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The investigation of chitosan-based hydrogels for intratumoral delivery of immunotherapeutic cytokines

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by

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This thesis is approved.

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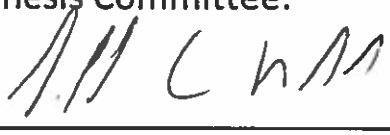

Abstract

Controlled and localized delivery of Interleukin 12 (IL-12) is critical to effective and non-toxic cancer immunotherapy. Current delivery protocols for pre-clinical anti-tumor trials utilize solutions of chitosan, a mucoadhesive and bioeliminable biopolymer, to slow the release of IL-12 into the tumor microenvironment. In order to further increase IL-12 retention, we have developed and characterized two chitosan-based *in situ* gelling hydrogels for IL-12 immunotherapy. One hydrogel, composed of heparin and chitosan, is herein described from initial conception through multiple stages of characterization including isolation of a range of suitable hydrogel formulations and observational physical and chemical properties. A second hydrogel is primarily composed of modified chitosan polymer. This hydrogel was characterized in previous studies, but the release kinetics (*in vitro* and *in vivo*) were not defined. In order to better understand this hydrogel, we performed both *in vitro* and *in vivo* protein release studies that validate this platform as a candidate for improved IL-12 therapy. Results indicate that the hydrogel and modified chitosan solution elicit protein retention at least twice as well as the chitosan solution currently used. Future studies will further examine release kinetics of the heparin-chitosan hydrogel and the anti-tumor response in mouse models using both hydrogels. If successful, these IL-12/hydrogel formulations will provide new clinical treatments for patients with solid tumors.

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1. Introduction

There were approximately 14 million new cancer diagnoses and 8.2 million cancer-related deaths worldwide in 2015.[1] Cancer and its associated ailments are the second-most deadly diseases in the US, only barely outnumbered by heart disease. In 2011, direct medical costs (exclusive of moneys spent on cancer research) associated with cancer treatment reached nearly 90 billion dollars in the US, signifying a significant—and in some cases futile—consumption of our current technologies and a need for further development.[1] For these reasons, the demand for improving and innovating diagnostic techniques and treatments is well warranted; interest in improving cancer diagnosis and treatment capabilities benefits both the global healthcare system and economy.

Unfortunately, many cancers go unchecked until they are large enough to cause physiological side effects that prompt the patient to seek a doctor. Because many primary tumors often have to grow quite extensively before being noticed, prognosis is often—but not always—poor by the time help is sought.[2] Treatment options for cancer patients include surgical excision, chemotherapy, radiotherapy, hormone therapy and immunotherapy.[3] These modalities are typically used in concert; for example, the majority of a breast cancer lumps could be surgically removed, but then the subject would undergo rounds of radiotherapy to eradicate the remainder of the cancerous cells in the tissue.[1] While this seems simple in theory, the clinical outcomes are far from ideal, resulting in millions of deaths worldwide every year. Furthermore, even if primary treatment is successful, current treatments cannot target and eliminate metastases and prevent recurrence.

Cancer immunotherapy is a rapidly advancing approach that is defined as the mobilization of the host's immune system to eliminate relieve, or suppress disease.[4] The potential of immunotherapy as a standalone or joint treatment option has become more attractive in recent years; the last decade has yielded 163,000 publications on cancer immunotherapy, up from 52,600 in the previous decade.[5]

At the most basic level, immunotherapies function by modification of immunological pathways. Cancer immunotherapy can be divided into four modalities—checkpoint modification, mature immune cell therapy, cell-specific vaccination, and immune system modulation—that can be implemented alone or in concert with each other—and with non-immunotherapeutic approaches— to varying effect.[4]

Immune system modulation requires the delivery of cytokines, co-factors, and other signaling molecules to mobilize the immune system. These modulators include a class of molecules called 'interleukins'. Interleukins such as interleukin 2 (IL-2) and interleukin 12 (IL-12) are integral to the immunological pathways of adaptive immunity, and can help the body recognize, fight, and eliminate solid primary tumors and their subsequent metastases.[6] In many cases, after tumor elimination, a 'memory' is established to prevent disease remission. Immunotherapy is not without its challenges. Outside of the treatment's effective therapeutic window, the therapy can be either ineffective or hypereffective. Hypereffective treatment leads to systemic autoimmune toxicity, organ failure, and death. For this reason, controlled delivery of therapeutics is crucial to effective treatment.

Drug delivery mechanisms range from drug-releasing nanoparticles to hydrogels that release the drug into the microenvironment as the structure breaks down. A variety of

materials and tools provide the ability to create site- and drug-specific platforms for delivering an effective quantity drug to a very specific target over an appropriate amount of time.[7]

The discovery, characterization, and implementation of novel delivery mechanisms are core research themes in the University of Arkansas's Laboratory for Vaccine and Immunotherapy Delivery (LVID). Much of the LVID's past publications are focused on immune system modulation using co-formulations of interleukin 12 (IL-12) and chitosan (CS), a viscous biopolymer derived primarily from crustacean shells and fungi.[8,9] Chitosan's functional groups, physicochemical properties, and average molecular weight (length) are highly variable, making it attractive for drug delivery applications, as it can be fine tuned for specific needs such as oral drug delivery and wound healing.[10] IL-12 is a cytokine, a cell-signaling molecule, that is released in the presence of infection and promotes differentiation of T cells (a type of immune cell) into Th1 cells. Th1 cells secrete interferon gamma (IFN-γ), IL-2, and lymphotoxin, which result in further cell-mediated offense.[11] After the infection has been eliminated, some of the activated B cells will differentiate into memory cells that re-stimulate the immune system if the same tumor cells types begin to grow again.[12]

Previous publications from the LVID demonstrate intratumoral injection of IL-12/chitosan mixtures and the subsequent anti-tumor effects (for some cell types) and cell-specific immunity that follows disease eradication.[8,9] However, these mixtures have been less effective against triple-negative breast cancer cells (TNBC) and other aggressive cancer cell lines.[9] It is believed that by further increasing and retaining concentrated intratumoral IL-12 will provide sufficient immune stimulation for tumor eradication of these more resilient tumors.

