Analysis and Novel Characterization of Synthetic and Biologically Enhanced Complexation Hydrogels for Oral Protein Delivery

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Dedication

To my Mother, Father and Brother for being there always and pushing me to never give up and fulfill my dreams.

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First and foremost I would like to give a special thanks to my family. I would not have come this far without their encouragement and love. They have been there for me and helped me push forward when times were difficult and for that I can never express enough gratitude.

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Graft copolymer networks have shown promise as devices for oral delivery of proteins. By increasing adhesion of these networks at the delivery site of the upper small intestine by utilizing small chemical linkages caused by the novel addition of a synthetic or biological functional groups we can make them more viable. The synthetically functionalized aldehydes bind covalently by way of a condensation reaction with the amines of the amino acids found in the glycoprotein network of the mucus layer of the small intestine to form imines. The biologically grafted SlpA protein adhesin from *L. acidophilus* binds to human epithelial cells, mucus and fibronectin. To investigate the effectiveness of these bonds, P(MAA-EG) copolymers are prepared with varying percentages of grafted aldehyde modified PEG or SlpA protein adhesin and characterized. When swollen in buffered solution, results indicate that all formulations

with the aldehyde modified PEG or SlpA protein adhesin maintained the desired pH sensitivity and transition as those formulations without modification. Bulk adhesion testing was carried out through the use of a mechanical testing apparatus for aldehyde modified hydrogels in contact with mucus. Adhesion results show an increase to the already present adhesion of the copolymers due to increased percentages of the aldehyde modified PEG tethers where the highest modified formulation had the largest increase over both control formulations. Molecular adhesion testing was performed on the aldehyde modified hydrogels utilizing atomic force microscopy as a novel *in vitro* testing method. The new method was developed to gauge the intricacies of adhesion. After, using a new technique to build hydrogel capped AFM testing tips, experiments were performed with mucus with results indicating an overall increase of adhesion across all formulations when compared to bulk tensile testing. Bulk adhesion testing for the SlpA modified hydrogels with Caco-2 cells also showed an increase to the already present adhesion of the control copolymers.

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Chapter 1: Introduction

Using injectable proteins has been an important method of therapy since they first started being used in the early part of the 20th century to help those with ailments such as Diabetes Mellitus (DM). Numerous research studies have been performed on understanding the various ailments and the subsequent protein's role in combating the diseases. However, there has been little headway in administration options for proteins, and to this day, the main course of administering proteins is through injection. This is a painful experience patients must endure severely limits patient compliance and can cause other severe problems that manifest in ways such as blindness and organ failure.

Patient compliance is a problem for all injectable proteins, such as insulin, calcitonin, interferons, and human growth hormone. The lack of compliance with insulin patients alone is of great concern due to the 14.6 million people diagnosed with diabetes and an estimated 6.2 million yet to be diagnosed in the United States alone. With the numbers of those that are taking insulin and other injectable proteins growing annually, there is an obvious need for a change in administration methods in order to increase patient compliance. Therefore, the development of novel oral delivery systems could be the solution to many people's problems.

Oral drug delivery has several obstacles that need to be overcome before it is a viable solution, such as pH and enzyme conditions that degrade the proteins, and difficulties with the transport across the epithelial cell layer. These obstacles and others limit the overall drug bioavailability but, the development of novel biomaterials to be used as innovative drug delivery devices have started to unlock ways around the problems that are encountered with oral drug delivery. The performance of these devices has been improved by synthesizing polymers that

have desirable chemical, physical and biological properties and favorable interactions with biological materials.

Recently, the pharmaceutical community has become very interested in the development of new and improved muscoadhesive biomaterials with an objective of targeting specific parts of the body. This has led to the discovery of new mucoadhesive systems to apply to the nasal, buccal, ocular, gastrointestinal, urethral, rectal, and vaginal tissues with the objective of using them as delivery devices for therapeutic agents.

Some of the significant advantages of mucoadhesive drug delivery devices in comparison to traditional pharmaceutical dosage forms are that they maintain the drug delivery system in a specific location in the body; they have a prolonged duration of contact with the tissue; and they increase the treatment efficiency since the drug is locally maintained at the site of action. This is important since localizing the drug at a targeted site of absorption and transporting the drug across the intestinal epithelial layer are two problems that have previously been discussed with low bioavailabilities. This strategy has already been used in many topical treatments and improvement has been successfully proven since the early stages of the mucoadhesion field [1-4].

Creating contact between the protein delivery system and the mucosa for an extended period is essential and should allow for modulated drug release and increase the absorption of the therapeutic agent, resulting in a higher drug bioavailability. One area of research investigates the attachment of these mucoadhesive devices to the buccal, nasal, rectal, and vaginal tissues in order to avoid any gastrointestinal degradation [5]. In other cases, research has been focused on developing a mucoadhesive delivery device for the oral administration of therapeutic agents to avoid intravenous therapy [6-8].

Approaching the problems previously mentioned by using oral delivery requires the use of an intricately designed, novel carrier such as hydrogels. There are numerous applications for hydrogels, especially in the medical and pharmaceutical sectors [9-11]. Hydrogels resemble natural living tissue because of their high water content and soft consistency similar to that of natural tissue[9]. Furthermore, the high water content of the materials contributes to their biocompatibility. Thus, hydrogels can be used as contact lenses, membranes for biosensors, linings for artificial hearts, materials for artificial skin, and drug delivery devices [9-13].

Some hydrogels exhibit pH-sensitivity. This renders them suitable candidates for oral drug delivery of proteins because of their ability to respond to their environment. We have developed hydrogels composed of high molecular weight poly(ethylene glycol) (PEG) grafted on poly(methacrylic acid) (PMAA), which will be designated as P(MAA-g-EG). Acrylic-based polymers have been extensively used for their mucoadhesive applications since they exhibit very high adhesive bond strengths in contact with tissues [14-16]. The mucoadhesive nature should give the increased residence time we are looking for and makes them a great selection as the desired delivery carrier.

Adding PEG as a tether can increase the adhesive properties, allowing for interpenetration of the polymer chains at the interface, which is important for mucoadhesion. When the polymer comes into contact with the mucosa, the concentration gradient at the interface provokes the spontaneous diffusion of the polymer chains into the mucus layer and the diffusion of the mucin glycoproteins that compose the mucosa into the polymer.

While these devices have shown promise, improvements must be made before they become viable for protein delivery. One possible solution is to incorporate adhesive site specific targeting properties into a drug delivery device. There are numerous applications for adhesive

site-specific hydrogels, especially in the pharmaceutical sector [9-11]. This site-specific targeting can be used to increase adhesion by creating stronger bonds between functional groups of the hydrogel and the mucosal and cellular interface than those of secondary and interpenetrative forces. Several different functional end groups have already been investigated for their adhesive and selective properties. One such group is that of an aldehyde. The aldehyde functional group has promise for increasing mucoadhesion through the use of a Schiff base reaction that occurs under mild aqueous conditions between aldehydes and amines. In the upper small intestine, the aldehyde functional group can be brought into contact with amines found on the glycoprotein network of the mucus lining the epithelial cells. This chemical linkage between the two should increase the mucoadhesive abilities of the hydrogel [17-19]. The enhanced adhesion is unlikely to occur in the stomach because the tethers would be inside of the hydrogel in its complexed state.

Engineering specific mucoadhesive interactions into the carrier is very attractive. Another way to pursue this goal will be to use the adhesion mechanism of bacteria in the small intestine. Bacterial adhesion to the mucosa is initiated through pili or fimbriae which are cell-surface fibers that project an adhesin away from the bacterial surface. Adhesins allow bacteria to adhere to the intestinal mucosa through binding to mucosal glycans. Adhesins from nonpathogenic intestinal bacteria, such as *Lactobacillus acidophilus*, could be incorporated into P(MAA-g-EG) hydrogels. These adhesins will allow for direct interaction with the mucosa and cellular lining in a manner similar to the attachment of bacteria naturally present in the intestine. Choosing to study adhesins from the probiotic *Lactobacillus* should avoid issues with the immunogenicity associated with proteins from pathogenic bacteria. *Lactobacillus* is usually present in the GI tract of most

humans and is a probiotic included in many dairy products to boost the immune system and gastrointestinal function.

Overall, the nature of the interaction between the delivery system and mucus layer is not yet fully understood. Yet, there have been several *in vitro* methods put forth to try to gauge this interaction in various ways. All of which have some limitation [20, 21]. This complex interaction calls for a better understanding of how things take place and interact in all stages of adhesion on a molecular level. One way of doing this is by developing an improved model for *in vitro* testing. The use of atomic force microscopy (AFM) could deliver these answers that are needed.

AFM has been primarily used for imaging purposes in its short history. AFM can also be used as a powerful tool used to investigate and probe the mechanical and viscoelastic properties of thin films on the nanoscale [22]. It can measure forces with remarkable sensitivity and positional precision called force spectroscopy. Quantitative physical properties can be obtained from this data, such as local elasticity, surface forces, surface charge, and hydrophobicity, and to measure inter- and intramolecular interactions. This can ultimately provide new insights into the molecular bases of processes such as protein folding and receptor–ligand interactions [23, 24].

This method would allow for new insight into the molecular interactions between the hydrogel and the substrate at each point of adhesion on the molecular level by allowing the hydrogel to initiate the interaction and begin bonding before being pulled off to record its adhesive nature. These measurements can gauge the affect of the physical and chemical bonds on the molecular level which can then be compared on a bulk scale as needed.

The purpose of this research was to use these new tethers to improve the adhesive characteristics of the desired carrier while maintaining the pH sensitivity of the well known

mucoadhesive material, PMAA and to gauge these interactions more accurately by developing a new *in vitro* method.

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Chapter 2: Background

The idea of mucoadhesive drug delivery has had an increase in attention and research in the past decade [1]. These delivery systems exploit the attraction between mucus layers and the drug polymer carriers. The main advantages of mucoadhesive drug carriers are the localization of the carriers within a specific site within the body and prolonged residence time. Both are believed to increase the drug bioavailability. Increasing and optimizing these advantages is imperative to the future of these delivery systems.

Here, we first describe the basic concepts of bioadhesion followed by discussing the structure of the small intestine from cell layer to mucus layer and its main component, mucin. We continue by discussing the multiple theories of mucoadhesion and the synthetic and biological forms that have been used to improve adhesion. Finally, we discuss the methods used and needed for transmucosal delivery, and methods for determining adhesion.

2.1 Bioadhesion

The definition of adhesion is when two substances come into molecular contact with one another and are held in place, as seen in Figure 2.1.This can also happen due to the presence of a third substance between the other two, which acts as an adhesive. Bioadhesion is defined as the phenomenon where two materials are held together for extended periods of time by interfacial forces with at least one of the materials being biological in nature. When the adhesive attachment is to mucus or a mucus membrane, this phenomenon is known as mucoadhesion and cellular adhesion when attached to cells [2-10].

As stated previously, bioadhesion usually consists of a soft tissue or a synthetic material that is often used to adhere to another soft tissue. However, bioadhesives have been used successfully in hard tissue applications in such areas as orthopedics and dentistry, with some

common examples being dental restoration fixatives, skin adhesives, and systems for bacterial colonization. Yet, there has recently been more interest in developing more effective bioadhesives in other fields such as in tissue engineering [11-13] and also as carriers for drug delivery systems [5-10]. Synthetic and natural macromolecules are all materials that have been used in bioadhesive applications. These materials can be found on biological components such as cell tissue, blood, and bacteria.

Further understanding of the mechanism of cellular adhesion has been spurred on by the desire to create biomimetic materials to aid in developing effective tissue-engineering scaffolds as well as biomaterials resistant to biofilm formation. Better cell attachment could be attained with the ability to produce synthetic materials that exhibit molecular structures recognized by specific cellular components, which would lead to a more favorable response and thus create a more biocompatible scaffold [4]. This will be discussed in further detail in a later section.

Over the last two decades, mucoadhesion has garnered growing interest for its potential to improve localized drug delivery. This can be done by retaining a dosage form at the site of action such as the small intestine or systemic delivery, or by retaining a formulation in contact with the absorption site such as the nasal cavity. Successful mucoadhesive polymers include hydrogels. Muco- and bioadhesion of hydrogels is the result of a combination of surface and diffusional phenomena that contribute to the formation of adequately strong interchain bridges between the polymer and the biological medium [14]. Ultimately, the need to deliver challenging molecules such as biopharmaceuticals (proteins and oligonucleotides) has increased interest in this field. Mucoadhesive materials could also be used as therapeutic agents to coat and protect damaged tissues like gastric ulcers or to act as lubricating agents in the eye or vagina [3].

2.2 Mucins and the Mucosal Layer

Mucus is produced in the ear, nose, and mouth, and also coats the respiratory, gastrointestinal, and reproductive tracts [15]. It is important to our bodies for many reasons, but primarily for the protection and lubrication of the underlying epithelium. It also serves specialized functions; for example, human cervical mucus plays an integral role in both conception and contraception. As shown in Figure 2.2, the mucus layer is also the primary site with which drug delivery devices interact [8]. Thus, it is essential to understand the structure and physical chemistry of mucus if it is to be exploited as a site for bioadhesive controlled drug release [16].

The mucosa in the human stomach has a mean thickness of approximately 190 μm, while, in the duodenum, it has a thickness which ranges from 10 to 400 μm [17]. Gastrointestinal mucus aids in the passage of food and boluses through the alimentary canal and also helps shield the epithelium from shear forces caused by peristaltic waves, and resists autodigestion. These characteristics are supported by the constant secretion and replenishment of the mucus due to losses from turbulence and degradation. When there is an irritant, such as alcohol or bile salts, or a diseased state, several things occur that cause an increase in accelerated mucin release. The amount of acidic side chains in the glycoprotein increases from 50% to 80%, resulting in more negatively charged mucins. Then, the submucosal gland layer increases in depth and there is an increase in the number of goblet cells. There is an increase in the total content of nondialysable solids and pH and the mucus is thickened in the GI tract due to DNA and albumin [16].

Mucus is a mixture of several components and its composition depends on several things such as the type of animal, location, and physiological conditions. The composition of the mucus layers is up to 95wt% water, usually from 0.5% to 5wt% mucin, about 1wt% inorganic salts,

with the remainder being carbohydrates and lipids [18]. Mucus may be either constantly or intermittently secreted [19, 20].

Mucin makes up more than 80% of the organic components of mucus [21] and is the main factor in controlling its gel-like structure [18]. Electron microscopy results indicate that the persistence length of mucin in solution is approximately 100 nm [22] and the effective diameter of mucin molecules is around 5 nm. Due to the existence of loops, kinks, and turns, mucin is thought to have significant flexibility [15]. Mucins are O-linked glycoproteins [23] directly responsible for the gelling properties of mucus and the cohesion properties of the gel. Mucins are also block copolymers with branched and unbranched blocks with both types having protein backbone chains. However, there is one noticeable difference in that the branched blocks have highly branched oligosaccharide chains attached to them. These oligosaccharide branches are attached to 63% of the protein core with the remainder of the core consisting of unglycoslyated terminal regions [16]. The subunits are coupled by peptide linkages and intramolecular cysteine-cysteine disulphide bridges.

In rat goblet cells, mucin exhibits 34 disulphide bridges per molecule while porcine intestinal mucin has 28 bridges per molecule, i.e., very similar to the density of disulphide bonds in human mucin [16]. The main amino acids in the branched protein blocks are serine, threonine, and proline. The serine and threonine residues dominate the amino acid composition, with both making up 25–40% of the total amino acids [23]. However, in the non-branched blocks, the composition of amino acid is average compared to the strong serine and threonine composition in the branched blocks [23] where the branched blocks make up 75% of the length of the protein backbone chains [24]. The oligosaccharide branch chains are what make up 50% by weight of the mucin [23].

At pH > 2.6, the sialic acid and sulphate residues of the branches are fully ionized, resulting in a net negative charge to the entire molecule. Since over half of the oligosaccharides chains contain acid groups, the charge interactions may have a significant effect on the behavior of mucus glycoproteins [16]. Also, it has been shown that the branched and backbone chains are charged in appropriate environments, thus increasing the extent of stretched conformation [25]. When an aqueous environment is present, the backbone proteins are neutral or hydrophobic; however, the branched sugar chains are highly hydrophilic so that if the branched density is high and the branched chains are long enough, other molecules only contact the hydrophilic sugar chains and not the backbone segments.

It is important to understand the interactions between mucins and the interactions between mucus and polymer. Mucin molecules have both glycosylated hydrophilic and unglycosylated hydrophobic peptide blocks which cause the blocks to configure into segregated domains. If there is a disruption of the bare peptide blocks by breaking disulfide bridges or by peptide proteolytic enzymes, then the gels are no longer able to form and they dissolve into aqueous solutions [26].

2.3 Intestinal Cell Layer

Under the layer of mucus lies the small intestine. The small intestine is made up of three segments, the duodenum, jejunum, and ileum. The first portion, duodenum, is a tube 10 inches in length.[27] The next two lengths are the jejunum with the remaining being the ileum. The duodenum is the shortest, widest, and where many nutrients are taken into the blood stream.

The intestinal lining is composed of serous, muscular, areolar, and mucus layers with the mucus and areolar being important to drug delivery with most nutrients being transported through both and taken into the blood stream. The transport of nutrients occurs because of cell

layers, called villi and microvilli, seen in Figure 2.3. The villi and microvilli protrude from the mucosal layer and into the open area of the intestine and absorb a majority of the nutrients that are present.[27]

The cell lining is composed of epithelial cells, goblet cells, and endocrine cells which are renewed every four to five days. Goblet cells are scattered in rather small numbers but are important because of their ability to produce the mucus that creates the mucus lining. They secrete mucus essentially continuously but can increase their rate due to increased irritation. Endocrine cells are also scattered throughout the small intestine and are used mostly to synthesize and secrete the various hormones such as gastrin that aid in the digestive process. Epithelial cells compose the majority of the intestinal cell layer. The primary functions of epithelial cells are to protect tissue that lies beneath, the regulation and exchange of fluids and chemicals, and the secretion of enzymes. The epithelial cells secrete many different types of enzymes and proteins such as fibronectin, which is used in cell adhesion and the formation of the extracellular matrix.

Between cells, there is a junctional complex. This layer helps prevent free flow from the cells of the lumen to the basal lamina or blood stream side. These tight junctions are important to the transport of large molecules that do not have transporters or receptors on the epithelial cells surface. They have a series of fusion sites that cause several barriers instead of just one.

2.4 Oral Delivery Systems

To date there have been numerous strategies for delivering proteins orally with varying levels of success. These strategies can be grouped into three basic categories, modification of physiochemical properties, functionalization of macromolecules, and improved carrier systems.

There are two main methods for modifying the physiochemical properties. The first involves inhibiting enzyme degradation of the protein macromolecule which would thus increase

the amount available for absorption. Most common inhibitors such as camostat mesilate, sodium glycocholate, and bacitracin have been shown to be effective at increasing the absorption of insulin in several studies, however the use of them in long term therapy is in question due to the possible absorption of unwanted proteins and increased protease secretion. [28, 29] The second method utilizes penetration enhancers to modulate tight junction permeability to increase paracellular transport across the cellular membrane. Substances like bile salts and EDTA cause the tight junctions to open and allow water soluble proteins, such as insulin, to pass through. The drawback is a lack of specificity and can allow unsavory substances such as toxins and pathogens to have access to the bloodstream and in some instances can even damage the cell membrane. [28, 29]

Making chemical modifications allows for increased drug solubility and stability which can improve transport across the epithelium. This seems to be more effective for peptides than for proteins due to the structural complexity of proteins and can result in loss of pharmacological activity. There has been some success with PEGylating insulin. Studies have shown that PheB1-mPEG-insulin maintain its biological activity and is a viable option to induce a drop in blood glucose levels. [30]

A most common approach has been to find an improved carrier system that will protect the drug from degradation, deliver it to the site of administration, and aid in transport across the epithelia. There have been several methods used to try to accomplish this. The pulmonary route delivers a dose orally to be absorbed by the large surface area present in the lungs and into the bloodstream. Exubera®, an insulin inhaler, was placed on the market but was removed not long after due to the limitations and low market share. People who smoked or had lung diseases were unable to use the inhaler and the overall bioavailability was inconsistent for those who could use it.

Drugs have also been incorporated into liposomes, erythrocytes, and nanospheres for delivery. Liposomes are artificial phospholipid membranes produced by naturally occurring lipids such as lecithin and cholesterol. This allows the drug to be delivered selectively and can protect against unfavorable conditions. Carrier erythrocytes are prepared by separating the erythrocytes from plasma. Drugs are inserted inside the erythrocites and they are readministrated. The loaded erythrocytes serve as circulation depots, can be surface-modified to improve target specificity, and can prevent drug degradation. Nanospheres, or nanoparticles for drug delivery, are submicron colloid particles which can be composed of polymers or lipids and can range between biodegradable to environmentally responsive. Release usually occurs by diffusion, swelling, erosion, or degradation. This form of delivery has several advantages including high carrier capacity, can incorporate both hydrophobic and hydrophilic substances, and can be used for a sustained release. Each delivery method discussed has had limited success but will require further study to increase bioavailability before they become viable. [28, 29]

Hydrogels are cross-linked networks that absorb large amounts of water and swell, thus resembling natural living tissue and contributing to their biocompatibility. They have many uses, but are very favorable for oral delivery of proteins and peptides due to their ability to protect drugs from the harsh surroundings of the GI tract and react to their environment. This occurs specifically with pH sensitive hydrogels. This type of hydrogel stays complexed at low pH due to hydrogen bonding between functional groups. In this state, the drug is protected from the harsh conditions of the environment most typically found in the stomach. Upon entering more neutral conditions, like those of the small intestine, we see an ionic repulsion causing decomplexation, water uptake and swelling of the hydrogel allowing for the release of the drugs. It has also been shown that they can increase transport across the intestinal mucosa for improved absorption into the bloodstream. There has been limited bioavailability already with acrylic acid based hydrogels

but an increase in residence time at the site of absorption through increased adhesion could provide the increase needed to become viable.

2.5 Transmucosal Delivery

Medical science has found treatments for many diseases but the ailments still linger and the alleviation of symptoms is the only thing left to be achieved. The emerging practices of combinatorial chemistry, along with a growing knowledge of the biochemistry of the human body, have led to an ever-increasing number of therapeutic proteins in the treatment of diseases. However, these proteins are often not as durable as the more traditional small molecule pharmaceutics. Several therapeutic agents, such as aspirin or simple antibiotics, can be orally administered with little problem reaching the bloodstream intact, but the larger and more delicate proteins must be injected directly into the bloodstream. The stomach prevents this from happening with its harsh conditions that destroy a large number of the proteins and prevents them from reaching the bloodstream. In the case of insulin, less than 0.1% of the orally administered insulin actually reaches the blood stream intact so there is a need for injections [31]. This problem demonstrates the need for an alternate route of delivery. One such route is oral delivery.

Oral administration has many barriers with the harsh conditions of the stomach and the transport barrier present in the intestines being the most difficult to overcome. The stomach contains an acidic environment and many proteolytic enzymes which are the cause of proteins being denatured or destroyed in the stomach, rendering them useless. Any proteins that make it past these conditions must then somehow be transported across the intestinal epithelium into the bloodstream. This process can be hindered even further due to layers of mucus. All of these

barriers ultimately lead to a low bioavailability. Thus, it is essential to overcome these barriers before oral delivery is even remotely thought of as an alternate route.

One way of accomplishing this is through developing copolymers that are capable of protecting a protein during transit through the stomach and also aiding in increasing the protein's transport across the cellular barrier in the upper small intestine [32].

The stomach is the main digestive organ of the body and contains many digestive enzymes as well as a very low pH. The pH of the stomach has been measured from 1.4 to 2.1 but changes to nearly 4.0 when food is present [33]. When proteins are delivered to this harsh environment without protection, destruction and denaturation occurs. After the stomach, there is the small intestine which has three regions, as stated previously. The amount of nutrients taken into the bloodstream decreases the further down the small intestines. The entire length of the small intestine is 5 m with a residence time typically ranging from 2 to 4 h. The linings of the small intestine are composed of the serous, muscular, areolar, and mucus layers but only the mucus and areolar layers are important with respect to drug delivery. The transport of nutrients into the body happens through the mucosal layer and into the areolar layer before the nutrients are finally transported into the bloodstream. There are cell layers that stick out of the mucosal layer and into the open area of the duodenum. These cell layers are where most of the nutrients are absorbed into the body [32].

The transport of nutrients across the cell layer and into the bloodstream can occur through any of four different transport mechanisms [34, 35]. The first mechanism, seen in Figure 2.4, is transcellular transport and is mainly used for the transport of smaller molecules such as oxygen. The molecule diffuses from one side of the barrier, through the cell, and to the other side uninhibited since it is either too small or neutral in charge.

The second of the four mechanisms of transport is transcytosis. This occurs when molecules approach the cellular barrier and interact with the cell membrane. When this happens, the membrane forms a pocket of lipid bilayer around the material, which is called a vesicle. The vesicle detaches from the cell membrane and passes inside the cell where it is either moved to the other side of the cell and released, or digested inside the cell [32].

The third mechanism is carrier-mediated transport. This happens when a molecule interacts with key groups on the surface of the lipid bilayer. When the molecule comes in contact with the bilayer, it reversibly binds to the complexes in it. The bound molecule-complex can then cross the lipid bilayer to the inside of the cell. Afterwards, the molecule-complex disassociates and moves to the other side of the cell where a similar process occurs that allows the molecule to be placed outside of the cell and across the cellular barrier.

The fourth mechanism of transport is paracellular transport. Paracellular transport occurs when the molecules pass through by moving between adjacent cells and is the main route used by hydrophilic and charged molecules. The available space and environment between the cells dictate the movement. If the area available between the cells was increased, the molecules would move easily across the layer.

Figure 2.5 shows the junctional complex. This complex is the junction between adjacent cells, possessed by the cell layer. Without this complex, materials would freely flow between the cells from the lumen to the basal lamina or bloodstream side. The junctional complex is divided into three regions. The abluminal component is the macula adherens or spot desmosome. The second or intermediate region is known as the zonula adherens or intermediate junction. The third is the zonula occludens which is the region with the most lumen and is also known as the occluding or tight junctions.

