8-2012

ORAL DELIVERY OF PEPTIDE DRUGS FOR MITIGATION OF CROHN'S DISEASE

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ORAL DELIVERY OF PEPTIDE DRUGS FOR MITIGATION OF CROHN’S DISEASE

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Bioengineering

by
Paul Arthur Blichmann
August 2012

Accepted by:
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Dr. Narendra Vyawahare
ABSTRACT

Protein drugs are typically administered intravenously, but this practice has clear disadvantages such as widespread circulation and swift clearance from the body. Orally delivered protein drugs are not yet available but potentially offer improved distribution, retention, and activity by use of protective matrices and cell-specific targeting. Much work has been done on delayed release formulations for the upper intestine, but there has not been overwhelming evidence of protection of peptides from gastric conditions. In many instances the large intestine may be a better release target due to lower proteolytic activity. Afflictions of the colon such as Inflammatory Bowel Disease and colon cancer would benefit most directly from colon targeted drug release.

Orally delivered colon-targeted protein drugs need protection and a release mechanism to withstand the upper GI and be dispersed along the inflamed areas of the lower intestine. This sort of delayed release has been proven with low molecular weight drugs, but has yet to be shown with peptides which also need protection from the acidic and proteolytic conditions of digestion. This work aims to distribute protein drugs throughout the colon while retaining protein activity.

Pellets containing active peptides are synthesized from dextran modified with methacrylate. Dextranase, formed by bacteria and found in increasing amount in the lower intestines, gradually degrades these pellets allowing steady transport of drug from the pellet. These pellets are further coated in an acrylic copolymer (supplied by Evonik) designed to protect from acidic pH of the stomach and degrade within the upper intestines. Polylactide nanoparticles loaded in these constructs were found to release steadily under conditions simulating the colon over 25-40 hours.

Lysozyme was incorporated into pellets as a model protein drug and showed an estimated 10% activity after a complete digestion simulation. α-MUC1 was utilized similarly and imaged on epithelial cells, demonstrating feasibility of delivering antibody drugs orally. As many cancers overexpress MUC1 and inflamed cells are believed to as well, MUC1 is a potential drug target for CD and colitis. In vivo data using a mice colitis model showed mitigation of symptoms when administered with antacids. Overall this drug delivery platform has demonstrated colon targeting and potential efficacy of orally delivered enzyme, antibody, and other complex peptide drugs.
ACKNOWLEDGEMENTS

Heartfelt thanks are extended to Igor Luzinov and Bodgan Zdyrko for polymer synthesis, Stephen Tomlinson and Xiofeng Yang and Emily Paulling for protein synthesis, Fei Qiao and Michelle Elvington for animal work and histology, Polina Kocherginskaya for preliminary work, and Andrew Honeycheck for providing samples from Evonik.

In vitro microscope images were captured at the Jordan Hall Imaging Facility of Clemson University. Special thanks to Dr Terri Bruce for all assistance.
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SPECIFIC AIMS

Crohn's Disease (CD) and other forms of Inflammatory Bowel Disease (IBD) are characterized by chronic inflammation of the gastrointestinal tract and cause distress to an estimated 1.4 million Americans. Current treatments include 5-aminosalicylic acid as a first step and corticosteroids as an additional measure. Newer treatments aim to inhibit mechanisms within the inflammatory response, most notably by blockers of TNF-alpha (Infliximab). These drugs are approached with caution as they can only be administered intravenously, causing a systemic immune suppression and vulnerability to serious infections such as tuberculosis, fungal infections, urinary tract infections, and lymphoma. Newer drugs which also serve as blocks in the inflammation cascade, such as complement inhibition, will also need to be injected and can be expected to have the same problems stemming from systemic immune suppression. A more localized drug delivery is desirable for limiting these effects, and in this case oral delivery will be quite convenient by bringing drug to the colon directly without high circulation levels. Protein drugs are not yet available orally as they are susceptible to degradation during digestion and inefficient release profiles, but with appropriate degradable hydrogels and protective coatings it should be possible to maintain some protein activity for effective disease treatment.

Goal 1: Develop a drug release system which releases gradually and specific to only colon conditions.

Drug delivery should target the colon with great efficiency, with no drug released early into the stomach or upper intestine and no drug being passed with the stool. As lesions could be present at any point in the colon, a gradual drug release is best to cover all areas and this requires 24-48 hours. Hydrogel made from a modified dextran is the basis of the system and drug release is dependent on colon-specific enzymes. Drug release profile was modeled with a release of nanoparticles from the gels.

Goal 2: Demonstrate protein efficacy after digestion simulation with enzyme and antibody examples

Measurable protein activity after digestion simulation indicates that the drug delivery system is capable of releasing active peptides at the disease sites. In separate experiments lysozyme and α-MUC1 were loaded into the dextran hydrogels which were coated with a pH-protective acrylic polymer. After digestion simulation and drug release by dextranase, the final products were tested for antibacterial activity and epithelial cell attachment, respectively.

Goal 3: Achieve remission of symptoms in an Inflammatory Bowel Disease animal model.

