

**AN ASSESSMENT OF BSA PROTEIN HYDROGEL
BIOCOMPATIBILITY IN THE VERTEBRATE
INTESTINAL TRACT**

by

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ABSTRACT

AN ASSESSMENT OF BSA PROTEIN HYDROGEL BIOCOMPATIBILITY IN THE VERTEBRATE INTESTINAL TRACT

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The fields of biomedicine and pharmacology have a mission to design methods to treat disease while minimizing adverse side effects using novel drug delivery systems. In developing new therapeutic systems, it is crucial to test that drug delivery systems target pathological cells and tissue and is non-toxic in healthy tissue. One promising method for targeted drug delivery is the use of hydrogels as carriers. Here, we studied the effects of bovine serum albumin (BSA) hydrogel consumption to assess the potential for hydrogel use in treating intestinal disease via oral administration. We investigated intestinal architecture and cell populations following hydrogel treatments in adult zebrafish. Our studies revealed that consumption of BSA hydrogels results in normal intestinal villi architecture and bowel wall integrity. Furthermore, we demonstrated that intestinal goblet cell appearance and abundance did not change, and eosinophil populations remain constant over the course of treatment compared to control tissue. We confirmed this by comparing control- and hydrogel-fed tissue to tissue with chemically-induced inflammation. This study is important for the future development of biocompatible drug delivery systems using hydrogels.

To my wife, parents, and brother, whose constant care
and support have helped make this work possible

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LIST OF ABBREVIATIONS

| | |
|--------------|------------------------------|
| AP-1 | activator protein 1 |
| APS | ammonium persulfate |
| BSA | bovine serum albumin |
| Ctrl | chymotrypsin-like |
| Dex | dexamethasone |
| DNA | deoxyribonucleic acid |
| EF1 α | elongation factor 1 alpha |
| FDA | Food and Drug Administration |
| GFP | green fluorescent protein |
| GI | gastrointestinal |
| H&E | hematoxylin & eosin |
| hpf | hours post fertilization |
| IBD | inflammatory bowel disease |
| IgA | immunoglobulin A |
| IgG | immunoglobulin G |
| IL1 β | interleukin 1 beta |
| IL8 | interleukin 8 |
| IL10 | interleukin 10 |
| M-cell | microfold cell |

| | |
|----------------|--|
| MMP9 | matrix metalloproteinase 9 |
| MPS | mononuclear phagocytic system |
| MUC2 | mucin 2 |
| NF- κ B | nuclear factor kappa-light-chain-enhancer of activated B cells |
| PAS | periodic acid – Schiff |
| PBS | phosphate buffered saline |
| PEG | polyethylene glycol |
| PFA | paraformaldehyde |
| Prss1 | serine protease 1 |
| PTFE | polytetrafluoroethylene |
| RES | reticuloendothelial system |
| RNA | ribonucleic acid |
| Slc15a1b | solute carrier family 15 member 1b |
| TNBS | trinitrobenzenesulfonic acid |
| TNF α | tumor necrosis factor alpha |

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CHAPTER 1

INTRODUCTION

A. GENERAL INTRODUCTION

In 2018 about 5.8 billion prescriptions were dispensed in the United States alone, a 2.8% increase from 2017, and a figure that is projected to continue to rise in the coming years (IQVIA, 2019). As the quantity of prescription drugs administered continues to rise, increased pressure is placed on the pharmaceutical industry to improve the time- and cost-intensive processes of drug discovery and development, pre-clinical and clinical testing, and Food and Drug Administration (FDA) approval. Bringing new treatment options to the market requires the collaboration of academia, the biotechnology and pharmaceutical industries, and government through every phase from drug discovery to approval, a process which often spans 10-15 years (Paul et al., 2010).

The rate of success is another obstacle the pharmaceutical industry faces. Of the therapeutic compounds that entered clinical testing in the United States from 1993 to 2004, only 16% passed approval (DiMasi et al., 2010). Between 2003 and 2011, 10.4%, or about one-in-ten drugs, successfully transitioned from the clinical testing phase to FDA approval (Hay et al., 2014). Improvements to clinical trial experimental design and the use of more animal models in earlier toxicology screens, with new technologies in targeted drug delivery have been proposed to increase success rates.

In recent years, the use of novel drug delivery systems to spatially and temporally target disease is one way the pharmaceutical industry has already begun to circumvent the lengthy, costly process of drug development and approval. While the financial burden of conventional medicine research and development has threatened the industry's business model and sustainability, the emergence of novel therapeutics has the potential to reverse downward trajectory and increase margins (Gautam and Pan, 2016; Hogan and Tangney, 2018; Kinch and Moore, 2016). More importantly, the advent of molecular therapeutics specifically targeting diseased tissue coupled with reduced adverse side effects has the potential to benefit public health and treatment of disease on a global scale.

B. TARGETED DRUG DELIVERY SYSTEMS

Targeted drug delivery is the method of precise and controlled drug delivery toward a specific site or tissue while reducing off-target effects. The conceptual foundations of targeted drug delivery originated over 100 years ago. In the late 1800s and early 1900s the German microbiologist, Paul Ehrlich, developed the 'magic bullet theory' beginning with observations that different chemical dyes would interact with a variety of biological structures (Ehrlich, 1878). These histological observations later served as the basis of research in immunology where Ehrlich hypothesized that receptors had the capacity to specifically bind to drugs similar to the toxins of various dyes. By screening various organic compounds, he later discovered the anti-microbial drug that would be used to treat syphilis (Ehrlich, 1910). This pioneering work was rewarded with the 1908 Nobel Prize for Physiology or Medicine and continues to influence modern chemotherapy and

drug discovery. These findings have remained the foundation for rapid advancements in the biomedical and pharmaceutical industries as we continue to develop and pursue powerful and highly-selective therapeutics to combat human disease.

Current technologies utilizing modern drug delivery methods are rapidly advancing. Today, drug discovery represents fields in pharmacology and biotechnology responsible for identifying and testing potential therapeutic candidates for the treatment of diseases. One of the earliest steps in the drug discovery process is target selection (Paul et al., 2010). A target in this context can be defined as a biochemical molecular structure; most often an enzyme, receptor, channel, or transporter; that a drug will interact with and consequently have a clinical effect (Imming et al., 2006). Therefore, much of successful management and treatment of disease is dependent on the efficiency and effectiveness of the drug-target interaction, and the route in which it is delivered. Many factors can be affected by the route of drug delivery including release time, metabolism, absorption, toxicity, and target specificity. Additionally, as the discovery of new molecules and agents emerge for therapeutics, challenges in understanding effective and safe drug delivery routes are also created.

Therefore, targeted drug delivery systems are continuously being employed and newly engineered in efforts to manage and increase efficacy of therapeutics and site-specific actions. This approach to drug delivery has shown promise over the last decades of research but the pharmaceutical industry still faces challenges with optimization of these systems. In theory, *in vivo* targeted drug delivery systems distribute precise

therapeutic concentrations exclusively to diseased cells or tissue. However, this has been an obstacle because drug delivery has proven difficult to control by two primary reasons, target selection and mode of delivery.

The major hurdle in targeted drug delivery is determining methods that maximize selective activity in pathological cells or tissue with high affinity while minimizing its introduction to healthy tissue. Advancements in DNA sequencing and completion of the human genome have facilitated deeper understanding of the genetic basis of disease. This allowed researchers to pinpoint expression patterns in diseased organ systems and direct efforts in a ligand-targeted drug delivery approach (Choi et al., 2009; Worthey et al., 2011). Often though, detectable expression patterns are variable in patients with the same disease, suggesting targeted delivery could be more beneficial in a personalized medicine approach. Additionally, biomarkers located in pathological tissue are often not restricted to the targeted tissue type and can lead to off-target or global effects (Bae and Park, 2011). Regarding modes of targeted delivery, conventional routes of administration remain problematic with respect to drug stability and degradation rates (Goldberg and Gomez-Orellana, 2003). For example, while oral administration has long been the primary and preferred route, proteolysis by digestive enzymes, patient-to-patient disparities in gastric pH, and unique microbiota environments within the small intestine leads to decreased drug permeability across the intestinal epithelia and consequently, limited bioavailability and diminished targeting (Banerjee et al., 2016). These obstacles highlight some of the major difficulties contributing to the instability of targeted delivery systems.