Tight junctions are needed for transport of large molecules that are without transporters or receptors on the epithelial cell's surface. These junctions consist of a zone between 100–600 nm in depth where the lateral membranes of adjacent epithelial cells are closely apposed [36]. Tight junctions contain a series of fusion sites that create several barriers to flow across the epithelium. These tight junctions oppose less flow and are the leakiest in the duodenum but get tighter as the small intestine reaches the colon. Thus the duodenum provides an optimal site for the release and absorption of proteins

2.6 Theories of Adhesion

There have been various attempts to improve the adhesive properties of polymers since the concept of mucoadhesion was established many years ago. Several of which include the following: using linear poly(ethylene glycol) as an adhesion promoter [37], neutralizing ionic polymers [38], using a sustained hydration process for mucoadhesion [39] and developing polymer-adhesin conjugates [40, 41] to provide specific binding to the epithelia. The increase in adhesion caused by these systems can be attributed to the formation of non-covalent bonds such as hydrogen bonds, van der Waals forces, and ionic interactions which are just several of the theories of bioadhesion [42].

There have been numerous theories developed to describe the phenomenon of bioadhesion and it is important to understand them all and how they are used to improve adhesion. There is no single theory that is widely accepted as the only mechanism for which bioadhesion occurs, but rather a combination of theories is used in describing the phenomena. The theories are the following: electronic theory, fracture theory, wetting theory, adsorption theory, and diffusion theory.

The electronic theory states that at the interface between the bioadhesive and the tissue, there is a double layer of electrical charge. This is caused by a transfer of electrons upon contact. The electron transfer occurs due to the difference in structure between the bioadhesive and the glycoprotein chains in the mucus. For this theory, bioadhesion is due to an attraction across the electrical double layer [43, 44].

Through many years of being developed, the adsorption theory has become seen to suggest that bioadhesion is due to secondary forces, such as van der Waals forces and hydrogen bonding [45]. The fracture theory of bioadhesion relates the force necessary to separate two surfaces to the adhesive bond strength [44].

The wetting theory, which is associated mostly with liquid bioadhesive systems, evaluates a liquid's ability to spread over a biological surface [46]. Analysis of the spreading coefficient of a liquid bioadhesive over a tissue is used to describe the displacement of the surrounding gastric fluid. A calculation has been presented to describe the interfacial tension between the bioadhesive liquid and the tissue [47]. Afterwards, the interfacial tension was shown to be proportional to $\chi^{1/2}$, where χ is the Flory polymer–polymer interaction parameter. Low values of this parameter correspond to structural similarities between polymers and an increased miscibility. [48, 49]

The diffusion theory describes the interpenetration of the polymer chains in the interfacial region as seen in Figure 2.6 [50]. In bioadhesion, the polymer is first brought into contact with the mucus for an extended period of time. After contact is made, there is a diffusion of the chains of the bioadhesive into the mucus layer and also the diffusion of the glycoprotein chains of the mucus into the bioadhesive polymer. This diffusion is due to the concentration gradient across the interface between the two surfaces. The chemical potential gradient and the diffusion

coefficient of a macromolecule through a crosslinked network will determine the diffusion rate. The chains that diffuse are used as a form of anchor or tether to semipermanently secure the bioadhesive into place. The interpenetration distance needed for good bioadhesion is roughly equal to the end-to-end distance of the macromolecular chains [44].

2.7 Interdiffusion and Interpenetration

Potential bioadhesives have been evaluated for their adhesive properties, durability, and biological inertness [20]. A number of mechanical tests can be used to compare the adhesive strength of bioadhesive formulations and can be used in both *in vivo* and *in vitro* experiments. Previous research found that if free poly(ethylene glycol) (PEG) chains were incorporated, mucoadhesion improved significantly [51]. This occurs due to the penetration of the free PEG chains across the interface between the mucosa and polymer. This idea was first proposed with a study of the adhesion of two similar gels incorporating free PEG chains [37, 52]. Mucoadhesion of hydrogels occurs because of a combination of surface and diffusional phenomena. Both of these phenomena are contributors to the formation of strong interchain bridges that form between the polymer and biological medium [53].

It is suggested that increasing the chain interpenetration will cause the mucoadhesion to be increased. Studies of the mucin interpenetration at the poly(acrylic acid)/mucin interface using ATR/FTIR spectroscopy have been completed [54]. The results from this study showed an increase in the concentration of mucin inside the poly(acrylic acid) polymer over time. Peppas and coworkers have proposed the use of adhesion promoters based on this mechanism to increase chain interpenetration and, as a result, mucoadhesion [55-57]. One way of incorporating adhesion promoters into the hydrogel is by grafting chains onto the polymer surface. These graft chains or tethers have one end chemically attached to the hydrogel surface while leaving the

other end free to diffuse and interpenetrate. Once contact occurs, interactions at the interface form due to physical entanglements and hydrogen bonds [8]. When the hydrogel is brought into intimate contact with the mucus surface, there is diffusion of the tethers across the interface caused by the concentration gradient. The loss of chains is prevented by the covalent bond that is formed between the tethers and the backbone of the hydrogel structure. The tethers may penetrate deep enough into the mucus and act as a bridge between the tissue of the upper small intestine and the mucoadhesive device. Figure 2.7 shows a scheme of polymer chains grafted onto the backbone of a hydrogel.

The diffusion at polymer-polymer interfaces has been shown to affect the polymer concentration profile and their interfacial thickness [58-61]. There have been multiple techniques [62] used to study polymer-polymer interdiffusion such as: neutron and ion scattering X-ray fluorescence [63-65], electron microscopy [66-68], Raman and infrared spectroscopy [69-75], and light scattering [76, 77]. These studies have been used to show that temperature [78], molecular weight [79], molecular weight distribution and composition [80, 81] are important for interdiffision at the polymer-polymer interface. These studies also involve the slow mode and fast mode theories. These theories indicate that interdiffusion at the polymer-polymer interface is controlled by the slower diffusing component when it is below the glass transition temperature. However, above the glass transition of the slow diffusing component, the faster diffusing component will dictate what takes place [82-86].

A theoretical analysis was used to study the interpenetration of free chains in mucoadhesion [55-57]. This analysis concluded that the mobility of the diffused chains depends on the chain length as well as the gel volume fraction. Free PEG chains were used as adhesion promoters with crosslinked poly(2-hydroxethyl methacrylate) particles. These particles resulted

in an increase in the mucoadhesive properties of the polymer [51]. This observation was cited as being a consequence of the penetration of the free PEG chains across the interface. The idea of surface-anchored polymers has also been used in other fields [87, 88]. Gel-gel adhesion by tethered polymers has been studied through the use of the single-chain mean-field (SCMF) theory with the results providing guidelines for how to design new biomaterials with tethered polymer chains [89].

The questions involved with using promoters in mucoadhesion can be addressed by developing a hydrogel carrier with PEG chain grafts. Copolymer networks of poly(methacrylic acid) grafted with poly(ethylene glycol) (henceforth designated as P(MAA-g-EG)) have been developed for protein delivery and exhibit reversible, pH-dependent swelling behavior. This swelling behavior occurs because of the formation of interpolymer complexes between protonated pendant acid groups and the etheric groups on the graft chains. When introduced into an acidic surrounding, the complexes form due to protonation of the pendant groups which keep the gel from imbibing much fluid. However, when introduced into media where the pH is more neutral or basic, the complexes dissociate. This happens because of the ionization of the pendant groups, resulting in the gels imbibing water and swelling to a high degree. It is believed that in such systems, the free PEG chains that have been grafted onto the gel act as mucoadhesive anchors, causing an increase in mucoadhesion and making these carriers very promising as systems for protein delivery [53].

2.8 Synthetic and Biological Forms of Adhesion

One final way to increase adhesion is by creating strong covalent bonds between functional groups of the polymer and the mucus and cell layer. This can be accomplished by synthetically generating functional end groups or by more biological means by linking the drug

delivery vehicle to known adhesive segments found in nature. Several different functional end groups have already been investigated for their adhesive and selective properties with more garnering increased interest.

One synthetic group is that of an aldehyde. The aldehyde functional group can increase mucoadhesion through the use of a Schiff base covalent reaction in which aldehyde groups undergo an acid catalyzed condensation reaction under mild aqueous conditions with amines to form a covalent link, giving an imine group with the elimination of water as seen in the reaction scheme in Figure 2.8. Even without the elimination of water, the two groups will covalently link into an equilibrium state of a hemiaminal through alkylimino-de-oxo-bisubstitution. This will be useful in the upper small intestine where the aldehyde can bind with amines found on the glycoprotein network of the mucus lining the epithelial cells. [90-92]

Adhesive segments found in nature are being studied more and more as scientists try to use this information about microorganisms for more beneficial effects in pharmaceutics. This is mostly done by determining ways to interrupt adhesion and thus prevent infection. Some are looking in a different direction and trying to mimic at least the initial stages of adhesion of these microorganisms. Before using these biological options for beneficial purposes it is important to understand how microorganisms adhere to materials and subsequently spread. Initial attachement for microorganisms depends on the characteristics of the bacterium, the material surface and the interface between the two.

Just as stated previously in theories of bioadhesion, there are several forces responsible for initial attachment including van der Waals, electrostatic and other specific interactions. [93] It is also important to be aware of the receptor/binding molecule interactions because they have implications with how diseases are spread. This is seen with certain strains of influenza where

human-to-human transmission is linked to the binding preferences to certain carbohydrates present in the epithelium. [94] To make biomaterials more successful they need to interact with the environment more specifically. One critical step in doing this is by achieving successful cytoadhesion, which involves cell attachment, cell spreading, and formation of focal adhesions. This can be made more effective with ligands that interact specifically with integrins present on the cell surface.[95-97] One such approach is with the RGD (Arg-Gly-Asp, Arginine-Glycine-Aspartic acid) peptide which targets integrin receptors on the cell surface.[97]

Heparin-binding peptides and lectins can also be used in cytoadhesion. The peptides target proteoglycans while lectins target carbohydrates which are easily found in mucins and cell membranes. These have been introduced as ways around the loss of first generation bioadhesives that are sloughed off with the mucus and other components present in the GI tract.[98-100] Of note is the tomato lectin which has shown an affinity for enterocytes along human Caco-2 cell cultures but also reactivity to mucin indicating the binding to the cell surface that is desired may be hindered.

Adhesive drug delivery systems are now evolving to employ segments of fimbriae, long filamentous protein projections found on the surface of bacteria, as specific binding moieties. [101] These fibers project bacterial lectins, also called adhesins, away from the bacterial surface, towards specific glycan receptors on the host cell. In most cases they are found to employ adhesins to exploit the diversity and virtually unlimited combinatorial potential of their carbohydrate receptors to ensure selective and finely tuned pathogen–host interactions [102].

Lactobacillus acidophilus is a probiotic that is part of the bacterial microbiota of the GI tract of many humans and animals. It has been used in supplements and milk products and has had its genome sequence studied extensively for use in various medicinal purposes. One

sequence of paracrystalline protein called surface layer protein A (SlpA) has been discovered to be of vital importance in *L. acidophilus* ability to adhere and colonize human tissue. While the mechanism of attachment is still not fully understood, this sequence has been found to be adhesive to mucus, epithelial cells, and also parts of the extracellular matrix like fibronectin.

With more knowledge of the processes involved with the different types of adhesion, there can be more effective biomaterials. More effective adhesion will aid in success and subsequently result in better treatment and increased quality of life.

2.9 Methods of Determining Adhesion

Oral drug delivery has had several breakthroughs throughout the last several decades but has also been less successful than other delivery methods due to the limits associated with the physiology of the gastrointestinal tract. It is important to understand these interactions to improve adhesion and thus the efficacy of oral delivery. For this, a large variety of methods for evaluating mucoadhesive properties of polymeric materials have been developed over the last several decades but there has been little breakthrough in this area of analytical measurements. Mucoadhesion is a very complex phenomenon when dealing with the different properties of the polymer, tissue, and environment. Overall, the nature of the interaction between the system and mucus layer is not yet fully understood. Yet, there have been several *in vitro* methods put forth to try to gauge this interaction in various ways, all of which have some limitation [103, 104].

Tensile testing is a staple amongst adhesive testing. Figure 2.9 shows how this method gauges the tensile force needed to separate a cylindrical disk from animal mucus, which in turn is used to calculate the overall work of adhesion.[105] The falling film technique is a simple method where microparticles are placed along a portion of small intestine before having a solution poured over it, as seen in Figure 2.10. The eluted particles are determined and are an

indication of the mucoadhesive properties.[106] Tensile tests and the trough method give a good indicator of this bulk adhesion needed for detachement but have a forced initial interaction not seen in the small intestine [107, 108].

The flow channel method uses polymer particles placed on a mucus surface. Laminar air flow is passed over the particle with photographs taken to analyze the behavior [109]. This method doesn't give quantitative results and results from a forced interaction between particle and mucus layer.

Both the BIACORE™ and mucin particle methods give indications of the affinity that a polymer particle has with mucin in the initial stages of attraction but both fail to give any data of the strength of the bond formed and thus the ability of the particle to maintain adhesion despite various forces that act upon it in the GI tract.

To develop an optimal carrier for oral protein delivery, a more comprehensive, molecular-scale understanding of these complex interactions is necessary. One way of doing this is by developing an improved model for *in vitro* testing.

Atomic force microscopy (AFM) is a promising technique capable of providing valuable information about molecular-scale interactions between tissue and polymeric drug carriers. AFM is unique for its ability to provide three-dimensional images of biological structures in ambient conditions as well as under physiological conditions with impeccable resolution. Figure 2.11 shows a diagram of the AFM. The oscillations of the cantilever are reduced and energy lost once the probe contacts the surface. This reduction in oscillation as the laser reflects off the cantilever and into the photodiode is used to identify and measure features along the surface of the substrate. [110, 111]

While AFM has predominantly been used as an imaging tool, the method lends itself to molecular adhesion measurements. These measurements can gauge the affect of the physical and chemical bonds on the molecular level which can then be compared on a bulk scale as needed. Other works have used AFM in this fashion while modifying AFM probes to measure the adhesion characteristics of substrates such as living cells. However, these modified tips are chemically made with small covalently bonded molecules as opposed to larger crosslinked particles.[112]

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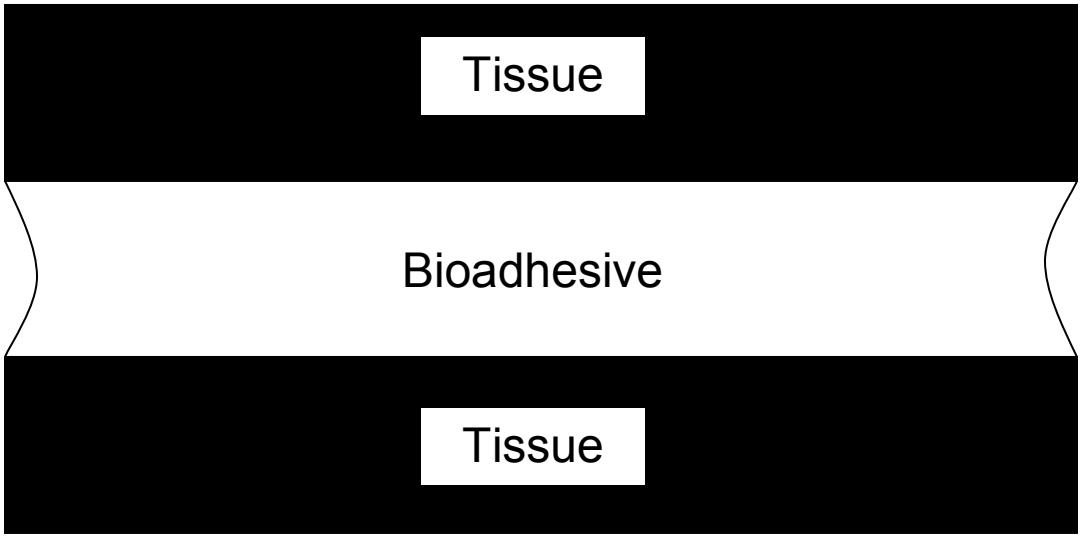

Figure 2.1.The sketch shows bioadhesion with molecular contact (adherence) of two tissues through the application of a bioadhesive between the two surfaces.

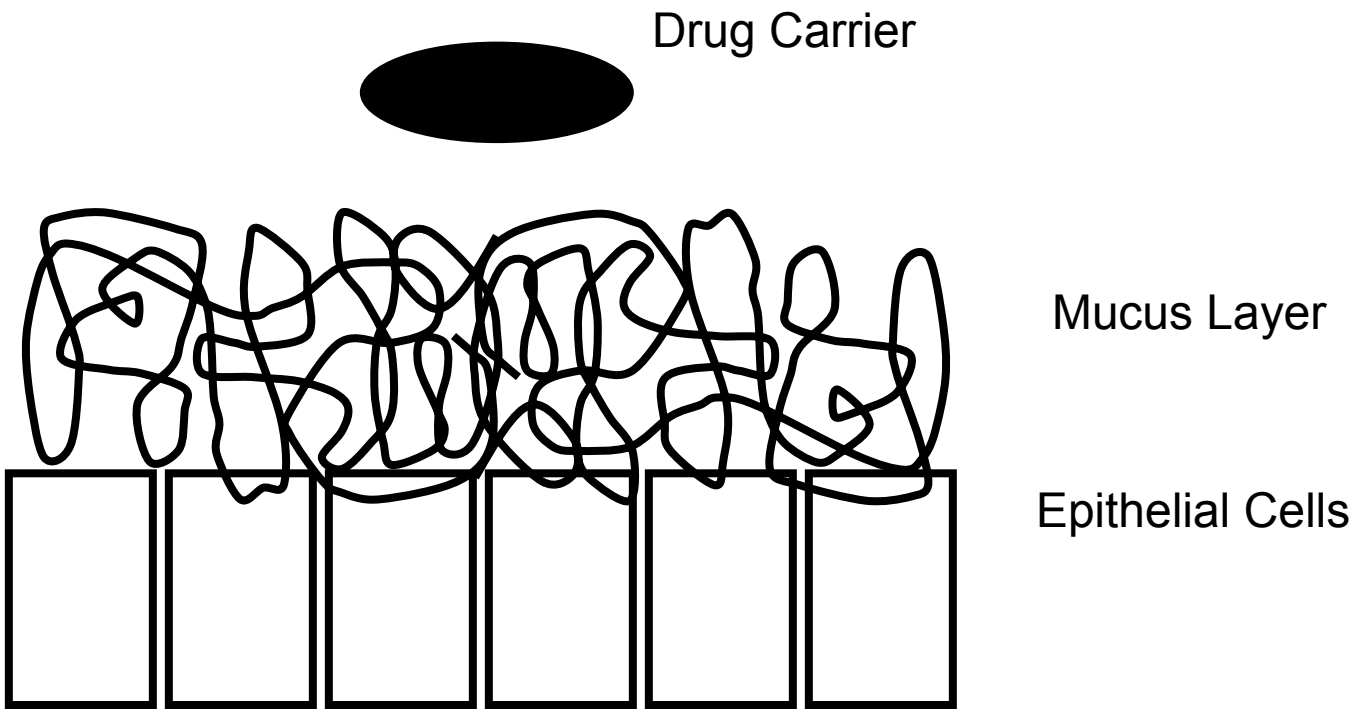

Figure 2.2. The sketch shows bioadhesion with molecular contact (adherence) of two tissues through the application of a bioadhesive between the two surfaces.

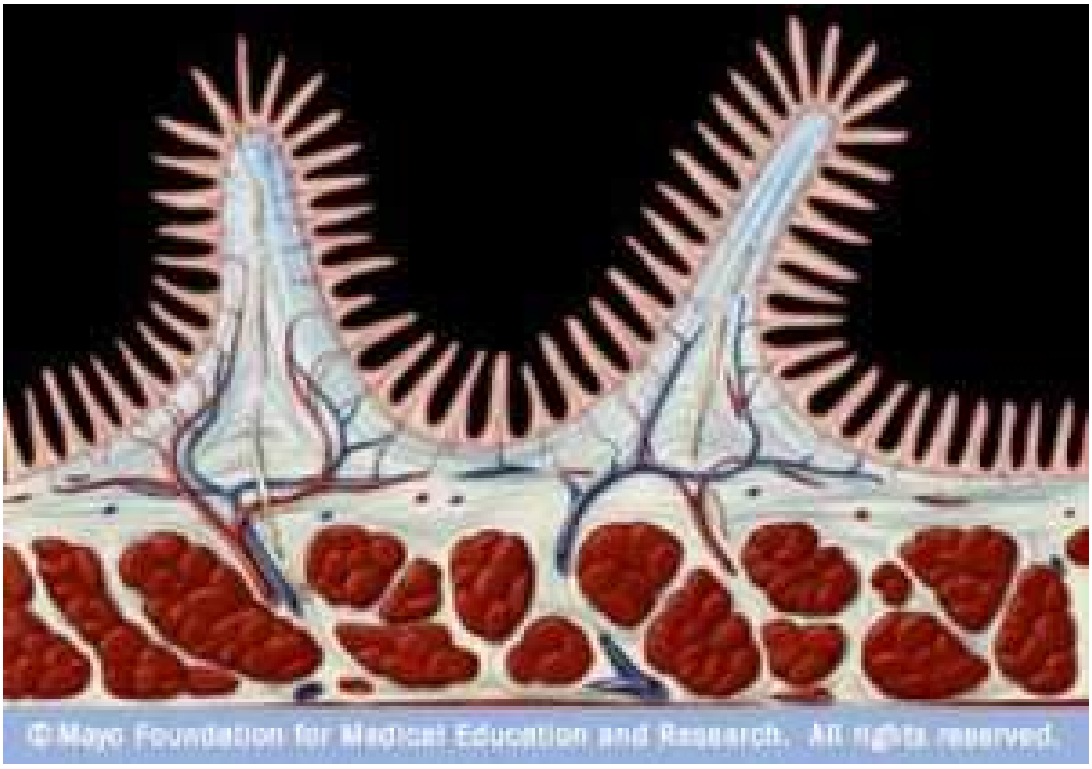

Figure 2.3. Picture of villi and microvilli present in the small intestine [113]

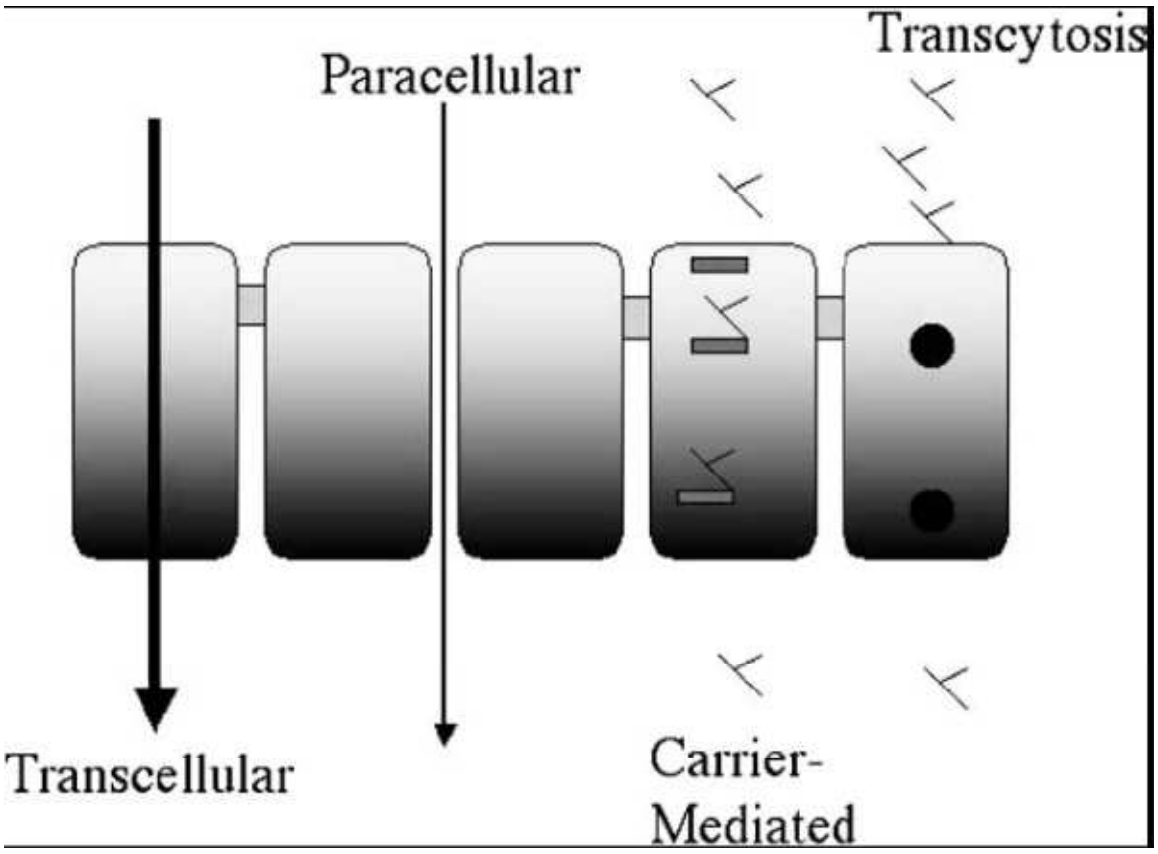

Figure 2.4. This sketch shows the four mechanisms of transport across a cell monolayer. The first is paracellular transport. Next is transcellular transport. Third is carrier-mediated transport, and the last is endo/transcytosis transport.

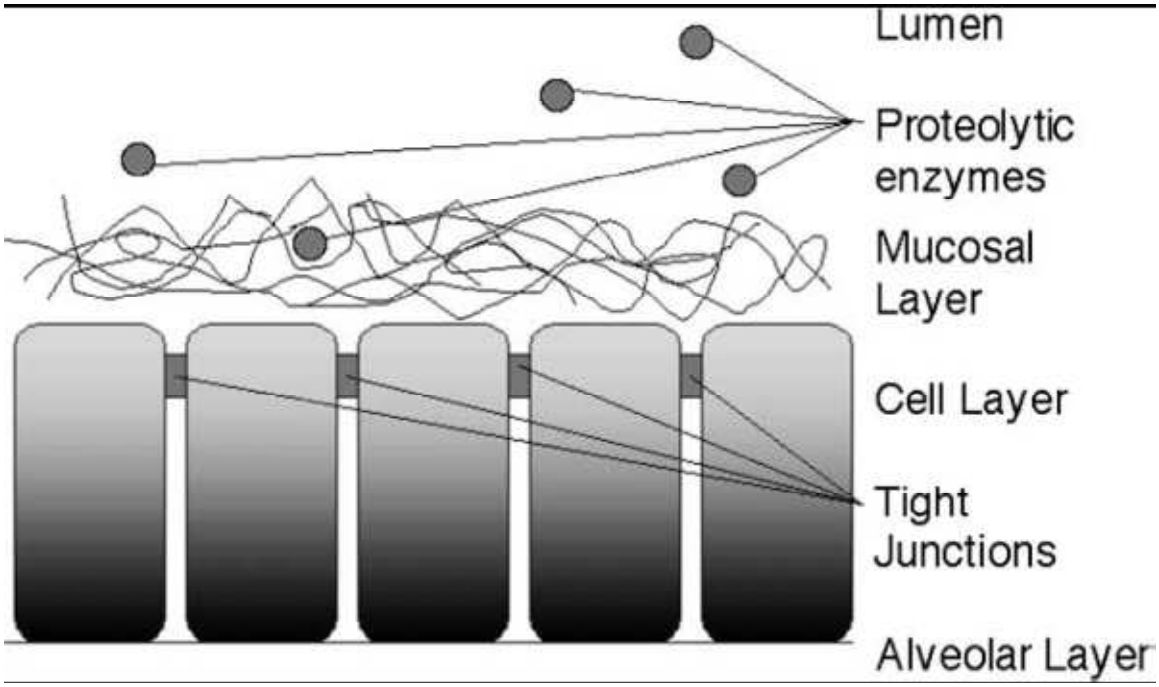

Figure 2.5. This sketch shows the environment and components of the upper small intestine.