A new drug combining complement inhibitor cryr and complement breakdown product targeting moiety CR2 has been successful as an injection in mouse studies. cryrCR2 was delivered orally within very small particles of the coated dextran hydrogel to colitis-induced mice and the health measures of weight, colon length, and intestinal histology were compared.
CHAPTER 1: BACKGROUND

Crohn’s Disease

Crohn’s Disease (CD) is a form of Inflammatory Bowel Disease (IBD) characterized by mucosal inflammation in patches or lesions along the GI tract, while Ulcerative Colitis (UC) has similar symptoms but with inflammation starting within the colon and proceeding more continuously to the rectum. Symptoms remit and return unpredictably and include swelling in the intestine, abdominal pain, diarrhea, weight loss, and fever. Although remissions can last as long as years, there is no cure and the mechanism of IBD is still speculated[1]. It is well known that certain foods can aggravate symptoms in some patients, but no single type of food has been singled out. The leading theory for the disease is an immune attack on bacteria, food, or other substance in the intestine. The most common and conservative prescription for CD/UC is 5-aminosalicyclic acid (5-ASA), an anti-inflammatory shown to inhibit pathways including PGE2, PGD2, IL-1, IL-2, TNF-α, and provide protection from reactive oxygen species [2]. It is available in topical and oral form and found to be most effective in combination. Should this fail to induce remission, it is standard to prescribe corticosteroids, although the severity of side effects limits this method to a few months of use.

Protein drugs are the key to future treatments, as cytokines and monoclonal antibodies are exceeding chemical drugs in anti-inflammatory roles. The most targeted component of the inflammation process is Tissue Necrosis Factor (TNF) alpha, such as Infliximab which is successful for many in treating CD, arthritis, and other autoimmune diseases[3]. There are several different injected monoclonal antibodies which act as TNF alpha
blockers, but they all have similar problems. With a major regulating factor of the immune system silenced, patients are at high risk of serious infection including urinary tract, fungal, and bacterial infections and elevated risk of lymphoma and cancer[4]. A different drug delivery platform is needed to make the most of TNF-alpha blockers and any new immune suppression cytokines.

**Desirability of Oral Administration**

The current drug delivery method for protein drugs is parenteral, whether given subcutaneously, intramuscularly, or intravenously. While this is more direct and efficient than absorption in the digestive tract by enteral administration, the pharmacokinetics of a large, systemic dose can be far from ideal. Following injection, a large amount of drug is widely distributed in the body, harming cells and tissues outside the targeted area of treatment. The toxicity of a drug limits the peak concentration of the drug, which could mean a dose falls below a therapeutic concentration within a short time of injection. Any injected drug will be cleared from the system by renal filtration, drug instability/insolubility, or immune recognition; this can only be countered by higher, more toxic doses, inconveniently frequent doses, or a continuous infusion[5]. Concerns with toxicity, health risks, quality of life, and high demands on medical attention limit this type of action with injected drugs, especially for a chronic condition. By using orally delivered enteral formulations, drug may be more gradually absorbed over a longer period, advantageous for permanent or chronic conditions.

In the case of TNF-alpha blockers prescribed against CD and IBD, oral delivery can limit the side effects of systemic immune suppression by bypassing circulation and acting
primarily around the inflamed epithelial wall. Newly developed treatments will likely also impede the immune system and would benefit for the same reason. Proposing to deliver protein drugs orally requires a thorough look at the available drug delivery materials.

**Oral Protein Drug Delivery - Insulin**

Insulin has become the most studied protein drug for oral delivery. With diabetes being a very common disease with huge commercial significance, insulin was one of the first proteins to be isolated in pure form and recombinantly cloned and produced. Many of the materials for oral drug delivery were developed with oral insulin delivery as a goal. Approaches include prodrugs or substances that cleave chemically to produce insulin, encapsulation in microparticles, and loading in hydrogels of natural or synthetic polymers. Materials include poly(methacrylic-g-ethylene glycol) hydrogel microspheres[6, 7], calcium phosphate-insulin-PEG-casein particles[8], vitamin B12 prodrugs[9], and chitosan-4-thiobutylamidine[10], to name a few. All reports have shown some degree of potential, and several have shown a hypoglycemic effect in rodents lasting 8 or more hours. However no oral format has proven sufficiently reproducible and dose-dependent in clinical trials, and new drug delivery materials are needed to increase both protein protection and bioavailability.

*Formulations for Oral Administration*

Oral drug formulations must maintain pellet integrity and isolate the contents from local conditions until the desired region of absorbance is reached. Acrylic polymers are a common capsule coating for controlling drug release. Sensitive to pH, they are
hydrophobic at low pH isolating the contents then swell, become porous, and delaminate over time as they become hydrophilic at higher pH. There are many commercially available acrylic copolymers, and Evonik Industries offers a wide series based on the pH level of swelling and duration of that process. There are even some extended release formulations which are slow enough to be marketed as colon targeting, but these depend on a pH of 7 or higher inside the intestines, which IBD patients may not reach[11-14]

Eudragit L100-55 (supplied by Evonik Industries) is a commercial acrylic copolymer appropriate for this purpose, providing impermeability at low pH and gradual swelling at pH values over 5.5. This coating is intended to isolate the pellet from stomach acid and start a gradual swelling in the duodenum, providing some degree of isolation from small intestine conditions before exposing the underlying hydrogel by the time of reaching the cecum. This is useful for IBD use as it doesn't depend on high intestinal pH like many extended release formulations. Eudragit acrylic copolymers have been demonstrated to delay release when coated onto drug tablets, but the ability to protect peptides is yet to be fully proven[15, 16]. It is likely that the hydrophobicity of acrylic at low pH forms a protective barrier in acidic stomach conditions, and acrylic polymers have been suggested to inhibit the intestinal proteases chemotrypsin and trypsin to protect insulin[17]. In vitro experiments are needed to demonstrate the degree of peptide activity which can be retained by using these materials.
**Fig 1. Desirable characteristics for oral delivery materials.** The release profile must target the desired area and peptide activity must be maintained for in vivo oral peptide delivery to be feasible.