Strategies and types of targeted drug delivery

The crucial components in development of drug delivery systems are to specifically deliver therapeutics to target regions, minimizing off-target and toxic effects, and retaining bioavailability and compatibility in a complex organism. Recently, there has been an increasing variety of strategies that show promise for the targeted release of therapeutics to combat disease. Currently, there are a variety of strategies employed to improve targeted drug delivery technologies that fall into three categories: passive, inverse, and active targeting. Passive delivery systems exploit the body's reticuloendothelial system (RES) and allow for the accumulation of drugs in those regions, primarily the liver and spleen when the delivered drug reaches system circulation (Almeida et al., 2011). These delivery systems are mainly utilized and designed to target hepatic and lymphatic tissues. On the cellular level, the mononuclear phagocytic system (MPS) is made up of monocytes and macrophages. Drugs targeted toward the MPS are taken up into cell bodies within this system to carry out their therapeutic function aimed toward the adaptive immune system (Chrastina et al., 2011). Utilization of drugs for passive targeting is primarily delivered to the therapeutic site by polyethylene glycol (PEG)-coated nanoparticle attachment or conjugation, allowing the delivered drug to remain in circulation for extended periods of time without clearance (Veronese and Pasut, 2005). Inverse targeting delivery systems utilize a similar approach to passive systems, but instead specifically target the RES or MPS for saturation using lipid-based carriers. This allows for an administered drug to concentrate in a desired area of the body normally deposited in the RES for clearance (Muro, 2012). Lipid-based carrier technology has been used for enhanced

biodistribution of DNA-targeting chemotherapeutics (Ghosh et al., 2016) and to increase intestinal bioavailability for oral administration (Goncalves et al., 2016). The most well characterized delivery system is active targeting. While passive and inverse targeting strategies are based on the length of time the pharmaceutical carrier remains in systemic circulation and the drugs accumulation at its pathological site, active targeting design relies on the attachment of specific therapeutic ligands to carriers for recognition and binding of cells in pathological tissue (Muro, 2012).

Targeted drug delivery for inflammatory bowel disease

Inflammatory bowel disease (IBD) is a chronic autoimmune disorder affecting the digestive tract. Genetic predisposition, diet, and environmental factors are known to play important roles in IBD pathogenesis, with over 3 million people in the United States having received diagnosis (Dahlhamer et al., 2016; Jostins et al., 2012). Ulcerative colitis and Crohn's disease are two forms of IBD and are characterized by dysfunctional interactions between the host and resident enteric bacteria. Severe interactions between host and the microbiota contribute to aberrant immune responses that compromise the intestinal epithelial barrier and disrupt healthy gut homeostasis. Conventional therapies for IBD are limited and designed to systemically target the immune system. These treatments include immunomodulation and vaccines, and while these treatments can maintain episodes of remission, they often induce significant adverse systemic effects (Celiberto et al., 2018). Additionally, around 70% of IBD patients require surgical intervention (Hua et al., 2015). Therefore, alternative therapeutic strategies utilizing targeted drug delivery aim to enhance bioavailability and

minimize systemic and off target effects are being developed to improve quality of life and prevent recurrence of symptoms.

There are many challenging factors that need consideration for intestinal targeted drug delivery. First, digestive system transit time should be considered for site-specific delivery. Small intestine transit time is 2-6 hours generally and vary significantly in the colon (5-70 hours). In patients with IBD, transit time has been shown to be delayed compared to healthy individuals (Rana et al., 2013). Changes in luminal pH is also important in determining effective drug delivery to the GI tract. In healthy individuals, pH rises to pH 6 in the duodenum from the highly acidic stomach and continues to increase through the small intestine to pH 7.4 in the ileum. Colonic pH decreases below pH 6 at the cecum and again slowly rises over pH 6.7 in the terminal rectum. With IBD there is little change in small intestine pH, but the colon is significantly lower from pH 5.5 in the proximal and as low as pH 2.3 in the distal colon (McConnell et al., 2008). Lastly, the microbiome is a crucial aspect in assessing approaches for treatment of IBD where an estimated 500-2000 distinct bacterial species contributing to digestion, metabolism, and health reside within the GI tract (Sartor, 2008). While there is little evidence showing reduced bacteria load in IBD patients, studies show decreased bacterial diversity which is thought to contribute to bacterial permeability, mucosal inflammation, and alterations in digestive physiology (Linskens et al., 2001).

Improvements to delivery systems for effective release at therapeutic sites in IBD patients have begun to account for all of these factors. Nanoparticle systems have been

engineered to both passively and actively target sites of inflammation. For example, reduced size-dependent systems have been shown to allow for the selective delivery of therapeutics to inflamed epithelia and immune cells and avoiding recognition and rapid elimination (Beloqui et al., 2013). Other strategies have exploited changes in pH levels for site-specific delivery. Based on the site of inflammation these carrier systems can be designed with polymeric, biodegradable coatings that dissolve in a pH-dependent manner for drug delivery specifically to small intestinal tissue where pH is generally unaltered in patients with IBD, or for dissolution and release of therapeutics within the colon at significantly lower pH (Asghar and Chandran, 2006). Interestingly, intestinal inflammation and depletion of the mucosal layer results in build-up of eosinophils, proteins, and antimicrobial peptides that carry a positive charge (Ramasundara et al., 2009; Tirosh et al., 2009). *In vivo* studies have already shown promising results utilizing negatively-charged delivery systems to adhere to regions with positively-charged build-up in colitis murine models (Beloqui et al., 2013). Another unique system has emerged for IBD drug delivery using negatively-charged carriers in the form of hydrogels. In one study, the commonly-used corticosteroid dexamethasone (Dex) was encapsulated in negatively-charged hydrogel polymer fibers and favorably adhered to inflamed intestinal mucosa over healthy tissue (Zhang et al., 2015). In addition, administration was performed in two murine colitis models exhibiting different systems, and both result in decreased systemic exposure compared to Dex alone. This novel system strategy demonstrated selective therapeutic release at sites of inflammation with reduced systemic absorption using hydrogel drug delivery.

C. HYDROGELS IN DRUG DELIVERY SYSTEMS

Hydrogels are three-dimensional aggregates of crosslinked polymer networks with varying structure and characteristics (Jonker et al., 2012). Technologies have recently emerged in therapeutic applications using hydrogels as a drug delivery system, but study of their properties as a biological tool have been observed for over 50 years. Hydrogels were first recognized for their capacity for rapid polymerization, ease of manipulation, hydrophilic nature, and importantly, biocompatibility (Wichterle and LÍM, 1960). Since then, hydrogels have been studied in the variety of fields including mechanobiology and biophysics. In these studies, the folding events and biomechanical properties of hydrogels have been observed for the assessment of chemical and mechanical stabilities under various conditions. Additionally, methods have been established using force-clamp spectroscopy and rheometry for precise single-molecule measurements of protein hydrogels (Khoury et al., 2018a; Ott et al., 2017; Popa et al., 2013). In biomedical and pharmaceutical applications hydrogels have used for wound healing and implanted transepithelial drug delivery (Bessa et al., 2008; Tellechea et al., 2015). In these studies, hydrogels were engineered with varying characteristics for precise spatiotemporal drug delivery, making them ideal for administering localized therapeutics (Ashley et al., 2013; Tibbitt et al., 2016). Additionally, use of hydrogel drug delivery systems have been employed via injection and surgical implantation techniques displaying the versatile routes of administration for successful and precise targeting (Gratton et al., 2008; Liu et al., 2008). The advances in hydrogels designed for controlled drug delivery are expected to continue to change the approach toward safer and more effective therapeutics.

D. ZEBRAFISH MODEL ORGANISM

Intestinal tract development

Zebrafish (*Danio rerio*) intestinal formation has been characterized into three distinct stages over the first five days of development. In stage one, the primitive gut begins to form around 26-30 hours post fertilization (hpf). At this stage of early development, a single sheet of endodermal tissue extends anterior to posterior from the future mouth to the future anus. Between 30 and 52 hpf is a period of patterning and rapid intestinal morphogenesis and development of posterior enteroendocrine cells. Additionally, at 48 hpf digestive system accessory organs have formed and the developing lumen is surrounded by enteric neuron and smooth muscle progenitor cells. Stage two is defined by intestinal epithelia polarization and uniform proliferation between 52 and 76 hpf, and the appearance of enteroendocrine cells scattered throughout the intestinal tract. In stage three, between 76 and 126 hpf, global remodeling and epithelial differentiation occur. In this stage, the intestinal lumen is compartmentalized into three distinct regions: the intestinal bulb, midgut, and hindgut, and by 126 hpf the three regions of the tract are functional. (Ng et al., 2005; Wallace et al., 2005; Wallace and Pack, 2003).