To attempt to increase retention, we aim to develop, characterize, and implement novel hydrogels that can be loaded with IL-12 and injected directly into the tumor. Christopher Wallace and Dr. Bhanu Koppolu of the LVID developed one hydrogel of interest.[13] The hydrogel is composed of modified chitosan (mCS) and crosslinking agents that form rapidly upon mixing. This hydrogel is proposed to follow a cross-linked polymer chain model, as described in the figure below (**Figure 1**). In this model, crosslinking molecules conjoin the side groups of the primary polymer to form a mesh network.[14]

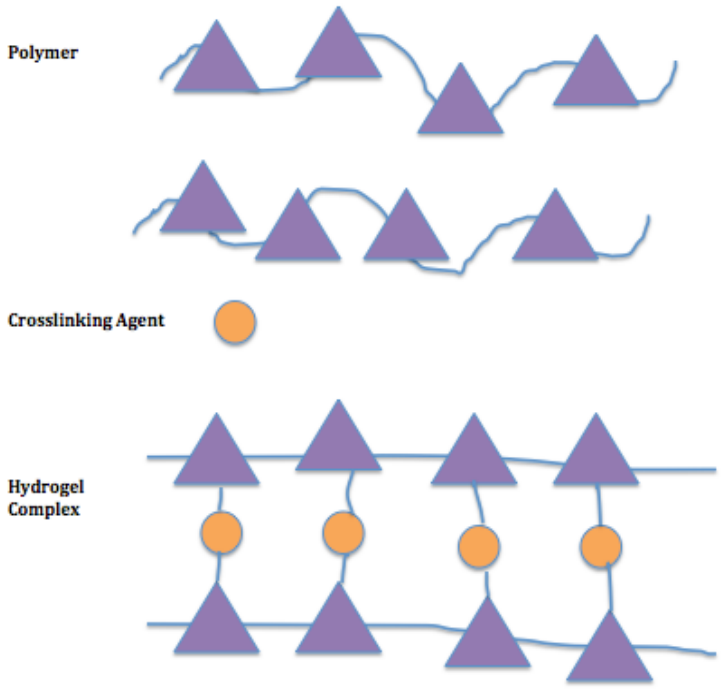

Figure 1: Cross-linked Hydrogels

The gelation parameters, hydrogel modulation of IL-12 bioactivity, and anti-tumor activity were previously assessed.[13] Investigations of this hydrogel included in this article include the development and implementation of a protocol to determine relative release kinetics, both *in vitro* and *in vivo*, and develop a pathway for further investigation.

A second chitosan-based hydrogel that co-formulates chitosan with heparin (Hep), a biopolymer typically used in medicine as an anticoagulant.[15] Heparin contains protein-binding sites very specific to IL-12 and other cytokines; using this polymer in the hydrogel matrix may further inhibit IL-12 dissemination and thus increase immune response and treatment capability.[16] This heparin-chitosan (Hep-CS) hydrogel forms instantaneously, as the oppositely charged polymer chains electrostatically attract and irreversibly bind. This hydrogel is classified as an ionic hydrogel, as the electrostatic forces between the polymers are the driving force of gelation.[14] The figure below (**Figure 2**) demonstrates this phenomenon.

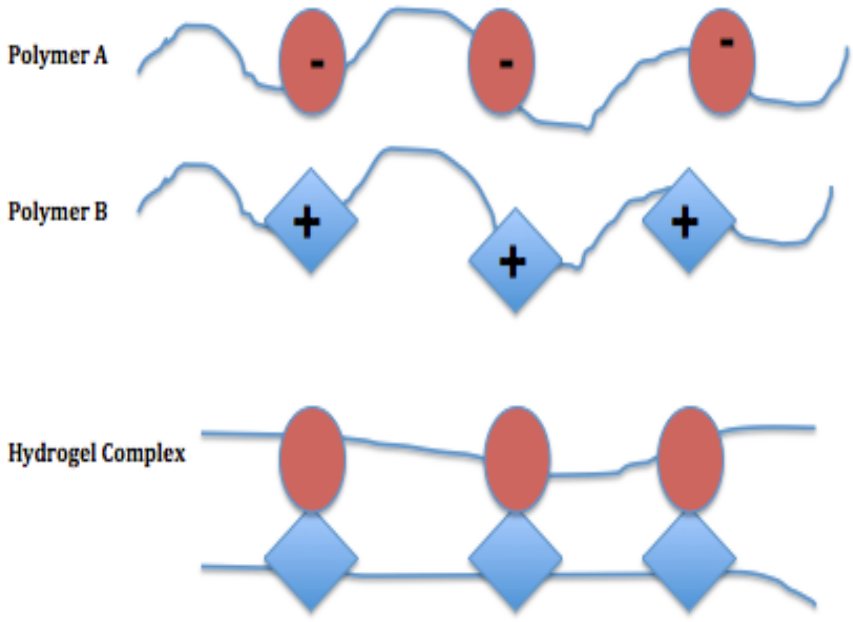

Figure 2: Ionic Hydrogels

Investigations include the determination of how component concentrations contribute to hydrogel formation, the quantification of hydrogel formation using different methods, and determination of a key physico-chemical property of the hydrogel, the lower critical solubility temperature (LCST).

The methods, data, and analysis herein describe the processes, results, and conclusions regarding the investigation of these two novel hydrogels and their potential for intratumoral delivery of IL-12. If successful, these studies will substantiate the belief that robust hydrogels are solid potential candidates for pre-clinical anti-tumor trials.

2. Materials and Methods

2.1 Heparin-Chitosan Hydrogel

2.1.1 Hydrogel Preparation

Initial hydrogel preparation was entirely experimental. Solutions of heparin (H3393, Sigma-Aldrich) were prepared in DPBS at 105, 50, 25, 15, 10, 7.5, 5, 2m, 1, and 0.1mg/mL. Chitosan (Chitoclear, Primex) solutions of different viscosities (2-20cP, 20-200cP, 200-600cP, and 600-2000cP) were prepared in 2% acetic acid at 5, 2.5, 1, and 0.1mg/mL. Equal volumes of heparin and chitosan were combined. Concentrations and chitosan viscosities were varied and the resulting hydrogels were observed. General bounds for gelation were established by visual confirmation of gelation; select combinations were used for future studies. See **Protocol 1** for details.

2.1.2 Colorimetric Assay for Hydrogel Optimization

Total hydrogel formation can be optimized by selecting chitosan and heparin concentrations that, when reacted, do not leave excess polymer in solution. Hydrogels were prepared per **Protocol 1** in triplicate using low viscosity (2-20 cP) chitosan at 5, 2.5, and 1 mg/mL and heparin at 15, 10 5, and 1 mg/mL. Samples were homogenized using a benchtop vortex and then centrifuged to pellet the hydrogel particles.

The remaining supernatant was removed and used in the assay. Dilutions of heparin and chitosan were prepared as controls. This spectrometric detection assay was adapted from Badawy et al. using 2-thiobarbituric acid (Sigma-Aldrich), sodium nitrite (Sigma-Aldrich), and sodium hydroxide (Sigma-Aldrich); see **Protocol 2** for details.[17]

2.1.3 Absorptivity Assay for Hydrogel Optimization

The hydrogel formation reaction results in Hep-CS hydrogels non-homogenous white coloration to varying degrees, dependent on the concentrations of the constituents. If formation of white coloration is a measure of heparin-chitosan interaction, increasing coloration indicates a more reacted hydrogel.