**Large Intestine Delivery**

Many pH dependent coatings and tablets have been reported[18-21], using the rise in pH from the stomach into the small intestine to initiate swelling which continues until the large intestine is reached and drug release rate increases. While these products may be quite reliable in healthy and normal individuals, patients of Inflammatory Bowel Disease (IBD) and colon cancer experience lower pH in the intestinal lumens[11, 12] and widely varying gastric emptying times[22], which makes the proper release of drug impossible[14, 20, 21, 23]. Furthermore, in several studies the in vitro colon drug release can be at least partially contributed to changes other than pH such as a change in buffer systems[21, 23, 24]. With this knowledge we will turn to drug delivery systems with bioresponsive or biodegradable foundations, which may prove to be more flexible to
conditions. The carbohydrate and protein-metabolizing enzymes released by colon-specific flora can provide site-specific release by biodegradation.

Biotransformation Capabilities of the Colon

The enzymatic conditions within the colon can initiate azoreduction, hydrolysis, acetylation, ring opening, and many other chemical reactions[25]. Azoreduction is the most commonly utilized, activating prodrugs such as prontosil and neoprontosil. The common Crohn’s Disease treatment 5-aminosalicylic acid is formed from acetylation of the prodrugs sulfasalazine, balsalazide, and olsalazine. In addition to releasing active compounds from prodrugs, bacterial enzymes can be used for colon specific degradation of natural materials to initiate drug release, such as gels made the polysaccharides chitosan, guar gum, inulin, and dextran. Polysaccharides are safe, inexpensive, nontoxic, can be readily chemically modified to reduce solubility and form crosslinked networks, and degrade well. Chitosan has been made into gels and microcapsules to provide colon-specific degradation but was found to dissolves quickly in acid[22, 26], requiring enteric coatings. It has been encapsulated along with alginate and shown to protect bacteriophages in vitro[27] and used in thiolated form in insulin delivery studies [10]. Much research on guar gums has been on reducing solubility, as the gels formed often have too high a swelling ratio to contain water soluble drugs but may work in delivering water insoluble drugs[28]. A colon-targeting material of azo-bonded methacrylated inulin hydrogel was intended to degrade by both inulinase and azoreductase, but was found to release drug entirely before reaching the large intestine[29]. Premature drug release from inulin gels was again observed in another more recent study, wherein the
rheological properties of methacrylated crosslinked inulin gels were extensively examined but were found to have an immediate burst release in the stomach [30]. While the premise of a polysaccharide gel for drug release remains attractive, most attempts have been found too unreliable for practical use.

Dextran

Dextran is among the most promising of these polysaccharides, having served as a prodrug anchor in well cited studies with naproxen [31] and ketoprofen [32], and recently for glucocorticoids [33]. The polysaccharide gels are safe enough for implantation, as gels of crosslinked dextran have seen success as implantable disks releasing EGF and bFGF in animal studies [34]. The availability, nontoxicity, and success in oral formulations make dextran an attractive candidate for a hydrogel-based oral drug delivery system, and the degradation of dextran is colon-specific by the bacterial enzyme dextranase. Dextran has been made into hydrogels in many accounts, usually modified with methacrylate for easy crosslinking [35-38] but occasionally using other methods like crosslinking with diisocyanate [39]. Between all of these studies with dextran hydrogels it was common to find a degree of swelling that allowed degradation while still preventing premature drug release, whereas this was difficult in studies with polysaccharide gels from other materials such as inulin and guar gum. While some studies have already indicated colon-specific release, none have the gradual release desired in this project and the protection of peptide drugs has not been proven for these materials.
With chemical addition of vinyl groups, dextran can be made crosslinkable and hydrogels can be made. By an epoxide ring opening reaction, glycidyl methacrylate groups can be substituted into the dextran backbone, creating crosslinkable methacrylated dextran:

![Chemical substitution of glycidyl methacrylate groups onto dextran chains](image)

Fig 2. Chemical substitution of glycidyl methacrylate groups onto dextran chains[38]. The resulting product is crosslinkable and can be used to make hydrogels for drug delivery.

This is the material developed by Dijk-Wolthuis and used by several over recent decades. The modified dextran can absorb drug by swelling in aqueous solutions and is crosslinkable using a free radical photoinitiator. Rate of degradation is somewhat controllable by changing the degree of substitution of methacryl groups on the dextran backbone, thus changing the crosslinkability. This material is responsible for containing the active drug and controlling its release. For CD a gradual release is desired as lesions of inflammation can be anywhere in the colon, and for any drug with preventative purpose a thorough distribution is needed to ensure remission. A desirable hypothetical release profile would begin release immediately in colon conditions and continue for at least 24 hours. One study of dextran hydrogels has shown this but with using dextranase
concentrations far beyond physiological values[40], another study with different
crosslinking method shows drug release as long as 50 hours[41]. None of these studies
referenced has examined protein protection against digestion simulations.

Protein Protection Assessments

While dextran hydrogels have been shown to have desirable degradation and small
molecule release properties, there has yet to be a demonstration of its ability to shield
fragile contents from any acidic or enzymatic trial. Acrylic coatings are also unproven
but should help in this regard, as the hydrophobicity at low pH likely forms a protective
barrier in acidic stomach conditions. Acrylic polymers are also suggested to inhibit the
intestinal proteases chemotrypsin and trypsin to protect insulin[17].