Conservation of the adult zebrafish intestinal tract

In the adult zebrafish intestinal tract there is conservation of architecture and anatomy (Figure 1). In zebrafish, the intestine is a folded tube that takes up a large space within the abdominal cavity. While they lack a stomach, the anterior intestinal tract in zebrafish has an enlarged lumen, which serves as a reservoir similar to that of the mammalian stomach (Figure 1A) This region is known as the intestinal bulb, and from anterior to

posterior, the midgut and hindgut. Although the zebrafish does not have the discrete segments of its intestinal tract found in humans, there is anterior to posterior functional homology. The innermost epithelial layer in zebrafish also lacks intestinal crypts, but instead there are homologous folds, or villi, found along its intestinal tract (Wallace and Peck, 2003). These folds are most abundant and larger in the anterior regions of the zebrafish tract and decrease in size and number from anterior to posterior. Similar to humans, there are differentiated epithelial cells within the zebrafish epithelial folds. Enteroendocrine cells are predominately found in the anterior region, absorptive enterocytes are located in the anterior bulb and midgut, and mucous-secreting goblet cells are found in all regions of the intestinal tract. Additionally, while the zebrafish do not have Paneth cells, there is evidence of microfold-like (M-like) immune cells scattered throughout the intestinal tract (Brugman, 2016; Ng et al., 2005; Wallace and Peck, 2003) (Figure 1B). Genomic studies in zebrafish have shown conserved metabolic and digestive system-specific genes (Wang et al., 2010). Additionally, the regulatory transcriptional networks of intestinal epithelial cells along the digestive tract are conserved between zebrafish and human. It has been established that the same regulatory networks that define the mammalian segments are conserved with the zebrafish intestinal regions (Lickwar et al., 2017). Conservation of cellular responses to toxicity and inflammation have also been identified in zebrafish. It has been shown that pro-inflammatory markers tumor necrosis factor-alpha ($TNF\alpha$), interleukin 1-beta ($IL1B$), and matrix metalloproteinase 9 ($MMP9$) increase in response to inflammation in zebrafish (Marjoram et al., 2015). These similarities in intestinal architecture, gene expression, cell types, and inflammatory responses allow for the toxicological

assessment of intestinal architecture disruption and expression patterns using the adult zebrafish model.

E. THESIS STATEMENT

Can hydrogel-based drug delivery systems emerge as safe alternatives to conventional treatment methods? This is the long-term question that initiated and drove the work in this thesis. We aimed to understand if oral administration of BSA hydrogels would produce intestinal inflammation and toxic effects over different treatment periods.

For this pilot investigation we utilized the adult zebrafish model to assess intestinal toxicity and inflammation. We used the zebrafish because of its small but highly conserved vertebrate intestinal architecture and cell types, and willingness to consume hydrogels over an extended period of time.

We asked whether hydrogel consumption would: 1) be lethal, 2) cause changes in overall body weight, 3) cause inflammation and disruption to intestinal architecture, and 4) result in changes to intestinal-specific cell populations, over the course of three treatment periods. By feeding hydrogels to adult zebrafish over three treatment periods we found that ingestion was non-lethal up to thirty days of consumption. We tracked changes in weight over the different treatment periods and determined that overall body weight was not affected by hydrogel ingestion when compared to controls over the same time points. Furthermore, general observations of fish over these treatments showed normal eating habits and behavior, and no phenotypical changes.

We adapted previously published analyses for our assessment of intestinal tissue and cellular populations following hydrogel treatments. Using these methods, we found that intestinal architecture remained unaffected by hydrogel consumption over the different treatment times. Additionally, cell populations specific to the intestinal tract were not altered following treatment. Because we did not observe architectural or cellular changes in hydrogel-fed animals, we analyzed the same parameters in chemically induced zebrafish. Here, we observed disruption to intestinal villi, bowel wall, and cell populations that we did not see with hydrogel consumption. These results suggest that hydrogel is a non-toxic, biocompatible material that can continue be investigated for biomedical and pharmaceutical applications.

With our study, we have shown that zebrafish are a useful model organism for testing the potential of hydrogels as a biocompatible drug delivery system. We can build on these findings to discover and test further applications for hydrogels as smart pharmaceuticals for the treatment of diseases.

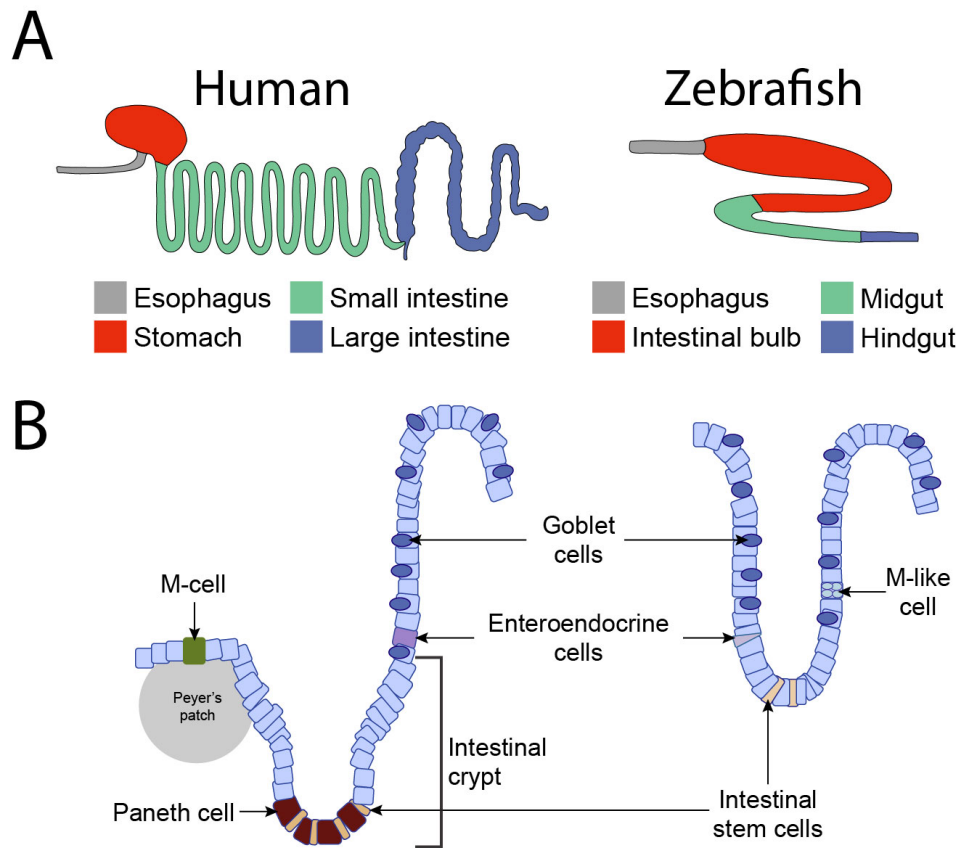


Figure 1. Conservation of digestive system architecture and intestinal cell types.

(A) The digestive system is composed of distinct but continuous organs in both the human (left) and zebrafish (right). Each system consists of a folded tube with three homologous regions. (B) Three principle cell types in human intestinal epithelia are found in zebrafish. Zebrafish lack intestinal crypts, M-cells, and Paneth cells; however, vacuolated M-like cells are thought to deliver intestinal luminal contents to immune cells. Images adapted from (Lickwar et al., 2017; Wallace and Pack, 2003).

CHAPTER 2

PROJECT DESIGN AND RESULTS

A. EXPERIMENTAL DESIGN

Our objective for this initial study was to test the safety of BSA hydrogels for use via direct ingestion as a potential drug delivery system. In order to accomplish this objective, we developed an experimental plan to feed BSA protein hydrogels to adult zebrafish. Zebrafish were chosen due to their ease in manipulation and high conservation of digestive system anatomy, metabolic and intestinal-specific genes, and cellular responses to toxicity and inflammation. Following hydrogel feeding over different time periods, animals were weighed and intestinal architecture was examined for signs of toxicity and inflammation. We characterized bowel wall thickness, villi length, and analyzed specific cell populations including goblet cells and eosinophils. We hypothesized that the intestinal architecture and cellular populations would remain normal through hydrogel treatments over different time periods. Specifically, we fed BSA protein hydrogels to animals once a day for 1, 10, or 30 days. During the study, animals were weighed regularly and at the end of each experiment animals were weighed, then the intestinal tract was dissected and examined (Figure 2A). Intestinal architecture and cell populations were examined using H&E staining and Alcian Blue-PAS staining (Figure 2B).