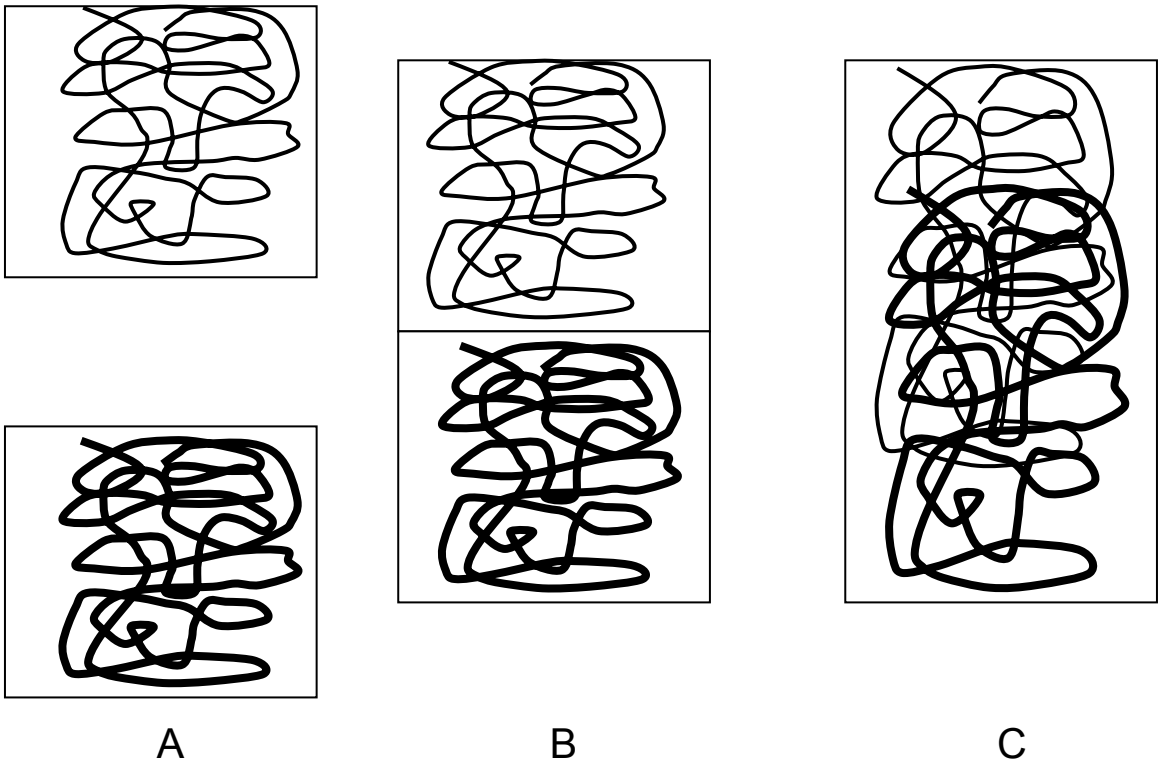

Figure 2.6. Upon contact, the interface between two polymer systems disappears and chain interpenetration or diffusion occurs. The sketch shows the interdiffusion of polymer chains in adhesion. (A) Top polymer layer and bottom layer before contact; (B) right after contact; (C) the interface becomes diffuse after contact for a period of time.

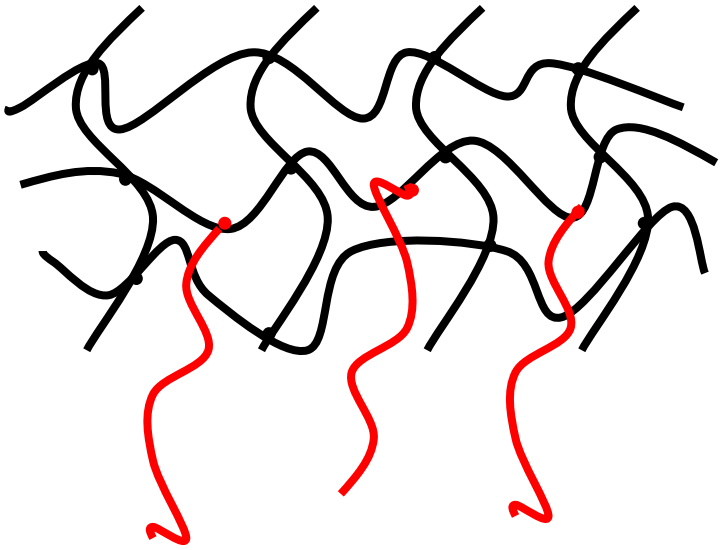

Figure 2.7. The sketch is a representation of polymer chains incorporated in hydrogel matrices as adhesion promoters. The polymer chains (red) are grafted onto the hydrogel backbone (black).

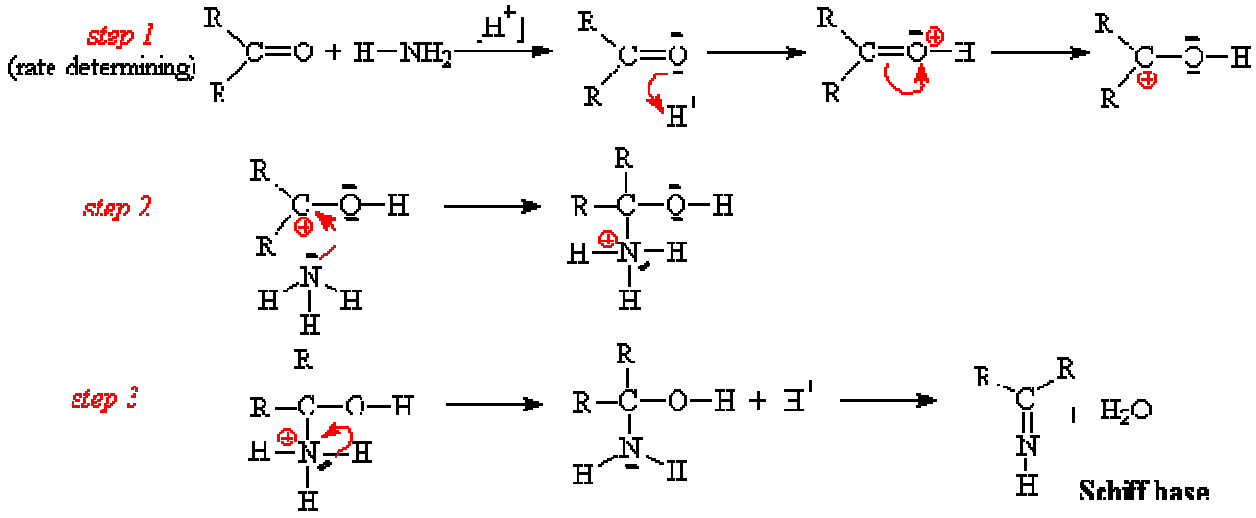

Figure 2.8. Chemical formula showing the chemical reaction between an aldehyde and an amine to form a Schiff base. [114]

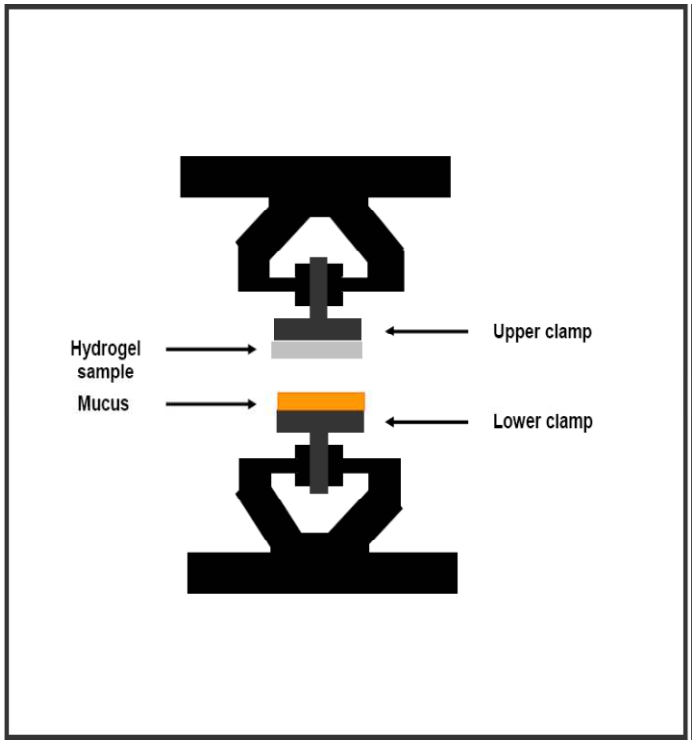

Figure 2.9. Representation of an Instron tensile testing apparatus with a hydrogel sample on the upper clamp and mucus on the lower clamp.

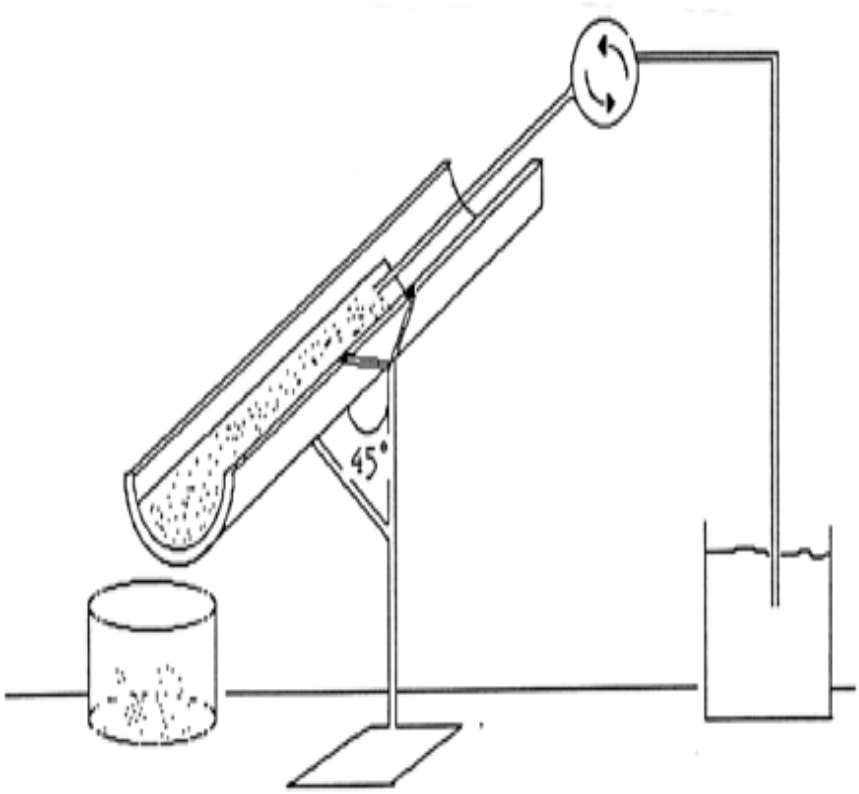

Figure 2.10. Picture of the trough adhesion test.

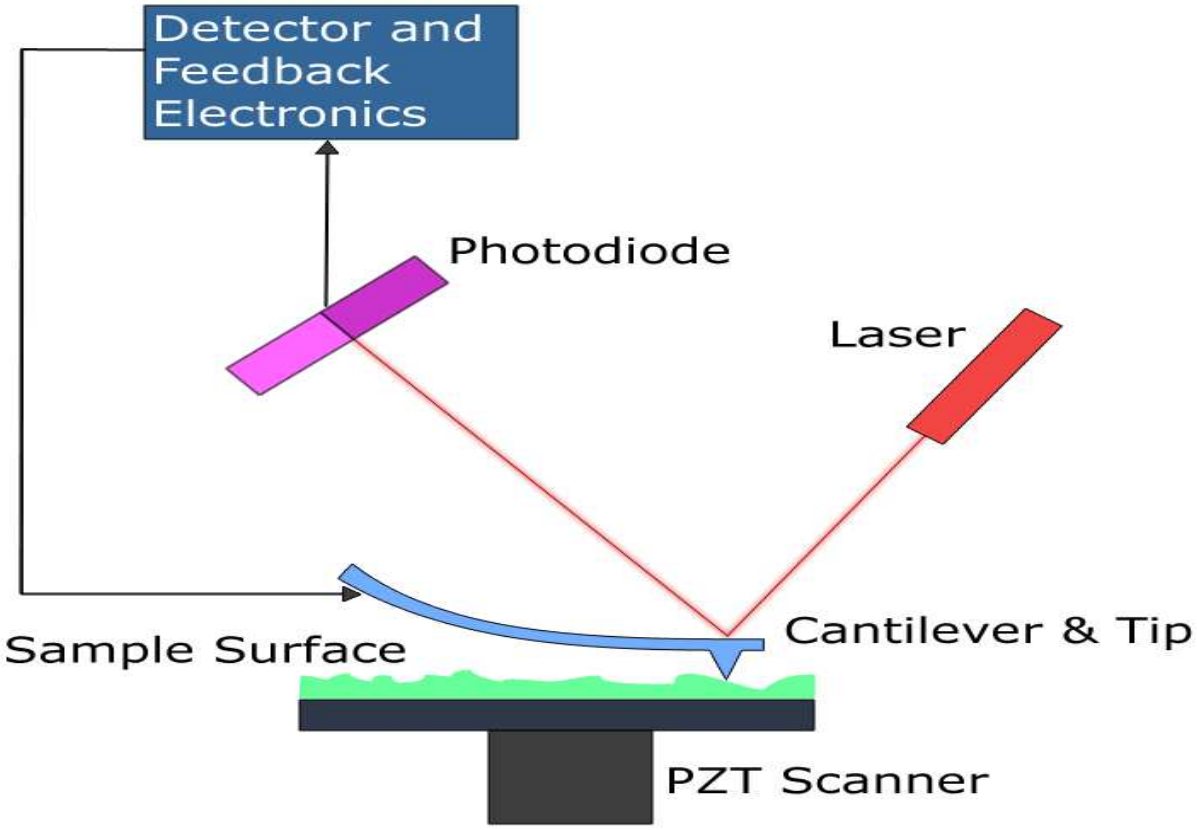

Figure 2.11. Diagram of how an AFM works.[115]

Chapter 3: Research Objectives

The purpose of this research was twofold: first, to improve upon an already established oral delivery vehicle by improving the system's adhesive characteristics while maintaining its pH sensitivity and second, to improve upon conventional *in vitro* adhesive testing and protocols. In this research, we use both synthetic and biological types of tethers grafted onto the poly(methacrylic acid) (PMAA) hydrogel with additional polyethylene glycol (PEG) grafts. This system has been designated as P(MAA-g-EG).

Acrylic-based polymers have been extensively used for mucoadhesive applications since they exhibit very high adhesive bond strengths in contact with tissues, increasing residence time and drug bioavailability in most cases. Adding PEG as a tether and other modified tethers can increase the adhesive properties, allowing for interpenetration of the polymer chains at the interface and in the case of the modified tethers create stronger covalent bonds to the tissue.

I hypothesize that better adhesion can be achieved with these modified tethers by creating stronger bonds to the mucus and epithelium of the small intestine. The first tether includes an aldehyde end group synthetically modified to the already grafted PEG tethers. This tether will bind to the amines found in the glycoprotein network of the mucus layer. The second tether links a purified adhesive protein found in the benign intestinal bacteria *Lactobacillus acidopholus* to the P(MAA-g-EG) hydrogel. This protein has been shown to be responsible for the attachment of the bacteria to the epithelium of the intestinal wall. I further hypothesize that atomic force microscopy can be utilized to give a better-defined adhesive profile of the hydrogels versus conventionally accepted *in*

vitro experimentation. The following chapters indicate that experiments I performed and the results obtained to verify my hypothesis.

Chapter 4 describes synthesis and characterization of the aldehyde modified PEG tethered hydrogels. The objective was to examine if the addition of the synthetic functional group increases the mucoadhesion of the methacrylic hydrogels while maintaining the desired characteristics. The amount of aldehyde modified PEG tethers was varied with unmodified PEG to investigate these parameters.

Chapter 5 describes the characterization and subsequent protocols for *in vitro* adhesion testing through force plot analysis with an atomic force microscope. The objective was to investigate a new method for *in vitro* adhesion testing that will avoid the limitations seen in most other accepted techniques. This new testing procedure was compared against a more established method using hydrogels developed in chapter 4.

Chapter 6 describes the synthesis and characterization of the *L. acidophilus* derived SlpA bacterial protein tagged hydrogel microparticles. The objective was to examine if the addition of the biologically derived protein adhesin increases the cellular adhesion of the methacrylic hydrogels while maintaining the desired characteristics.

Chapter 7 presents conclusions based on the previous chapters' results and recommendations for future work.

Chapter 4: Synthesis and Properties of Poly(Methacrylic Acid) pH Sensitive Hydrogels with Aldehyde Modified PEG Tethers

Introduction

Administration of proteins has been an important unsolved problem of drug delivery. While scientists race to discover better treatments for the diseases that plague societies throughout the world, administration of many of these treatments pose problems that can be quite severe. Many who need specific proteins to help with their afflictions must take multiple injections daily. Many are unwilling to go through this ordeal and those who do still see other side effects. Oral delivery of proteins, such as insulin for diabetics would prove to be convenient, improve patient compliance, and have better mimicry of dosage into the portal vein.[1] However, oral delivery has several obstacles that need to be overcome before it is a viable solution. These problems range from enzymes that digest the proteins to difficulties with the transport of large molecules across the mucosa and epithelial cell layer. These obstacles limit the overall drug bioavailability to ranges that are not commercially acceptable. However, contributions by pharmaceutical and biomaterials scientists have led to the development of novel carriers to be used as innovative drug delivery systems. The performance of these systems was improved by synthesizing polymers that have desirable chemical, physical, and biological properties and favorable interactions with biological materials. These advancements have started to unlock ways around the problems that are encountered with oral drug delivery.[2-6]

Incorporating mucoadhesive properties into a drug delivery system has many significant advantages compared to traditional pharmaceutical dosage forms. The

mucoadhesive properties can maintain the drug delivery system in a specific location in the body, have a prolonged duration of contact with the tissue, and increase the treatment efficiency since the drug is locally maintained at the site of transport. This is important since localizing the drug at a targeted site of absorption and transporting the drug across the intestinal epithelial layer are two problems associated with the low bioavailabilities that often plague oral protein delivery. Increased contact of the drug delivery system with the mucosal absorptive membranes for an extended period could increase the absorption of the therapeutic agent, resulting in a higher drug bioavailability. This strategy is already used in many topical treatments, and improvement was successfully proven since the early stages of the mucoadhesion field.[2-6]

Hydrogels with pH-sensitivity make excellent candidates for the oral delivery route because of their ability to respond to their environment, they resemble natural living tissue, are biocompatible, and provide the protection of proteins needed in harsh environments.[7-11] Hydrogels composed of high molecular weight poly(ethylene glycol) (PEG) grafted on poly(methacrylic acid) (PMAA), which will be designated P(MAA-g-EG), have been made and investigated. Briefly, the hydrogels swelling behavior occurs because of the formation of interpolymer complexes between protonated pendant acid groups and the etheric groups on the graft chains. This interpolymer complexation is a thermodynamically favorable event and is reversible.[12, 13] Acrylic-based polymers have been extensively used for mucoadhesive applications because they exhibit high adhesive bond strengths in contact with tissues.[14-16] This increases residence time and drug bioavailability in most cases. Researchers believe that in such

systems, the free PEG chains that were grafted onto the gel act as mucoadhesive anchors, causing an increase in mucoadhesion and making these carriers very promising as systems for protein delivery.[17]

One way to increase adhesion is through creating stronger bonds between functional groups. Several different functional end groups have already been investigated for their adhesive and selective properties with more garnering increased interest. One such group is that of an aldehyde. The aldehyde functional group has promise for increasing mucoadhesion through the use of a Schiff base covalent reaction in which aldehyde groups undergo an acid-catalyzed condensation reaction under mild aqueous conditions with amines to form a covalent link, giving an imine group with the elimination of water. Even without the elimination of water, the two groups will covalently link into an equilibrium state of a hemiaminal through alkylimino-de-oxo-bisubstitution. In the upper small intestine, the aldehyde functional group can be brought into contact with amines found on the glycoprotein network of the mucus lining the epithelial cells. This chemical linkage between the two should increase the mucoadhesive abilities of the hydrogel yet still be discharged from the body when the mucus layer of the small intestines is renewed every six hours.[18-20] These interactions should be prevented in the stomach because the tethers should be inside of the hydrogel in its complexed state.

In this research, we propose the use of aldehyde modified PEG tethers for improved adhesion to the upper small intestine to address the barriers that should ultimately lead to meeting the ultimate goal of improved bioavailability.

Materials and Methods

Synthesis of Aldehyde Modified PEG

PEG aldehyde was synthesized by a reaction outlined by Dr. Steven Ley.[21] Briefly, PEG monomethacrylate monohydroxyl (PEGMA, Sigma, St. Louis, MO) was dissolved with Dichloromethane (DCM, Sigma, St. Louis, MO), 4 angstrom sieves (Sigma, St. Louis, MO), and N-methyl morpholine N-oxide (NMO, Sigma, St. Louis, MO). The solution was purged with N_2 for 10 minutes and the catalyst, tetra-n-propylammonium per-ruthenate (TPAP, Sigma, St. Louis, MO), was added. Following this step, the solution was purged for an additional 10 minutes with N_2. The solution was stirred for 30 minutes until a change from dark green to black was observed, indicating the reaction was successful.[21] After mixing, DCM was added before several salt washes were performed (potassium chloride, copper (II) sulfate, sodium sulfate) in a separatory funnel. The remaining organic layer was dried over several days to remove any remaining solvents. A Schiff's base assay composed of fuchsin sulfurous acid (Sigma, St. Louis, MO) was used to detect aldehydes on a UV/VIS spectrometer. A standards curve was made using glutaraldehyde (Sigma, St. Louis, MO) and the Schiff's base assay. Figure 4.1 shows the structure of aldehyde modified PEGMA.

Hydrogel Synthesis

The methacrylic acid (MAA) monomer was passed through a dehibit column to remove the inhibitor. The polymer films were prepared by free radical UV polymerization. The diluted monomer mixture was composed of methacrylic acid (MAA, Sigma, St. Louis, MO) and both PEGMA and aldehyde modified PEGMA with a

molecular weight of approximately 500. Irgacure® 184 (1-hydroxy-cyclohexyl-phenylketone, Ciba-Geigy, Hawthorne, NY), which was used as a photoinitiator, and tetraethylene glycol dimethacrylate (TEGDMA, Polysciences Inc. Warrington, PA), which was used as a crosslinking agent was also incorporated. Deionized water and ethanol were used as solvents. The monomer solution was made in a ratio of 1:1, MAA:EG units, with three formulations containing aldehyde modified PEGMA in percentages of 0.06%, 0.6%, and 3.3%, incorporated with unmodified PEGMA as well as solutions of P(MAA) with no PEG tethers and P(MAA-EG) with no aldehyde modified PEGMA. TEGDMA was added as 1 mol% of the total monomer and Irgacure® 184 added as 1 wt% of the total monomer.

The monomer solution was dissolved with a 50:50 monomer to solvent weight ratio. The total solvent weight was composed of equal parts deionized water and ethanol. Once all components were added to the monomer mixture, the solution was sonicated until all materials were dissolved into solution. The mixture was purged with nitrogen for 20 minutes to remove oxygen, which acts as a free radical scavenger. The solution was pipetted between two glass slides (75mm X 50mm X 1mm) separated by a 0.8 mm Teflon® spacer. The slides were placed under a UV light (Efos Corporation, Ultracure 100ss, high pressure mercury lamp, Mississauga, Ontario, Canada) and allowed to polymerize for 30 minutes.

After polymerization was complete, the thin films were washed with deionized water daily for one to two weeks to remove all unreacted components. After washing, the films were cut into 12mm diameter disks and dried under a vacuum (**Heraeus VTR-**

5036, Heraeus Instruments GmbH., Hanau, Germany) for two days at approximately 30° C. Fourier transform infrared spectroscopy (FTIR) was used to determine if the Schiff's linkage was still taking place in the hydrogels with aldehyde modified PEGMA.

Swelling Analysis

Dynamic swelling studies were performed using dimethylglutaric acid (DMGA, I=0.1M, Acros Organics, NJ) buffers. Ten buffers, with a pH range of 3.2 to 7.6 were prepared. The ionic strength of the buffers was controlled with 0.1M sodium chloride (NaCl, Sigma Aldrich, St. Louis, MO). The polymer disks were placed in 50 mL of the first DMGA buffer with a pH of 3.2 for five minutes at 37° C. Afterward, the disks were taken out of the buffer, blotted to remove excess buffer, and weighed to determine the water uptake. The disks were then placed in the DMGA buffer of the next highest pH and the process was repeated up to the buffer with a pH of 7.6.

For equilibrium swelling, polymer disks were swollen in five different pH solutions for a span of 48 hours at 37° C. One disk was placed in each of the five different DMGA buffers with a pH range between 3.2 and 7.6. Afterward, the swollen disks were blotted to remove excess buffer and weighed in ambient air to determine the water uptake.

The following equation was used to determine the weight swelling ratio, Q:

$$Q = W_{swollen} / W_{dry} \quad (1)$$

$W_{swollen}$ and W_{dry} refer to the weights of the copolymer disk in swollen and dry states, respectively. These values were then used to plot weight swelling ratio, Q, vs. swelling pH.