Hydrogels were prepared in triplicate using 2-20 cP chitosan at 5, 2.5, and 1 mg/mL and 105, 50, 25, 15, 10, 5, and 1, 0.5, and 0.1 mg/mL heparin. Hydrogels were vortexed for 5 seconds upon combination of reactants, then again for 10 seconds prior to distribution to a 96-well plate. Dilutions of chitosan and heparin were prepared and used to differentiate between reacted and unreacted hydrogels. PBS was used to determine background absorbance of the plate. Absorbance spectra of samples were read using a spectrophotometer.

2.1.4 Thermosensitivity Assessment, LCST

Hydrogels were prepared in triplicate using 10mg/mL heparin and 5mg/mL chitosan of viscosity 20-200 cP. Immediately after combination of heparin and chitosan, samples were incubated for 1 hour in environments of 70, 55, 37, 27, and 4C. Samples were removed, photographed, and observed upon completion.

2.2 mChitosan Hydrogel

2.2.1 Hydrogel Preparation

Hydrogels were prepared using a proprietary formulation composed of the primary modified chitosan polymer (mCS), crosslinking agent (X), and a catalyst. This protocol was established in a previous study at the LVID and is not listed herein.

2.2.2 In Vitro Release Kinetics

Using fluoroisothiocyanate-tagged bovine serum albumin protein (FITC-BSA)

Triplicates of release solutions (PBS, 0.5% m/v mCS, and mCS hydrogel) were loaded with 50 ug FITC-BSA (Sigma-Aldrich) and injected into 1 mL Float-A-Lyzer (100kd MWCO) floating dialysis tubes (Spectrum Labs). Loaded tubes were placed in 15 mL PBS in a 50 mL centrifuge tube and incubated in a 37C shaker at 200 rpm. 1 mL samples of PBS dialysate were removed and 1 mL fresh PBS was added at 3, 6, 12, 24, 48, 72, 96, 120, 144, and 168 hours. Samples were kept at room temperature until study was concluded, then fluorescence of all samples were read using a BioTek Synergy 2 spectrofluorometer with a FITC excitation and emission filter set.

Post-study investigations confirm fluorescent instability and recoverability of the FITC tag.

Using Alexa Fluor 488 (AF488)

Prior to release study, pH stability and transient stability of AF488 (Life Technologies) were examined.

50 micrograms of hIL-12/AF488 was released 300kD MWCO Float-A-Lyzers into 25mL PBS dialysate. Release solutions were PBS, mCS (0.5% w/v), and mCS hydrogel. Tubes were incubated at 37C at 200 rpm. 200uL samples of dialysate were taken at 3, 6, 12,

24, 48, 72, 96, 120, 144, and 168 hours. Samples were kept at room temperature until the study was concluded. 100 uL of the 200 uL sample were read using a BioTek Synergy 2 spectrofluorometer with a FITC excitation and emission filter set. See **Protocol 3** for details.

2.2.3 In Vivo IL-12 Retention in mCS hydrogel, mCS solution, and PBS

mIL-12 (Arkansas Biologics Center) was first tagged with AlexuaFluor 660 (AF660) (Life Technologies, USA) following standard product protocol. Mice (n=3 per group) were subcutaneously injected with 100 uL of mCS, PBS, and mCS hydrogel loaded with 1 ug mIL-12/AF660. Mice were imaged using an IVIS Lumina Pre-Clinical Imaging System minutes after injection and 1, 2, 3, 4, 6, 7, 10, and 14 days later, or until fluorescence was no longer significant. Regions of interest (ROIs) were drawn around injection site in the digital images, and radiant efficiency for each mouse at each time point was calculated using the image capture software and tools. Data was processed and graphed using Microsoft Excel.

2.2.4 In Vivo Comparison of mCS and Protasan

mCS and Protasan solutions were prepared and loaded with 1 ug mIL-12/AF660. Mice (n=3 per group) were subcutaneously injected with 100 uL of either mCS-IL12/AF660 or Protasan-IL12/AF660. Mice were imaged using an IVIS Lumina Pre-Clinical Imaging System minutes after injection and 1, 2, 3, 4, 5, 6, and 7 days later. Regions of interest (ROIs) were drawn around injection site in the digital images, and radiant efficiency for each mouse at each time point was calculated using the image capture software and tools. Data was processed and graphed using Microsoft Excel.

3. Results

3.1 Heparin-Chitosan Hydrogel

3.1.1 Hydrogel Preparation

Figure 3 (below) shows four prepared samples using 1:5, 2:1, 10:1, and 21:1 heparin-to-chitosan ratios. Degree of gelation was determined visually by inversion, by density of white coloration, and gel volume fraction. Order of decreasing degree of gelation is as follows: 2:1→10:1→21:1→1:5

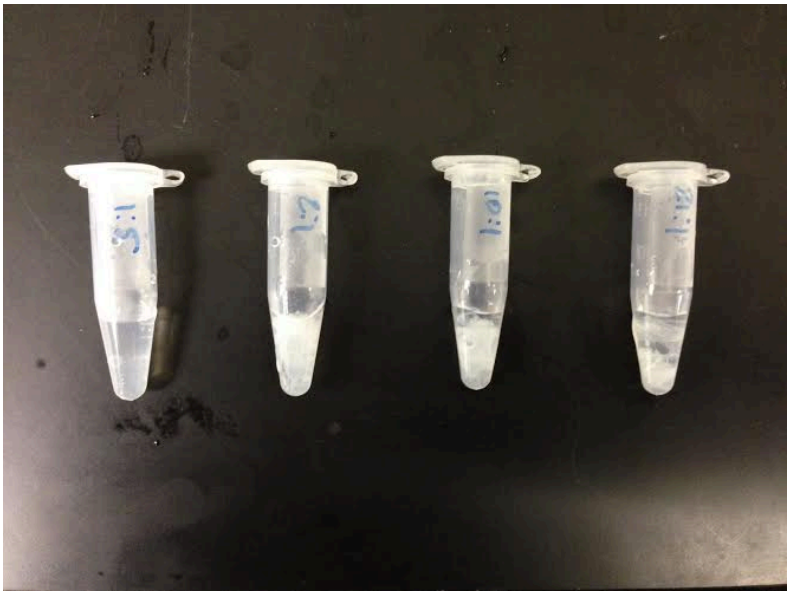

Figure 3: Heparin-Chitosan Hydrogel Examples. Heparin:Chitosan ratios from left to right are 1:5, 2:1, 10:1, and 21:1. The 2:1 hydrogel was observed to be the most robust and fully-reacted.

3.1.2 Colorimetric Assay for Hydrogel Optimization

Figure 4 shows a 96-well plate containing 3 concentrations of heparin and 3 concentrations of chitosan. Each sample concentration has been diluted into four buffers with different pH. It was determined that chitosan and heparin cause similar changes in color across a range of pH values (4-7). Similar coloration is observed at between the two reacted polymers. As pH increases (from left to right, starting at

columns 1, 5, and 9), becomes less reactive and heparin becomes more reactive. Chitosan and heparin yield the same absorbance peak to varying degree, depending on concentration and pH, making mixtures of the two solutions impossible to discern.