We demonstrate that overall body weight is normal over the three different hydrogel treatment periods. We further demonstrate that intestinal architecture is unchanged with hydrogel consumption up to 30 days of treatment and we show normal intestinal specific cellular populations throughout hydrogel treatments. Additionally, we generated positive control data using previously established methods to assess induced inflammation in the adult zebrafish intestine (Brugman and Nieuwenhuis, 2017). As expected, our positive control induced inflammation experiments demonstrated disrupted intestinal architecture and variations in cell populations. Our data demonstrate that consumption of BSA hydrogels does not induce gross, toxic effects within the vertebrate digestive tract for the times examined. These findings provide evidence toward the potential to utilize protein hydrogels as a drug delivery technology.

B. MATERIALS AND METHODS

Zebrafish husbandry, maintenance and strains

Standard protocols were used for zebrafish (*Danio rerio*) maintenance and husbandry (Westerfield, 2007). Wildtype AB and *TgBAC(tnfa:GFP)^{pd1028}* (Marjoram et al., 2015) adult zebrafish were used for all experiments. Equal numbers of male and female zebrafish were used for all experiments and treatment groups. This study was performed under approval from the University of Wisconsin-Milwaukee Institutional Animal Care and Use Committee.

Adult weight measurements

To obtain weight data, adult zebrafish were anesthetized using a 4% Tricaine (MS-222) (Sigma, E10521) solution until loss of equilibrium and response to mild touch. Zebrafish were quickly weighed before being placed back into separate post-anesthesia recovery tanks and then returned to the husbandry system. All fish were closely monitored for one hour after anesthesia.

BSA protein hydrogels

For all studies, bovine serum albumin (BSA) protein hydrogels were freshly made one day prior to feeding to adult zebrafish. Hydrogels were made according to Khoury et al, 2018 (Khoury et al., 2018b). Briefly, 2 mM BSA hydrogels were prepared by dissolving BSA powder (66.5 kDa; Rocky Mountain Biologics, BSA-BAF) in Tris solution (20 mM and 150 mM NaCl, pH 7.4). Ammonium persulfate (APS) (Sigma, A3678) and tris(bipyridine)ruthenium (II) chloride ($[\text{Ru}(\text{bpy})_3]^{2+}$) (Sigma, 544981) were prepared by

dissolving APS and $[\text{Ru}(\text{bpy})_3]^{2+}$ powder into Tris to a final concentration of 1 M and 6.67 mM, respectively. 2 mM BSA solution was mixed with the solutions of APS and $[\text{Ru}(\text{bpy})_3]^{2+}$ at a volume ratio of 15:1:1. Next, the photoactive reaction mixture was injected into 10 cm PTFE tubes (see Figure 2D). The loaded tubes were placed under a 100 W mercury lamp to prevent protein denaturation and irradiated for 30 minutes at room temperature to polymerize the solution into the protein hydrogels (see Figure 2D). Following polymerization, hydrogels were extruded and stored in a Tris-NaCl solution until feeding (see Figure 2E).

Inflammation induction

To induce intestinal inflammation for positive control experiments we used oxazolone induced inflammation by following the protocol published by Brugman and Nieuwenhuis, 2017. (Brugman and Nieuwenhuis, 2017). Briefly, oxazolone solution was prepared by dissolving 20 mg oxazolone (4-Ethoxymethylene-2-phenyl-2-oxazolin-5-one) powder (Sigma, E0753) in 5 mL 100% ethanol. This solution was then added to 5 mL PBS (0.8% NaCl, 0.02% KCl, 0.02 M PO_4 , pH 7.3). Ethanol vehicle control solutions were prepared as 50% ethanol solution in PBS. For induction of inflammation, zebrafish were anesthetized in 4% Tricaine solution until loss of equilibrium and response to mild touch. Zebrafish were placed ventral side up on a wet injection mat with system water and ethanol control or oxazolone solutions were administered by intrarectal injection at 0.6 μL per 0.1 g zebrafish body weight using 50 mL fine tip pipette. Following injection, zebrafish were transferred to a clean post-anesthesia recovery tank and monitored for one hour. Fish remained in recovery tanks for the following 8 hours to allow for the

onset of inflammation after the injection. After 8 hours, adults were euthanized for intestinal dissections and analyses.

Intestinal dissection and tissue collection

For intestinal dissections we followed the procedure published by Gupta and Mullins, 2010 (Gupta and Mullins, 2010). Briefly, adult zebrafish were anesthetized using a 4% Tricaine solution and then euthanized by ice water incubation for 10 minutes. Zebrafish were then pinned to a dissection mat through the caudal fin and eye socket. Dissections were performed by cutting along the ventral side from the anterior anal fin to the operculum and peeling back skin and muscle to expose the underlying organs and digestive tract. The digestive tract was removed in its entirety and placed in PBS. For histological analysis, tissue was placed in a 4% paraformaldehyde (PFA) (Alfa Aesar, 43368) in PBS at 4°C. For qPCR analysis tissue was placed in Trizol (Invitrogen, 15596026) and stored at -80°C until RNA extraction.

Histology sectioning and analysis

Dissected intestinal samples were washed in PBS and fixed in 4% PFA in PBS overnight at 4°C. Samples were transferred to 20% sucrose in PBS overnight at 4°C for cryopreservation then frozen in HistoPrep embedding media (Fisher Scientific, SH75-125D). Frozen 5-micron sagittal sections from the intestinal bulb and anterior midgut regions were generated for staining procedures and analysis. Sections were stained with either hematoxylin (Harleco, 65067-75) and eosin (Fisher Scientific, E511-100) (H&E) to analyze tissue architecture or with Alcian Blue-Periodic acid-Schiff (PAS)

(ThermoFisher, 87023) to evaluate intestinal cell types per the manufacturer protocols. Stained sections were imaged using a Nikon Eclipse E4000 microscope at 10X magnification and ToupView imaging software. For quantitative histological analysis, measurements were taken from 6 fish per treatment group for all experiments. For evaluation of bowel wall thickness, 15 representative measurements were taken from at least 3 H&E stained sections per adult tissue sample. Measurements were then averaged for each intestinal sample. For evaluation of intestinal villi length, 2-6 measurements per intestinal villi in each of 3 sections per adult tissue sample were analyzed and averaged for each sample. We adapted an established scoring system for Alcian Blue-PAS staining from (Brugman et al., 2009) to evaluate cell populations (Brugman et al., 2009). For each adult intestinal sample, at least 3 sections were analyzed for goblet cells and infiltrating eosinophils (see Table 1). Each section was scored independently for goblet cells and eosinophils and given a score ranging from 1-3. The sum of the two scores was calculated and referred to as the total intestinal score per section. All of the total intestinal scores for each section were averaged, resulting in one overall intestinal score per sample. For quantification, overall intestinal scores were averaged for each treatment group.

Statistical analysis

All data are shown as mean \pm standard deviation. Statistical analyses were carried out by Student's *t*-test. Student's *t*-tests were performed between control and hydrogel-fed groups at individual time points and between ethanol vehicle and oxazolone treated

adults. A P-value of $p < 0.05$ (*) was considered significant. All analyses were performed using R 3.5.1 software.

C. RESULTS

Generation of BSA hydrogels

In order to test for hydrogel-associated toxicity following ingestion, bovine serum albumin (BSA) protein hydrogels were generated the day before feeding for all experiments. We generated hydrogels daily to ensure that the treatment was consistent over each treatment period. We generated BSA hydrogels in a photoactive redox reaction for polymerization as published in Khoury et al, 2018 (Khoury et al., 2018b). The photoactive BSA-polymerization mixture was injected into 10 cm PTFE tubes (Figure 2C). The loaded tubes were placed under a 100 W mercury lamp to prevent protein denaturation and irradiated for 30 minutes at room temperature to polymerize the solution into the protein hydrogels (Figure 2D). Following polymerization, hydrogels were extruded and stored overnight at 4 °C before feeding in a Tris-NaCl solution (Figure 2E). For all hydrogel treatments, adult fish consumed 5 μ L of 2 mM BSA hydrogel each day. Adult zebrafish readily ate the BSA hydrogels over the course of all treatments (Supplemental Video 1). This observation suggested to us that the hydrogels were not perceived as toxic by the hydrogel-fed adult animals.

Overall body weight does not change with hydrogel consumption

We first assessed the effects of hydrogel consumption on overall body weight (Figure 3). In the control diet condition, adult wild-type zebrafish were fed a normal diet twice daily and in the test condition, adult zebrafish were fed a BSA protein hydrogel once daily and their normal diet once daily. Fish were fed the control or hydrogel diets for 1 day, 10 days, or 30 days. To determine if overall body weight was affected by this

regimen, fish were anesthetized and weighed pre-treatment (Day 1), again at the halfway point for the 10- and 30-day studies (Day 5 and Day 15, respectively), and finally post-treatment for all groups prior to sacrificing the animals and collecting intestinal tissue for further analysis (Figure 3).

