomforts to participants.** They will be required to wear gloves to insure that removal of the formulation from their hands is an easy task. This will also aid in minimizing allergic reactions to the oils in the formulations.

2. Benefits

   a. Describe any potential direct benefits to study participants. If none, indicate so. ***Important Note:*** *Please do not include compensation/payment/extra credit in this section, as these are "incentives" and not "benefits" of participation in research; any incentives must be described in Section G.4.* **The research participants will be given an opportunity to gain a basic understanding of designing a research project. Their input will aid in the design of the final formulation.**

   b. Describe the potential benefits to society or humankind. **This project has been designed to determine if there is a relationship between bioadhesion and viscosity. Other parameters are also manipulated to determine if they play a role in this process. The current formulation was designed to treat aquatic life forms, but it maybe useful in treating human wounds.**

3. Risk/Benefit Analysis

   a. Indicate how the risks to the participants are reasonable in relation to anticipated benefits, if any, to participants and the importance of the knowledge that may reasonably be expected to result from the study *(i.e., How do the benefits of the study outweigh the risks, if not directly to the participants then to society or humankind?).* We do not view this project as presenting risks to the subjects.

4. Sensitive or Illegal Activities

a. Will study collect any information that if disclosed could potentially have adverse consequences for participants or damage their financial standing, employability, insurability, or reputation *(includes but not limited to sexual attitudes, preferences, or practices; HIV/AIDS or other sexually transmitted diseases; use of alcohol, drugs, or other addictive products; illegal conduct; an individual's psychological well-being or mental health; and genetic information)?*

No

b. If yes, explain how the researchers will protect this information from any inadvertent disclosure.

5. Reportable Information

a. Is it reasonably foreseeable that the study will collect or be privy to information that State or Federal law requires to be reported to other officials (e.g., child or elder abuse) or ethically might require action (e.g., suicidal ideation, intent to hurt self or others)? No

b. If yes, please explain and include a discussion of the reporting requirements in the consent document(s).

Section L: DATA SECURITY AND FUTURE USE OF INFORMATION

1. Data Security

Check the box that applies.

☒ **Anonymous –** The data and/or specimens will not be labeled with any individually-identifiable information (e.g., name, SSN, medical record number, home address, telephone number, email address, etc.), or labeled with a code that the research team can link to individually-identifiable information.

☐ **Confidential –** The responses/information may potentially be linked/traced back to an individual participant, for example, by the researcher/s (like in face-to-face interviews, focus groups). **If necessary, provide additional pertinent information.**

☐ **Confidential – Indirect identifiers.** The data and/or specimens will be labeled with a code that the research team can link to individually-identifiable information. If the data and/or specimens will be coded, describe below how the key to the code will be securely maintained.

- ☐ **Paper records will be used.** The key to the code will be secured in a locked container (such as a file cabinet or drawer) in a locked room. The coded data and/or specimens will be maintained in a different location.
- ☐ **Computer/electronic files will be used.** The key to the code will be in an encrypted and/or password protected file. The coded data file will be maintained on a separate computer/server.
- ☐ **Other (please specify), or provide additional pertinent information.**

☐ **Confidential – Direct Identifiers.** The data and/or specimens will be directly labeled with the individually-identifiable information.

- ☐ **Paper records will be used.** The information will be secured in a locked container (such as a file cabinet or drawer) in a locked room.
- ☐ **Computer/electronic files will be used.** The information will be stored in an encrypted and/or password protected file.
- ☐ **Other (please specify), or provide additional pertinent information.**

If "Confidential" is marked, please answer all the following:

Explain why it is necessary to keep direct or indirect identifiers.

Identify who will have access to the individually-identifiable information and/or the key to the code.