Insulin Loading and Release Analysis

Hydrogel films were dried, crushed, and sieved with a mesh size of 45μm. Images of particles were taken on a light microscope and processed using an image processing program. Particle size distribution was found to be 8.5 mm with a standard deviation of 5.8mm. All glassware was treated with Sigmacote® (Sigma Aldrich, St. Louis, MO) to prevent insulin adsorption on the glass wall. Approximately, 140 mg of crushed hydrogel particles were dispersed into 20 ml of human insulin solution (500 μg/mL in phosphate buffered saline (PBS) at pH = 7.4) at 37° C and stirred for four hours. Afterwards, 10 ml of 0.1M hydrochloric acid (HCl, Sigma Aldrich, St. Louis, MO) was added to the solutions to collapse the hydrogel network. The particles were filtered and rinsed with 50 ml of 0.1 M HCl and 50 ml of DI-water to remove any surface bound protein. The loaded particles were then lyophilized.

The concentration of insulin in the loading filtrate was determined by reverse phase high performance liquid chromatography (RP-HPLC). The amount of insulin loaded was based on the amount of insulin remaining in the loading filtrate to the initial insulin in the loading solution. Briefly, aliquots of 0.2 ml of the loading filtrate were taken using a 1 ml syringe with a 0.45 μm filter. The mobile phase for the RP-HPLC was composed of a mixture of mobile phase A [water with 0.1% TFA (v/v)] and mobile phase B [acetonitrile with 0.1% TFA (v/v)] on a Waters Symmetry300™ C18 column (5 μm, 3.9 x 150 mm). Samples of 20 μl were injected to Waters 2695 separations module equipped with a 996 Photodiode Array detector (Milford, MA) at 1 ml/min. A gradient from 25-38% mobile phase B was run for 10 minutes. Concentrations were determined

based on the area under the curve compared to insulin standards with detection at 254 nm.

Release studies using the insulin loaded polymer (ILP) were performed by adding 15 mg of the ILP to 30 ml of PBS at pH = 7.4 into a Distek Dissolution System 2100B (New Brunswick, NJ). The solutions were stirred with 0.8 ml aliquots withdrawn using a syringe equipped with a 0.45 μm syringe filter at time intervals of 10, 30, 60, 90, 180, 360, and 1440 minutes. A constant release volume was maintained by addition of 0.8 ml of fresh PBS. These studies were done in triplicate. The protein concentrations for the release time points were determined using a Micro BCA™ Protein Assay Kit (Thermo Scientific, Rockford, IL), measured by a microplate reader at 570 nm.

Adhesion Analysis

The adhesion of the polymers in contact with mucin was measured with a mechanical testing apparatus (Instron 4442, 50 N load cell, Canton, MA). The hydrogels were swollen for 10 minutes in a PBS solution (pH 7.4) and fixed to the upper support with cyanoacrylate medical adhesive. One milliliter of 2 wt% porcine mucin (Sigma Aldrich, St. Louis, MO) in DI water was deposited on the lower mount of the tensile tester. The 2 wt% was chosen to mimic the mucin percentage found in the small intestine. The two surfaces were brought into contact with an impingement force of approximately 0.05N-0.1N. The upper clamp was raised at a constant rate of 6 mm/min until complete detachment occurred while the detachment force (N) vs. displacement (m) was measured. The work of adhesion (μJ) was taken to be the area under the curve. This procedure was also performed in an aqueous environment with 10ml of PBS buffer (pH 7.4) in ambient

conditions, which is to say at room temperature and open to the atmosphere. To prevent dissolution in the PBS buffer, the mucin solution was made at 20 wt%. The added 10 ml of PBS bringing the overall solution back to 2 wt% of mucin solution.

Results

Analysis of Chemical Structure

FTIR spectroscopy was performed to investigate the functionality of the aldehyde modified PEGMA after polymerization to the pH sensitive hydrogel. Spectra were taken of the Schiff's reagent, a dry sample of the 3.3% aldehyde modified PEGMA formulation, and a 3.3% aldehyde modified PEGMA formulation soaked in Schiff's reagent and washed in DI water to remove excess unreacted Schiff's reagent as seen in Figure 4.2a and b.

Figure 4.2a shows a broad peak between 3000-3500 (cm-1) for the Schiff's reagent spectra. This broad peak distinguishes the amine and aromatic groups found in the fuchsin sulfurous acid of the Schiff's reagent, which is noticeably absent from the dried 3.3% sample but is evident in the soaked and washed 3.3% sample. This indicates that the aldehyde bound to the Schiff's reagent since there are no aromatics or amines present in the initial hydrogel sample.

In the washed 3.3% sample of Figure 4.2b there are two distinct peaks at 1175(cm-1) and 1300 (cm-1) that are absent in the dried 3.3% sample. These two peaks are shifted due to the steric hindrance with such large molecules but indicate the presence of sulfurous acid. These double peaks indicate that the aldehyde is bound to the Schiff's reagent since there is no sulfurous acid present in the initial hydrogel sample. The

aldehyde modified PEGMA polymerized to the hydrogel and maintained functionality throughout the polymerization process.

This study allows us to confirm that the aldehyde modified PEG tethers are still functional. This indicates that they survived the harsh conditions of the polymerization process and remain active to be utilized in the adhesion to the gastrointestinal tract.

Hydrogel Swelling

It is important to understand the phenomenon of complexation through hydrogen bonding. Interpolymer complexation forms between electron deficient moieties and moieties containing regions of high electron density. This interpolymer complexation is a thermodynamically favorable event and is reversible. In acidic surroundings, the copolymer is in a collapsed state because of the hydrogen bonding between the ether group of the PEG chain and the carboxylic group of the PMAA.[12, 13] The pH must be sufficiently below the pKa to allow for sufficient protonation of the carboxylic acids group. As the pH values reach the pKa value of MAA (4.8), the carboxylic acids are ionized. This ionization causes the dissociation of the hydrogen bonds, resulting in a repulsive interaction between the PEG chains and the MAA molecules. This phenomenon is commonly known as decomplexation.

While there is hydrogen bonding between the PMAA and the PEG chain, the bonds can be disrupted. It has been shown that the methyl group of PMAA acts as an electron receiver and stabilizes the carboxylic acid group. The increase in electron receiving due to the methyl group causes an increase in the stability of the carboxylic acid's hydrogen bonding, further stabilizing the complexation.[22]

Dynamic and equilibrium swelling studies were performed to investigate the pH sensitivity and water uptake of these samples with the incorporation of the aldehyde modified PEGMA in comparison to two control samples of PMAA and P(MAA-EG). The objective being to indicate that incorporation of the new aldehyde modified PEG tethers has no negative effect on the swelling or pH sensitivity of the hydrogels. The dynamic water uptake for each formulation is shown in Figures 4.3a and b. All formulations maintain pH sensitivity with a transition around a pH of 5.8. All formulations have similar swelling ratios with high points of 1.5 and low points of 1.0 with one exception. The PMAA formulation has a maximum of 3.3 and a minimum of 1.4 due to the lack of increased hydrogen bonding brought by the PEG tethers.

The equilibrium water uptake for each formulation is shown in Figure 4.4. All formulations maintain pH sensitivity with a transition around a pH of 5.7. All formulations have similar swelling ratios with a maximum of 6.8 and minimum of 1.4, with one exception. The PMAA formulation has a maximum of 13.9 and a minimum of 2.8 due to the lack of increased hydrogen bonding brought by the PEG tethers. The aldehyde modified PEGMA formulations for both dynamic and equilibrium swelling show continued pH sensitivity, as seen with the control formulations.

All compositions in both the dynamic and equilibrium study showed pH sensitivity at pH of 5.8 and 5.7, respectively. There was also an increase in water uptake as pH increased, indicating that the addition of the aldehyde moiety does not affect the bonds that cause complexation and decomplexation. Of note is the drop between the weight swelling ratio of the PMAA formulation. This was to be expected with fewer

ether group sites due to the lack of PEG chains, thus decreasing the hydrogen bonding and the carrier's ability to keep fluid out.

This study allows us to confirm that the new tethers do not affect the desired properties of the pH sensitive hydrogels. Thus, we expect that the new carriers could maintain the same characteristics through the oral delivery process.

Insulin Loading and Release Analysis

Insulin load and release studies were performed to determine how incorporation of the aldehyde modified PEGMA tethers would affect release characteristics. To determine any detrimental effect by the new aldehyde tethers, load and release studies were performed on the highest aldehyde modified PEGMA tethered formulation (3.3%). This has implications because of the success of prior work with pH sensitive hydrogels.[23] As discussed previously, RP-HPLC was used to determine that the amount of insulin loaded was at 93%. The fractional insulin released is shown in Figure 4.5. The graph shows a minimum of 0.19 after 10 minutes at the first time point and then an exponential increase to 0.73 at the last time point of three hours.

This study allows us to confirm that insulin release is still possible with the new tethers of the pH sensitive hydrogel. It is expected that these systems could maintain significant insulin release with potentially more adhesion.

Adhesion Analysis

Adhesion studies were performed to investigate the effectiveness of the chemical Schiff's linkage in aiding mucoadhesion when compared to the interpenetration and hydrodynamic forces seen in the control formulations in both dry and wet conditions.

During the mucoadhesive experiments, the hydrogel disks were previously swollen in a buffer solution of pH 7.4 for 10 minutes. The first step necessary for the formation of a mucoadhesive bond between a polymer and the mucus is the wetting and swelling of the polymer. This step is crucial since the amount of water imbibed by the polymer at the time of adhesion will affect the interaction of the system with the mucus at the interface. Ten minutes of exposure to a buffer solution was sufficient to swell the system to conditions similar to those in the upper small intestine. This value simulates the physiological situation of a hydrogel traveling through the GI tract to the upper small intestine, where it is unswollen under the conditions described above, to the target site of adhesion, where it starts swelling and decomplexing.

Different PEG-tethered designs were studied. The objective was to improve the mucoadhesive performance of a crosslinked poly(methacrylic acid) hydrogel, a well-known mucoadhesive polymer, by decorating it with PEG tethers that have different adhesive moieties. The PEG tethers in this research were decorated with an aldehyde functional group. The basic P(MAA-EG) hydrogel utilizes many secondary chemical interactions. These secondary chemical interactions include ionic bonds, van der Waals interactions, and hydrogen bonding, with hydrogen bonding probably being the most important secondary interaction in mucoadhesion. Some functional groups that form hydrogen bonds are hydroxyls and carboxyls, which are both present on the basic

P(MAA-g-EG) system. However, while these types of forces are weak, numerous interaction sites led to strong mucoadhesion.[24] Upon entering the high pH conditions of the upper small intestine, hydrogen bonds are formed between the hydrogel and the mucus. There are some ionic interactions but not a significant amount. These secondary forces hold long enough for swelling and, as a result, interdiffusion will take place. This happens because the PEG chains are not interacting with the poly(methacrylic acid) backbone at the higher pH conditions. Consequently, the PEG chains are able to diffuse through the mucus and enhance mucoadhesion. The aldehyde moiety will complement these interactions with the addition of another possibly stronger chemical reaction with that of a Schiff's base covalent linkage. This occurs when the aldehyde forms a chemical linkage with an amine functional group similar to those found on the N-terminus of the glycoproteins that help compose the mucus layer that coats the small intestine.

Figures 4.6a and b show typical graphs of the force of detachment (N) vs. displacement (m) curves. The two graphs are of the lowest and highest order results retrieved from testing, respectively. This can be seen in the overall increase in the magnitude of the maximum force on the y-axis with Figure 4.6a peaking at 0.09 N and Figure 4.6b peaking at 0.35 N. Figure 4.6a shows the curves obtained in the tensile experiment present three different regions indicated with the letters A, B, and C. From point A to B, the disk is completely in contact with the mucin solution and the graph peaks at a maximum force as the two surfaces begin to detach. A large force is necessary for starting the detachment process. In the second portion of the curve, from point B to C, the graph begins decaying as the mucin solution detaches and moves to the exterior

portion of the disk. The graph begins to tail off, corresponding to the only contact with the disk, being through a taut string of mucin. Point C indicates the break point, where the two surfaces completely detach causing the graph to return to zero, ending the experiment. The work of adhesion (μJ) was calculated to be the area under the curve.

Figure 4.7 shows the work of adhesion (μJ) for PMAA, P(MAA-EG) and all three weight percentage ratios of aldehyde modified PEGMA (0.06%, 0.6% and 3.3%) in ambient surroundings with 2 wt% mucin solution and PBS buffered bath with 20 wt% mucin solution. Figure 4.7 shows a minimum of 10.7 μJ for the PMAA formulation in ambient surroundings and a maximum of 48.1 μJ for the 3.3% aldehyde modified PEGMA formulation in PBS buffered bath surroundings.

The data shows a trend of increasing adhesion from no tethers in the case of PMAA to those with tethers, as well as an increase in adhesion with an increase in the amount of aldehyde-modified PEGMA. This is the case for both ambient and buffered bath conditions, although the trend is not seen with the buffered bath conditions until reaching the higher percentages of aldehyde modified PEGMA. This indicated that interpenetration forces cause an initial increase in adhesion from PMAA to P(MAA-EG) but that the addition of the added chemical bonding through the Schiff's linkage causes a greater increase in the adhesion and adhesion will only increase with more aldehyde modified PEGMA due to the availability of more bonding sites.

Also of note is the overall increase in the force of adhesion between the ambient and PBS buffered bath solutions, especially for the PMAA formulation. This occurs due to the added swelling that takes place within the water bath. As stated previously, the

PMAA formulation has a larger water uptake than that of any other formulation. This is due to the lack of the hydrogen bonding that takes place between PEG and PMAA in all tethered formulations. These hydrogen bonds cause for tighter junctions and smaller uptake of fluid, thus swelling. Since PMAA does not have these interactions, it has more uptake of fluid and more swelling, which is still taking place in the water bath during the mucoadhesion experiments. The increased swelling allows for more of a wicking action to take place between the hydrogel and the mucin substrate, causing an increase in adhesion.

This study allowed us to determine the increased adhesion the aldehyde modified PEG tethers provide for. With increased adhesion over other formulations, these new materials could potentially improve drug delivery due to increased residence time in the upper GI tract.

Conclusions

The present work was performed to determine the effectiveness of using chemical linkages by utilizing a reactive functional group, in this case an aldehyde moiety, to increase the limited adhesion of the hydrogel. The better adherence to the small intestine would lead to increased residence time, release at the delivery site, and would presumably affect the bioavailability of the protein. Hydrogels with and without PEG tethers and with varying percentages of aldehyde modified PEG tethers were synthesized and characterized to determine any improved adhesive capacity without affecting the overall characteristics of the oral delivery device. Swelling studies showed that the aldehyde functionalized hydrogels retained their pH sensitivity at pH=5.5. Release

studies showed the aldehyde functionalized hydrogels maintained the fractional insulin release seen in previous work with nonfunctionalized hydrogels. Widely accepted *in vitro* tensile testing showed improved adhesive capacity with increased chemical linkages with the aldehyde moiety in both dry and wet conditions. These findings indicate the potential these functionalized hydrogels have for improved oral drug delivery.

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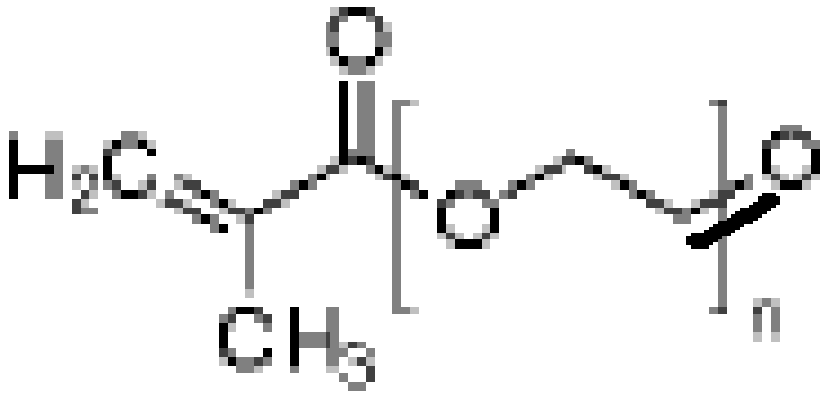
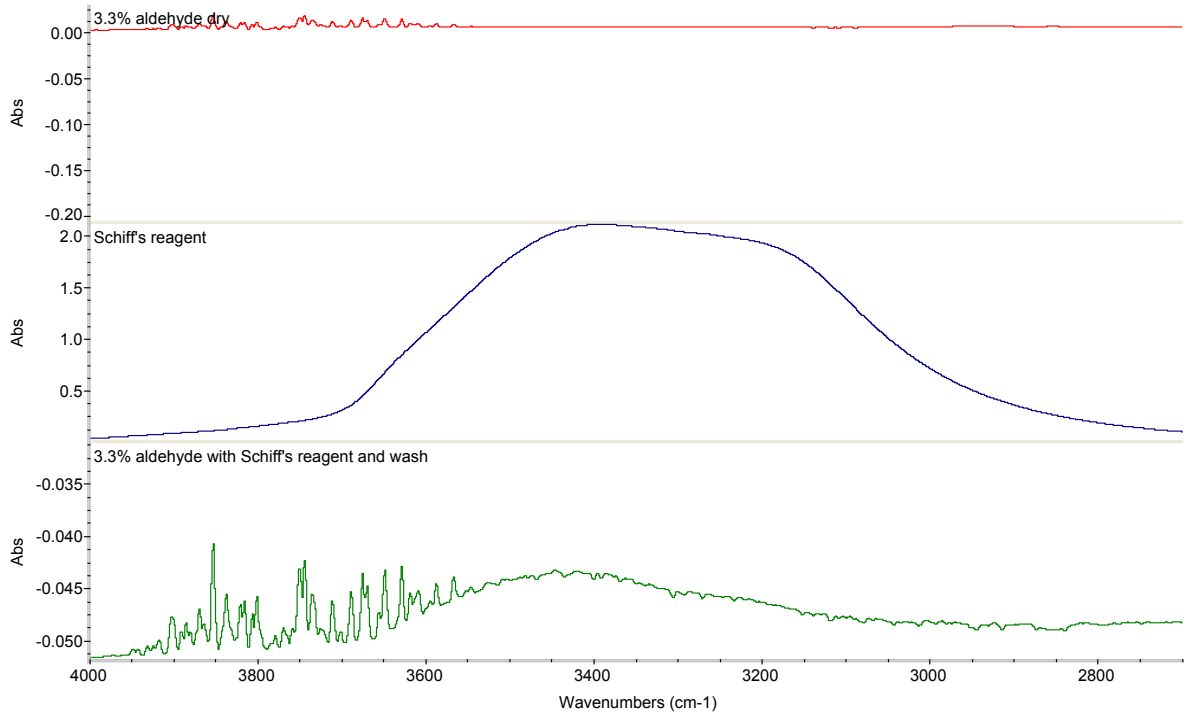
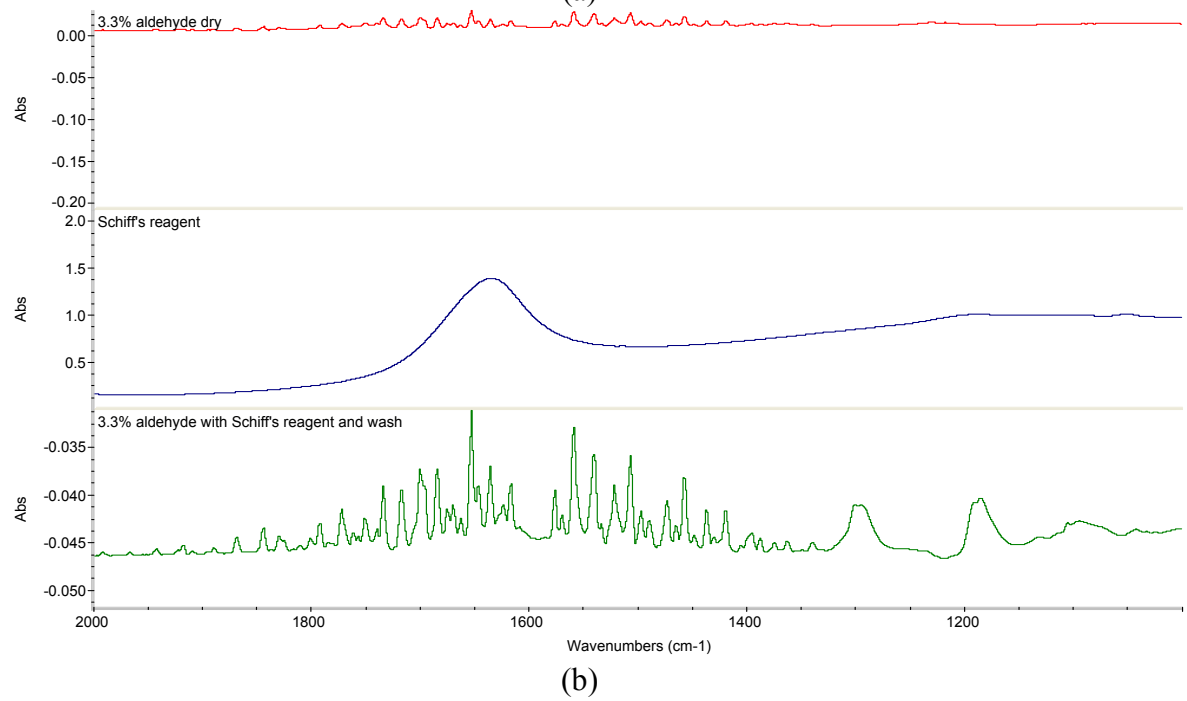


Figure 4.1. Structure of aldehyde modified PEGMA

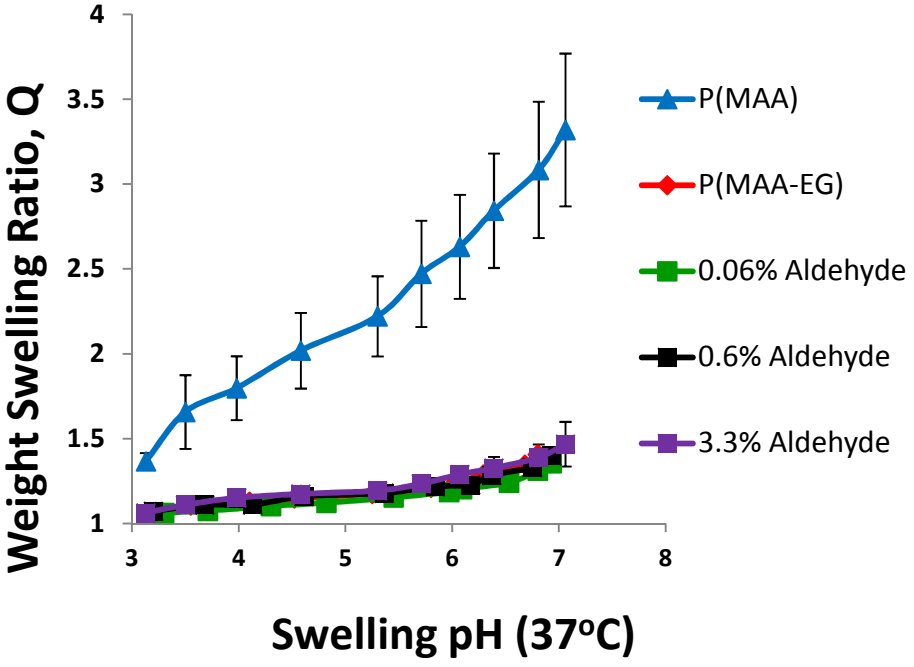


(a)

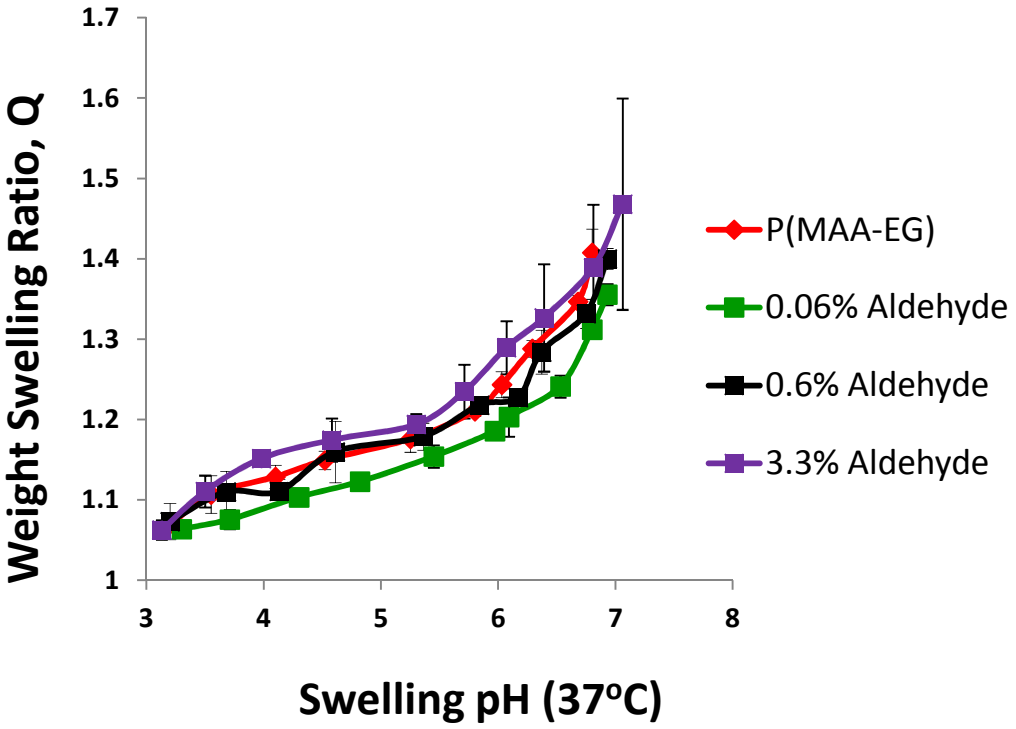


(b)

Figure 4.2a and b. FTIR spectra (top to bottom): P(MAA-EG) hydrogel with 3.3% aldehyde modified PEGMA in dry state, Schiff's reagent for aldehydes and P(MAA-EG) hydrogel with 3.3% aldehyde modified PEGMA after being in Schiff's reagent and washed in DI water.