No significant differences in body weight were found between control and hydrogel-fed fish after 1 day, over 10 days (Figure 3A), or 30 days (Figure 3B; 1-day study not shown). Additionally, upon observation of hydrogel-fed animals, we did not detect any changes in feeding habits, swimming behavior, or social behavior. We did not observe phenotypic changes in emaciation, pigment changes, or decreased opercular movement. These data demonstrate that ingestion of hydrogels over the specific time points examined is non-lethal and does not compromise animal weight or behavior. These results suggest that ingestion of BSA hydrogels is not grossly toxic and that hydrogel ingestion does not have a significant effect on the metabolic state of the animals.

Analysis of intestinal villi structure and bowel wall thickness following hydrogel treatment

In order to assess the effect of hydrogel consumption on intestinal architecture, we used H&E histological analysis of dissected intestinal tissue from control-fed, hydrogel-fed, and positive control induced inflammation samples. Intestinal damage has previously been characterized in zebrafish using bowel wall thickness and intestinal villi length in chemically-induced inflammatory models (Brugman et al., 2009; Geiger et al., 2013).

We used this method of analysis to examine intestinal villi length and bowel wall thickness in control-fed and hydrogel-fed animals after 1, 10, and 30 days of feeding, and to compare these results with our analysis of villi length and bowel wall thickness in induced inflammation positive controls. These results are shown in Figure 4 with representative H&E images and quantification.

After 1-day hydrogel treatments (one feeding), villi lengths were the same in the hydrogel-fed tissue compared to controls (Figure 4A-B, I). In 10-day hydrogel-fed samples villi lengths were also the same between hydrogel-fed tissue and controls with no significant difference detected when quantified (Figure 4C-D, I). Following the 30-day hydrogel treatment, villi length of hydrogel-fed tissue was normal compared to control-fed (Figure 4E-F, I). Due to the lack of effect with hydrogel-feeding we wanted to confirm our ability to detect abnormal intestinal architecture and inflammation.

Therefore, we used a well-established method of oxazolone injection to induce intestinal inflammation. We found that in both ethanol and oxazolone-induced positive control samples, there was noticeable disruption to intestinal villi including a loss of villi and decrease in length (Figure 4G-I). In addition, we quantified a significant decrease in villi length between ethanol vehicle and oxazolone treatments in the positive controls.

These results are consistent with previous reports in chemically induced zebrafish models showing disruption in intestinal epithelial integrity (Brugman et al., 2009).

Therefore, results from our positive controls confirmed the observation that hydrogel-fed samples have normal tissue villi length up to 30 days of treatment.

It has been established that the thickness of the bowel wall will increase due to inflammation (Brugman et al., 2009). Therefore, we next measured bowel wall thickness in control, hydrogel-fed, and inflammation induced positive control samples using H&E stained sections (Figure 4). For the 1-day treatment period we did not observe changes in bowel wall thickness between control and hydrogel-fed tissue (Figure 4A-B, J). Surprisingly, the 10-day hydrogel-fed bowel wall tissue was significantly thicker than that of 10-day controls (Figure 4C-D, J). The difference in bowel wall thickness at this time point may have been due to the small sample size, the size of animals independent of the treatment, or an acute response to hydrogel consumption after 10 days. In the 30-day treatment group, bowel wall thickness was normal between control and hydrogel-fed samples (Figure 4E-F, J). As expected, observations of ethanol and oxazolone-induced samples showed an overall increase in bowel wall thickness when compared to control- and hydrogel-fed at 1, 10, or 30 days of treatment (Figure 4G-H, J), again indicating ethanol alone is toxic to the intestinal epithelia and confirming the inflammatory response of the bowel wall to injury (Figure 4G-H, J). Together, these data demonstrate an overall non-toxic response to protein hydrogel consumption after 30 days of treatment with minimal disruption to bowel wall thickness, villi architecture, and overall luminal structure.

Examination of intestinal cell types following hydrogel treatment

Following our assessment of intestinal architecture, we wanted to test the effect of hydrogel consumption on cell types within the intestinal tract. To test this we performed detailed analysis of the appearance and number of mucus-producing goblet cells in

intestinal villi, and the presence of inflammatory-responsive eosinophils within the bowel wall and along villi. Variations in goblet cell appearance and number and increases in eosinophil infiltration have been shown to be characteristic of an intestinal inflammatory response in zebrafish colitis models (Brugman et al., 2009). Therefore, we adapted this previously published scoring system to assess and quantify goblet cells and eosinophil infiltration in control-fed, hydrogel-fed, and positive control tissue samples. To perform these experiments, we stained intestinal sections from 1, 10, and 30 day treated samples, and positive control samples with Alcian Blue and PAS. Alcian Blue allows for observation of goblet cells, and when counterstained with PAS allows for visualization of infiltrating eosinophils (Brugman et al., 2009). An overall intestinal score was calculated for each treatment group by assessing goblet cell appearance and the infiltration of eosinophils in each sample (Table 1). Each treatment sample was scored independently for goblet cells and eosinophils (each with a score ranging from 1-3). The sum of the two scores was calculated and averaged per sample resulting in an overall intestinal score (Figure 5).

Within the intestinal tract in 1-day control and hydrogel-fed samples, we observed normal intestinal goblet cells lined along villi and minimal PAS-positive eosinophils in the bowel wall (Figure 5A-B, I). We observed a similar cell population pattern with the 10-day treatments (Figure 5C-D, I). Finally, as we hypothesized, the 30-day control and hydrogel-fed samples had normal goblet cells and eosinophil populations (Figure 5E-F, I). Similar to our observation in H&E stained tissue, Alcian Blue-PAS-stained ethanol and oxazolone-induced positive control samples showed severe disruption and

widening of villi, variations in goblet cell number and increased clusters of eosinophils (Figure 5G-H, I). Quantification using the scoring system described in Table 1 demonstrated that the overall intestinal score in chemically induced tissue was increased compared to control and hydrogel-fed tissue at all timepoints. Significant differences between control-fed and hydrogel-fed intestines at 1, 10, or 30 days of consumption were not detected (Figure 5I).

Summary

Together, these data indicate that consumption of BSA protein hydrogels results in minimal disturbance to adult zebrafish gut architecture, goblet cell, and eosinophil cell populations. Additionally, our positive control results are supported by previous work in chemically induced models using similar techniques and scoring systems to evaluate intestinal disturbances (Brugman et al., 2009; Geiger et al., 2013). Our evaluations of villi length, bowel wall thickness, intestinal cell populations support our hypothesis that hydrogel consumption is non-toxic and does not grossly affect intestinal tract integrity.

