

Analysis of Novel Carboxymethyl Chitosan/Sodium Alginate Microspheres as a  
Potential Drug Delivery System

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### **Abstract**

Although protein therapeutics are physiologically successful, over 75 percent of drugs are still administered via a subcutaneous shot; many patients are unsusceptible to this type of treatment. In this study, a combination of carboxymethyl chitosan and sodium alginate were studied as a potential coating for protein therapy, designed to be administered through the oral route in the form of a microsphere. Carboxymethyl chitosan and sodium alginate were selected due to their high biocompatibility, low toxicity, and low swelling properties. Human serum albumin (HSA) was encapsulated in microspheres. The release rate of the protein was studied in pH 2 and pH 7.5 to mimic conditions of the gastrointestinal tract by UV spectroscopy. Degradation of HSA was analyzed using HPLC. The results showed that the HSA was released at a slower rate in pH 2 compared to pH 7.5.

## Introduction

Scientists have identified over seven thousand naturally occurring peptides and they often play a crucial role in human physiology. Peptides are generally selective and efficacious signaling molecules that trigger intracellular effects when they bind to specific cellular receptors. Given their function and intrinsic properties, peptides represent a new class of therapeutics that are safe, tolerable, efficient, and specific. But most peptide drugs are only available through the parenteral route, therefore further research is needed in order to increase availability and convenience to patients.

Peptide therapeutics are recognized as a great source of potential for treating metabolic disorders and other medical disorders. They offer many advantages over conventional small molecule drugs:

1. Proteins are highly specific and carry out specialized functions that cannot be mimicked by chemical compounds.
2. Due to their specificity, proteins are unlikely to cause adverse effects in the body due to interactions with biological processes.
3. The body already naturally produces the proteins used in therapeutics so it is unlikely to ~~illicit~~ elicit an immune response
4. Protein therapeutics is a safer alternative to gene therapy treatments of diseases involving damaged genes.
5. The FDA is more likely to approve protein therapies (Muheem et al., 2016).

There are now a few commercially sold protein and peptide therapeutics due to the

research efforts in both academic and industrial laboratories. The peptide drug market has had great success: in 2011, the peptide-based medicine Lupron™ from Abbott Laboratories achieved over 2.3 billion USD for treatment of prostate cancer (Kaspar & Reichert, 2013). Clearly, peptide therapeutics are not only successful in maintaining health, but also are successful in the global market. Additionally, the therapeutics industry is expected to increase at a compound annual growth rate of 10.3 percent and total 47 billion USD in revenue by 2025 (Fosgerau & Hoffman, 2015). Obviously, there is a large market for protein therapy, yet there are not many formulations available due to many failed potential attempts at creating new drugs.

A number of challenges hinder the development and use of protein pharmaceuticals. Not only are there intrinsic weaknesses, but also a combination of poor permeability characteristics and clearance mechanisms results in low bio-availability from oral and non-oral mucosal routes (Hamley, 2007). As a result, about 75 percent of peptide drugs are administered by the parenteral route (Transparency Research Market, 2015). The parenteral route involves a subcutaneous injection which significantly lowers the number of barriers the peptide must overcome through other administration methods. However, penetrative peptide formulations require repetitive dosing due to short half-lives. Even successful formulations can face problems. For example, according to a study done by the American Diabetes Association found that only 24.4% of patients with Type 2 diabetes were very willing to receive insulin therapy, a drug that is administered through the parenteral route. Almost 50 percent of those surveyed cited anticipated pain from needles and restrictiveness as reason for unwillingness (Polonsky et al., 2005). In

spite of the fact that insulin therapy helps diabetics achieve glycemic control, patients still shy away due to the administration form's implications. Additionally, some studies have shown that daily fluctuating blood glucose, which is influenced by insulin therapy, may be associated with lower cognitive performance (Abbatecola, 2006). If the drug did not require frequent dosage due to its short half-life, then fluctuating blood glucose and its adverse effects may be lessened. Frequent injections, patient acceptability, and changing drug concentration in the blood creates many drawbacks for an otherwise advantageous drug. Researchers are looking to formulate other forms of peptide therapy that will be less invasive and have a higher efficacy.

The most attractive form of drug delivery is through the peroral route. Otherwise known as the oral route, peroral administration is when the drug is placed in the mouth and swallowed (Sultatos, 2007). Benefits from oral delivery include ease of administration and patient acceptability. Studies have shown that patients have a higher chance of accepting treatment when delivered in an oral form because drugs are easy to administer (Weidman Evans, Metz, & Evans, 2014). Doctors also find it easier to prescribe oral drugs since patients do not have to have as regular checkups. Additionally, drugs that are orally administered tend to have extended release, which allows for a longer duration of action (Evans & Krentz, 1999). Clearly, oral peptide drugs would benefit not only the patient, but also the doctor.