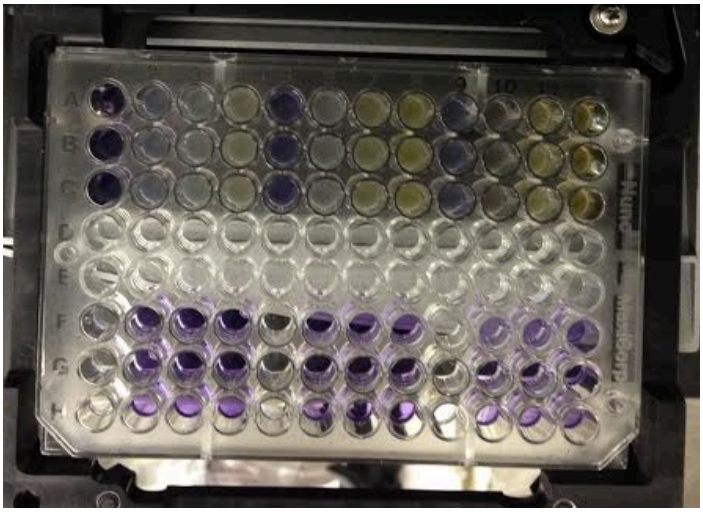

Figure 4: Calorimetric Assay

3.1.3 Absorptivity Assay for Hydrogel Optimization

Hydrogel absorption at 300nm was used to quantify degree of gelation. According to absorptivity curves in **Figure 5**, optimization occurs at 1-2:1 m/m heparin to chitosan; additionally, higher concentrations of chitosan result in higher absorbance values overall.

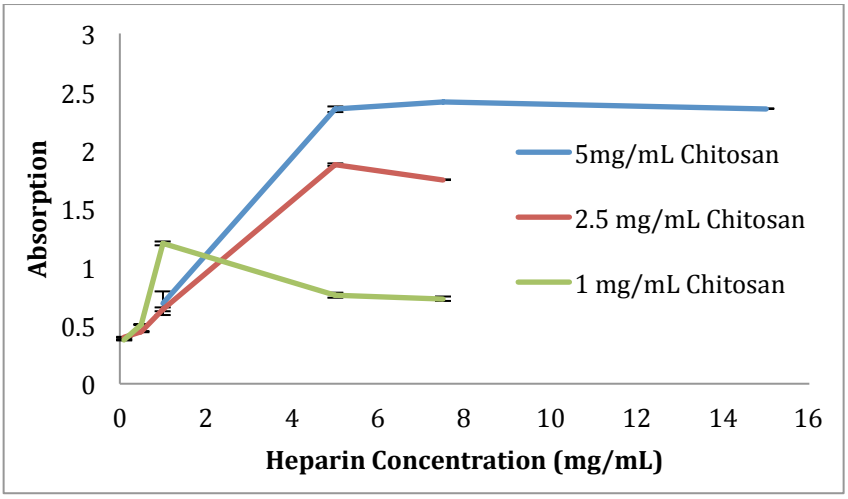

Figure 5: Absorption of Varying Hydrogels at 300nm

3.1.4 Thermosensitivity Assessment

Figure 6: Hydrogel Samples at Extreme Temperatures. Immersion temperatures decrease from left to right. Sample 1 was immersed in 70C, sample 2 was immersed in 55C, sample 3 was in 37C, sample 4 was in 27C, and sample 5 was in 4C.

Hydrogel collapse—characterized by dense white hydrogel— is observed above the structure's lower critical solution temperature. Partial collapse is observed at 55C, with total collapse at 70C. LCST, then, is approximately 55C. An exact LCST is under evaluation.

3.2 mChitosan Hydrogel

3.2.1 Hydrogel Preparation

Figure 7 displays samples of hydrogel components (three leftmost tubes) and hydrogel (right) loaded with FITC-BSA, a fluorescent marker, and inverted. The hydrogel retains shape and remains at the 'bottom' of the tube, whereas liquid components do not.

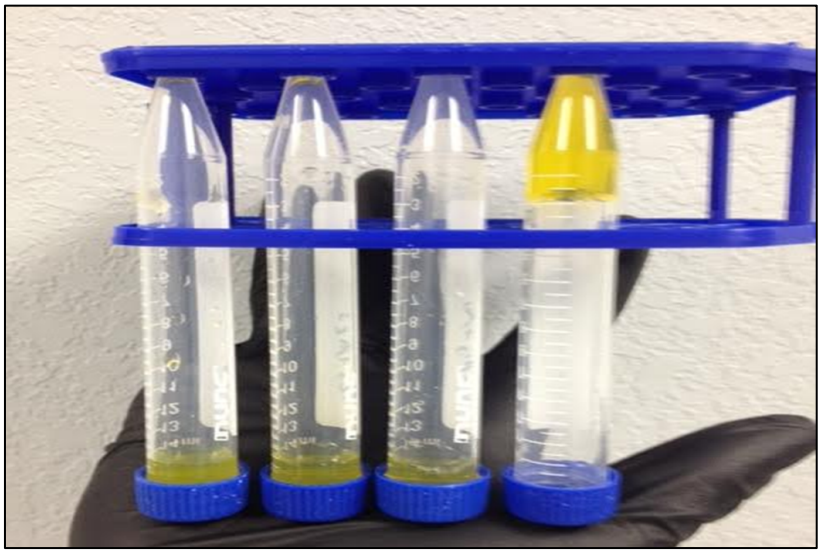

Figure 7: mCS Hydrogel Preparation

3.2.2 In Vitro Release Kinetics

The initial release study using FITC-BSA was deemed unsuccessful from the total lack of measured fluorescence exemplified in **Figure 8**.