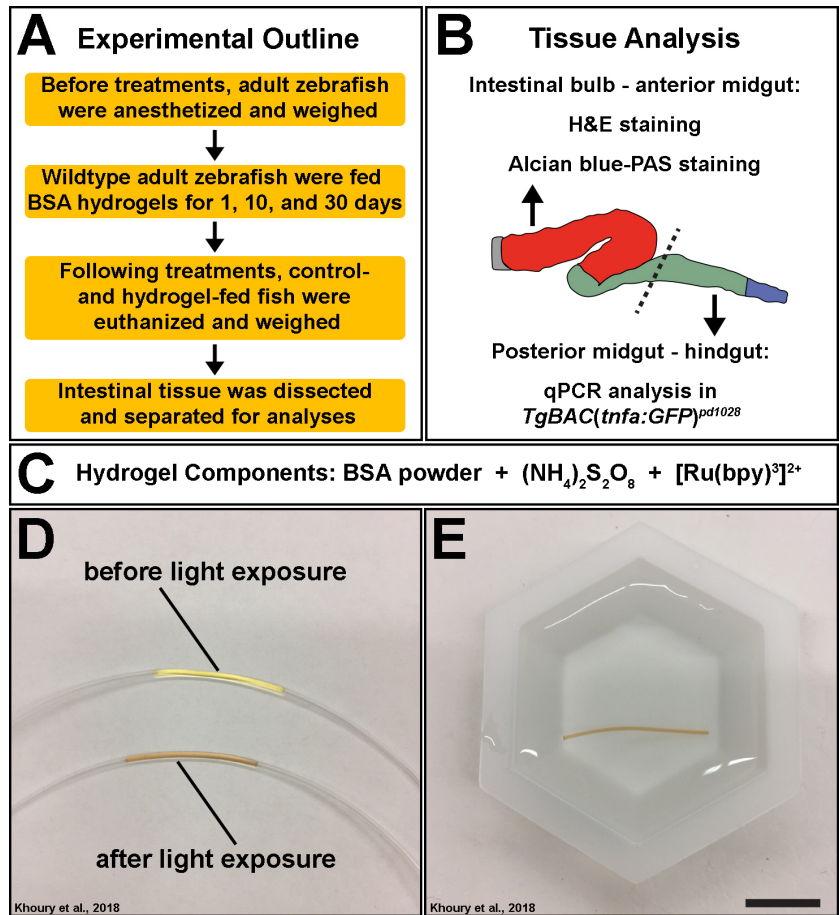


Figure 2. Experimental outline, tissue analyses, and hydrogel polymerization. (A) Experimental outline of hydrogel treatments, intestinal dissections, and sample preparation for analysis. (B) Intestinal bulb-anterior midgut tissue was used for H&E and Alcian-Blue-PAS staining techniques for all experiments. Posterior midgut-hindgut tissue was used for TNF α expression analysis. (C) 2 mM BSA, ammonium persulfate (APS, $(\text{NH}_4)_2\text{S}_2\text{O}_8$) and $[\text{Ru}(\text{bpy})_3]^{2+}$ were mixed for hydrogel polymerization. (D) Representative hydrogel mixture before (top) and after (bottom) 30-minute light exposure for complete polymerization. (E) Representative polymerized 2 mM BSA hydrogel stored in Tris-NaCl used for treatments. (D-E) Images adapted from Khoury et al., 2018. Scale bar: 1 cm.

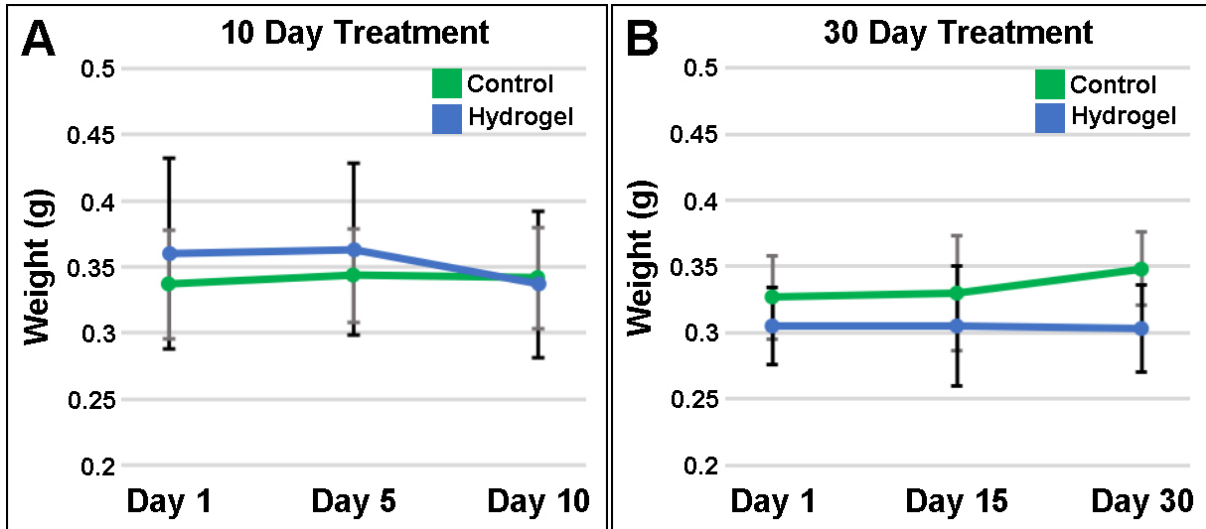


Figure 3. Mean body weight of adult zebrafish during hydrogel treatments. (A) Average body weight of control-fed (green) and hydrogel-fed (blue) zebrafish before, during, and after 10-day treatment. (B) Average body weight of control-fed (green) and hydrogel-fed (blue) zebrafish before, during, and after 30-day treatment. The same six zebrafish are represented in each condition. Data points represent the average weights for each condition. Data are represented as mean \pm s.d. Control-fed error bars, grey. Hydrogel-fed error bars, black. $n=6$ for all conditions.

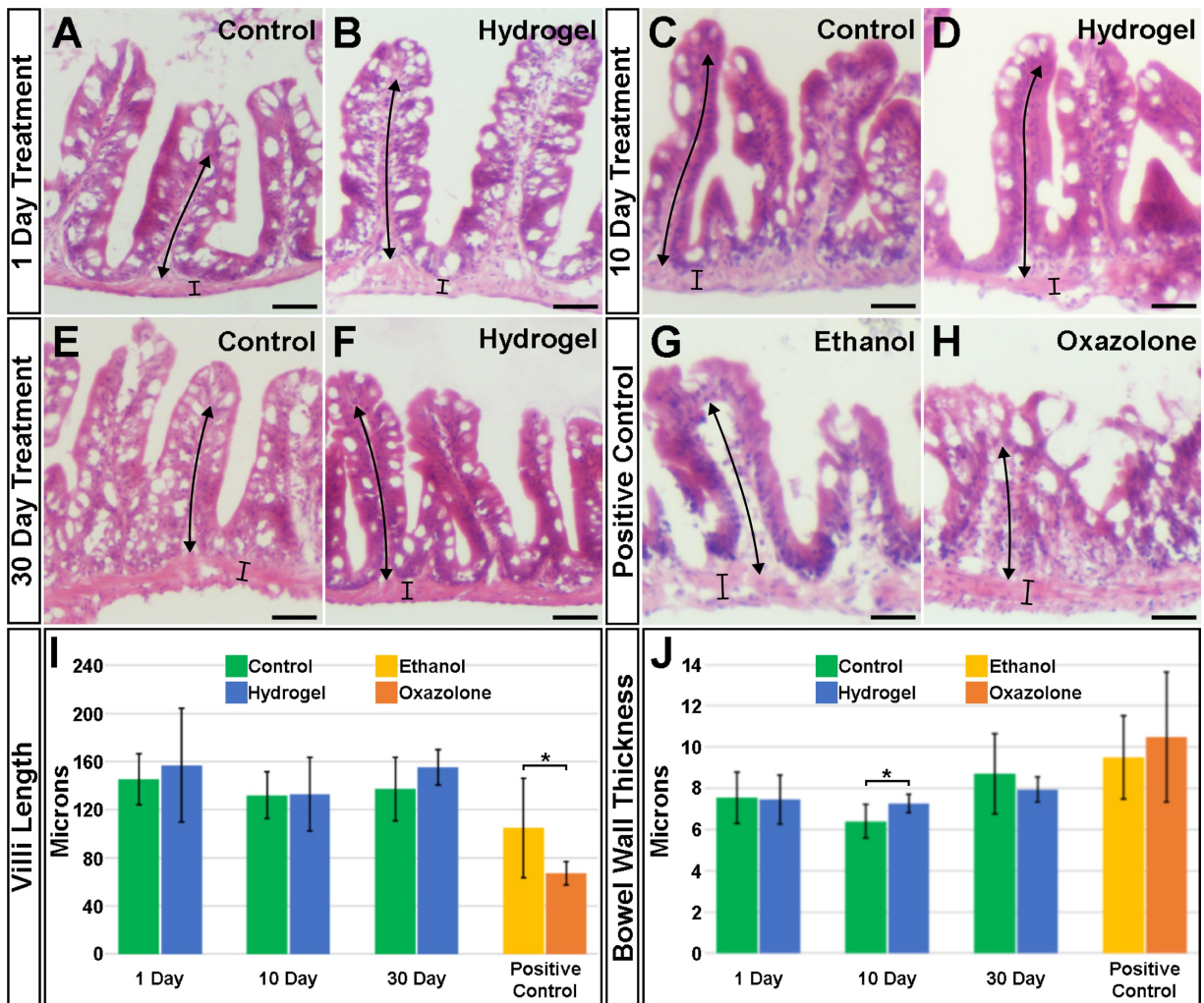


Figure 4. H&E staining and quantification of intestinal architecture. (A-F)

Representative H&E stain after 1 day (A, B), 10 days (C, D), and 30 days (E, F) of

control- and hydrogel-fed treatments. (G, H) Representative H&E stain of ethanol

vehicle (G) and oxazolone-induced inflammation (H) tissue. (I) Quantification of

intestinal villi length for each condition. Arrowed lines in A-H indicate representative villi

length measurements. (J) Quantification of bowel wall thickness for each condition. Bar

lines in A-H indicate representative bowel wall measurements. Data represented as

mean±s.d. * $P < 0.05$. Scale bars: 50 μ m. $n = 6$ for all conditions.

| Parameter | Score 1 | Score 2 | Score 3 |
|---|---|---|--|
| Goblet Cell Appearance | Dense, distinctive along villi edges | Scattered along villi edges, some indistinct | Few and indistinct, or complete loss of cells in villi |
| Infiltration of PAS-positive Eosinophils | Some scattered cells, few present in villi (<3) | Clusters in some villi and at the base of folds | Large clusters in several villi and at base of folds |

Table 1: Intestinal cell type scoring parameters. Scoring method to quantify intestinal-specific cell types following hydrogel and positive control treatments in Alcian blue-PAS stained tissue. A score of 1-3 is assigned for goblet cell appearance and separate score of 1-3 is assigned for infiltration of eosinophils assessed in each sample. Distinct goblet cells were characterized by circular cells along the villi. Clusters of infiltrating eosinophils were characterized by groups of 3 or more cells within close proximity. Total intestinal score is the sum of the Goblet Cell Appearance score and Infiltration of PAS-positive Eosinophils score (possible range = 2-6).