Antibody Delivery

Protection of orally delivered antibody drugs would enable monoclonal antibody drugs
like Infliximab to be given orally. It is likely that future and developing immune
suppressants will take a similar form. Research in antibody drug conjugates would also
be enabled for the unique colon and inflammation conditions. The vast majority of
antibody drug conjugate research has focused on targeting cancer cells with cytotoxic
drugs from systemic circulation; however oral delivery can avoid rapid degradation and
clearance of antibody drug conjugates while giving an improved localized distribution in
diseases of the gastrointestinal system like colon cancer and IBD. Establishing colon
specific drug release is a good basis, but further adherence and retention on the epithelial
wall can effectively increase the concentration and duration of a dose. Due to the cost
and fragile nature of protein drugs, it is desirable to target all molecules as precisely as possible to make the most out of every dosage.

Anti-inflammatory or other mitigating drugs could be localized to affected epithelial cells by targeting moieties and be absorbed gradually through the mucosa, providing a more permanent drug presence and reduced side effects compared to large untargeted doses. One potential target is ICAM-1, a luminal cell adhesion molecule involved in adhesion and migration of neutrophils and monocytes, which is expressed during inflammation and IBD[42, 43]. CD68 targeting would bind to macrophages present at inflamed areas[44]. The glycoprotein MUC1 was chosen for more investigation due to some unique properties described below.

**MUC1**

Systemic absorbance in the intestines is somewhat hampered by the slow transport across the mucus and water layers surrounding the epithelial layer[13], believed to cause poor bioavailability in some research[8, 10]. However for diseases like IBD and colon cancer these layers provide a natural substrate for prolonged retention, potentially evening out the local concentration of drug between doses and keeping the drug in proximity to affected epithelial areas. MUC1 is a large, glycosolated protein expressed on the apical membrane healthy epithelial cells with an extracellular domain extending 300-500 nm from the cell, much further than the normal glycocalyx[45]. MUC1 targeting is a popular approach in cancer research, as it is found to be overexpressed in over 50% of tumors[46]. The glycoprotein is also upregulated during infection and believed to play a
role in preventing infection of mucosal cells[47]. Overexpression has been also observed due to inflammation, leading many to speculate on the links between IBD and colon cancer[48]. This molecule is worthy of investigation as a ligand for colon inflammation targeting.

Significance

The current treatments for CD are insufficient to mitigate symptoms inflammation symptoms long term. Immune suppression drugs like Infliximab and newer pathway inhibitors need improved drug distribution to limit side effects, which is why we turn to oral delivery. Oral drug delivery systems currently available have been proven to delay release in a myriad of small molecule drug formulations, but nothing available fits the requirements for protein delivery. Furthermore most developments have focused on insulin and not considered colon targeting. Polysaccharide gels have colon-targeting potential, with crosslinked dextran being the most robust candidate. Acrylic coatings may impart an additional level of isolation in acidic stomach conditions.

Our proposed solution is a capsule base of methacrylated dextran, containing appropriate proteins in a hydrogel. A capsule coating of methacrylate: ethyl acrylate alternating copolymer imparts some impermeability to acid and swells to delaminate in the upper intestines. This formulation is intended to isolate contents from stomach and upper intestine conditions and release gradually through the length of the colon. The type of release profile desired has never been shown for these specific materials and will need to be demonstrated in vitro.
Peptide protection is critical for delivering active peptides, and studies with the dextran and acrylic materials chosen have not yet proven sufficient peptide protection. To quantify peptide protection the enzyme lysozyme was incorporated into coated pellets and tested for antibacterial activity after digestion simulations. Further supporting data was found by treating an antibody to MUC1 in the same manner as lysozyme and assessing its binding.

With appropriate release characteristics and sufficient protein protection demonstrated, this drug delivery system is capable of orally delivering a variety of therapeutic protein drugs. To verify this, in vivo experiments in mice used drug loaded pellets to treat colon inflammation.
CHAPTER 2: RESEARCH METHODS

1. Structure of Protective System

The drug delivery system used in all experiments included dextran hydrogel and acrylic coating. Methacrylated dextran and photoinitiator were added to protein solutions and polymerized into droplets by UV activation. The resulting gels were covered in an aqueous suspension of acrylic copolymer.

1.1 Dextran Modification

Dextran gels were prepared using the procedure similar to that reported by Dijk-Wolthuis [38]. Dextran (MW 70,000) was coupled to glycidyl methacrylate to introduce vinyl group that is necessary for crosslinking dextran molecules. For coupling, glycidyl methacrylate and (N,N-dimethylamino) pyridine was added to dextran solution in
dimethylsulfoxide (DMSO). The solution was stirred at room temperature for 48 h. The product was isolated by 3 times rinsing with acetone solution followed by centrifugation. Modified dextran contained 4 methacrylic acid residues per 100 anhydroglucoside units (DS=4, determined by NMR spectra).

1.2 Pellet Formation

Lyophilized modified dextran (described in 1.1) was added to solutions of the relevant peptide to make a 25% dextran solution and AIBN was added at .75% of dry polymer weight. The suspension was pipetted onto paraffin film to form hemispheres and crosslinked by UV lamp (365 nm, 84 watts) for 12 minutes, producing 20% wt dextran hydrogels.

1.3 Eudragit Coating

Coating solution was an aqueous suspension of 12.5% Eudragit L100-55 with 6.25% talc to reduce tackiness and 1.5% triethyl citrate as a plasticizer. Dextran hydrogels were coated in prepared Eudragit suspension, drained of excess, and the surfaces was dried briefly with warm air. All pellets received three coats in this method.