Although the idea of oral protein therapeutics is promising, there are many barriers that hinder the development of these drugs. Two major barriers are enzyme degradation and intestinal permeability. To begin, in the gastrointestinal tract (GI tract),

there are many peptidases, enzymes that hydrolyze peptide bonds and work together to degrade protein (Woodley, 1994). They function to break down the food people eat. In order to develop a usable protein drug, researchers must find a way to prevent proteins from being hydrolyzed. If the protein were to be degraded, then the function of the protein would change, leaving the drug useless. Some solutions include inhibiting proteolytic enzymes or providing protection for the drug. Another barrier is intestinal transport issues. Most therapeutic peptides and proteins are hydrophilic, so they would not be able to follow the transcellular route of absorption in the intestines through passive diffusion (Camenisch et al., 1993). If the drugs cannot be absorbed, then they cannot reach the blood stream and induce a response in the body. A permeation enhancer could be used to increase the chances of the drug being absorbed into the blood stream. Although many of the barriers that inhibit the development of protein therapeutics can be solved, there are still questions about whether the solutions to those problems (use of enzyme inhibitors or permeation enhancement) are beneficial in long term treatment because of possible absorption of unwanted proteins, disturbance of digestion, and uncontrolled feedback regulation (Shah et al., 2002).

In order to combat the barriers of oral protein therapeutics, novel carriers for protein delivery including emulsions, nanoparticles, microspheres, and liposomes have been used to protect the drugs from the environment of the GI tract. One of the most promising carriers is microspheres. They function by coating the protein with a substance, usually a polymer. By restricting the release of the protein to favorable areas of the GI tract, microspheres are a valuable drug formulation carrier vehicle to consider.

Some drawbacks are that microspheres have difficulty with precise control of release and avoidance of particle aggregation (Muheem et al., 2016).

In this experiment, a novel microsphere was tested as a potential drug carrier. The materials used were carboxymethyl chitosan and sodium alginate. Chitosan is a

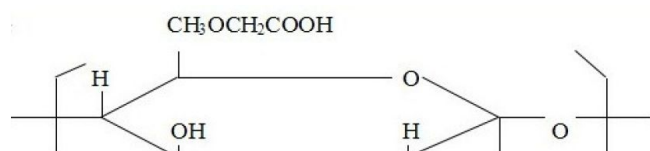
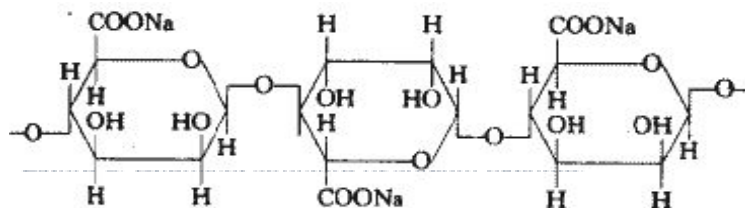


Figure 1. Chemical structure of carboxymethyl chitosan.

derivative of chitin and is a biopolymer that has high bioavailability and low toxicity. It was chosen for the microsphere because of its ability to open

tight junctions in the intestinal epithelial cell and facilitate the transport of drugs (Derakhshandeh & Fathi, 2012). The addition of the carboxymethyl group in place of the hydroxyl groups increase the

water solubility of the drug while maintaining its other characteristics (Upadhyaya et al., 2014). Derived from algae,



SA is nontoxic and biodegradable when given orally. SA shrinks at low pHs, such as the gastric environment, and thus the encapsulated drugs are not released (Lupo et al., 2014). The higher the swelling degree, the more protein is released from the microspheres. HSA was chosen as the model protein because of its stability. The release kinetics from the microspheres were tested in pH 2 and pH 7.5 to mimic the conditions of the gastrointestinal tract and structural integrity of HSA was analyzed.

### **Question**

Can carboxymethyl chitosan/sodium alginate microspheres serve as a potential drug delivery system by demonstrating extended release properties and maintaining structural integrity?

### **Hypothesis**

Human serum albumin encapsulated in carboxymethyl chitosan and sodium alginate will be released from the microspheres and will maintain structural integrity in low pH.

### **Materials**

#### **Reagents**

- Sodium Alginate was obtained from Sigma Aldrich.
- Carboxymethyl chitosan was purchased from Santa Cruz Biotechnology. The deacetylation degree of the chitosan was 90%.
- TWEEN® 20, also called Polyethylene glycol sorbitan monolaurate from Sigma Aldrich
- Tris-hydrochloride ( $\text{NH}_2\text{C}(\text{CH}_2\text{OH})_3 \cdot \text{HCl}$ ) buffer from Sigma Aldrich
- Albumin, Human Serum, 25% Aqueous Solution in Tris-HCl buffer (HSA) from



Sigma Aldrich. Purity is >95% by agarose electrophoresis.