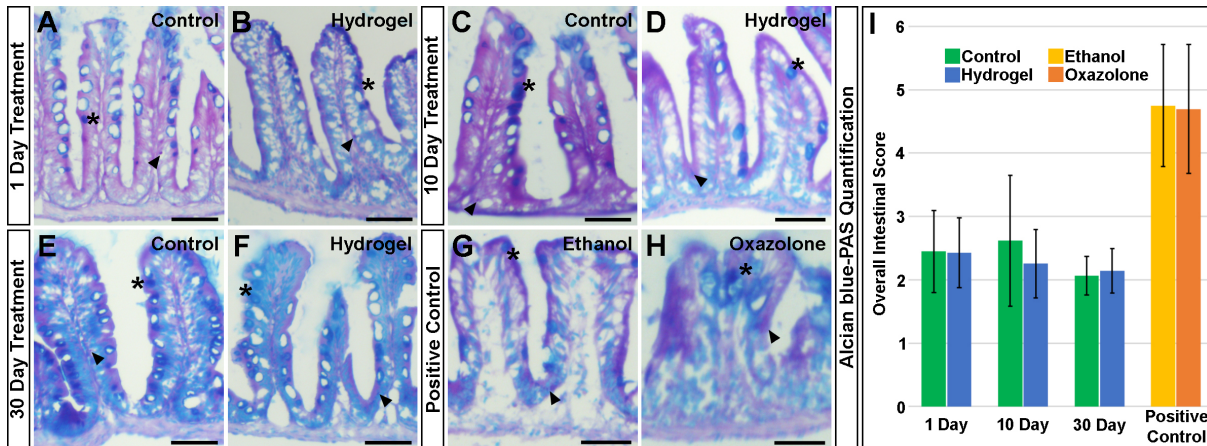


Figure 5. Alcian Blue-PAS staining and intestinal score for goblet cells and infiltrating eosinophils. (A-F) Representative Alcian Blue-PAS stain after 1 day (A, B), 10 days (C, D), and 30 days (E, F) of control- and hydrogel-fed tissue. (G, H) Representative Alcian Blue-PAS stain of ethanol vehicle (G) and oxazolone-induced inflammation (H) tissue. (I) Cumulative intestinal score for each condition. Asterisks in A-H indicate representative goblet cells. Arrowheads in A-H indicate representative PAS-positive eosinophils. Data represented as mean \pm s.d. All comparisons between treatment groups were not significant. Scale bars: 50 μ m. $n=6$ for all conditions.

CHAPTER 3

CONCLUSIONS, DISCUSSION, AND FUTURE DIRECTIONS

A. CONCLUSIONS AND DISCUSSION

The overall objective of this project was to evaluate the effects produced by consumption of bovine serum albumin (BSA) protein hydrogels on intestinal epithelium using the adult zebrafish as a model. This project served as a pilot study in our long-term research initiative to develop and test protein hydrogels as a targeted drug delivery system for the therapeutic treatment of intestinal disease, in particular, inflammatory bowel disease (IBD). We hypothesize that hydrogels can be engineered to specifically adsorb and bind to aberrantly-generated antibodies or antibody-tagged microorganisms and pathogens within the gastrointestinal tract for clearance.

In the pilot study presented here, we tested hydrogels for potential toxicity. Adult zebrafish were fed control diet or BSA hydrogels for 1, 10, and 30 days (Chapter 2, Figure 3). We examined body weight at the beginning, middle, and end of the study and found that overall body weight was not affected by hydrogel consumption. Next, we focused on intestinal tissue histology following treatments at each time point. We found that intestinal villi length was unaffected by hydrogel treatment compared to controls. Bowel wall thickness was unchanged at 1- and 30-days post treatment; however, we found increased thickness in hydrogel-fed samples after 10 days (Chapter 2, Figure 4). Using a scoring system for intestinal goblet cells and eosinophil abundances, we found

no changes between control and hydrogel-fed samples at all time points (Chapter 2, Figure 5).

Together, these data suggest that BSA hydrogel consumption is non-toxic to adult zebrafish and support the idea to further explore the potential for continued use of hydrogels as a drug delivery system for intestinal disease therapy.

Overall body weight after hydrogel consumption

We hypothesized that consumption of BSA protein hydrogels would not alter overall body weight. We tested this by weighing adult zebrafish before treatments, at the halfway points for the 10- and 30-days treatments, and at the conclusion of each treatment period. We found that total body weight remained unaffected at all time points through the course of treatment and at the conclusion. Additionally, general observations of the treatment groups did not reveal gross changes in phenotype or animal behavior. There are no known data on the effects of normal diet replacement with protein polymers, it has been shown that adult zebrafish starved for 18 days did not have significant changes to body weight (Novak et al., 2005). Therefore, analysis of overall body weight following treatments may not have provided the best approach in assessing toxic effects of protein hydrogel consumption. Additional experiments are necessary to understand the absorption, metabolism, and detoxification of protein hydrogels within the vertebrate system and to more accurately predict their potential for toxicity. It is known that the cytochrome p450 (CYP) superfamily of drug-metabolizing enzymes in zebrafish are highly conserved (Saad et al., 2016). Therefore, it would be

beneficial to analyze the CYP family of proteins following hydrogel consumption for continued risk assessment and toxicology screening studies. This could be done using qPCR and Western analysis of CYP family members from intestinal epithelium, liver, and kidney tissues.

Consumption of BSA hydrogels and intestinal damage or inflammation

We also hypothesized that ingestion of hydrogels over our experimental time points would not induce damage to intestinal tissue or cause an inflammatory response. With H&E staining techniques, we did not find significant changes in villi length between hydrogel-fed and control-fed tissue. Bowel wall thickness was also unaffected at 1- and 30-days post treatment. Interestingly, at the 10-day time point bowel wall thickness was significantly greater in hydrogel-fed tissue, suggesting a potential acute inflammatory response after 10 days. However, this response is likely to be transient since the animals did not demonstrate the same phenotype after the 30-day treatment. Furthermore, power analysis using G*Power 3.1.9.4 revealed that the probability distribution for the sample sizes in these studies was too large. This indicated that the small sample size would result in a higher probability of obtaining a significant result. Therefore, a larger sample size may be necessary to statistically demonstrate a significant change in bowel wall thickness following hydrogel treatment at 10 days. After 30 days of treatment we found healthy villi architecture, bowel wall thickness, and overall intestinal morphology.

Our positive control experiments highlighted our ability to detect inflammation (Chapter 2, Figure 4) and were consistent with previous reports in colitis zebrafish models chemically induced with oxazolone (Brugman et al., 2009) and 2,4,6-trinitrobenzenesulfonic acid (TNBS) (Geiger et al., 2013). Consequently, our data revealed no significant changes to gross intestinal architecture, but further analysis of epithelial integrity is crucial in determining effects of hydrogel consumption.

In this study we demonstrated that hydrogels do not disrupt intestinal villi in healthy, control animals, but all of these experiments were conducted using healthy, wild-type animals. However, practical use of this therapy would be treatment of patients experiencing inflammation. Therefore, it would be essential to test for toxicity of hydrogel-feeding in previously damaged tissue. In order to test this, we could induce inflammation, as demonstrated by our positive controls, in wild-type animals and subsequently feed them BSA protein hydrogels. Then we would evaluate intestinal epithelial integrity and cell populations. This would allow us to determine if protein hydrogels induce any additional damage to previously inflamed tissue.