(a)



(b)

Figure 4.3a and b. Dynamic swelling results to gauge pH sensitivity. (a) Dynamic swelling of all formulations (b) close up of four formulations

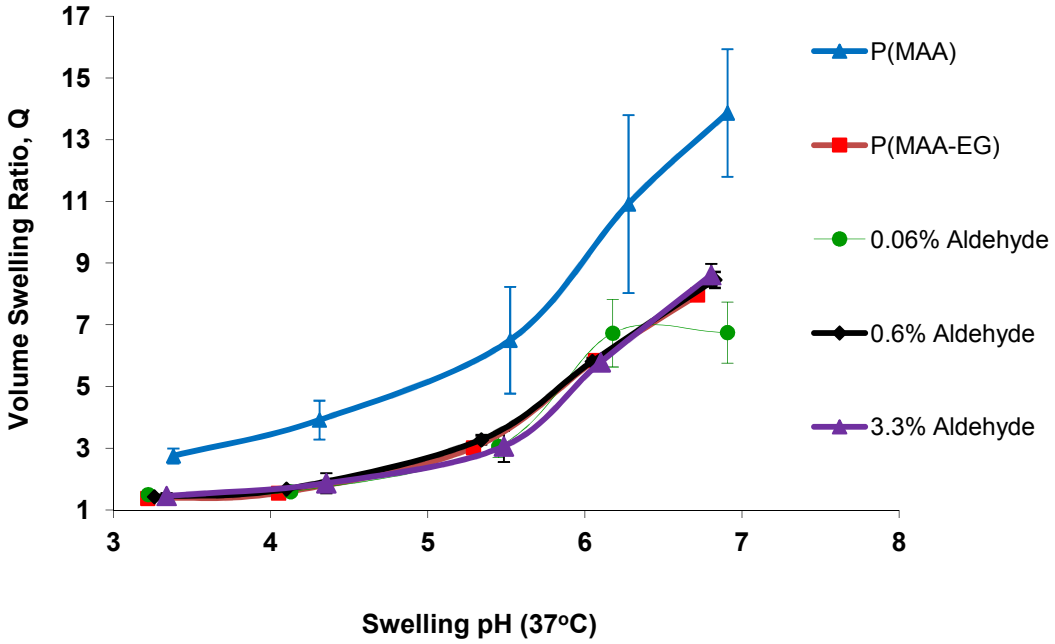


Figure 4.4. Equilibrium swelling of polymer discs of all formulations after spending 48 hours in each of five buffers to gauge pH sensitivity

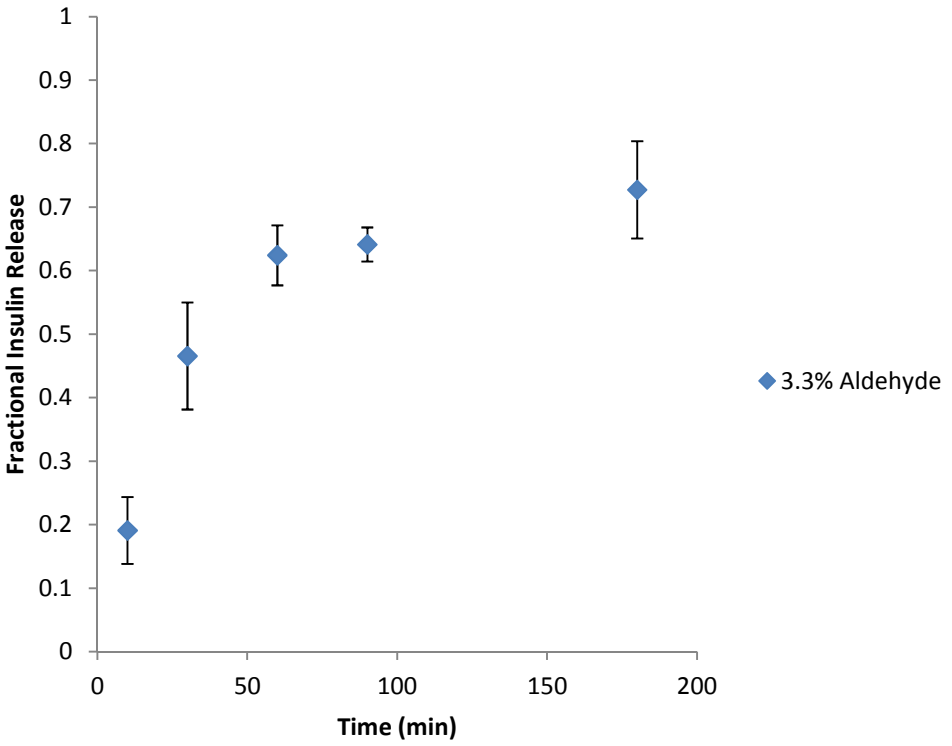
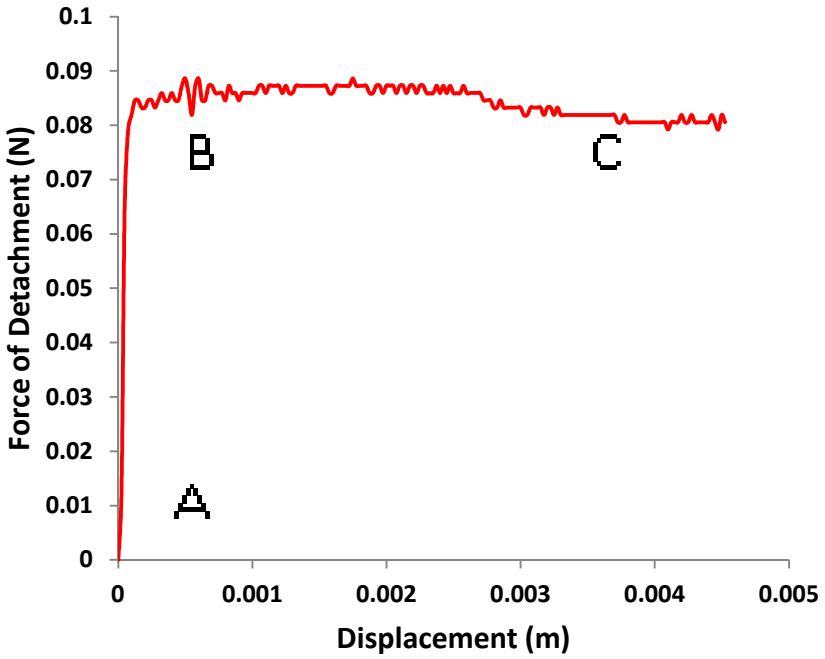
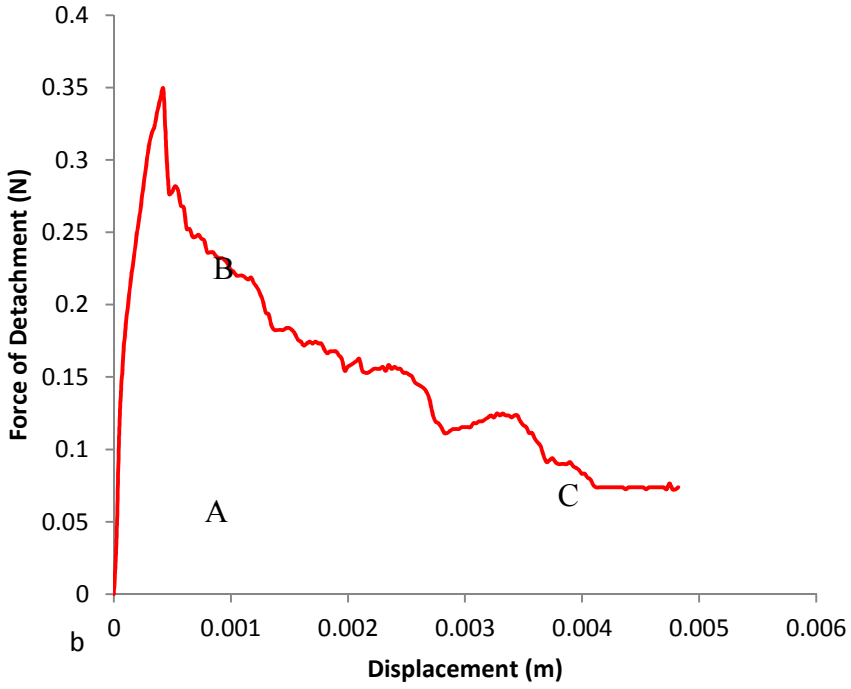


Figure 4.5. Fractional insulin release of 3.3% aldehyde modified PEGMA hydrogel formulation



(a)



(b)

Figure 4.6a and b. Force of detachment vs. displacement from instron test with highest and lowest order results. (a) shows the lowest order force curve of a P(MAA) disk, (b) shows the highest order force curve of a 3.3% aldehyde disk.

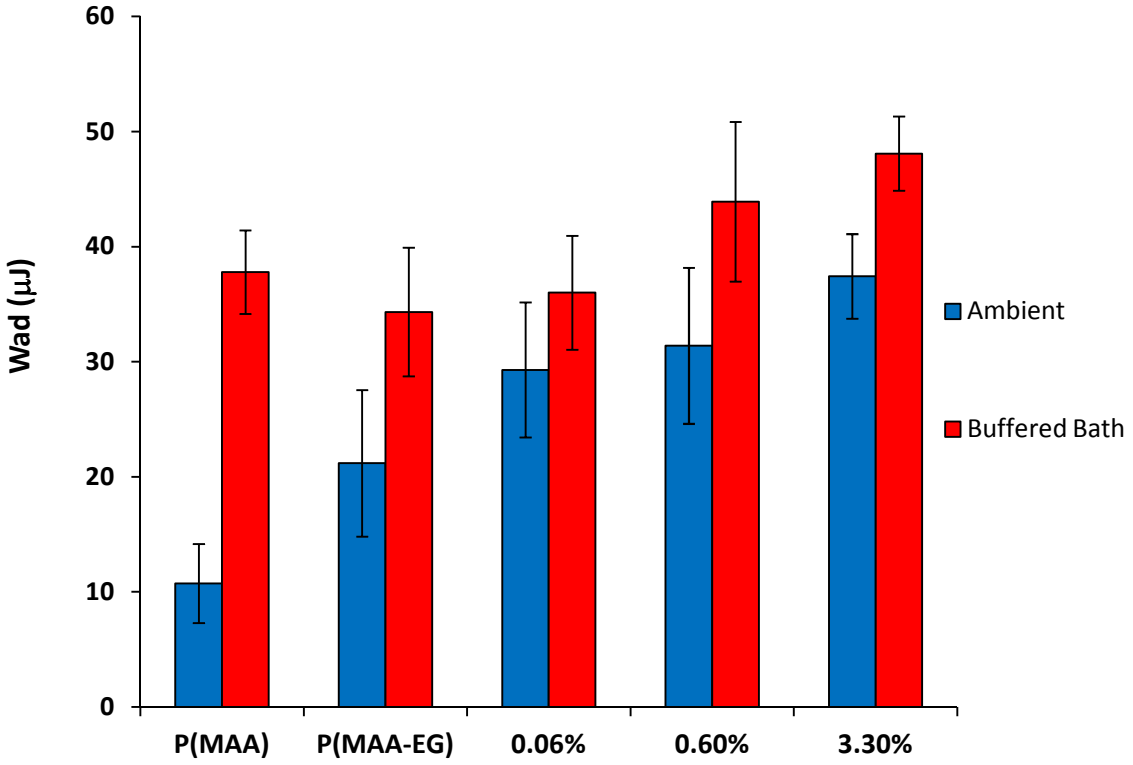


Figure 4.7. Work of adhesion (μJ) from instron testing with ambient and buffered bath data for all formulations

Chapter 5: Atomic Force Microscopy as a Tool for Determining Adhesion of Poly Methacrylic Acid pH sensitive Hydrogels *In Vitro*

Introduction

There have been many advancements in oral drug delivery but it has also met less success than other delivery methods due to the limits associated with the physiology of the gastrointestinal (GI) tract. This limits the effectiveness of the delivery system due to difficulties with transport across the lumen of the intestine and low bioavailability. One way to combat this is through improved adhesion at the site of delivery. To do this, it is important to understand these interactions between the polymer interface and mucus surface to improve adhesion and thus the efficacy of oral delivery.

Mucoadhesion is known to be a very complex phenomenon when dealing with the different properties of the polymer, tissue and environment. There have been numerous theories developed to describe the phenomenon of mucoadhesion. There is no single theory that is widely accepted, but rather a combination of theories typically used in describing the phenomena. The theories range from the adsorption theory to the diffusion theory, both of which are two of the main theories we have focused on thus far in this research. Briefly, the adsorption theory deals with adhesion through secondary forces and the diffusion theory describes the interpenetration of polymer chains into the interfacial region. There are also other avenues of adhesion, such as creating stronger covalent bonds like that of the Schiff's base reaction, which utilizes an aldehyde bonded to an amine group. Overall, the nature of the interaction between the system and mucus layer is not yet fully understood [1-4].

There have been several *in vitro* methods put forth over the last several decades to try to gauge this interaction in various ways. There has been little headway with all methods having some limitation [5, 6]. Looking at the methods used in this research, the tensile testing is a staple amongst adhesive testing which gauges the tensile force needed to separate a cylindrical disk from animal mucus and used to calculate the overall work of adhesion [7]. The falling film technique places microparticles along a portion of small intestine before having a solution poured over it. The eluted particles are determined and are an indication of the mucoadhesive properties [8]. Tensile tests and the trough method are used to give a good indicator of the bulk adhesion but have a forced initial interaction not seen in the small intestine [9].

The flow channel method uses polymer particles placed on a mucus surface. Laminar air flow is passed over the particle with photographs taken to analyze the behavior [10]. This method does not give quantitative results and as with the tensile testing and trough method have a forced interaction between particle and mucus layer.

Both the BIACORE™ and mucin particle methods give indications of the affinity that a polymer particle has with mucin in the initial stages of attraction but both fail to give any data of the strength of the bond formed and thus the ability of the particle to maintain adhesion despite various forces that act upon it in the GI tract.

Since adhesion is so complex, there is a need for a better understanding of how substances interact through every stage of adhesion on a molecular level. Improved knowledge of molecular level interplay will enable further understanding of bulk level adhesion. One way of doing this, is by developing an improved model for *in vitro* testing.

Atomic force microscopy (AFM) is primarily used for imaging purposes, but it can also be a powerful tool used to investigate and probe the mechanical and viscoelastic properties of thin films on the nanoscale [11]. Scientists have used AFM as a new avenue for providing images at a resolution higher than that obtained by light microscopy. The use of AFM has grown increasingly in the biological sciences and has now been established as a versatile tool to address the structure, properties and functions of biological specimens [12, 13]. AFM is unique for its ability to provide three-dimensional images of biological structures in ambient conditions as well as under physiological conditions with impeccable resolution. This is done by moving a sharp probe mounted on the end of a flexible cantilever across the surface of the substrate. In the conventional contact mode AFM, the probe is in continuous contact with the surface as the substrate is scanned. Tapping mode AFM, causes the cantilever to be oscillated at a high frequency, resulting in only intermittent contact between the probe and cell surface. The oscillations of the cantilever are reduced and energy lost once the probe contacts the surface. This reduction in oscillation is used to identify and measure features along the surface of the substrate [14, 15].

In addition, it can also measure forces with remarkable sensitivity and positional precision. This mode is called force spectroscopy. In this mode, the cantilever deflection is recorded as the tip is pushed towards the sample and retracted from it. Quantitative physical properties can be obtained from this data, such as local elasticity, surface forces, surface charge and hydrophobicity, and to measure inter- and intramolecular interactions.

This can ultimately provide new insights into the molecular basis of processes such as protein folding and receptor–ligand interactions [14, 15].

For the purposes of this research, this method would allow for new insight into the molecular interactions between the hydrogel and the substrate at each point of adhesion on the molecular level by allowing the hydrogel to initiate the interaction and begin bonding before being pulled off to record its adhesive nature. These measurements can gauge the effect of the physical and chemical bonds of the polymethacrylic acid, the grafted PEG tethers, and various other attached moieties on the molecular level which can then be compared on a bulk scale as needed. Other works have used AFM in this fashion while modifying AFM probes to measure the adhesion characterisitic of substrates such as living cells. However, these modified tips are chemically made with small covalently bonded molecules as opposed to larger crosslinked particles [16, 17].

The goal of this research is to develop a new *in vitro* testing method for understanding the adhesive molecular interactions between copolymers, such as those used in drug delivery vehicles, and the substrates they are to adhere to throughout the body such as the mucus layer. To begin, we will be exploring the interactions between previously made pH sensitive hydrogel microparticles and mucus solution. This would be the first instance of either material being use in AFM force modulation.

Materials and Methods

Hydrogel Synthesis

The methacrylic acid (MAA) monomer was passed through a dehibit column to remove the inhibitor. The polymer films were prepared by free radical UV

polymerization. The diluted monomer mixture was composed of methacrylic acid (MAA, Sigma, St. Louis, MO) and both PEGMA and aldehyde modified PEGMA [18] with a molecular weight of approximately 500. Irgacure® 184 (1-hydroxy-cyclohexyl-phenylketone, Ciba-Geigy, Hawthorne, NY), which was used as a photoinitiator, and tetraethylene glycol dimethacrylate (TEGDMA, Polysciences Inc. Warrington, PA), which was used as a crosslinking agent, was also incorporated. Deionized water and ethanol were used as solvents. The monomer solution was made in a ratio of 1:1, MAA:EG units, containing 3.3% aldehyde modified PEGMA incorporated with unmodified PEGMA as well as solutions of P(MAA-EG) with no aldehyde modified PEGMA. TEGDMA was added as 1 mol% of the total monomer and Irgacure® 184 added as 1 wt% of the total monomer.

The monomer solution was dissolved with a 50:50 monomer to solvent ratio by weight. The total solvent weight was composed of equal parts deionized water and ethanol. Once all components were added to the monomer mixture, the solution was sonicated until all materials were dissolved into solution. The mixture was purged with nitrogen for 20 minutes to remove oxygen, which acts as a free radical scavenger. The solution was pipetted between two glass slides (75 mm X 50 mm X 1 mm) separated by a 0.8 mm Teflon® spacer. The slides were placed under a UV light (Efos Corporation, Ultracure 100ss, High pressure mercury lamp, Mississauga, Ontario, Canada) and allowed to polymerize for 30 minutes.

After polymerization was complete, the thin films were washed with deionized water daily for one to two weeks to remove all unreacted components. After washing, the

films were dried under a vacuum (**Heraeus VTR-5036, Heraeus** Instruments GmbH., Hanau, Germany) for two days at approximately 30° C. Hydrogel films were crushed, and sieved with a mesh size of 45μm. Images of particles were taken on a light microscope and processed using an image processing program. Particle size distribution was found to be 8.5 mm with a standard deviation of 5.8mm.

AFM Tip Mounting

The microparticles were mounted on the ends of tipless AFM probes (Veeco Probes, Camarillo, CA). The probes have two tips on each side, each corresponding to different *k* values. The tip with the *k* value comparable to what was calculated beforehand was used. First, the AFM probe was mounted onto a pipette tip, placed onto a micromanipulator, and adjusted until the desired tip was in view under the light inverted microscope. Next, the microparticles were mixed with a cyanoacrylate adhesive, placed onto the end of another pipette tip and attached to the opposing micromanipulator. The manipulators were used to set to fine mode to insure slow movement between tips and decrease the likelihood of breaking the probe. The microparticles were lowered over the tip in the negative *z* direction repeatedly until a microparticle was adhered to the tip. Pictures were taken with a camera attachment to the microscope at a magnification of 20 X for each tip and scale bars added to determine the diameter of each particle.

AFM Experiments

The previously made tip was placed into the AFM mount, secured, and brought into view under the microscope fixture. The laser used to read the deflection of the

cantilever was aligned with the tip according to the signal markers of the computer software. All sampling parameters were set to the default settings. The sample substrate was prepared and placed beneath the tip. A 20wt% mucin solution was made and a small amount added to a microslide (3 in X 1 in X 1 in) before being spin-coated at 2500 RPMs for 30 seconds. Once the sample was in place, the tip was lowered to within a few micrometers and engaged, allowing force curves to be sampled at different deflection settings. The data was recorded, resulting in the plotting of deflection (nN) vs. z position (nm). After normalizing the curve (subtracting the trace from the retrace curve) the trapezoidal rule was used to calculate the work of adhesion (nJ). This is discussed in further detail at the end of this section.

These tests were also carried out in wet mode, which simulates the conditions of the GI tract. The microparticle tips were hydrated previous to testing by the addition of PBS buffer (pH 7.4) for 10 minutes. All other procedures are the same. Tipless probes were also tested on clear glass slides and on mucin coated glass slides as controls.

K Value Calculations

In order to optimize the ratio between the stiffness (*k*-value) of the cantilever and the expected mechanical properties, an estimate was made on the predicted contact force between the sample and probe. These estimates were made from previously conducted Instron tensile tests with specimens of diameter = 14 mm and thickness = 0.8 mm. The Veeco probes (Number NP-010, Camarillo, CA) have *k*-values ranging from 0.06-0.58 N/m. From these tests the area of a 14 mm diameter disk used, $A_1 = 1.54 \times 10^{-4}$ mm^2 and the maximum force achieved, $F_{max} = 0.01$ N, was obtained.

These numbers were used to quantify the stress, σ,

$$\sigma = \frac{F_{\max}}{A_1} \ , \tag{2}$$

sustainable by the sample under tensile load. By estimating the contact area, A_2 of a 2000 μm^2 microparticle on a 100 mm long probe, deflecting by approximately 1 μm (x), we obtained a *k*-value of approximately 0.12 N/m with equations 3 and 4 indicating which tipless probe should be used. The probe used had a *k*-value of 0.12 N/m and had a nominal length of 205 μm, and width of 40 μm.

$$F_{new} = \sigma A_2 \tag{3}$$

$$k = \frac{F_{new}}{x} \tag{4}$$

AFM Analysis

The raw data from the AFM experiments was analyzed to give the work of adhesion. The data was recorded, resulting in the plotting of deflection (nN) vs. z position (nm) and presented in two sets, trace and retrace. The curve was normalized by subtracting the retrace from the trace data. With this done the adhesion curve is now in the positive regime. The trapezoid rule was used to determine the area under the curve and give the work of adhesion (nJ). This work of adhesion was then multiplied by the k value of the tip that was used on the AFM probe and then divided by the surface area of the particle that was used to normalize the data since different probes will have different-

sized particles attached. In the event that the adhesion curve was cut off it was pieced together by extrapolating the curve to a point and then carried out in the same way as just stated.

Results

AFM Tips

Pictures were taken of each microparticle mounted probe to confirm adherence to the tip and determine the diameter and topography of each microparticle that would be used in testing. This is important to know the appropriate estimated surface area in contact with the mucin substrate which will be used to normalize the data at the end of the calculations.

Three probes were used successfully comprised of two control samples of PMAA and P(MAA-EG) as well as the 3.3% aldehyde modified P(MAA-EG) sample were used with no preswelling, figures 6.1-6.3. Two more samples comprising P(MAA-EG) and 3.3% aldehyde modified P(MAA-EG) were used with the 10 minute preswelling before being used in the AFM, Figures 6.4 and 6.5. A PMAA sample was not used in preswelling testing due to its higher swelling ratio. The higher swelling ratio causes the hydrogel to increase to a size that makes interactions difficult with the substrate. In each picture, scale bars were used which gave diameters of 34, 41, 24, 34, and 45 μm respectively. The topography of each particle is spherical in nature even though it has some rough and jagged areas, leading us to use the surface area of a sphere to determine the surface area of the particles to be used in normalization of the final data analysis.

This allows us to confirm that the particles adhered to the tipless probes. Secondly, it gives us the dimensions of each particle and a closer look at the topography allowing us to determine the best way to calculate the surface area that would be in contact with the mucin in experimentation.

AFM Experiments

AFM experiments were run using the previously made tips. Raw data was gathered in the form of trace and retrace data plotted as deflection (nN) vs. z position (nm). These graphs are used for two reasons. The first is to determine that adhesion is occurring. The second is to use the graphical data to calculate the quantitative results of adhesion.

Determination of adhesion in AFM force modulation is proven by observing the trace and retrace data, Figure 6.6. Trace data is from point 1 to point 3 seen and the retrace data is from point 3 to point 6. Briefly, moving from point 1 to 2 the probe tip is brought closer to the surface until the natural attraction between the surface and the probe causes each to come into contact and bind. Upon contact, the cantilever deflection will increase from point 2 to 3 as the fixed end of the cantilever is brought closer to the sample, causing an increase in deflection in the positive regime completing the trace data. After loading the cantilever to a desired force value, it begins to withdraw, moving from point 3 to 4. We are able to determine if adhesion is present at this point as the retrace data moves past the initial contact point from the trace data indicating adhesion during contact with the surface. With two perfectly rigid, frictionless surfaces, the retrace line would follow the trace exactly. Point 4 to 5 occurs as the cantilever is pulled away and

adhesion is broken from the surface substrate. Points 5 to 6 are the cantilever moving completely away from the surface.

Samples from AFM force modulation testing performed with PMAA and 3.3% aldehyde modified P(MAA-EG) microparticle tips are shown in Figures 6.7 and 6.8 respectively. Each shows a distinct curve under the initial interaction point of the trace data indicating adhesion between the surface mucin and the microparticle tips did occur. After confirmation, each sample probe was tested in triplicate and used to determine quantitative results. This was done by subtracting the retrace from the trace data to give the adhesive curve in the positive regime. The area underneath this curve was found and determined to be the work of adhesion. This allows us to confirm that adhesion did occur with the particle loaded tips and gather the data for each particle to quantitatively gauge the work of adhesion.

AFM Analysis

AFM force modulation studies were performed to investigate first the effectiveness of using AFM in determining adhesive properties of hydrogels on a molecular level. This data can then be used in two ways. First, it can be used to determine the importance of such adhesive properties of secondary forces, interpenetrative forces and various other covalent linkages on a molecular level. Second, it can be scaled up and compared with previous bulk adhesion experiments.

During the AFM experiments, the hydrogel tagged tips comprised of PMAA, P(MAA-EG) and 3.3% aldehyde modified P(MAA-EG) were used dry and unswollen for one series of experiments and for a second set P(MAA-EG) and 3.3% aldehyde modified

P(MAA-EG) were preswollen with a buffer solution of pH 7.4 for 10 minutes. The first set of unswollen tips were used to give a proof of concept, but also to see what adhesive interactions occur without the hydrodynamic forces that are involved after swelling. The second set was preswollen because as stated in previous chapters, the swelling is a key first step in the formation of a mucoadhesive bond between a polymer and the mucus due to the wetting and swelling of the polymer. Ten minutes of exposure to a buffer solution was sufficient to swell the system to conditions similar to those in the upper small intestine.