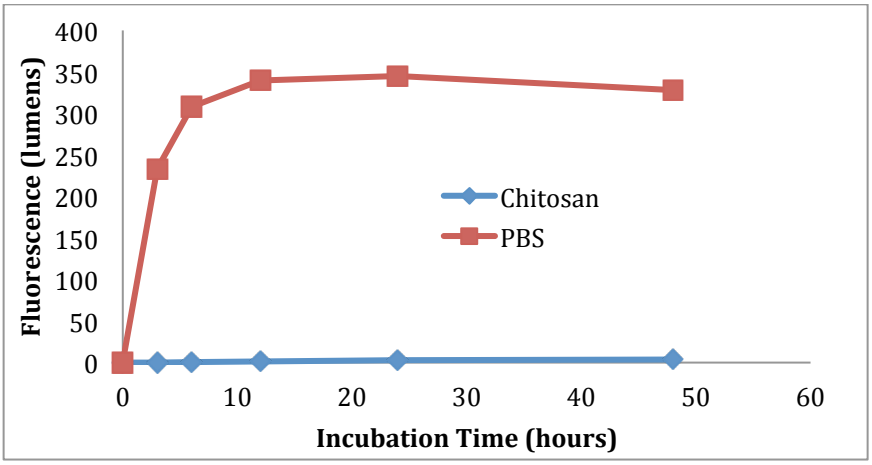

Figure 8: FITC-BSA Drug Release Kinetics from 100kD MWCO Float-A-Lyzer Tubes

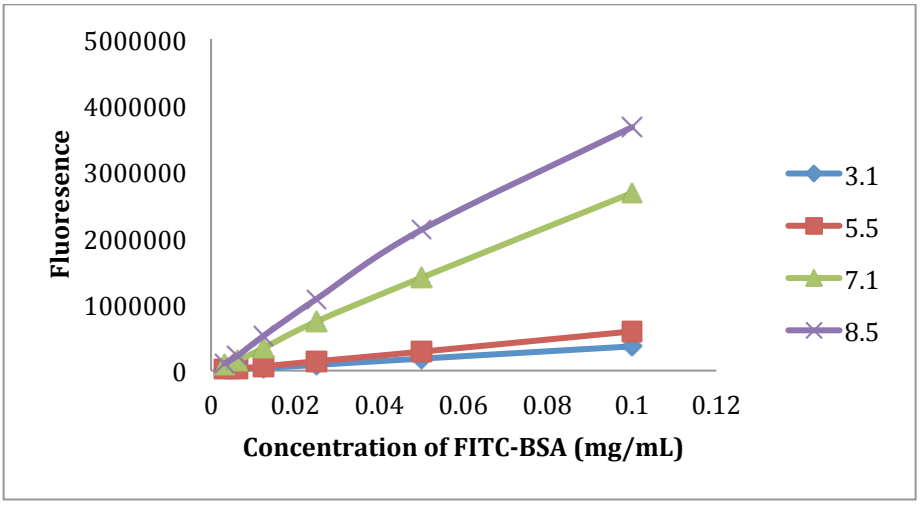

Figure 9: pH Dependence of FITC-BSA Fluorescence

Figure 9 indicates high variability of FITC fluorescence when incubated in different pH buffers. **Figure 10**, however, shows almost complete recovery in fluorescence when pH is adjusted from low to high. Fluorescence is almost completely depleted at a pH of 3, but recovers 86% of fluorescence when adjusted up to 8.

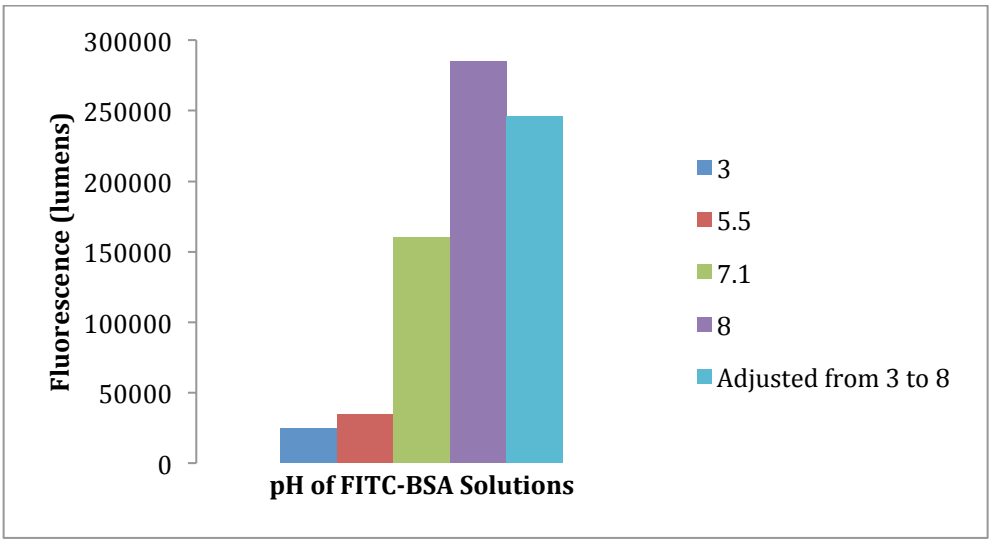

Figure 10: Fluorescence Recovery from Low pH

Figure 11 indicates that, between a pH of 5.6 and 7.9, fluorescence of IL-12/AF488 does not vary significantly, which indicates that it can be implemented in release studies using release solutions of varying pH.

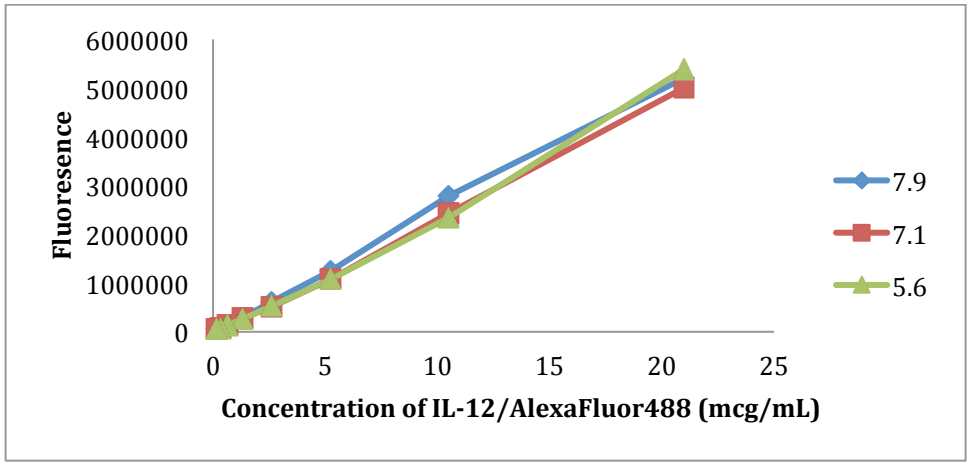

Figure 11: pH Dependence of IL-12/AF488 Fluorescence

Figure 12 shows about ±10% fluctuation from the initial and final fluorescence measurements over a period of one week.