☐ **Public. Information will be individually-identifiable when published, presented, or made available to the public.**

2. Future Use of Information

If individually-identifiable information and/or codes will be retained after completion of data collection, describe how the information will be handled and stored to ensure confidentiality. ***Check all that apply.***

☒ **All data files will be stripped of individually-identifiable information and/or the key to the code destroyed.**

☐ **All specimens will be stripped of individually-identifiable information and/or the key to the code destroyed.**

☐ **Individually-identifiable information and/or codes linking the data or specimens to individual identifiers will be retained.** ***If this box is checked, describe:***

a. Retention period.

b. Justification for retention.

c. Procedure for removing or destroying the direct/indirect identifiers, if applicable.

☐ Audio and/or video recordings (if applicable) will be transcribed/analyzed and then destroyed or modified to eliminate the possibility that study participants could be identified.

☐ Audio and/or video recordings (if applicable) will be retained. *If this box is checked, describe:*

a. Retention period.

b. Justification for retention.

☐ Other (please specify), or provide additional pertinent information.

Section M: CONSENT PROCESS

Important Note: *The IRB strongly recommends the use of consent templates that are available on the IRB website to ensure that all the elements of informed consent are included (per 45 CFR 116). If more than one consent document will be used, please name each accordingly.*

☒ The PI is attaching a copy of all consent documents that participants will sign.

☒ The PI is requesting that the IRB waive requirement to document informed consent. A signed consent form may be waived if one of the following criteria is met, ***check the box that applies.***

☐ 1. The only record linking the participant and the research would be the consent document and the principal risk would be potential harm resulting from a breach of confidentiality. Each participant will be asked whether the participant wants documentation linking the participant with the research, and the participant's wishes will govern; or

☐ 2. The research presents no more than minimal risk of harm to participants and involves no procedures for which written consent is normally required outside of the research context.

The consent script or cover letter that will be used in lieu of a consent form is attached. Yes

☐ The PI is requesting that the IRB approve a consent procedure which does not include, or which alters, some or all of the elements of informed consent set forth in 45 CFR 116, or waive the requirement to obtain informed consent. An informed consent may be waived if the IRB finds that all of the following have been met:

1. The research involves no more than minimal risk to the participants;
2. The waiver or alteration will not adversely affect the rights and welfare of the participants;
3. The research could not practicably be carried out without the waiver or alteration; and,
4. Whenever appropriate, the participants will be provided with additional pertinent information after participation.

Provide justification for requesting a waiver.

Describe how, where, and when informed consent will be obtained from research participants (or permission from parent/s or guardian/s and assent from minor participants), if applicable.

Section N: VULNERABLE AND/OR SPECIAL POPULATIONS

1. Check if some or all of the targeted participants fall into the following groups. ***Important Note:*** *Some targeted populations require compliance with additional Subparts and the completion of an Appendix or of specific section (see last column).*

Population Type	Required to Complete
☐ Pregnant women, neonates, or fetuses	Appendix for Subpart B
☐ Prisoners	Appendix for Subpart C
☐ Minors	
☐ Mentally-disabled/cognitively-impaired/severe psychological disorders	
☐ Physically-disabled	
☐ Terminally ill	
☐ Economically/educationally-disadvantaged	
☐ A specific group based on religion, race, ethnicity, immigration status, language, or sexual orientation	
☐ UGA Psychology Research Pool/Other UGA students/employees	
☐ Other (please describe)	

2. Explain justification for including the group(s) checked above in this particular study.
3. Is there a working relationship between any researchers and the participants (e.g., PI's own students or employees)?
   No

a. If yes, please describe.

4. Describe any additional safeguards to protect the rights and welfare of these participants and to minimize any possible coercion or undue influence. *For example, amount of payment will be non-coercive for the financially disadvantaged, extra-careful evaluations of participants' understanding of the study, advocates to be involved in the consent process, or use flyers to recruit participants instead of directly approaching own staff or students.*

Section O: COLLABORATIVE PROJECT OR OUTSIDE PERFORMANCE SITE

Check one of the two boxes below:

☒ This project does not involve any collaboration with non-UGA researchers or performance in non-UGA facilities.