The hydrogels used in this research have several different avenues for creating adhesive bonds. The basic P(MAA) and P(MAA-EG) hydrogels utilizes many secondary chemical interactions including ionic bonds, van der Waals interactions, and hydrogen bonding, with hydrogen bonding probably being the most important secondary interaction in mucoadhesion. However, while these types of forces are weak, numerous interaction sites lead to strong mucoadhesion.[19] Upon entering the high pH conditions of the upper small intestine, these secondary forces hold long enough for swelling and, as a result, interdiffusion will take place. This happens because the PEG chains are not interacting with the poly(methacrylic acid) backbone at the higher pH conditions. Consequently, the PEG chains are able to diffuse through the mucus and enhance mucoadhesion. The aldehyde moiety complements these interactions and, as shown previously, adds another stronger chemical reaction with that of a Schiff's base covalent linkage. This occurs when the aldehyde forms a chemical linkage with an amine functional group similar to those found on the N-terminus of the glycoproteins that help

compose the mucus layer that coats the small intestine. Using the AFM we can try to understand which of these forces is more significant for the overall adhesion of the hydrogel.

To separate theses forces from the data we have collected we must look at the first law of thermodynamics. This law states that energy is conserved. If we were to look at these experiments in a perfect scenario we would have a perfectly rigid cantilever or a perfectly elastic substrate that was also frictionless and a cantilever that was perfectly elastic. In either scenario, as the tip is brought into contact with the surface, the AFM laser move along a perfectly retraceable path. Of course, this is not seen because the interactions between the tip and substrate are not perfectly elastic and there is no non-zero coefficient of friction. These discrepancies can be attributed to the energy being dissipated as acoustic or thermal energy which increases more due to the addition of the deformable hydrogel to the tip. Bringing us back to the first law of thermodynamics, we should find that the summation of all chemical and mechanical energies is equal to the work done by the system or zero in this ideal scenario meaning that all mechanical energy that is dissipated is absorbed chemically via,

$$Ec + Em = 0\,, \tag{5}$$

where Ec is chemical energy and Em is mechanical energy.

We can further break this equation down into the three basic components of the system: the hydrogel, the mucin substrate, and the silicon of the cantilever tip. Where $E_{c,muc}$, $E_{c,HG}$, and $E_{c,si}$ are the chemical energies of the mucin, hydrogel and silicon

respectively and $E_{m,muc}$, $E_{m,HG}$, and $E_{m,si}$ are those same quantities for mechanical energies. Equation 5 then becomes,

$$\left(E_{c,muc} + E_{c,HG} + E_{c,si}\right) + \left(E_{m,muc} + E_{m,HG} + E_{m,si}\right) = 0\,, \tag{6}$$

In a nonideal scenario, such as the experiments in this research, equation (6) will be equal to the amount of energy absorbed by the sample and can be found by integrating the area of the curve of data given from the trace and retrace curves. To find the forces associated with the chemical bonding, we must determine the mechanical energies and subtract them from that force. $E_{m,si}$ is the mechanical energy of the deformation of the silicon cantilever. This can be determined by running a force curve on a tip with no hydrogel microparticle and bringing it into contact with a smooth glass surface. This value was found to be 2.95 nJ.

The other two mechanical energies, $E_{m,muc}$, and $E_{m,HG}$, can be determined by experiments with a tip with no hydrogel but with mucin as the substrate instead of just a glass surface. From this we can decouple these energies. This value was found to be 24.53 nJ. $E_{c,si}$ is zero since there are no chemical interactions with the silicon of the cantilever.

Table 6.1 shows the values for all formulations in the dry AFM testing and preswelling AFM testing along with their standard deviations. The previously found mechanical energies can now be subtracted from these values giving the forces associated with chemical bonding. These values can then be normalized by dividing by the surface area of each individual microparticle giving the normalized forces associated with chemical bonding. These new values can be seen in Table 6.2. There are several things to

note in these values. First, the work of adhesion of all formulations in dry testing produced negative values as opposed to the preswollen values which were all positive values. This could be due to the sample having more of a repulsive force acting upon it from the mucin substrate than an attractive force as seen with the preswollen values. The repulsive force could be due to the lack of sufficient bonding due to secondary forces with a lack of hydrated surroundings or swelling of the hydrogel itself. Also, a lack of swelling of the hydrogels could cause both the PEG tethers and aldehyde modified PEG tethers to remain on the interior of the hydrogels and prevent them from interacting with the mucin substrate also preventing any interpenetration or Schiff's base covalent bonding to occur. The lack of chemical bonding energies results in the mechanical energies doing more work in the system and thus the negative chemical bonding energies. The second thing of note is that in both the dry and preswollen values, the 3.3% aldehyde modified formulations all had a larger work of chemical bonding adhesion than either of the controls formulations. This maintains the trend we saw in earlier work with bulk testing on the Instron tensile testing machine.

To further compare these results to the bulk testing *in vitro* model used previously in this research, the normalized data is scaled up to a bulk level. This is done by multiplying the normalized values of the AFM data by the area of a uniform cylindrical disk used in Instron testing and converting to microjoules. The area of a cylindrical disk for PMAA was 9.5×10^{13} nJ while the other two formulations were 1.54×10^{14} nJ. A bar graph comparing all three formulations used in Instron testing, and the scaled up values for the dry and preswollen AFM testing, figure 6.9. The values for the Instron testing are

10.73 μJ, 21.17 μJ and 37.4 μJ for PMAA, P(MAA-EG) and 3.3% aldehyde modified P(MAA-EG) hydrogels respectively. The values for the dry AFM testing are -591.6 μJ, -792.49 μJ and -1361.43 μJ for PMAA, P(MAA-EG) and 3.3% aldehyde modified P(MAA-EG) hydrogels respectively. The values for the preswollen AFM testing are 1563.81 μJ and 2801.14 μJ for P(MAA-EG) and 3.3% aldehyde modified P(MAA-EG) hydrogels respectively.

Looking at the scaled up data, we can first see that the trend of increasing adhesion from the control formulations to the 3.3% aldehyde formulation holds true for all three testing methods. We see an overall increase of adhesion by 76%, 72% and 79% from the most adhesive control formulation to the 3.3% aldehyde formulation over Instron, dry AFM and preswollen AFM testing respectively. We also see that AFM values were two orders of magnitude greater than those found from the Instron tensile testing method. This is anticipated due to the increase in relative surface area of the microparticle in contact with the mucin substrate unlike that of the cylindrical disk used in Instron testing. This shows that we get as good, if not more, accurate adhesive results from this method since the actual administration of the drug delivery system would be through microparticles and not bulky cylindrical disks.

Finally, we can use these results to determine the anticipated values each form of adhesion separately brings to the overall work of adhesion. Since the dry AFM data seems to have little chemical bonding energies due to lack of the hydrated environment and swelling we are unable to utilize this data for these purposes and must focus on the preswollen AFM data. Since we were unable to perform tests with P(MAA) due to its

higher swelling, ratio causing the hydrogel to increase to a size that makes interactions difficult with the substrate we must focus on the affects of just the Schiff's base bonding from the aldehyde functional groups. Looking at the normalized data from Table 2, we see that there is an increase of adhesion of 8.03 X 10^{-9} nJ or an increase of the 79% we discussed previously from the tethered P(MAA-EG) to the 3.3% aldehyde modified formulation. The only difference between the two formulations is the addition of the aldehyde moiety signifying that this addition has a significant effect on the adhesive properties of the hydrogel outside the adhesive effects of secondary and interpenetrative forces.

This study allowed us to confirm the validity of AFM force modulation as an effective model for *in vitro* adhesion testing of drug delivery vehicles specifically pH sensitive hydrogels on a molecular level. Using the data gathered from testing we were able to show that the values were as accurate, or more so, than that of the bulk testing Instron model. We also showed that while there is more work to be done to determine the exact importance of the secondary and interpenetrative forces we gauged the increased normalized force of adhesion and overall percent increase of adhesion with the addition of the aldehyde moiety.

Conclusions

The present work determined that AFM force modulation is an effective model for *in vitro* adhesion testing of drug delivery vehicles specifically pH sensitive hydrogels on a molecular level. Utilizing a more accurate *in vitro* method would bring new insight into the methods of adhesion and further the work of improving oral drug delivery

vehicles. Determining how to better adhere to the small intestine would lead to increased residence time, release at the delivery site, and would presumably affect the bioavailability of the protein. Hydrogels with and without PEG tethers and with aldehyde modified PEG tethers were synthesized and used in this study and compared with work previously done using the Instron tensile testing method. Microparticle-tagged AFM tips were made and confirmed for use in experimentation. All sample tips were engaged and successful force curves were recorded. The data gathered showed an overall increase of adhesion across all formulations when compared with the bulk testing. Preswollen AFM data was utilized to determine that the aldehyde moiety alone has an increase of 8.03 X 10^{-9} nJ or a 79% increase on adhesion without the addition of secondary or interpenetrative forces. These findings indicate the potential this new method has for determining the effectiveness of both old and new forms of adhesion.

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AFM dry testing	Formulation	Work of adhesion (nJ)
	P(MAA)	4.86 ± 0.15
	P(MAA-EG)	0.30 ± 0.07
	3.3% aldehyde tethers	11.48 ± 0.04
AFM preswelling testing	Formulation	Work of adhesion (nJ)
	P(MAA)	N/A
	P(MAA-EG)	140.04 ± 3.14
	3.3% aldehyde tethers	155.05 ± 3.22

Table 5.1. Work of adhesion (nJ) for all three hydrogel formulations used in both dry AFM testing and preswollen AFM testing. These are the straight values after integration of the trace/retrace curves with no other calculations involved.

AFM dry testing	Formulation	Work of adhesion (nJ)
	P(MAA)	-6.23E-09 ± 4.11E-11
	P(MAA-EG)	-5.15E-09 ± 1.30E-11
	3.3% aldehyde tethers	-8.84E-09 ± 2.12E-11
AFM preswelling testing	Formulation	Work of adhesion (nJ)
	P(MAA)	N/A
	P(MAA-EG)	1.02E-08 ± 2.83E-10
	3.3% aldehyde tethers	1.82E-08 ± 4.59E-10

Table 5.2. Work of adhesion (nJ) for all three hydrogel formulations used in both dry AFM testing and preswollen AFM testing. These are the normalized values after integration of the trace/retrace curves, subtraction of the mechanical energies, divided by the surface area of each individual microarticle.

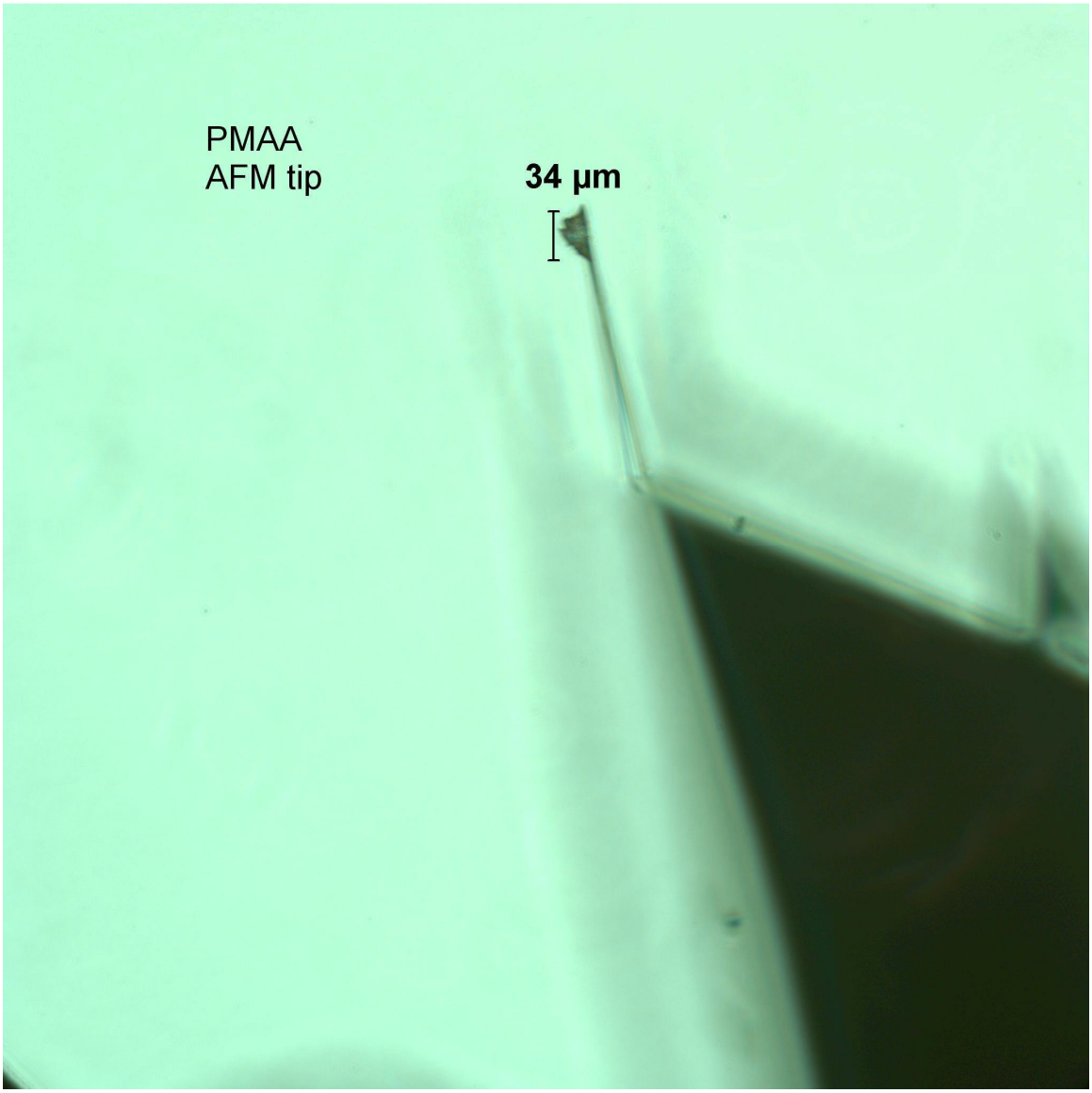

Figure 5.1. PMAA tagged tipless probe that was used during dry testing.

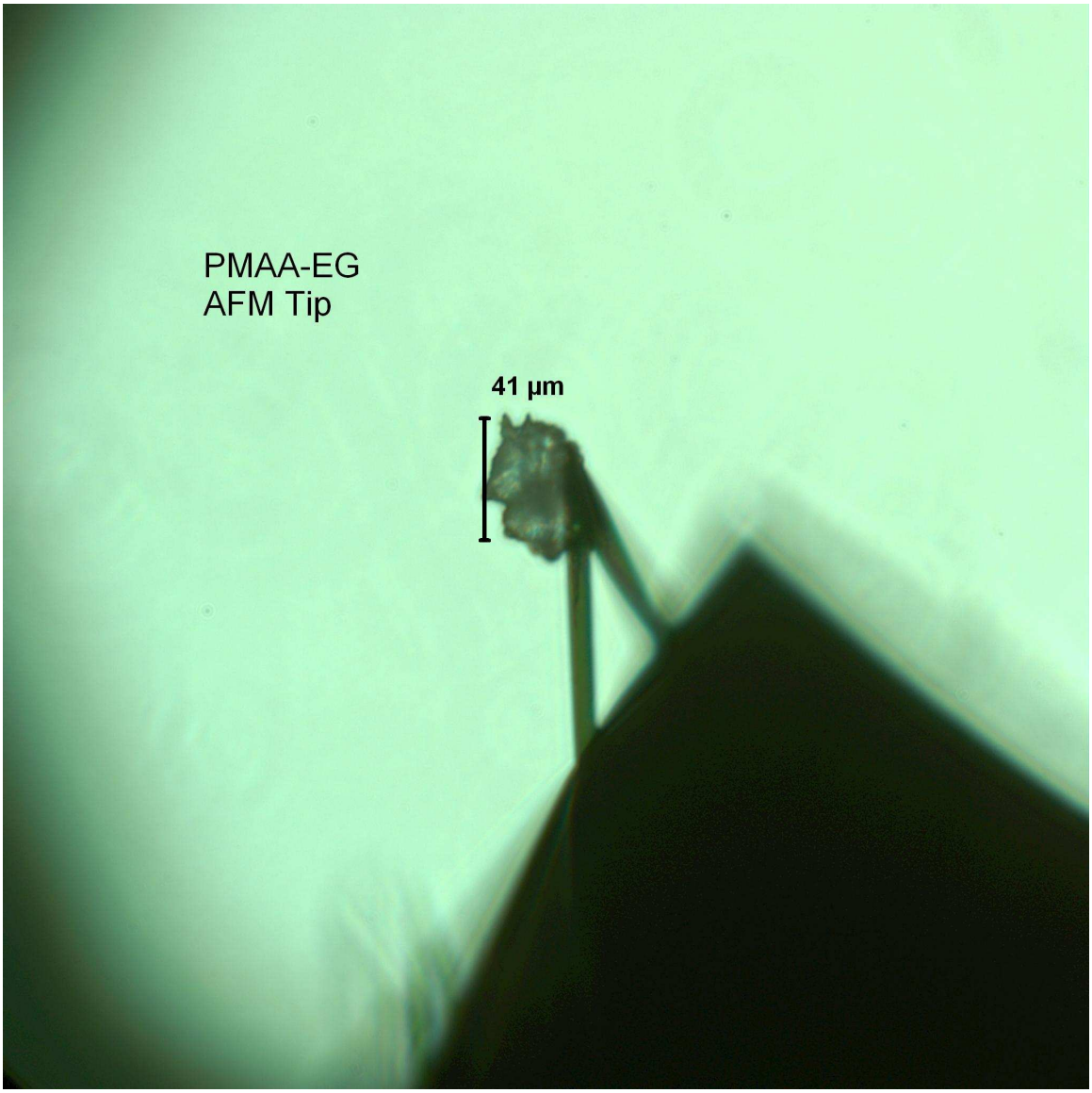

Figure 5.2. P(MAA-EG) tagged tipless probe that was used during dry testing.

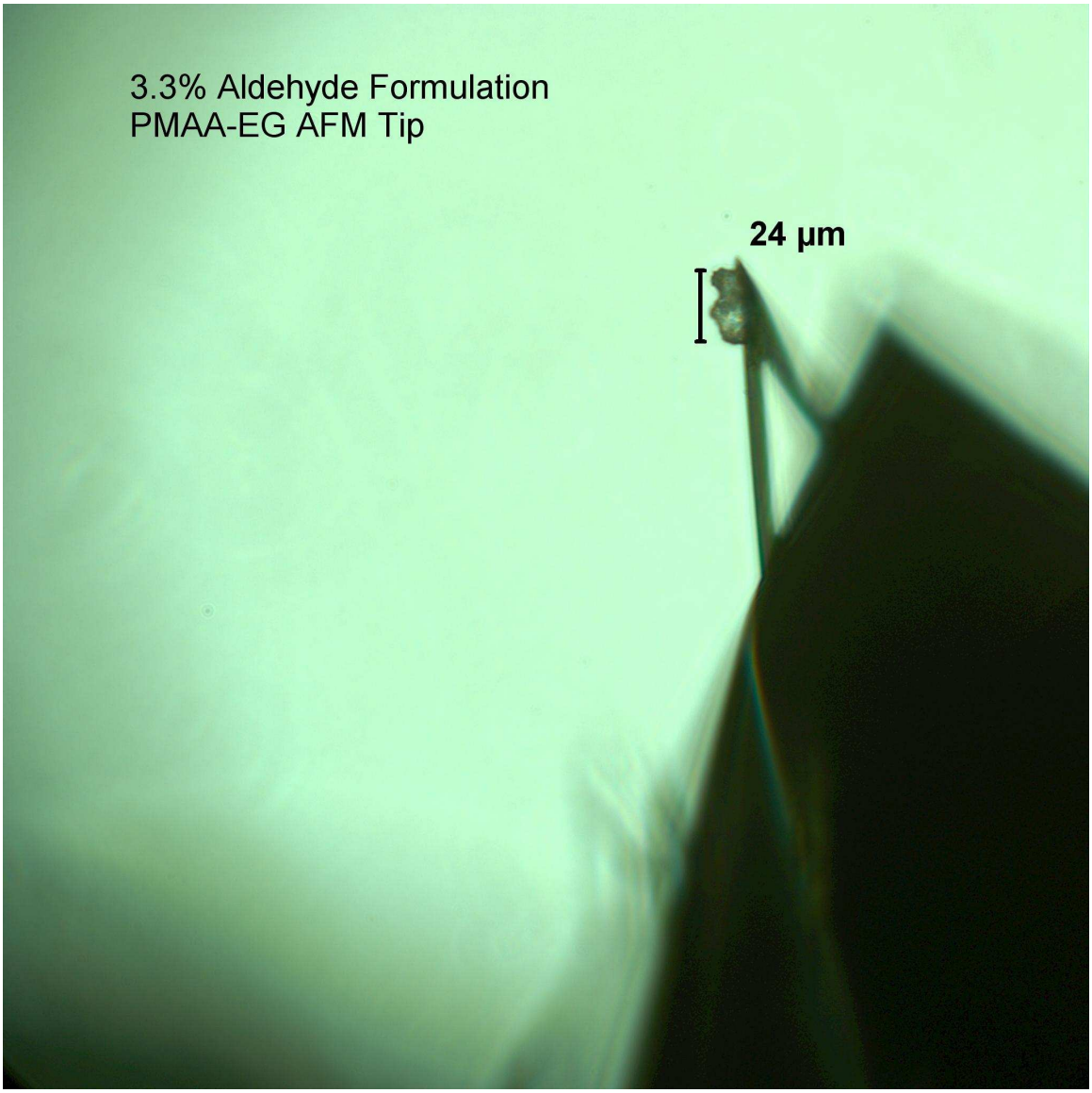

Figure 5.3. 3.3% aldehyde modified P(MAA-EG) tagged tipless probe used during dry testing.

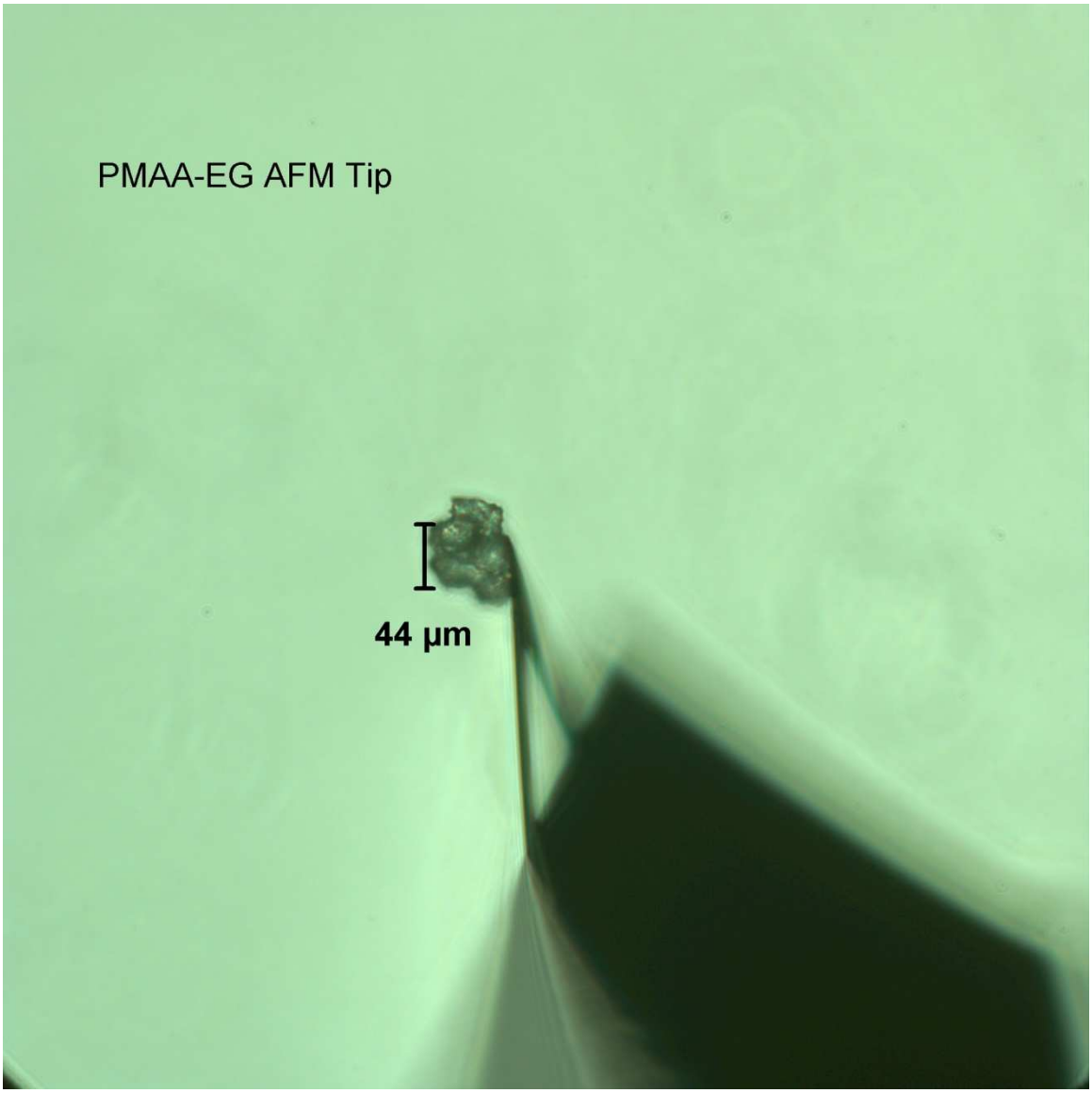

Figure 5.4. PMAA tagged tipless probe used during preswollen testing.