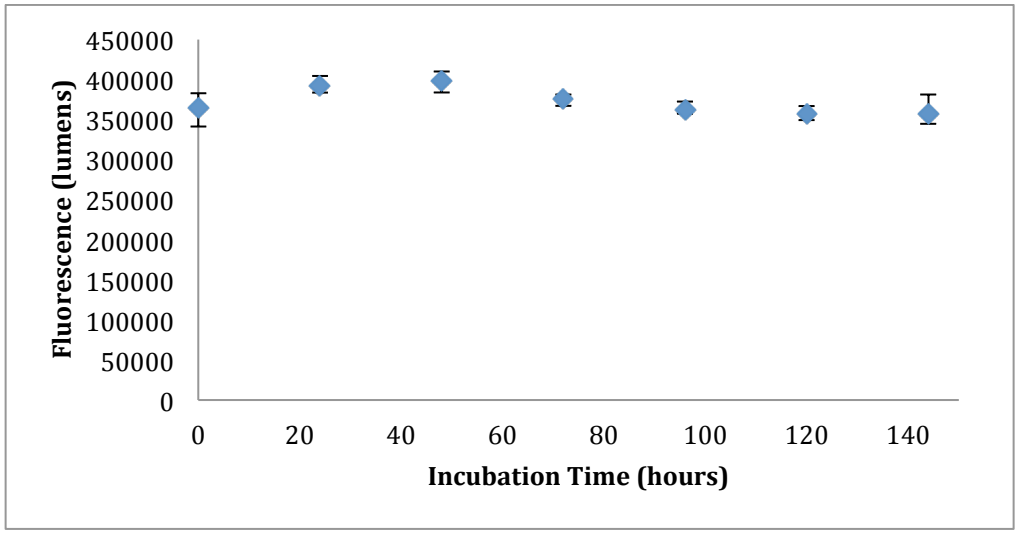

Figure 12: Transient Stability of IL-12/AF488

Release profiles of IL-12/AF488 from a study comparing PBS release from 300kD and 100kD MWCO Float-A-Lyzers are shown in **Figure 13**. It was determined that 300kD

MWCO Float-A-Lyzers release nearly twice as much protein as 100kD Float-A-Lyzers in a period of 48 hours.

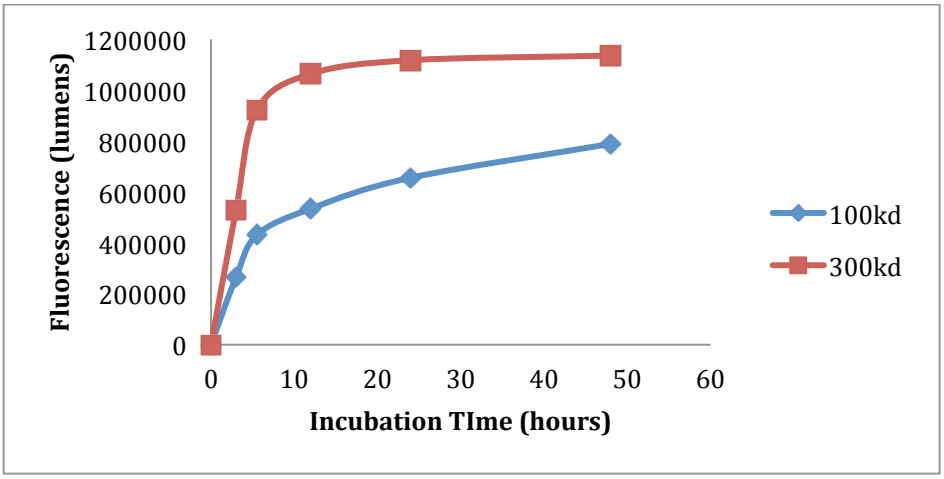

Figure 13: IL-12/AF488 Release from 100kD and 300kD MWCO Float-A-Lyzer Tubes

Final release profiles of IL-12/AF488 from PBS, mCS solution and mCS hydrogel are shown below in **Figure 14**. The PBS curve is relatively linear, with approximately 45% of protein released after one week. Modified chitosan solution inhibits IL-12/AF488 release, reducing cumulative release from 45% to 16%. The hydrogel displays kinetics similar to PBS for the first 24 hours, then prevents release for the remainder of the 7 days, with a total of approximately 8%.

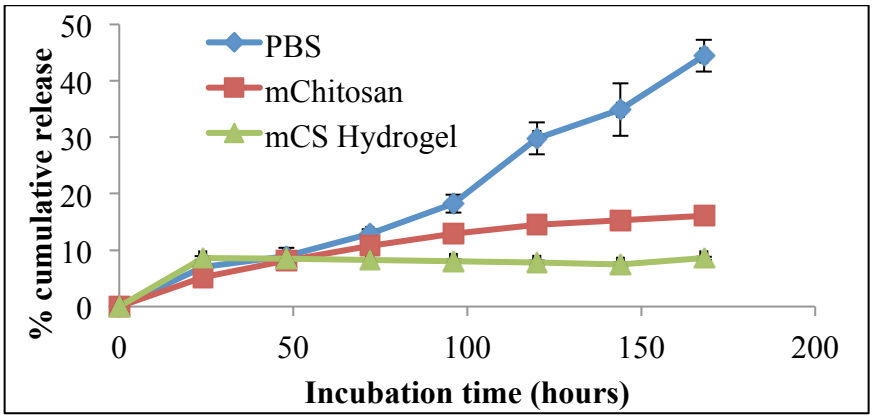

Figure 14. IL-12/AF488 Release Profiles from 300kD MWCO Float-A-Lyzer Tubes

3.2.3 In Vivo Hydrogel IL-12 Retention

Figure 15 displays retention of IL-12/AF660 calculated by comparing the radiant efficiency of each time point to the initial radiant efficiency (RE). The photos in **Figure 16** are scaled to the same RE upper and lower bounds for direct comparison between groups. **Figure 15** and **Figure 16** show the drastic dispersion of IL-12/AF660 from the PBS injections after 24 hours. The photos indicate a lower initial fluorescence in the hydrogel mice and that most of the protein is disseminated quickly. The graph, however, indicates that 15-20% of the protein is retained for seven days, and the remainder is lost by day 14; this elevated and potentially mis-representative value can be attributed to the relatively low amount of protein at the initial time point. Modified chitosan solution elicits much greater control on dissemination of protein. After 10 days, mCS solution injection has retained twice the amount of initial fluorescent protein than the mCS hydrogel. 14 days from initial injection, all fluorescent protein was gone from mCS hydrogel mice while mCS solution mice showed retention of 12%. The PBS and mCS solution mice start with approximately the same amount of protein at the injection site. Fluorescence is still visible in the mCS solution mice at day 20, where PBS mice have no remaining fluorescence after 24 hours and mCS solution mice have no remaining fluorescence at 10 days.