1.4 Digestion Simulations

All in vitro experiments used digestion simulations. After coating had dried, the stomach was simulated by 1 hour in PBS pH 1.5 and the small intestines were simulated by 5 hours in .1% trypsin. In some experiments a range of milder conditions were also tested in the same method, as listed later in the experimental section. The large intestine was simulated by the physiological level of .1 U/mL dextranase in neutral PBS for
determining the release profile, and all other experiments used .5U/mL dextranase for 3 hours to release the final product for testing.

2. **Nanoparticle Release Profile**

**Rationale**

This experiment determines the timecourse of drug release in the GI tract. Hydrophilic nanoparticles were used as tracers because they are easily measured and do not degrade in the test conditions. For this purpose we were interested in release with regard to time and condition without interference from peptide degradation, so nanoparticles were more appropriate than proteins for tracers. In early stages of the project, when this experiment was performed, nanoparticles were intended to be the base of a drug and targeting moiety complex. Although none of the peptides used in this thesis involved nanoparticles, future work may see nanoparticles incorporated into the controlled release system. As the release from the gels was believed to be more degradation-dependant than diffusion-dependant, nanoparticles are appropriate for a universal measurement.

2.1 **PLA-PEG Nanoparticle Synthesis**

Rhodamine-labeled PLA-PEG nanoparticles were made from of 75% poly(DL-lactide) (Aldrich) and 25% poly(ethylene glycol)methyl ether-block-polylactide (PLA ~5000 Mn, PEG ~5000 Mn, Aldrich) dissolved in 99.7% purity acetone to make a solution of 7.5 mg/mL polymer. The acetone solution was added dropwise to sonicating DI water and sonicated a further 30 minutes (Branson 5510 sonicator). Nanoparticles were sized at 80 nm by particle size analysis, purified by 3 centrifugations, and suspended in DI water.
2.2 Pellet Formation and Coating

Modified dextran was added to a nanoparticle suspension as described in Methods 1.2 to make 50 uL hemispheres. These were coated three times in the acrylic suspension as described in Methods 1.3.

2.3 Digestion Simulation and Release Tracking

The loaded pellets were subjected to the digestion simulation described in Methods 1.4 for stomach and small intestine. No measurements were made for these, as dye in the trypsin interfered with colorimetric readings. During colon simulation, the fluid above the pellets was tested for emission at 610 nm (Bio-Tek Synergy HT) and compared to nanoparticle suspensions to determine release percentage. This ratio exceeded 1.0 but was normalized to 100%, as the pellet had degraded completely by the final time point. Every several hours, dextranase was renewed to maintain activity and new readings were added to the previous.

3. Lysozyme Protection Assay

Rationale

Previous work on either dextran hydrogels or acrylic coatings has never demonstrated protein protection, much less in a well quantified form. The idea of this experiment is not to propose lysozyme as a therapy but as a model of enzyme delivery, incorporated into pellets and sustaining digestion to various observable and calculated degrees. Retention of enzyme activity is an excellent indicator of the protection provided by the drug delivery system, considering that enzymes are more fragile than other peptide classes. Within the body enzymes are renewed constantly as their activity quickly fades. Even
observing partial enzyme activity after facing digestion conditions will indicate success for more robust categories of proteins. Lysozyme serves this role perfectly, being well studied, widely available, and possessing easily measured antibacterial activity.

3.1 Pellet Formation and Experimental Conditions

Hydrogels were made from modified dextran in the same method described above except 20 uL in size and containing .5 mg/mL lysozyme rather than nanoparticles. All pellets were coated in as described in Methods 1.3. There were five different stomach and small intestine digestion simulation conditions tested:

<table>
<thead>
<tr>
<th>Treatment #</th>
<th>Stomach Simulation (1 hr)</th>
<th>Small Intestine Simulation (5 hr)</th>
<th>Quick Colon Digestion (3 hr)</th>
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<tr>
<td>1</td>
<td>none</td>
<td>PBS</td>
<td>1U/mL Dextranase</td>
</tr>
<tr>
<td>2</td>
<td>PBS pH 4</td>
<td>.1% Trypsin</td>
<td>1U/mL Dextranase</td>
</tr>
<tr>
<td>3</td>
<td>PBS pH 4</td>
<td>PBS</td>
<td>1U/mL Dextranase</td>
</tr>
<tr>
<td>4</td>
<td>PBS pH 1.5</td>
<td>.1% Trypsin</td>
<td>1U/mL Dextranase</td>
</tr>
<tr>
<td>5</td>
<td>PBS pH 1.5</td>
<td>PBS</td>
<td>1U/mL Dextranase</td>
</tr>
</tbody>
</table>

3.2 Antibacterial Assay

The final products were tested for antibacterial activity against *Micrococcus lysodeikticus*. The quick colon digestion was observed to release no more than ½ of loaded particles, but was chosen because longer incubations would risk degradation of enzyme activity. Based on this the control lysozyme solution was .025 mg/mL. Stock *Micrococcus* suspension was added to 96 well plates for an initial OD of .6. Antibacterial activity was recorded by absorbance at 405 nm using Bio-Tek Synergy HT plate reader under continuous shaking.
4. Antibody Protection Study

Rationale

Similar to the Lysozyme Protection Assay, this study will indicate peptide protection. In this case it is more relevant to mAb. drugs like Infliximab and to experimental targeting moieties. anti-MUC1 was chosen for a ligand for its expression on epithelial cells, relevance to IBD, and its availability/familiarity in the lab. Using α-MUC1 conjugation, it should be possible to target molecules to the epithelial wall with emphasis on the inflammation locations, where they will concentrate above the glycocalyx of epithelial cell and enhance retention between doses. α-MUC1 was incorporated into the drug delivery system, treated with digestion simulations, and tested for activity investigated by immunofluorescence and confocal microscopy. The NHBE cells used for imaging originate from the airway not the intestine, but as both cell types express MUC1, NHBE was sufficient for this purpose.