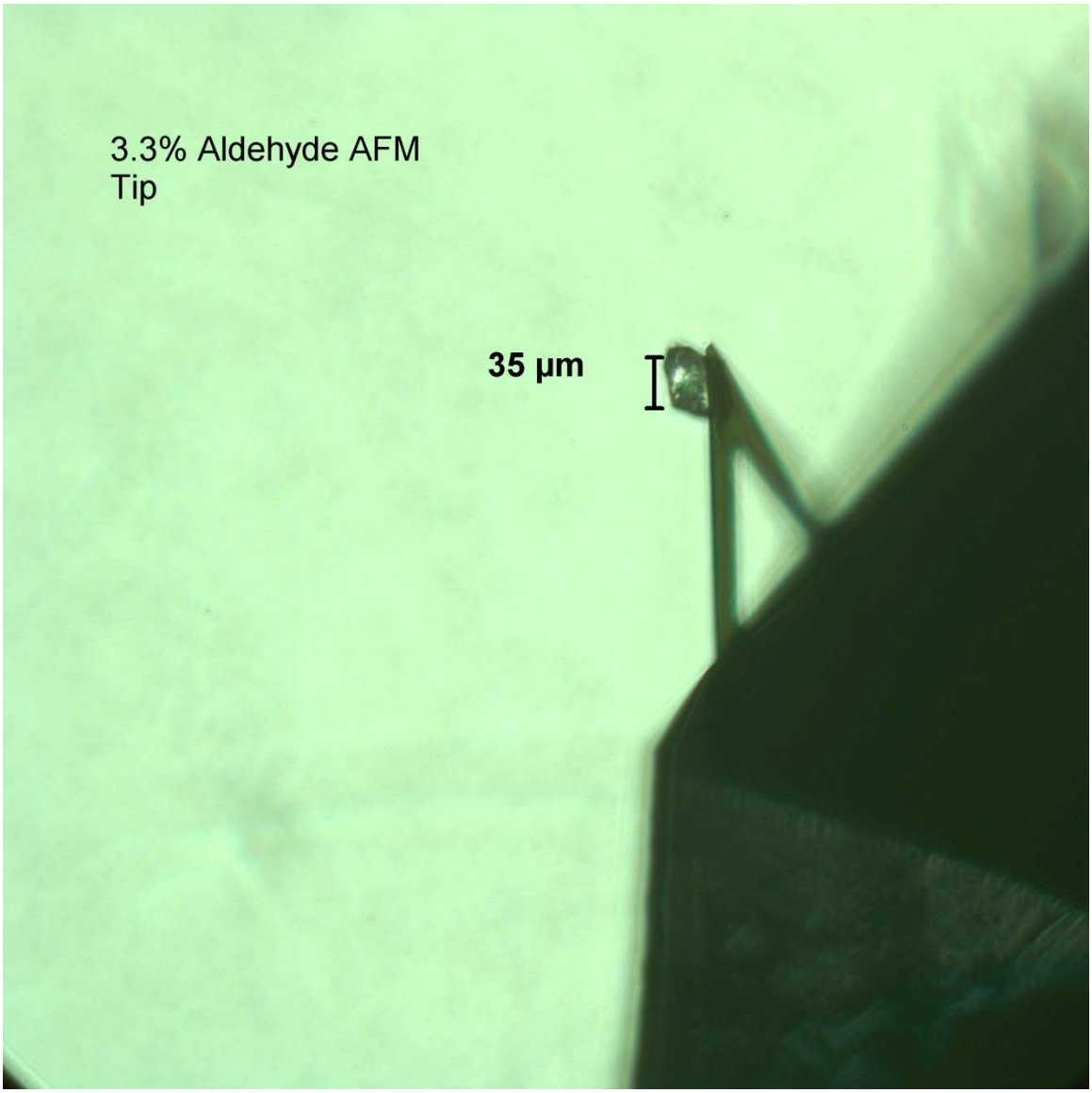

Figure 5.5. 3.3% aldehyde modified P(MAA-EG) tagged tipless probe used during preswollen testing.

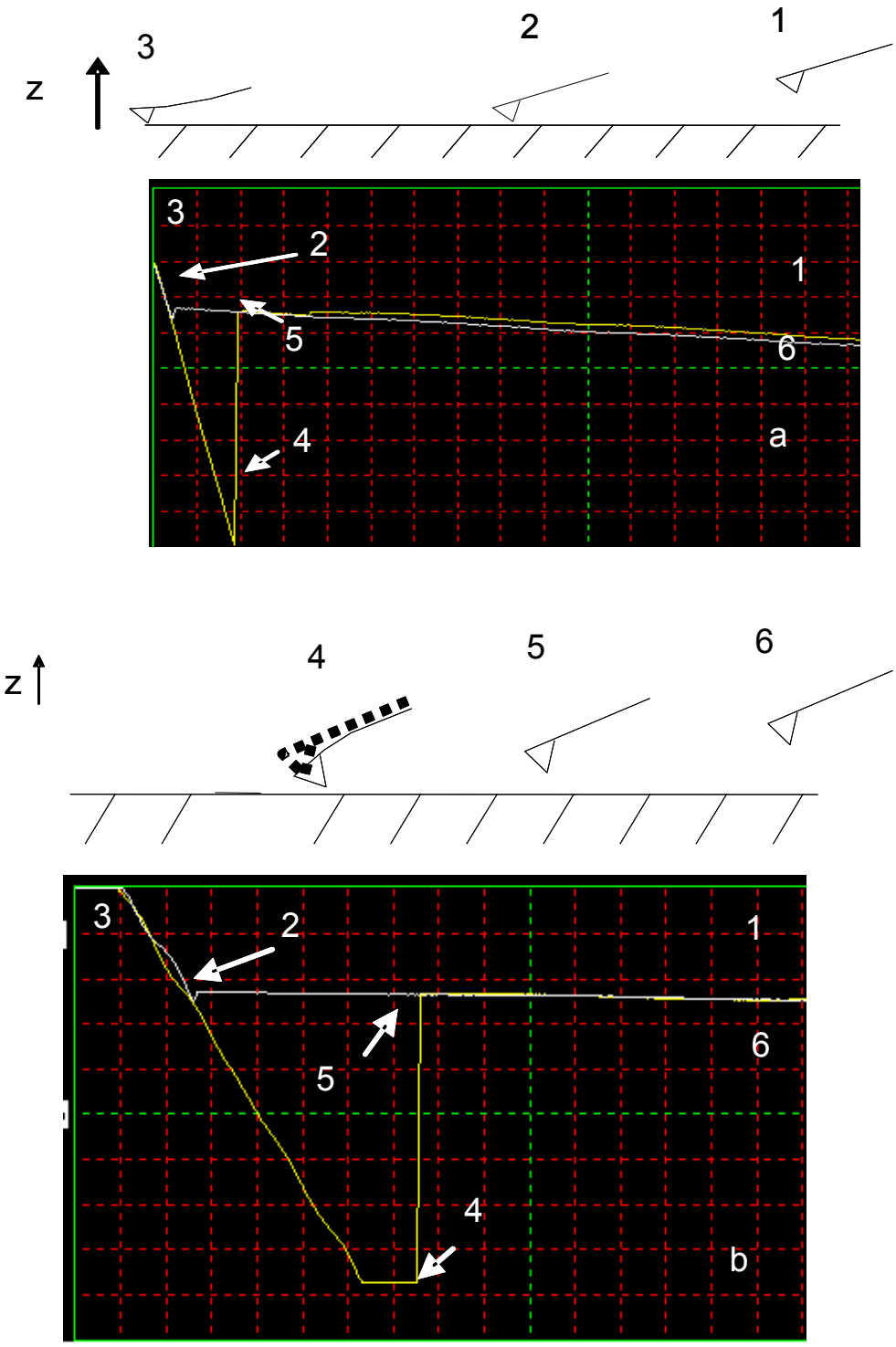

Figure 5.6a and b. Trace and retrace curves from AFM force measurements and the accompanying probe positions.

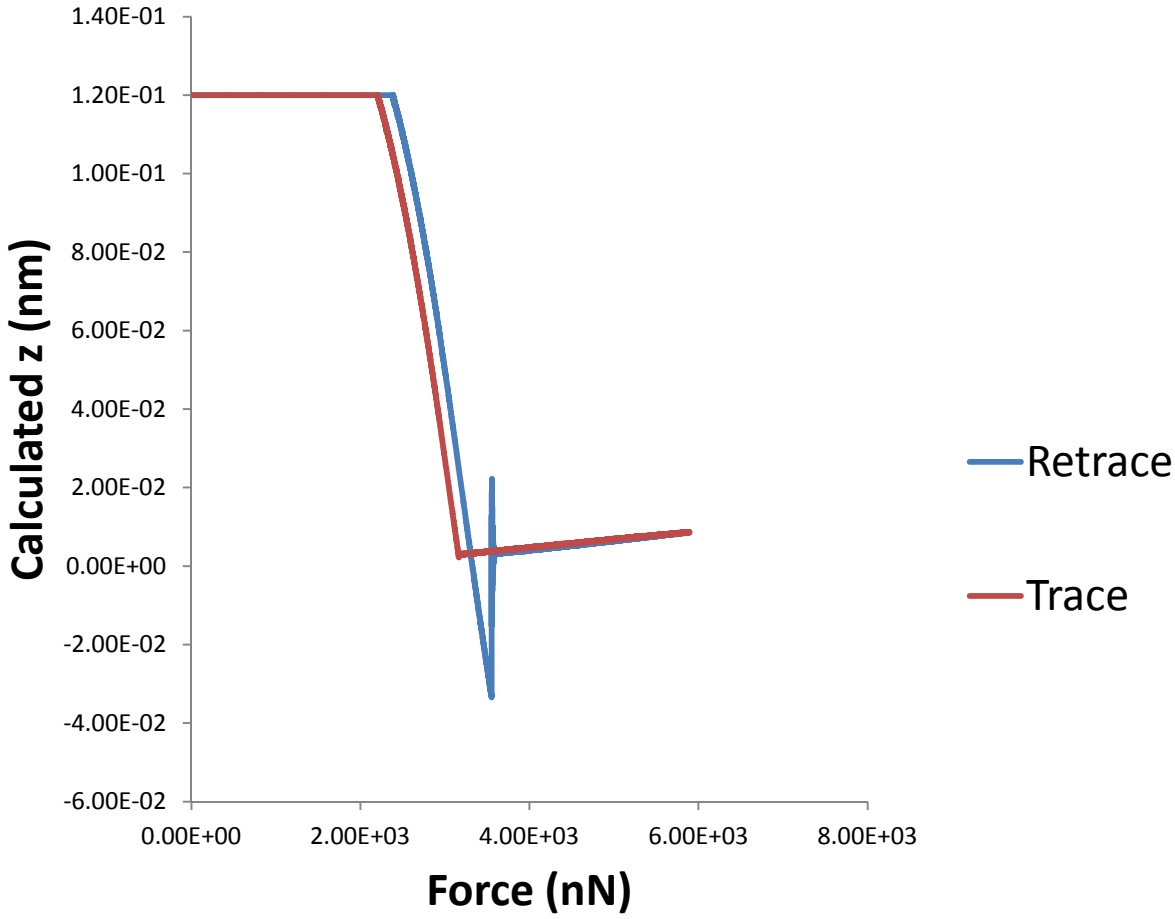

Figure 5.7.Trace/retrace data from AFM of PMAA hydrogel in dry mode.

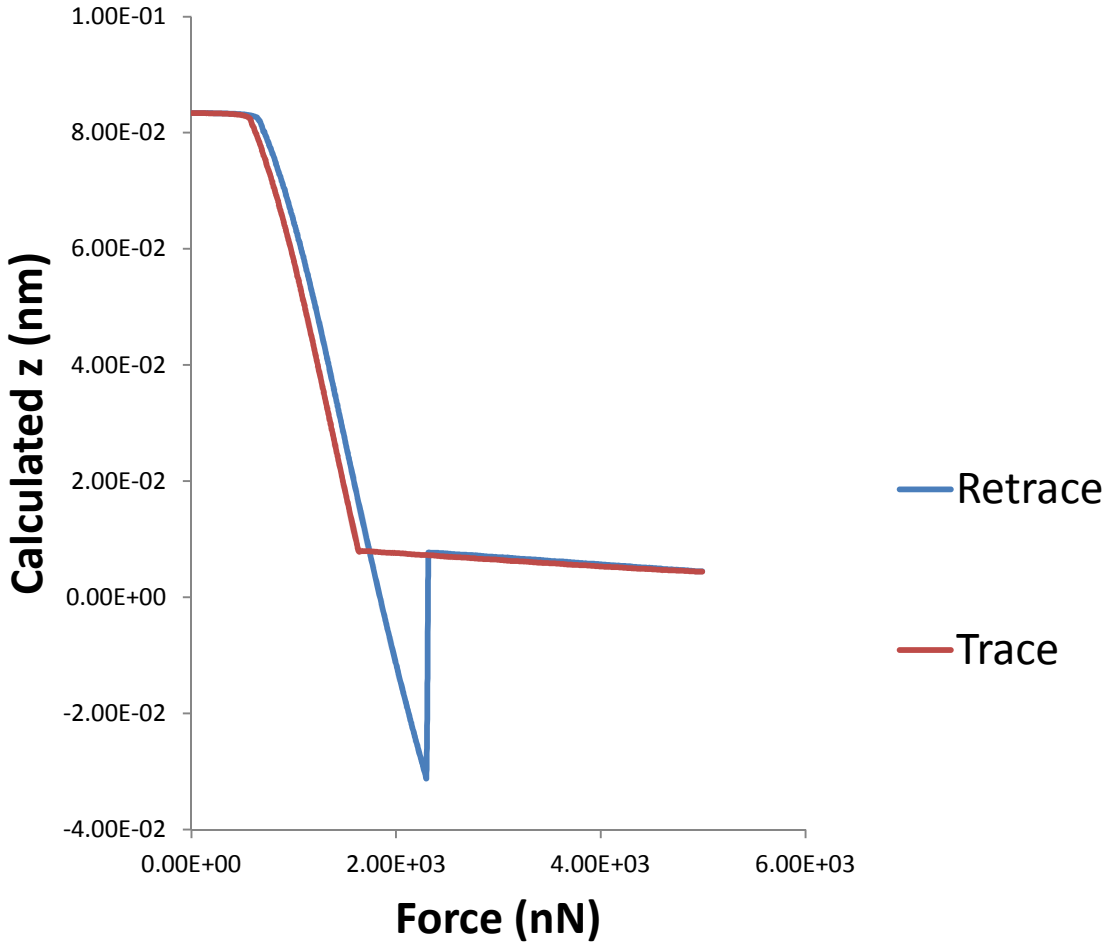

Figure 5.8. Trace/retrace data from AFM of 3.3% aldehyde hydrogel in dry mode.

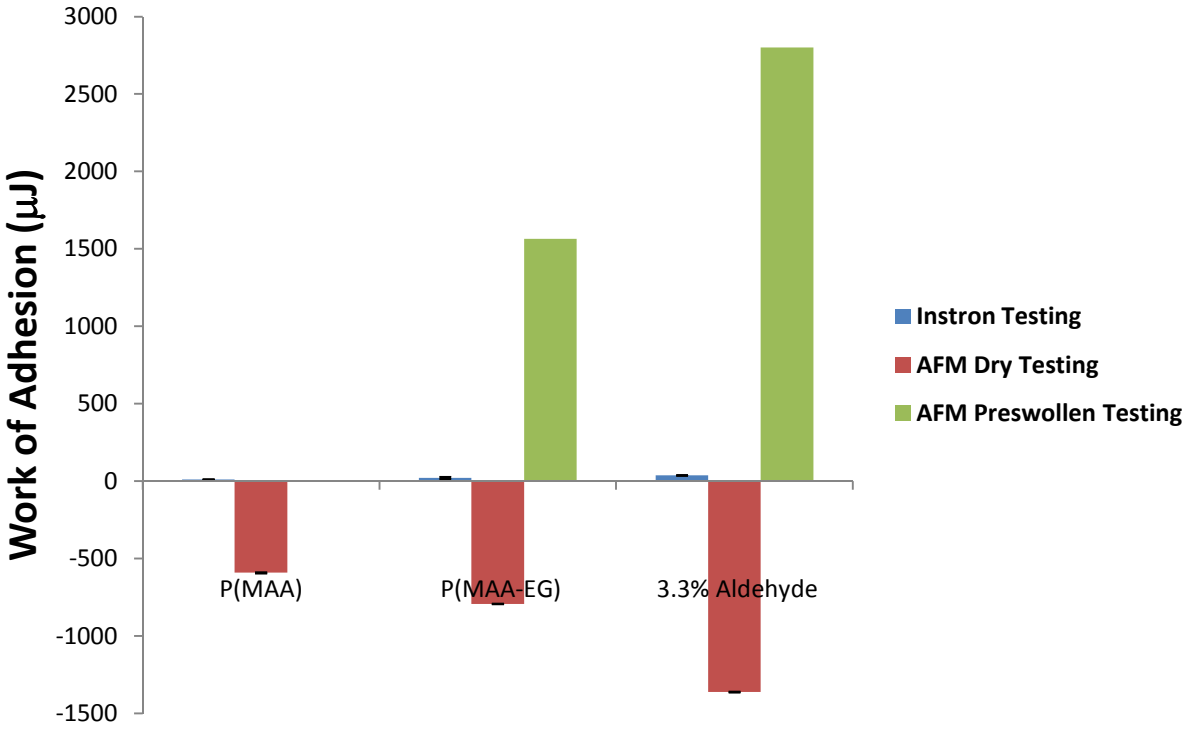

Figure 5.9. Work of adhesion for P(MAA), P(MAA-EG) and 3.3% aldehyde formulations scaled up to bulk level values for the Instron, dry AFM and preswollen AFM testing. For AFM testing n = 3. For Instron testing n = 5.

Chapter 6: Synthesize and determine properties of pH sensitive hydrogels modified with the SlpA bacterial protein found in *Lactobacillus Acidophilus*

Introduction

In this chapter, we investigate an alternate method of adhering the hydrogel microparticles to the small intestine. This method utilizes a biological technique that allows adherence to cells and mucus unlike the more synthetic route previously discussed in chapter 4. The improved adhesion and selectivity should allow further headway into overcoming the obstacles that limit the overall drug bioavailability to ranges that are not commercially acceptable. [1-5] These obstacles range from enzymes that digest the proteins to difficulties with the transport of large molecules across the mucosa and epithelial cell layer. Novel carriers have been developed with desirable properties to meet these challenges with an eye towards improving adhesion at the site of delivery which would maintain the drug delivery system in a specific location in the body, have a prolonged duration of contact with the tissue, and increase the treatment efficiency since the drug is locally maintained at the site of transport.

Bacteria have evolved over time into many types with various uses ranging from being destructive and causing infection, major illness, and even death, to those that are more beneficial by aiding digestion. The primary factors that dictate the tissue tropism of a microbe are its adhesive properties. When bacteria cannot adhere to target cells they are usually rapidly dispatched from the host. Adhesion may be the most important step in the colonization of mammalian tissues by both commensal and pathogenic bacteria. The adhesive process is initiated by cell-surface fibers called fimbriae or pili. These fibers

project bacterial lectins, also called adhesins, away from the bacterial surface, towards specific glycan receptors on the host cell. In most cases they are found to employ adhesins to exploit the diversity and virtually unlimited combinatorial potential of their carbohydrate receptors to ensure selective and finely tuned pathogen–host interactions [6].

Many are looking at ways of hindering this adhesive process of bacteria as a way to avoid the painful disease and infections that they can cause. [7-9] However, many have also used viruses and bacteria as a blueprint for how to improve drug delivery and other medical practices and treatments.[10, 11] The latter has allowed us to look at bacteria's ability to adhere and utilize that for the purposes of drug delivery. Incorporating adhesive properties into a drug delivery system has many significant advantages that have already been stated. One specific advantage with using these bacterial adhesins is the ability to localize the drug at a targeted site of absorption. Increased contact of the drug delivery system with the mucosal absorptive membranes of a specific site for an extended period could increase the absorption of the therapeutic agent, resulting in a higher drug bioavailability. [1-5] We accomplished this previously with a more synthetic functional group when we utilized aldehyde-modified PEG tethers incorporated into a pH sensitive hydrogel. We envision being able to have similar success utilizing a more biological route with a specific bacterium.

Lactobacillus acidophilus is a probiotic that is part of the bacterial microbiota of the gastrointestinal tract of many humans and animals. It has been used in supplements and milk products and has had its genome sequence studied extensively for use in various

medicinal purposes. [12-14] From these studies, one sequence of protein called surface layer protein A (SlpA) has been discovered to be of vital importance in *L. acidophilus's* ability to adhere and colonize human tissue. While the mechanism of attachment is still not fully understood, this sequence has been found to be adhesive to mucus, epithelial cells, and also parts of the extracellular matrix such as fibronectin. [12-16] The adhesive properties and the nonpathogenic nature of the bacteria are why this has been selected as the bacterium of choice for further testing. We were able to get improved adhesion to the mucosa with a synthetic aldehyde moiety, but we should be able to get even more selective adhesion using more intricate functional groups found throughout nature. The bonding of the SlpA adhesin, and that of any of the possible bonding sites of mucin, epithelial cells or substances found in the extra cellular matrix, should increase the adhesive abilities of the hydrogel yet still be discharged from the body when the mucus layer or cells are renewed between six hours to a few days. Much of the research around SlpA has been with proving what it can adhere to and how to prevent it. [15, 16] Most research revolving around bacterial therapeutics and the lactobacillus strains deal with utilizing probiotics or the entire bacterial unit itself to adhere and deliver drugs. [17] There has been limited research with bacterial adhesin mediated adhesion for drug delivery systems with units such as Type-1 and K-99 fimbriae of *E. coli* or the invasin of *Y. pseudotuberculosis* but could be problematic due to their toxic nature. [18-21] The use of the nonpathogenic SlpA as an adhesive agent opens a new avenue for oral drug delivery.

A P(MAA-g-EG) pH-sensitive hydrogel was again used as the model drug delivery system. Hydrogels with pH-sensitivity make excellent candidates for the oral delivery route because of their ability to respond to their environment, they resemble natural living tissue, are biocompatible, provide the protection of proteins needed in harsh environments, and exhibit high adhesive bond strength in contact with tissues. The latter should help to increase residence time even further after preliminary contact by the grafted bacterial adhesin. [22-29] An *ex vivo* testing method referred to as the trough method was used in determining adhesion. This method has been used by other researchers to determine the adhesive capacity of hydrogel delivery devices when in contact with cellular models. [30-32]

Materials and Methods

Bacterial Purification

The *Lactobacillus Acidophilus* strain NCK1962 was given by Dr. Todd R. Klaenhammer from North Carolina State University. Cultures were propagated overnight in MRS broth and harvested by centrifugation at 8000 X g for 20 minutes at 4^{o}C. The pellets were suspended in a 5 M LiCl solution in a 1ml:10mg ratio and stirred for 15 minutes at 4^{o}C. The solution was centrifuged again and the remaining pellet was dialyzed for 24 hours in distilled water at 4^{o}C using a Spectrapor 6-8000 membrane. A white precipitate formed after dialysis and was centrifuged at 40000 X g for 20 minutes at 4^{o}C. The pellet was resuspended in 1 M LiCl and stirred for 15 minutes at 4^{o}C before being centrifuged again at 40000 X g for 20 minutes at 4^{o}C and washed with 15 ml of cold deionized (DI) water. [12]

The purity of the S-layer protein was determined by SDS-polyacrylamide gel electrophoresis (PAGE). A western blot and coommassie stain were used. For the western blot, the antibody was obtained from Dr. Sandra M. Ruzal from the University de Buenos Aires in Argentina.

Hydrogel Synthesis

The methacrylic acid (MAA) monomer was passed through a dehibit column to remove the inhibitor. The polymer films were prepared by free radical UV polymerization. The diluted monomer mixture was composed of methacrylic acid (MAA, Sigma, St. Louis, MO) and PEGMA with a molecular weight of approximately 500. Irgacure® 184 (1-hydroxy-cyclohexyl-phenylketone, Ciba-Geigy, Hawthorne, NY), which was used as a photoinitiator, and tetraethylene glycol dimethacrylate (TEGDMA, Polysciences Inc. Warrington, PA), which was used as a crosslinking agent was also incorporated. Deionized water and ethanol were used as solvents. The monomer solution was made in a ratio of 1:1, MAA:EG units, P(MAA-EG) was made with unmodified PEGMA. TEGDMA was added as 1 mol% of the total monomer and Irgacure® 184 added as 1 wt% of the total monomer.

The monomer solution was dissolved with a 50:50 monomer to solvent weight ratio. The total solvent weight was composed of equal parts deionized water and ethanol. Once all components were added to the monomer mixture, the solution was sonicated until all materials were dissolved into solution. The mixture was purged with nitrogen for 20 minutes to remove oxygen, which acts as a free radical scavenger. The solution was pipetted between two glass slides (75mm X 50mm X 1mm) separated by a 0.8 mm

Teflon® spacer. The slides were placed under a UV light (Efos Corporation, Ultracure 100ss, High pressure mercury lamp, Mississauga, Ontario, Canada) and allowed to polymerize for 30 minutes.

After polymerization was complete, the thin films were washed with deionized water daily for one to two weeks to remove all unreacted components. After washing, the films were dried under a vacuum (**Heraeus VTR-5036, Heraeus** Instruments GmbH., Hanau, Germany) for 2 days at approximately 30° C. Hydrogel films were crushed and sieved with a mesh size of 45μm. Images of particles were taken on a light microscope and processed using an image processing program. Particle size distribution was found to be 8.5 mm with a standard deviation of 5.8mm.

The SlpA protein was covalently attached to the hydrogel instead of attached via PEG. This attachment occurred through a linkage between the carboxylic acid of the methacrylic acid units and the amine groups found on the bacterial protein. [33] Briefly, 2.5 ml of a 1mg/ml solution of the protein was treated with 200ml of a 200mg/ml solution of 4-(4,6-dimethoxy[1,3,5]triazin-2-yl)-4-methylmorpholinium chloride (DMTMM, Sigma Aldrich, St. Louis, MO) in distilled water. The solution was placed on a rotator for 40-60 minutes at room temperature. The mixture was added to Sephadex G-25M PD10 columns and eluted after centrifuging. This separated the DMTMM modified protein from free DMTMM. The modified protein was added to microparticles of P(MAA-EG) hydrogel previously made. The solution was sonicated, vortexed, and placed on a rotator overnight at room temperature. The particles were washed twice by centrifugation with 1ml of PBS to remove any unbound protein. SDS-PAGE was used to

determine percent protein linked. A western blot was used with the antibody obtained from Dr. Sandra M. Ruzal.

Swelling Analysis

Dynamic swelling studies were performed using dimethylglutaric acid (DMGA, I=0.1M, Acros Organics, NJ) buffers. Ten buffers, with a pH range of 3.2 to 7.6 were prepared. The ionic strength of the buffers was controlled with 0.1M sodium chloride (NaCl, Sigma Aldrich, St. Louis, MO). Polymer microparticles of P(MAA-EG) were compared against the protein linked microparticles by placing 50 mg in 10 mL of the first DMGA buffer with a pH of 3.2 for 10 minutes at 37° C. Afterward, the microparticles were centrifuged down out of the buffer. The supernatant was poured off and the microparticles were weighed to determine the water uptake. The next DMGA buffer of the next highest pH was added to the microparticles and the process was repeated up to the buffer with a pH of 7.6.

The following equation was used to determine the weight swelling ratio, Q:

$$Q = W_{swollen} / W_{dry} \quad (1)$$

$W_{swollen}$ and W_{dry} refer to the weights of the copolymer microparticles in swollen and dry states, respectively. These values were then used to plot weight swelling ratio, Q, vs. swelling pH.