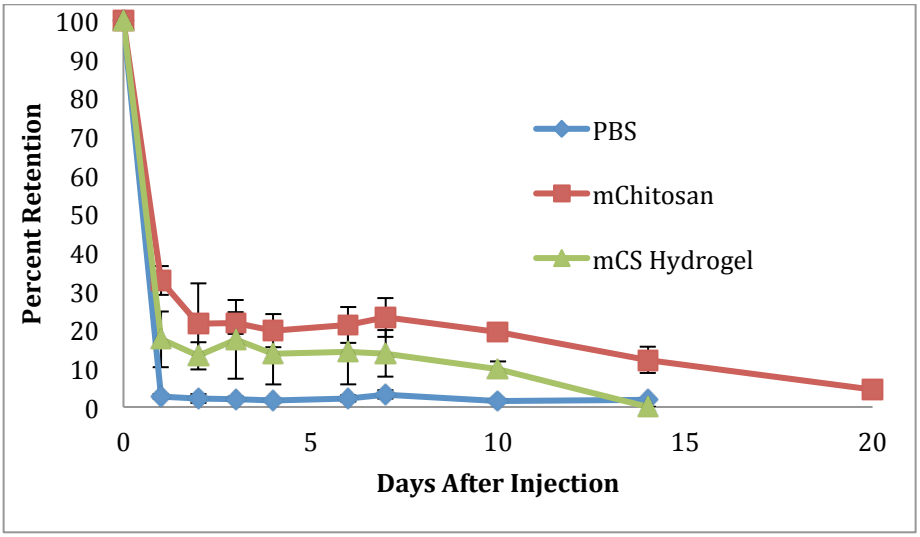

Figure 15 : Percent Retention IL-12/AF660 Retention *In Vivo*

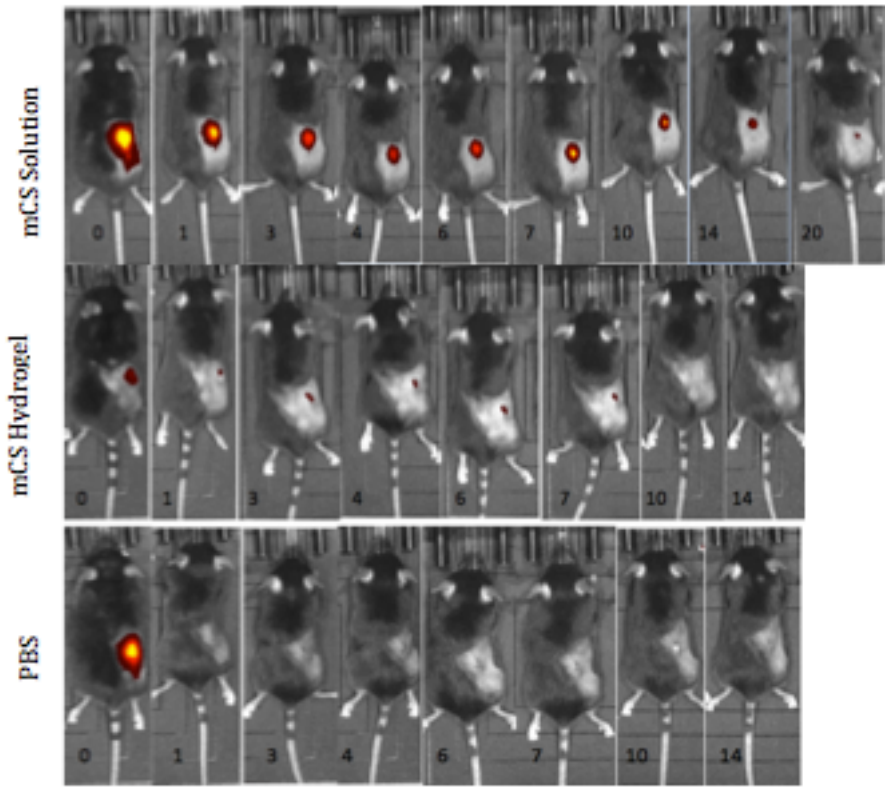

Figure 16: *In Vivo* IL-12/AF660 Retention: Photographs with Fluorescent Overlay

3.2.4 In Vivo Comparison of mCS and the LVID's Standard Practice Delivery System

This investigation revealed a nearly 5-fold increase in localized IL-12/AF660 retention by mCS solution over Protasan after 1 week. Percent retention was calculated point-by-point, as a comparison of the magnitude of radiant efficiency to the initial value at time of injection. After 7 days, mCS solution retained approximately 10% IL-12/AF660 while Protasan retained approximately 2%. The data for mCS does not agree with the retention results from the previous study so repeat studies will be conducted to confirm results.

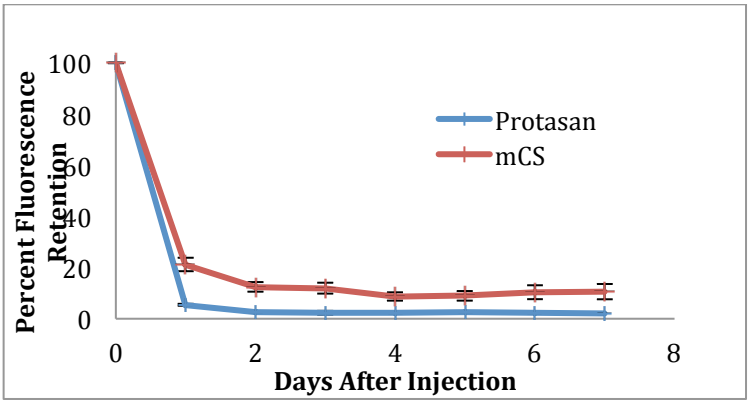

Figure 17: ***In Vivo IL-12/AF660 Retention of mCS and Protasan***

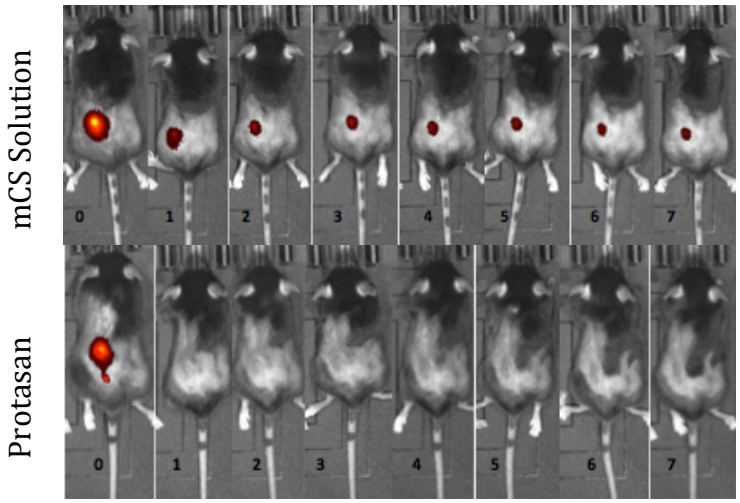

Figure 18: Mouse Photos With Fluorescent Overlays

4. Discussion

The two hydrogels have presented unique challenges to characterization and implementation in pre-clinical studies. Pre-clinical studies rely heavily on a base of characterization studies that lend credibility to the platform and suggest that experimentation is not without reason.

The heparin-chitosan hydrogel is in very early stages of investigation. The nature of early experimentation is innately slow and arduous. Though creation, manipulation, and visual observation of these hydrogels is simple, quantification of characteristics such as degree of gelation or reaction, determination of excess polymer in solution, and LCST is more complex, unclear, and non-resolute. We have isolated a range of concentrations and viscosities of heparin and chitosan that combine to make robust gels that do not degrade via hydrolysis or other non-biological pathways over the span of 3 weeks, even at 37C.