4.1 Sample Preparation

MUC1 primary antibody (rabbit polyclonal, Abcam, .2mg/mL) was incorporated into gels and coated or left unprotected and subjected to various acidic and trypsin treatments to simulate the stomach and small intestine stages of digestion. The target concentration was a 1:50 dilution. Large intestine incubation used a high dextranase concentration to
dissolve pellets quickly for testing. Final products were tested on fixed NHBE cells for MUC1 adherence. The following table summarizes the sample groups:

<table>
<thead>
<tr>
<th></th>
<th>Stomach Incubation (1.5 hr)</th>
<th>Small intestine incubation (5 hr)</th>
<th>Large intestine incubation (3 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pellet Sample 1</td>
<td>PBS pH 1.5</td>
<td>.1% trypsin</td>
<td>2.5 U/mL dextranase</td>
</tr>
<tr>
<td>Pellet Sample 2</td>
<td>PBS pH 4.0</td>
<td>.1% trypsin</td>
<td>2.5 U/mL dextranase</td>
</tr>
<tr>
<td>Pellet Sample 3</td>
<td>PBS pH 7.0</td>
<td>PBS pH 7.0</td>
<td>2.5 U/mL dextranase</td>
</tr>
<tr>
<td>Positive Control</td>
<td>PBS pH 7.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative Control</td>
<td>No Primary Ab.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

To verify the statement that the pellets had provided antibody protection, free MUC1 antibody was subjected to the same conditions as Pellet Sample 1 and compared to positive and negative controls, as detailed:

<table>
<thead>
<tr>
<th></th>
<th>Stomach Incubation (1.5 hr)</th>
<th>Small intestine incubation (5 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unprotected Antibody</td>
<td>PBS pH 1.5</td>
<td>.1% trypsin</td>
</tr>
<tr>
<td>Positive Control</td>
<td>PBS pH 7.0</td>
<td></td>
</tr>
<tr>
<td>Negative Control</td>
<td>No Primary Ab.</td>
<td></td>
</tr>
</tbody>
</table>

4.2 Cell Preparation

T75 flasks were coated with human placenta collagen for 4 hours before introducing normal human bronchial epithelial cells (NHBE, P3). Cells were grown for 5 days at 37° and 5% CO₂. The media used was BEGM (Lonza) and was changed on the second day
and every two days afterward. Cells were passaged into chambered coverglass using Accutase and trypsin inhibitor then left overnight before fixation and labeling.

4.3 Immunofluorescence Techniques

All chambers of NHBE cells were fixed by 4% paraformaldehyde for 15 minutes. Samples as described above were added to constitute the primary incubation. Blocking solution was 3% goat serum and 2% bovine serum albumin in 20 mM glycine PBS. The secondary antibody was Alexa Fluor 488-labeled goat α-rabbit (Invitrogen) at 1:200 for 30 minutes. Between all steps cells were rinsed three times with 20 mM glycine PBS.

All in vitro images were taken on a Nikon Eclipse TI.

5. CR2crry in vivo study

Rationale

The protein CR2crry was developed by S Tomlinson and combines complement inhibitor crry with targeting moiety CR2, which attaches to breakdown products at sites of complement activation. In murine autoimmune disease models it has been shown quite effective as low dose injections [49, 50]. While Infliximab would have been a more industrially relevant choice, this peptide is sufficient for in vivo demonstration because it is previously proven to reduce inflammation. CR2crry requires digestion protection for its immune and cytokine components, so success with CR2crry would prove that the peptide protection is also sufficient for Infliximab.
This drug was incorporated into small pellets and administered orally to mice. Symptoms of weight loss, colon shortening, and macrophage/neutrophil recruitment can be observed in rodents. Preparation of all pellets was performed by myself while at MUSC, and all animal handling work and data collection was performed by Michelle Elvington and Fei Qiao at MUSC. The experiment served the purposes of both parties so efficiency, expenses, and collaboration were part of the rationale.

5.1 Pellet Preparation
Using a 12 mg/mL solution of CR2crry, 2 uL dextran hydrogels about 1mm in size were formed as described in Methods 1.2. Control pellets were made omitting the CR2crry. Pellets were coated with Eudragit L100-55 as described in section 1.3 of Research Methods.

5.2 In Vivo Administration
In vivo work was conducted by Michelle Elvington and Fei Qiao of Dr Tomlinson's lab at MUSC. Ulcerative colitis was induced in mice with a dextran sodium sulfate (DSS) enema regime. In sample groups using CR2crry or control pellets, 6 mice were given 3 pellets per day for 5 days. Certain sample groups used omeprazole as an antacid for supplementary drug protection. Animal weight was recorded daily. Upon animal sacrifice colon length was measured and histological samples were stained with hemotoxylin/eosin and scored on severity of inflammation, mucosal/submucosal damage, and basal lamina damage. All 3 scores were added together for comparison between groups.
CHAPTER 3: RESULTS AND DISCUSSION

Nanoparticle Release

Nanoparticle release was tracked over the lower intestine portion of digestion simulation only as the colorimetric reading could not be taken in the trypsin solution. Considering that the expected full release was obtained and the acrylic coating lasted for most of the small intestine incubation, the release before colon conditions was negligible. At 3 hour intervals, the dextranase solution was changed for freshly prepared solution due to fading enzyme activity. After each exchange, fluorescence values were added to the previous cumulative release and compared to the control suspension. Fluorescence values were compared to a control nanoparticle suspension representing full release to determine percent release at each time point. Although this ratio exceeded 100%, it can be safely assumed that drug release finished at 100% as the pellets were dissolved completely at the final timepoint. Although it may not have been accurate to treat the fluorescence values from the plate reader as linear and additive, this data is sufficient to show expected duration of drug release.
Fig 4. Release of labeled nanoparticles from coated dextran hydrogel pellets during large intestine portion of digestion simulation relative to a control nanoparticle suspension. n=4.