The epithelium that lines the intestine provides the physical boundary between luminal contents and underlying tissue and is responsible for effective absorption of nutrients from food (Walton et al., 2016). The intestinal tract is exposed to a variety of pathogens and toxins; therefore, the epithelial barrier allows for selective uptake and regulation of fluids and solutes while maintaining the innate and adaptive immune systems and their responses (Sumagin and Parkos, 2015). Loss of intestinal epithelial barrier integrity can

lead to malabsorption and insufficient nutritional uptake to sustain homeostasis and health (Goulet et al., 2004). Therefore, it would be important to assess components of the intestinal epithelium that maintain its barrier integrity and selective permeability following hydrogel consumption. For example, we could use immunohistochemistry to analyze apical cell-to-cell adherens junctions by analyzing the localization and presence of N-cadherin. Another possibility would be to investigate tight junction proteins such as claudin, zonula occludens-1 (ZO-1), or associated regulatory proteins such as protein kinase C (PKC) that regulate the movement of solutes, fluids and ions (Lee, Moon and Kim, 2018).

Goblet cell and eosinophil abundances with BSA hydrogel treatment

We found that intestinal cell types, goblet cells and eosinophils, were unaffected with consumption of BSA hydrogels, suggesting that disruption of intestinal cell populations in chemically induced tissue is not found in hydrogel-fed tissue. Importantly, goblet cells secrete highly glycosylated mucins into the intestinal lumen as a first line of defense against environmental toxins, pathogens, and microbials. While this work revealed that there are no gross morphological changes to goblet cells following treatment, it would be important to determine if goblet cell function is maintained during hydrogel consumption. Three goblet cell specific proteins include, Mucin 2 (MUC2), Resistin-like molecule- β (RELM β), and Trefoil factor 3 (TFF3). MUC2 is the most abundant goblet cell mucin and its secretion is crucial for the organization of the intestinal mucous layers along the epithelial surface (Van der Sluis et al., 2006). RELM β has been shown to promote MUC2 secretions, inhibit intestinal parasite chemotaxis, and regulate intestinal

inflammatory responses through macrophage and T cell activation (Nair et al., 2008). TFF3 is a signaling molecule for epithelial repair and apoptosis resistance and contributes to mucin crosslinking for increased mucous layer organization and structure (Taupin, Kinoshita and Podolsky, 2000). Thus, in addition to overall morphology, qPCR, Western analyses, and immunostaining of each component could be beneficial to understand the impact of hydrogel consumption on goblet cell function.

B. FUTURE DIRECTIONS

We discovered that BSA protein hydrogels did not alter overall body weight, have grossly toxic effects on intestinal tissue, or cause changes in specific cell types within the digestive tract; however, the toxic threshold that hydrogel consumption may have remains unknown. The following questions address different hypotheses regarding hydrogel consumption and ideas to test potential toxic effects.

Does hydrogel consumption result in increased expression of pro-inflammatory markers?

Tumor necrosis factor-alpha ($TNF\alpha$) is one of the most well characterized pro-inflammatory cytokines that is known to have increase in expression in response to inflammation or tissue damage (Jones-Hall and Nakatsu, 2016). Therefore, we could examine *tnfa* gene expression following hydrogel feeding. We hypothesize that hydrogel consumption over an extended time period (>10 days) would result in no change in *tnfa* expression. To test this hypothesis we could use the *TgBAC(tnfa:GFP)^{pd1028}* transgenic zebrafish line. These fish provide a unique tool that would allow us to carefully examine *tnfa* expression in the intestine (Marjoram et al., 2015). To assess an inflammatory response to hydrogel consumption, we would collect intestinal tissue from zebrafish after feeding BSA hydrogels. As a positive control we would inject *TgBAC(tnfa:GFP)^{pd1028}* adults with either ethanol vehicle or oxazolone to induce intestinal inflammation. Additionally, we could analyze other pro-inflammatory cytokines that may change in response to hydrogel consumption such as interleukins *il1 β* , *il8*, and *il10*, which have been shown to be significantly increased in colitis induced zebrafish

models (Brugman et al., 2009; Geiger et al., 2013). We hypothesize that with hydrogel consumption, we would not see increased expression of pro-inflammatory cytokines.

Intestinal inflammation and epithelial damage found in immune-mediated diseases like IBD often leads to translocation of gut microbials, gut bacteria infiltration, and T-cell activation that can subsequently induce systemic inflammatory responses. Therefore, we could evaluate additional organs following hydrogel treatment to test for a systemic response to their consumption. We could perform qPCR analyses on various zebrafish tissues for immune-associated transcription factors in addition to pro-inflammatory cytokines. For example, we could evaluate expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) or activator protein-1 (AP-1) which are transcription factors that are activated by cytokines, stress, and infection, and subsequently control multiple cellular processes (Morales Fenero et al., 2016). These experiments would allow us to have a more complete profile of inflammation in response to consumption of hydrogels allowing us to improve their efficacy as a drug delivery system.

At what point would BSA hydrogel consumption have toxic effects?

Since BSA hydrogel consumption was non-lethal up to 30 days of ingestion and we did not observe significant effects suggesting toxicity, we could ask if a longer treatment period would cause toxic effects. We hypothesize that at the concentration and volume of BSA hydrogels fed to adult zebrafish in this study, there would not be toxicity over a longer treatment period. To test this, we could carry out similar experiments over a

longer time-period in a dose-responsive manner. For these studies, we could change the concentration of proteins in hydrogels, change the composition of the hydrogel polymers, or we could test long-term treatment in inflammation challenged animals. This would provide data to further support hydrogel consumption as a potential drug therapy for intestinal disease.

How are protein hydrogel polymers digested?

Currently we do not know how hydrogels are digested within the intestinal tract. This includes how they are broken down by digestive enzymes, absorbed by intestinal epithelial cells, or whether they are entirely cleared by the digestive system. We hypothesize that there would be some breakdown of hydrogels as they pass through the intestinal tract. To examine digestion of protein hydrogels we could perform qPCR analyses of intestinal tissue for expression of digestive enzymes including serine protease (*prss1*) or chymotrypsin-like (*ctrl*). We could also analyze expression of amino acid transport molecules (*slc15a1b*) to assess protein hydrogel absorption (Tian et al., 2015). Furthermore, we could perform RNA-sequencing for comprehensive transcriptome profiles of control and hydrogel-fed intestinal tissue for comparison (San et al., 2018) To test hydrogel clearance, we could isolate control and hydrogel-fed adult zebrafish and collect waste for protein analysis using mass spectrometry (Lichtman et al., 2013). Together, these studies would allow us to determine if hydrogel consumption alters normal digestion, nutrient absorption, and waste excretion.

What hydrogel composition would be beneficial for the treatment of intestinal disease?

Administration of protein hydrogels have previously been shown to have therapeutic effects. In zebrafish larvae, fibrin-based hydrogel injections promoted intestinal angiogenesis (Hsieh et al., 2017). The same study also showed self-healing of ischemic limbs in mice following intravenous injection (Hsieh et al., 2017). Therefore, we hypothesize that hydrogels may have beneficial effects by being able to bind endogenous proteins within the intestinal lumen for clearance. In IBD patients, epithelial damage, intestinal inflammation, and dysregulation of the mucosal immune response results in the production and release of Immunoglobulin A (IgA) and Immunoglobulin G (IgG) (Mitsuyama et al., 2016). Additionally, high levels of IgA and IgG-coated bacteria complexes have been detected in fecal matter of IBD patients (Lin et al., 2018). These findings have the potential to be exploited using protein hydrogel technology. Our collaborators have the capacity to synthesize protein hydrogels from diverse purified proteins, including bacterial surface proteins (personal communication, Dr. Ionel Popa, University of Wisconsin-Milwaukee, Department of Physics). Protein L is a bacterial surface protein found on *Peptostreptococcus magnus* that binds to the light chain of various classes of immunoglobulins with high affinity (Akerström and Björck, 1989). Therefore, we could synthesize Protein L hydrogels and feed to challenged zebrafish. We hypothesize that Protein L hydrogels would bind to immunoglobulins released within the intestinal lumen. After feeding, we could collect and analyze waste to determine *in vivo* hydrogel binding of intestinal antibodies. We predict that this technology has the potential to significantly impact the treatment of intestinal inflammation and disease.

CONTRIBUTIONS

The contributions for this thesis work are provided as follows: Drs. Ionel Popa and Jennifer H. Gutzman conceptualized this collaborative project. Dr. Jennifer H. Gutzman made contributions to design, optimization, and performance of experiments, analysis of data, and editing of this document. Dr. Luai Khoury made contributions to hydrogel synthesis. Intestinal scoring of Alcian Blue-PAS stained tissue was performed blinded by Dr. Mike Visetsouk, who also made contributions to analysis of data. Elizabeth Falat made contributions to primer design and optimization of qPCR methods. Data from Figure 1 was adapted from Lickwar et al., 2017 and Wallace et al., 2005. All other work was conducted by Ryan J. Garde.

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