The colorimetric assay was determined to be effective for determining the concentration of both heparin and chitosan solutions on a wide range. It was also determined that pH has a significant influence on the color-producing reaction. This means that excess chitosan, heparin, or mixtures of the two polymers will be indistinguishable in the supernatant. This assay was performed using 2-20 cP chitosan to enable homogenization and complete reaction; higher viscosity chitosan prevents homogenization and centrifugation, which is necessary to remove supernatant. Homogeneity of Hep-CS hydrogels is an obstacle for any light- or color-based quantification method; for this reason, the absorbance study should be used more as a guide than an absolute measurement of degree of gelation.

The modified chitosan hydrogel was previously described and partially characterized in research at the LVID. The primary difficulty with this hydrogel was the *in vitro* drug release study. Initial protocols and previous studies utilized FITC-BSA, a highly sensitive fluorescent protein, as the model protein for release studies. Multiple iterations of this study were inconclusive because of the sensitivity of FITC to varying pH. Sampling volume was an additional factor that may have confounded initial studies. Initial sampling volume was 10% of the dialysate, which caused drastic dilution by the end of the experiment. Mathematically, dilution factors aren't confounding, but the highly varying concentration gradients and introduction of new PBS is a variable that was later eliminated.

The final protocol utilized Alexa Fluor 488 (AF488) as the fluorescent marker conjugated to IL-12. AF488 is stable on the range of physiological pH values and did not appear to decay significantly over the span of one week. Furthermore, by releasing a tagged therapeutic protein of interest, the data will more accurately represent the true release kinetics of the hydrogel and modified chitosan solution and hold more value. While the IL-12/AF488 release study was replicated 4 times, only one of the graphs is represented in this review. Unexplained variance was observed from 24 to 72 hours in the other experiments, but final cumulative protein release was consistent across trials. Variance in fluorescence-based experiments inspired the use of ELISA (Enzyme-Linked Immunosorbent Assay) as a detection and quantification method. Experimental setup was held consistent with previous studies, but IL-12/AF488 was replaced with mouse IL-12 without AF488. Protein quantity in each sample was quantified using a colorimetric ELISA assay. Data was inconclusive and is not included in this report.

In vivo IL-12 residency studies successfully modeled protein retention of different release platforms. Results of the hydrogel's retention, however, is not as expected. The mCS hydrogel was hypothesized to retain the highest amount of protein, but the data suggests that mCS solution retains a greater amount of protein over a longer period of time. This may be attributed to the catalyst-driven decomposition of the hydrogel; it was determined that increasing volumes of catalyst increases the rate of degradation of hydrogel after initial formation. Modified chitosan solution was compared to Protasan, the standard IL-12 delivery solution used to eliminate tumors in mouse models, to evaluate whether or not results of these studies have thus far improved protein retention. The modified chitosan solution retained significantly more protein than Protasan over 7 days. There is discrepancy in the percent protein retention of modified chitosan between the two individual studies; the mCS solution retained more than twice the amount of protein in the hydrogel comparison study than in the Protasan comparison study. For this reason, a third study is ongoing to re-evaluate retention capabilities of the mCS solution, and PBS, and mCS hydrogel without catalyst to prevent rapid degradation.

Results of the investigations of both of these hydrogels provide a platform for future pre-clinical trials. These studies have been used to evaluate varying formulations of these two hydrogels and mCS solution for intratumoral delivery of IL-12. By understanding the properties of these hydrogels and controlling the release of IL-12, we may be able to increase localized immune stimulation, enhance elimination of solid tumors, and reduce widespread immunotoxicity. If successful, this conjugation could be directly translatable to the clinic as a low-cost and durable treatment for solid tumors.

5. Appendix

Protocol 1: Heparin-Chitosan Hydrogel Preparation

1. Prepare desired chitosan solution (2X desired final concentration)
    a. Dissolve chitosan (Chitoclear) solution in 2% acetic acid
2. Prepare desired heparin solution (2X desired final concentration)
    a. Dissolve heparin powder in PBS
3. Add 0.5 final volume heparin solution to a suitable centrifuge tube
4. Add 0.5 final volume chitosan solution to heparin solution, using fluidic forces to mix solutions together
5. Allow solutions to react; reaction should occur in fewer than 20 seconds

Protocol 2: Colorimetric Assay for Heparin and Chitosan Detection

1. Gel preparation
    a. Combine chitosan (Chitoclear, <20 cP, Primex) and heparin solutions using a pipette tip to stir
    b. Homogenize mixture using benchtop vortex
    c. Centrifuge gel (and counterbalance) at 6000 rpm for 3 minutes
    d. Remove 200 uL supernatant and add to new microcentrifuge tube
2. Control preparation
    a. Dissolve chitosan (Chitoclear, <20cp, Primex) at 5mg/mL in 2% acetic acid and create 8 2-fold dilutions
    b. Adjust pH of CS solutions to 5.5 using 10M NaOH

   c. Dissolve heparin at 10mg/mL in PBS and create 8 2-fold dilutions
   d. adjust pH of heparin solutions to 5.5 using 99.9% acetic acid
   e. Add 200 uL of each solution to microcentrifuge tubes
3. Add 100 uL NaNO2 (0.5M) to each microcentrifuge tube, lightly mixing solutions by rolling
4. Incubate microcentrifuge tubes in 80C water bath for 30 minutes
5. Remove solutions from water bath, allow to cool for 2 minutes
6. Add 200 uL NaOH(0.1M) and 500 uL thiobarbituric acid (0.4M), lightly mix by inversion
7. Incubate microcentrifuge tubes in 80C water bath for 10 minutes
8. Remove microcentrifuge tubes and allow to cool for 10 minutes
9. Transfer 80 uL of each solution into a labeled 96-well plate and measure absorbance at 555nm

Protocol 3: Release Kinetics Determination Using Fluorescent Proteins

1. Prepare release solutions (in triplicate) as prescribed desired and load with desired concentration of fluorescent protein
2. Load release solutions into Float-A-Lyzers (pre-treated per product sheet protocol)
   a. Float-A-Lyzers should be selected based on protein size. Molecular weight cutoff of protein should be greater than the molecular weight of the protein, but smaller than the molecular weight of the polymer or components of the release solution
3. Add 25 mL of 1X PBS to a 50 mL centrifuge tube for each Float-A-Lyzer

4. Add Float-A-Lyzers to centrifuge tubes, wrap tubes and lids in aluminum foil to prevent light exposure, and place in shaker at 37C and 200 rpm
5. Take 200 uL samples of the dialysate (PBS) at prescribed time intervals and add to black microcentrifuge tubes; keep samples at room temperature
6. Upon completion of study, add 100uL of each sample to a black-welled, clear-bottom 96-well plate and read fluorescence of samples using FITC excitation and emission filters.

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