The timeframe of release is suitable for dispersion throughout the lower intestine, as the 25-40 hour release period normally parallels the transit time in the lower intestines. A typical release could be expected to last at least 24 hours. This is a good baseline for delivery as it should cover a large area of the colon, but modifications are possible by varying the DS (degree of substitution) of methacryl groups on dextran, which will change the crosslinkability and likely the time for the hydrogel to digest. This possibility can be explored if some patients need release over a shorter timeframe due to a faster gastric emptying time. Ulcerative Colitis patients with inflammation extending beyond the reach of enema may benefit from using oral delivery with a more delayed release to emphasize more distal areas of the colon.

*Lysozyme Protection Assay*
The antibacterial property of lysozyme is indicated by a drop in bacterial OD. Samples are listed in the order of their activity:

<table>
<thead>
<tr>
<th>Treatment #</th>
<th>Stomach Simulation (1 hr)</th>
<th>Small Intestine Simulation (5 hr)</th>
<th>Quick Digestion (3 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control Lysozyme</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>none</td>
<td>PBS</td>
<td>1U/mL Dextranase</td>
</tr>
<tr>
<td>2</td>
<td>PBS pH 4</td>
<td>PBS</td>
<td>1U/mL Dextranase</td>
</tr>
<tr>
<td>3</td>
<td>PBS pH 4</td>
<td>0.1% Trypsin</td>
<td>1U/mL Dextranase</td>
</tr>
<tr>
<td>4</td>
<td>PBS pH 1.5</td>
<td>PBS</td>
<td>1U/mL Dextranase</td>
</tr>
<tr>
<td>5</td>
<td>PBS pH 1.5</td>
<td>0.1% Trypsin</td>
<td>1U/mL Dextranase</td>
</tr>
<tr>
<td>Control Cells</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2. Lysozyme Protection Assay test groups in order of activity, high to low.

Fig 5. Lysozyme activity of sample and control groups. Steeper drop in OD indicates higher activity. Samples 1-5 conditions are listed in the table above, with Sample 5 receiving harshest treatment and Sample 1 the mildest. Black squares: cell control. Red Circles: Treatment #5. Blue Triangles: #4. Green Triangles: #3. Orange Diamonds: #2. Purple Pentagons: #1. Brown Hexagons: LZ Control. n=4
By appearance on Figure 5, Samples 1-3 retained most activity, while samples that were exposed to PBS pH 1.5 in the stomach incubation lost more activity. Groups 4 and 5, representing harsh digestion conditions, retained an estimated 10% of lysozyme activity when compared to lysozyme control. When compared to the very mild conditions of Group 1, Groups 4 and 5 retained an estimated 30% of lysozyme activity. This suggests that if a protein drug was loaded into a pellet and taken orally, it would retain some activity upon reaching inflammation sites within the colon.

Fig. 6: Initial rate of lysozyme activity for all samples, in terms of OD/min. By this measure the most severe treatment group has one-third activity compared to the LZ control. n=4

By measure of the initial slope, it would seem that lysozyme from the Treatment #5 has one-third activity compared to the fresh lysozyme control. Still, from Fig. 5 it appears that Treatment #5 lags and the pellets are not sufficiently protected from acid. It's not possible to say whether the quality of coating is to blame or if the material itself does not perform in this manner.
**Antibody Protection Study**

Negative controls without primary antibody showed nearly no staining, and while the antibody sample treated without gel protection was detectable it was comparable to the negative control and far fainter than positive control of identical concentration. MUC1 antibody would not survive digestion without the aid of protective matrices, as seen in the following image set.

![Image](image1.png)

*Fig. 7: Clockwise from top left: Positive Control, Negative Control, Unprotected Antibody. Negative Control shows some nonspecific staining, but Unprotected Antibody is slightly brighter. Both pale in comparison to Positive Control, indicative of the damage an antibody experiences in unprotected digestion.*
Mean intensity was calculated over the area of complete cells. Again this shows a difference between unprotected antibody and negative control, but it is far below the positive control.

![Graph showing mean intensities](image)

Fig. 8: Mean intensities using free antibody (Positive), antibody subjected to digestion simulation (Treated), and images without primary antibody (Negative). While some antibody activity was observed in Treated samples, the damage was substantial when compared to the Positive control.

All samples from pellet digestion and dissolution exhibited green staining, indicating the antibody had retained activity while the unprotected antibody above did not. This indicates once again the ability of the pellet to protect delicate peptides, and furthermore demonstrates the possibility of immunotargeting with MUC1 conjugates. Sample 2 used a stomach incubation with pH 4.0 to simulate use of antacid. In the images gathered sample 2 was consistently successful, but sample 1 was also labeled sufficiently so an antacid regimen seemed unnecessary, although later studies would suggest antacids to be helpful.
Fig. 9: Confocal images of MUC1 binding using primary antibody from pellets after digestion simulations.
A: Positive control.
B: Negative control.
C: pH 7.0 and no trypsin treatments
D: pH 4.0 and .1% trypsin
E: pH 1.5 and .1% trypsin
When comparing mean intensity values, we see that all the treatment groups fared similarly to the positive control with antibody activity mostly intact even in Sample 1 with the harshest conditions.