Adhesion Analysis

Caco-2 cells were used between 30 and 40 passages. Cells were grown in a 5% CO_2 atmosphere. All reagents were obtained from Gibco (Gibco-Invitrogen Corp.,

Carlsbad, CA). Dulbecco's Modified Eagle Medium (DMEM) was used with 16.7% fetal bovine serum (FBS), and 1% each of non essential amino acids and sodium pyruvate. Cells were grown in 75 cm^2 BD Falcon tissue culture treated flasks (Thermo Fisher Scientific, Pittsburgh, PA). The medium was replaced every two days and adhesion experiments performed every seven days after plating. The cells were grown up to confluence, placed on a 45-degree incline and washed with DI water. 30 mg of dry particles were dispersed and left for 20 minutes in the incubator. Afterwards, the system was flushed with DI water for five minutes at 22ml/min. The detached particles were collected, dried and weighed with the adhesive capacity being the percentage of particles retained by the tissue.

Results

Bacterial Purification Analysis

SDS-PAGE was performed to determine the purity of the S-layer protein after the initial purification process from the bacteria cultures and later after the linking reaction with the hydrogel microparticles previously mentioned. A western blot and coommassie stain were used to certify the initial purification with subsequent western blots used to quantify SlpA linkage to the microparticles. For the western blot, the antibody was obtained from Dr. Sandra M. Ruzal from the University de Buenos Aires in Argentina. Figure 6.1 shows the western blot stain after initial purification of the *L. acidophilus* strain. A coommassie stain not shown was performed as a secondary check and was successful. The bands moving from slot 1 to 7 are different steps in the purification process. Slots 1-6 correspond to the following samples taken during the purification

process: sample after the 5M LiCl wash; sample of the supernatant after first centrifuge step; sample of pellet after first centrifuge step; sample after dilution; sample of surpernatant after second centrifuge step; and sample of supernatant after 1M LiCl wash and centrifuge step. We can see that bands increase in intensity and thus purity as we reach the final purification in the seventh slot. Both figures show a bright solid band at 50 kDa in the seventh slot which is where we anticipated the purified adhesin being according to the literature. [12]

After producing more SlpA adhesin, some of the purified samples were used to make several standards in DI water ranging in concentration. These standards were used with the residual filtrand from the final step to graft the adhesin to the microparticles in a western blot assay. This would allow us to quantify the amount of SlpA bound to hydrogel microparticles by creating a standards curve of SlpA concentrations to light intensity. We were able to accomplish this by gauging the light intensity of the different concentrations of the bands in the western blot assay with higher concentrations giving a brighter band. Each linking reaction was run to determine the percent SlpA bound of each new batch. The reactions produced multiple ranges of percent SlpA bound. These ranges were between 30% and 60%. No obvious reason was found for the differences in percent SlpA bound as all experiments were performed identically. These particles were then used in subsequent swelling and adhesion testing.

These studies allow us to confirm that the SlpA protein is successfully purified from the *L. acidophilus* strain and ready for use in the grafting reaction. Secondly, we were able to determine the amount of protein attached to the microparticles after going

through the reactions steps, giving a yield between 30-60%. This is of note due to the difficulty in binding biologics in polymerization and with being one of the first in the generation of bacterial improved drug delivery systems.

Hydrogel Swelling

Dynamic swelling studies were performed to investigate the pH sensitivity and water uptake of samples with the SlpA modified hydrogels in comparison to a control sample of P(MAA-EG). The phenomenon of complexation occurs through hydrogen bonding between electron deficient moieties and moieties containing regions of high electron density. This occurs most notably in acidic surroundings because of the hydrogen bonding between the ether group of the PEG chain and the carboxylic group of the PMAA. [34, 35] As the pH values reach the pKa value of MAA (4.8), the carboxylic acids are ionized causing the dissociation of the hydrogen bonds, resulting in a repulsive interaction between the PEG chains and the MAA molecules causing what is known as decomplexation. Due to the addition of a new moiety, we must determine if the additional functional groups present in the amino acids of the SlpA adhesin interferes with the interactions between the PEG chains and MAA molecules that result in complexation and gives the hydrogels its desirable pH sensitivity. Without this pH sensitivity there would be no drug release and thus it is imperative to maintain these characteristics. The highest percentage (60%) of linked SlpA was used to show that incorporating the new moiety at a high percentage would not adversely affect the swelling and pH characteristics of the hydrogel. The dynamic water uptake for each formulation is shown in Figure 6.2. Both formulations maintain pH sensitivity with a transition around a pH of 5.6. There was an

increase in water uptake as pH increased, indicating that the addition of the SlpA moiety does not affect the bonds that cause complexation and decomplexation. Both formulations have similar swelling ratios with high points of around 10 and low point of around 3.5. The pH transition is similar to the transition seen when hydrogels were cut into cylinders and used in dynamic swelling experiments seen earlier in Chapter 4, but the overall Q ratio is higher. This difference in Q ratios is easily explained with the increase in surface area exposed to the buffer with microparticles leading to more water uptake as opposed to compact cylinders.

This study allows us to confirm several things. First, that the microparticle method for dynamic swelling is valid and comparable to the method using cylinders. Second and most importantly, the incorporation of SlpA does not affect the desired properties of the hydrogel allowing it to maintain its pH sensitivity and not shifting the pH transition. Thus, we expect that the new carriers could maintain the same characteristics through the oral delivery process.

Adhesion Analysis

An *ex vivo* adhesion study utilizing Caco-2 cells also known as the trough method was performed to investigate the effectiveness of the bonding of the SlpA protein adhesion in aiding cellular adhesion when compared to the interpenetration and hydrodynamic forces seen with a control formulation. [30-32]

During the adhesive experiments, the microparticles were allowed to swell for 20 minutes in a CO_2 rich incubator after being administered to a T-flask of Caco-2 cells that had been grown to confluency. Caco-2 is a colonic carcinoma cell line that is commonly

used in cell experimentation. The cell line was originally isolated from human adenocarcinoma of the colon. These cells express many of the same markers associated with normal small intestinal cells and spontaneously differentiate postconfluence to display functional apical brush border microvilli and epithelial-cell polarization. The swelling time for the microparticles is important since, as stated previously, the initial formation of an adhesive bond between a polymer and the biological substrate is the wetting and swelling of the polymer. Just 10 minutes of exposure is sufficient to swell the system to conditions similar to those in the upper small intestine but 20 minutes was used to stay consistent with previously performed experimental procedures in literature.[31, 32] This value simulates the physiological situation of a hydrogel traveling through the GI tract to the upper small intestine, where it is complexed under the conditions described previously, to the target site of adhesion, where it starts swelling and decomplexing.

The objective was to improve the adhesive performance of a crosslinked poly(methacrylic acid) hydrogel, a well-known adhesive polymer, by decorating it with SlpA adhesins that allow the *L. acidophilus* bacterial strain to adhere to cell surfaces. First reviewing the properties of the basic P(MAA-EG) hydrogel there is evidence it utilizes many secondary chemical interactions from van der Waals interactions, to hydrogen bonding which is considered the most important secondary interaction in adhesion.[36] When entering the upper small intestine, the secondary forces are numerous giving an overall stronger adhesion that would be absent with just one small secondary interaction. These forces can hold long enough for swelling and, as a result, interdiffusion of the free flowing PEG chains to take place. The PEG chains are not

interacting with the poly(methacrylic acid) backbone at the higher pH conditions and are able to diffuse through the mucus and enhance adhesion. The SlpA moiety will complement these interactions much like the synthetic aldehyde moiety did in the previous chapter with the addition of another possibly stronger chemical linkage to receptors found on the cell surface throughout the small intestine.

Figure 6.3 shows the percent retained by the Caco-2 cells for the control hydrogel P(MAA-EG) and the 30% bound SlpA modified hydrogel. The lower percent of bound SlpA was utilized to show that increased adhesion is possible even with minimal amounts. Figure 6.3 shows a percent retained of 70.6% and 82.4% for the P(MAA-EG) and SlpA modified hydrogel respectively. The data shows an increase in adhesive retention of 16.7% of the SlpA modified hydrogel over that of the control hydrogel. This indicates that even in minimal amounts the addition of the added chemical bonding through the SlpA protein adhesins causes a greater increase in the adhesion to the cell surface than secondary forces and interpenetration alone with possibly even a further increase in adhesion possible with the addition of mucin and various extracellular matrix proteins that are present throughout the small intestine. The addition of the added chemical bonding through the SlpA adhesin causes a greater increase in the adhesion and adhesion will only increase with more SlpA due to the availability of more bonding sites.

Finally, it is important to note that it would be difficult to compare the results between the synthetically modified hydrogels that utilize the aldehyde moiety and that of the biologically modified hydrogels that utilize the SlpA adhesin since they involve different adhesive experiments and different adhesive substrates between mucin solution

and Caco-2 cells. Instead we can determine that both successfully upgrade the adhesive capacity of the normal control hydrogels of PMAA and P(MAA-EG) with relatively small amounts and that both have the ability to improve upon these results making each excellent candidates as oral delivery vehicles. However, between the two moieties the biological path may hold more potential due to the possibility of multiple adhesive pathways along targeted sites and the ability to possibly pinpoint the area of delivery unlike the previous synthetic route which binds to any amine in the chime, mucin, and cells of the small intestine.

Overall, this study allowed us to determine the increased adhesion of the SlpA modified hydrogels. With increased adhesion over the control formulation, this new material could potentially improve drug delivery due to increased residence time in the upper GI tract.

Conclusions

The present work was performed to determine the effectiveness of using chemical linkages by utilizing a protein found in an everyday probiotic bacteria, in this case SlpA from *L. acidophilus*, to increase the limited adhesion of the P(MAA-EG) hydrogel. The better adherence to the small intestine would lead to increased residence time, release at the delivery site, and would presumably affect the bioavailability of the protein. Hydrogels with PEG tethers and with varying percentages of SlpA modified hydrogels were synthesized and characterized to determine any improved adhesive capacity without affecting the overall characteristics of the oral delivery device. Western blot assays confirmed the purification of SlpA and percent bound after linking to P(MAA-EG)

hydrogel microparticles. Dynamic swelling studies showed that the SlpA functionalized hydrogels retained their pH sensitivity at pH of 5.6. A widely accepted *ex vivo* experiment showed an improved adhesive capacity of 16.7% with the increased chemical linkages of the SlpA moiety over that of the control formulation. [30-32] These findings indicate the potential these biologically functionalized hydrogels have for improved oral drug delivery and that they also compare favorably along with the swelling and adhesive characteristics of the synthetically modified hydrogels that used an aldehyde functional group.

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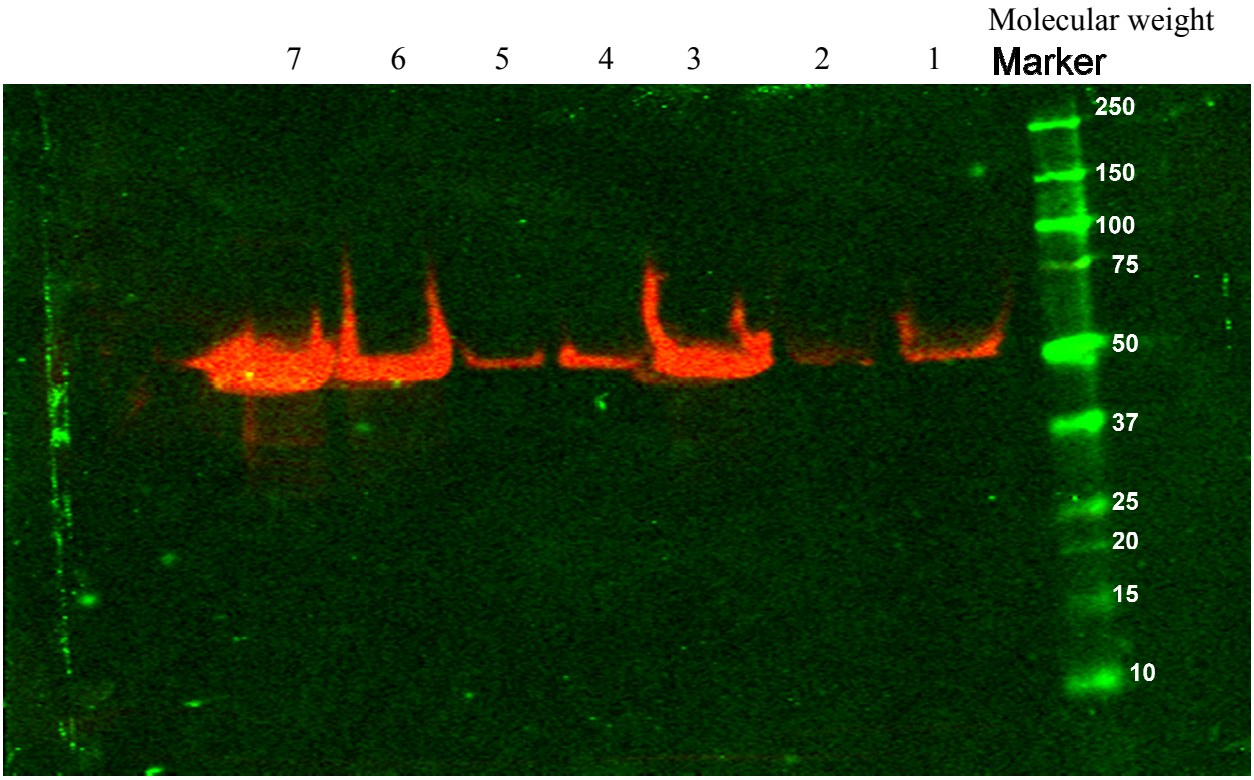

Figure 6.1. Western blot assay of samples taken during purification process of SlpA protein from L. acidophilus: Slots 1-7 correspond to the following samples taken during the purification process: (1) sample after the 5M LiCl wash, (2) sample of the supernatant after first centrifuge step, (3) sample of pellet after first centrifuge step, (4) sample after dilution, (5) sample of supernatant after second centrifuge step, (6) sample of supernatant after 1M LiCl wash and (7) centrifuge step and final purified sample.

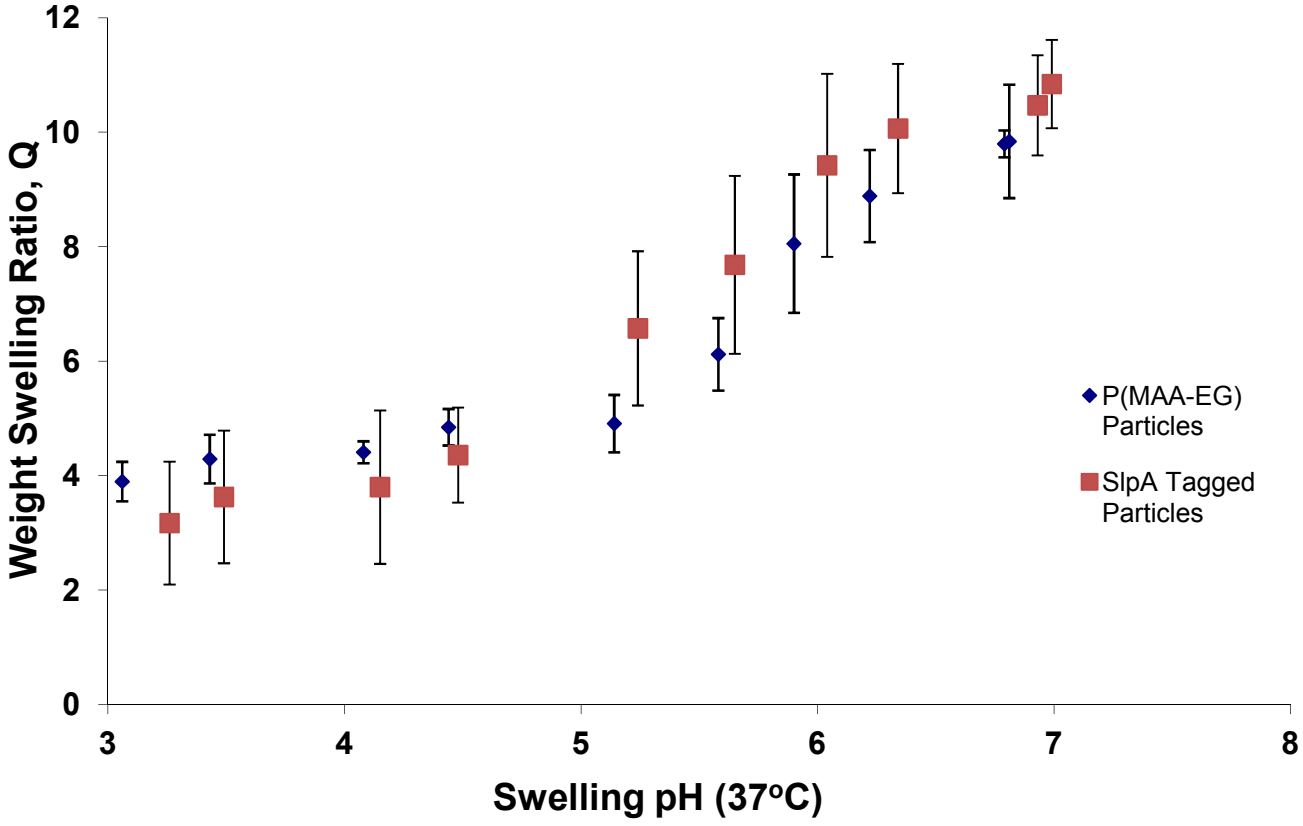

Figure 6.2. Dynamic swelling results of P(MAA-EG) and SlpA tagged microparticles after spending 10 minutes in each pH buffer successively to gauge pH sensitivity.

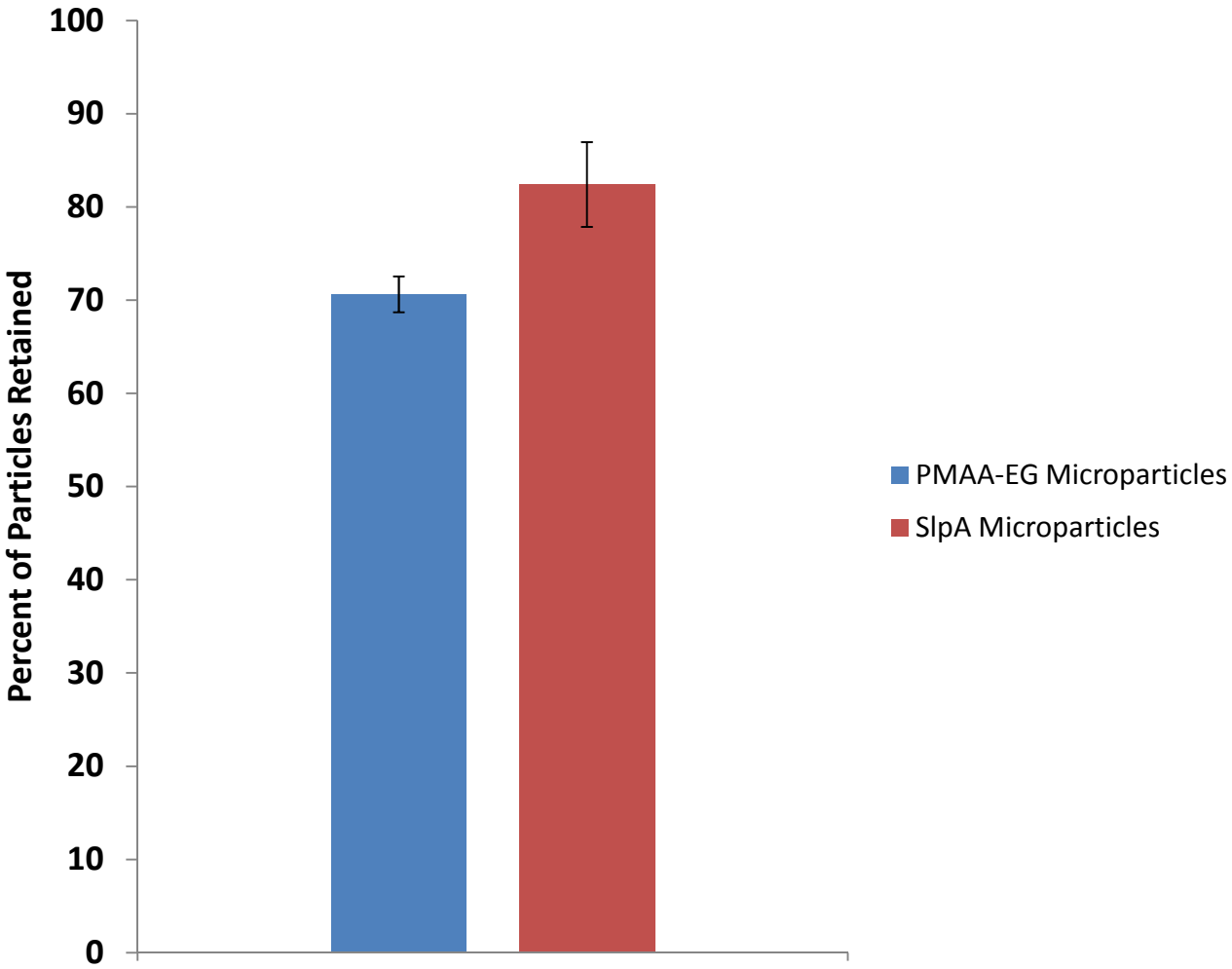

Figure 6.3. Percent of particle retention of P(MAA-EG) and SlpA tagged microparticles to seeded Caco-2 cells after DI water wash from the trough adhesive method. P value = 0.01.

Chapter 7: Conclusions and Recommendations

The field of bioadhesion is rapidly evolving with uses ranging from developing more effective drug delivery systems, to preventing and understanding disease transmission. One approach of great interest involves using bioadhesive materials in contact with mucosal surfaces (mucoadhesion) and cellular surfaces (celluar adhesion), to improve the efficacy of therapeutic treatments. While the understanding of adhesion needs further research the benefits gained by using adhesive biomaterials in the design of new drug delivery systems are essential to the efficacy of this area of research. There are several advantages that adhesive drug release devices bring to the controlled release field, but the main one is the ability to maintain the delivery device at a specific location in the body for an extended period of time. These features are able to provide higher efficiency of local and systemic therapies, resulting in a higher bioavailability of the drug in the body.

The novel functionalization of pH responsive hydrogels were evaluated in this work to understand their application in bioadhesive controlled release drug delivery systems. The present work was performed to determine the effectiveness of using synthetic and biological linkages in improving and understanding adhesion of the drug delivery vehicle. In the first part of this thesis, a synthetic aldehyde moiety was used, to increase the limited adhesion of the hydrogel. The better adherence to the small intestine would lead to increased residence time, release at the delivery site, and would presumably affect the bioavailability of the protein. Aldehyde was functionalized onto PEG tethers by catalytic reaction with TPAP and grafted onto hydrogels in varying

percentages. Swelling studies showed that the aldehyde functionalized hydrogels retained their pH sensitivity while release studies showed that the addition of the aldehyde moiety did not hinder the insulin release seen in previous work with nonfunctionalized hydrogels. Widely accepted *in vitro* tensile testing showed improved adhesive capacity with increased chemical linkages with the aldehyde moiety in both dry and wet conditions.

In the second part of this thesis, a novel *in vitro* adhesive testing method was generated. AFM force modulation was used as an effective model for *in vitro* adhesion testing of drug delivery vehicles specifically pH sensitive hydrogels on a molecular level. Utilizing a more accurate *in vitro* method would bring new insight into the methods of adhesion and further the work of improving oral drug delivery vehicles. Hydrogels with and without PEG tethers and with aldehyde modified PEG tethers were used in this study and compared with work previously done using the Instron tensile testing method. Microparticle tagged AFM tips were made, confirmed for use in experimentation and showed an overall increase of adhesion across all formulations when compared with bulk testing. Preswollen AFM data was utilized to determine that the aldehyde moiety alone shows an increase of adhesion without the addition of secondary or interpenetrative forces. Overall, a new method of *in vitro* testing for adhesive capabilities was established.

The third part of this thesis was performed to determine the effectiveness of using biological linkages by utilizing a protein found in an everyday probiotic bacteria, in this case SlpA from *L. acidophilus*, to increase the limited muco and cellular adhesion of the P(MAA-EG) hydrogel. Hydrogels with PEG tethers and with varying percentages of

SlpA modified hydrogels were synthesized and characterized to determine any improved adhesive capacity without affecting the overall characteristics of the oral delivery device. Western blot assays confirmed the purification of SlpA and percent bound after linking to P(MAA-EG) hydrogel microparticles. Dynamic swelling studies showed that the SlpA functionalized hydrogels retained their pH sensitivity and a widely accepted *ex vivo* experiment utilizing Caco-2 cells showed an improved adhesive capacity with the increased chemical linkages of the SlpA moiety.

To summarize the work contributed to the understanding of the drug delivery systems capable of modulating adhesion. Both the synthetic and biological modified hydrogels were shown to be effective in maintaining the pH responsive nature of the systems and increasing the overall muco and cellular adhesion respectively. Also, the capabilities of AFM force modulation as a novel *in vitro* tool was explored and found to accurately determine the adhesive capabilities of drug delivery vehicles on a molecular level. Future recommendations would involve developing a sanitary setup for Caco-2 covered slips to be used in AFM force modulation testing to explore SlpA modified hydrogels. Furthermore, further research involving other biologically based adhesive properties should be considered especially those with properties that affect transport across the epithelia.

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Chapter 8: Vita

F. Michael Marks III was born on July 9, 1982, to F. Michael Marks Jr. and Carolyn L. Marks. He grew up in Roxboro, NC, where he graduated high school from Roxboro Christian Academy in 1999. Michael went on to major in chemical engineering at the North Carolina State University, and he earned his Bachelor's Degree in Chemical Engineering in May 2005, graduating Magna Cum Laude. While at NCSU, he researched self assembled monolayers (SAM's) on gold substrates for Dr. Peter Kilpatrick, he co-oped with Eastman Chemical Company in Kingsport, TN and interned at Talecris Biotherapeutics in Sanford, NC.

After graduation, Michael joined the University of Texas at Austin as an IGERT Research Fellow working with Professor Nicholas A. Peppas. While at UT-A, he performed a summer research internship at Martin Luther University of Halle-Wittenberg working under the supervision of Karsten Maeder and earned his Masters' Degree in Chemical Engineering in December 2006.

Michael proceeded to Drexel University as a GAANN Research Fellow working with Professor Anthony M. Lowman working towards a Ph.D. in Chemical Engineering.

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