☐ This project involves collaboration with non-UGA researchers or performance in non-UGA facilities (e.g., local public school, participants' workplace, hospital). ***If this box is checked, list all sites at which you will conduct this research.*** Attach authorization/permission and/or current IRB approval. *Checkboxes below are not clickable so place "X" before or over the box. To add a row, bring cursor to outside of last row, press "enter" key, and copy/paste the previous cells.*

Name of Institution	Location (County/State/Country)	Authorization/permission letter and/or current IRB approval.	
		☐ Attached	☐ Pending
		☐ Attached	☐ Pending

IMPORTANT NOTE: **If none of the following applies to your research, this is the END of the application form.**

Section P: METHODS AND PROCEDURES THAT REQUIRE ADDITIONAL INFORMATION

Section P: METHODS AND PROCEDURES THAT REQUIRE ADDITIONAL INFORMATION

Check all that apply. ***Important Note:*** *The items listed below are **NOT** an inclusive list of methods and procedures that may be used in research studies. Some procedures require the completion of an Appendix or of specific sections (see last column).*

Method/Procedure	Required to Complete
☒ Student research (For student's thesis/dissertation/others)	Section Q (below)
☐ Deception, concealment, or incomplete disclosure	Section R (below)
☐ Internet research	Section S (below)
☐ Blood sampling/collection	Section T (below)
☐ Clinical trial (Drugs, biologics, or devices)	
☐ Genetic analyses	
☐ Data/Tissue repository	
☐ HIPAA (Protected health information)	
☐ DXA/X-RAY	
☐ MRI/EEG/ECG/NIRS/Ultrasound	
☐ Other (please describe)	

Section Q: STUDENT RESEARCH

Important Note: *The IRB recommends submission for IRB review only after the appropriate committee has conducted the necessary scientific review and approved the research proposal.*

1. This application is being submitted for: ☐ Undergraduate Honors Thesis ☐ Masters Thesis Research ☒ Doctoral Dissertation Research ☐ Other (please describe)

2. Has the student's thesis/dissertation committee approved this research? ☒ Yes ☐ No

Section R: DECEPTION, CONCEALMENT, OR INCOMPLETE DISCLOSURE

1. Describe the deception, concealment, or incomplete disclosure; **explain** why it is necessary, and how you will debrief the participants. ***Important Note:*** *The consent form should include the following statement: "In order to make this study*

a valid one, some information about (my participation or the study) will be withheld until completion of the study."

2. Debriefing Form is attached. ☐ **Yes** ☐ **No**; If no, please explain.

Section S: INTERNET RESEARCH

If data will be collected, transmitted, and/or stored via the internet, the level of security should be appropriate to the level of risk. Indicate the measures that will be taken to ensure security of data transmitted over the internet. *Check all that apply.*

☐ A mechanism will be used to strip off the IP addresses for data submitted via e-mail.
☐ The data will be transmitted in encrypted format.
☐ Firewall technology will be used to protect the research computer from unauthorized access.
☐ Hardware storing the data will be accessible only to authorized users with log-in privileges.
☐ Other (please describe), or provide additional pertinent information.

Section T: BLOOD SAMPLING / COLLECTION

If blood will be collected for the purpose of this research, please respond to all the following:

1. Route/method of collection (e.g., by finger stick, heel stick, venipuncture):
2. Frequency of collection (e.g., 2 times per week, for 3 weeks):
3. Volume of blood for each collection (in milliliters):
4. Total volume to be collected (in milliliters):
5. Are participants healthy, non-pregnant adults who weigh at least 110 pounds? (Choose YES or NO)
   a. If no, indicate if amount collected will exceed the lesser of 50 ml or 3 ml per kg in an 8-week period and if collection will occur more frequently than 2 times per week.
6. Will participants fast prior to blood collection(s)? (Choose YES or NO)
   a. If yes, describe how informed consent will be obtained prior to fasting.