![Graph showing mean intensity of cells from each sample. By this measure the antibody is retaining activity against all digestion simulations.](image)

This demonstrates the feasibility of orally delivering antibody drugs to the colon. If this level of antibody protection can be applied in vivo, oral formulation of Infliximab and similar drugs will certainly be possible.
**In Vivo: Mouse Colitis Model**

Colitis model mice that were subjected to DSS regime lost an average of 12.5% body weight, while mice receiving CR2crry pellets lost only 5% and mice receiving the antacid drug omeprazole along with the CR2crry pellets did not lose weight. As weight loss and malnutrition are symptoms of colitis, the anti-inflammation properties CR2crry appear to remain intact through oral delivery, especially in conjunction with omeprazole.

![Graph showing weight of mice over duration of test](image)

Fig. 11: Weight of mice over duration of test from before treatment/DSS (day -1) to sacrifice (day 5). DSS indicates colitis model and omeprazole indicates antacid use. Lack of weight loss indicates lack or remission of colitis symptoms. n=6

The same data is shown below in terms of total percentage weight change. CR2crry alone significantly mitigated the weight loss compared to DSS controls, but CR2crry with antacid did much better and had as little weight change as the sham controls. In this respect, oral delivery of CR2crry was successful when antacid was given simultaneously.
Shortening of the colon was severe in the DSS control group, losing about 40% of length compared to the sham group. In this measure the CR2crry pellets alone showed little effect, but with the addition of antacid colon shortening was much mitigated. Colon length data in Fig 13 demonstrates the benefit of administering antacids along with the peptide loaded pellets. It may be possible that some small flaws existed in the acrylic coating of the CR2crry pellets, and had the CR2crry pellets been coated by industrial sprayers rather than manual methods they would perform comparably to the manually prepared pellets with antacid.
Colon tissue samples taken after sacrifice were stained with H&E and scored using a cumulative scale using three parameters:

1. Severity of inflammation (0, none; 1, slight; 2, moderate; 3, severe),
2. Depth of injury (0, none; 1, mucosal; 2, mucosal and submucosal; 3, transmural),
3. Crypt damage (0, none; 1, basal one-third damaged; 2, basal two-thirds damaged; 3, only surface epithelium intact; 4, complete loss of crypt and epithelium)

These scores were added for each sample forming a 10-point scale. Representative images are shown below.

Fig. 13: Average colon length at sacrifice. Colon shortening was largely mitigated by the CR2crry with antacid, with very high significance compared to all controls and other treatments. n=6
Fig. 14: Representative colon sections stained by H&E. A: Sham, B: Pellet Control, C: DSS, D: DSS + antacid, E: DSS + CR2cry pellet, F: DSS + CR2cry pellet + antacid. These images were used to assign histological scores of tissue damage and inflammation.
Once again the group given CR2crry pellets administered with antacid proved as healthy as the sham group. Without antacid the benefit of CR2crry on histological score was not statistically significant. This confirms the advantages of using antacids with peptide drugs, or otherwise the need for a more consistent acrylic coating. When administered with an antacid, orally administered CR2crry pellets mitigated all measured colitis symptoms to the level of sham groups.

**CHAPTER 4: CONCLUSIONS**

The oral format is the most familiar, convenient, and safe way for patients to be medicated on a daily basis, and the experiments recorded here build the case that it will is feasible to orally deliver peptide drugs while retaining a useful amount of activity. The
coated hydrogels of modified dextran provided a drug release profile well-localized and well-dispersed, releasing under colon conditions over a course of at least 24 hours. The protective system also provided enough protein protection to support measurable enzyme activity after digestion simulation, with over 30% lysozyme activity observed.

Upregulation of glycoprotein MUC1 during inflammation may be present in CD and colitis lesions which would warrant the use of MUC1 immunotargeting for drug conjugates. This specific antibody-antigen combination was investigated and found to work well when provided the protection of the pellet. Future work may aim to determine the strength of affinity this immunotargeting can provide and to what degree drug nanocarriers could be retained in the glycohalyx of affected cells. Other targeting strategies relevant to IBD include macrophage targeting with cd86 and epithelial cell adhesion molecules such as ICAM-1, which may be worth investigating as variations in this project.

In vivo studies gave preliminary evidence that complex proteins can be delivered orally and remain effective. When combined with antacid, orally delivered CR2cry mitigated the weight loss, shortening of the colon, and intestinal inflammation associated with ulcerative colitis. Future work in this regard would focus on improving resistance to acid provided by the coated pellet, likely by improving the coating process to industrial standards. It would also be interesting to repeat this experiment using a commercially available drug like Infliximab.
This opens the possibility of clinical translation for a great wealth of hormones, cellular signals, genetic sequences, and other macromolecules that have been recently investigated for clinical use. Oral administration will aid convenience and compliance while avoiding the deleterious effects of systemic injection. With colonic drug delivery, IBD is possibly the disease most directly benefited but it may be determined that many protein drugs are better absorbed in the mild proteolytic conditions of the large intestine rather than the small intestine. In either case the mastery of oral delivery targeting the colon, complete with proven peptide protection, has immense therapeutic potential.

Future work on this project should include administering Infliximab in vivo and improving the pellet coating by using proper sprayer or bed coating equipment. Alternate targeting moieties and nanomaterial conjugations are other interesting aspects.
REFERENCES