- Insulin human, >95% (HPLC), semisynthetic, powder form from Sigma Aldrich.
- Paraffin oil analytical grade
- Calcium Chloride ( $\text{CaCl}_2$ ) analytical grade
- Acetic acid ( $\text{CH}_3\text{COOH}$ ) analytical grade
- Ethanol ( $\text{C}_2\text{H}_6\text{O}$ ) analytical grade
- Hydrochloric acid (HCl) analytical grade
- Deionized water ( $\text{diH}_2\text{O}$ ) analytical grade
- Trifluoroacetic acid ( $\text{C}_2\text{HF}_3\text{O}_2$ ) analytical grade
- Acetonitrile ( $\text{C}_2\text{H}_3\text{N}$ ) analytical grade

### **Instrumentation**

- Beckman Coulter DU-650 Spectrophotometer
- Aglient 1100 Series High Performance Liquid Chromatography (HPLC)
- Beckman Coulter Allegra 6 High Speed Centrifuge
- Corning® LSE™ High Speed Microcentrifuge

### **Methods**

#### **Preparation of Microspheres**

Carboxymethyl chitosan (CM-chitosan) prepared at 5% wt and 8% wt sodium alginate was prepared in 20 ml of deionized water. The mixture was stirred in a 250 ml Erlenmeyer flask for 30 minutes on a stirring plate at medium speed, until the solution

appeared to be homogenous. Then, 1 ml of 25% human serum albumin protein (250mg) was added to the solution. The mixture was then stirred for 15 minutes to create a blend solution. The microspheres were prepared by emulsion phase separation carried out at room temperature. The aqueous solution containing the blend solution and HSA was dispersed in 40 ml of paraffin oil containing 1% TWEEN-20. With a mechanical stirrer at 900 rpm, the solution was stirred for 3 hours, until a paraffin system was formed with water-in-oil emulsion. To harden the microspheres, an aqueous  $\text{CaCl}_2$  solution containing 1 g  $\text{CaCl}_2$ , 10 ml  $\text{dH}_2\text{O}$ , 7.5 ml ethanol, and 0.25 ml acetic acid was slowly dropped into the system. The dispersion was mixed for 2 hours.

### **Isolation of microspheres**

From the solution, the microspheres were removed from the flask and placed into a 50 ml Falcon tube. Only microspheres smaller than 10  $\mu\text{m}$  were removed. The microspheres were then centrifuged at 3000 rpm for 10 minutes. The remaining phases were removed from the tube, leaving only the microspheres.

### **Morphological Analysis**

To study the shape of the microspheres, the shape of the microspheres after each treatment was viewed under a microscope at 10x magnification. To determine the average size of microspheres, an average of three samples viewed under the microscope was analyzed.

### **Determining Encapsulation Efficiency**

To determine encapsulation efficiency, the following equation was used:

$$\text{Encapsulation efficiency} = \frac{\text{Amount of HSA released}}{\text{Loading amount of HSA}} \times 100$$

The amount of HSA released into the solution after 48 hours was assumed to be the total amount of protein encapsulated by the microspheres. The total amount of HSA loaded was the original amount added during the making of the microspheres.

### **HSA Release from Microspheres**

To test the release of the protein from the microspheres, 10 ml of isolated microspheres were added to a new 50 ml Falcon tube along with 15 ml of buffer (one sample 50 mM Tris-HCl at pH 2 and another sample 50 mM Tris-HCl buffer at pH 7.5). At selected time intervals, 750  $\mu\text{g}$  of the supernatant was collected and placed in an Eppendorf tube.

Before each collection the microspheres were centrifuged and after each collection the microspheres were lightly agitated to ensure the released protein was released into the supernatant. Sample retrieval was performed in replicate to improve accuracy.

To calculate the release, each sample was centrifuged at low speed for one minute to ensure a purity. Then, each sample was assayed using UV spectroscopy to measure its absorbance at 280 nm. The data was plotted using Microsoft Excel to analyze the data.

### **Analysis of Structural Integrity**

To determine whether the HSA was degraded in the acidic and neutral

environments, samples from the released microspheres were analyzed. Samples from 0.1 M HCl and Tris-HCl buffer were syringe filtered using a 2  $\mu\text{m}$  filter. By using reverse phase high performance liquid chromatography, samples from hours 0, 24, and 48 were analyzed. A Zorbax® 300 SB C<sub>18</sub> column (4.6x150 mm, 5 micron) was used. The mobile phase was run with a linear gradient of Solvent B (0.1% TFA in acetonitrile) from 0-100% in 30 minutes over Solvent A (0.1% TFA in H<sub>2</sub>O). Each injection was 100  $\mu\text{L}$  and the flow rate was 0.9 mL/minute. The temperature was 40 degrees Celsius. Peaks detected at 280 nm were analyzed for the detection of tryptophan groups in the HSA to determine degradation.

## Results

### Morphology of Microspheres

The surface and internal morphology of the CM-CS/SA microspheres are depicted in Figure 3a and 3b. The images depicted a smooth surface, but the shape of microspheres tended to be irregular when they contained HSA. Figure 3a shows microspheres treated in 50 mM Tris-HCl buffer (pH 7.5) after 48 hours whose average diameter was 9.2  $\mu\text{m}$ . Figure 3b shows microspheres treated in a 0.1 M HCl solution

*Figure 3a and 3b. a. The image to the left shows microspheres treated at pH 7.5 after 48 hours. B. The right shows microspheres treated in pH 2 for 48 hours.*

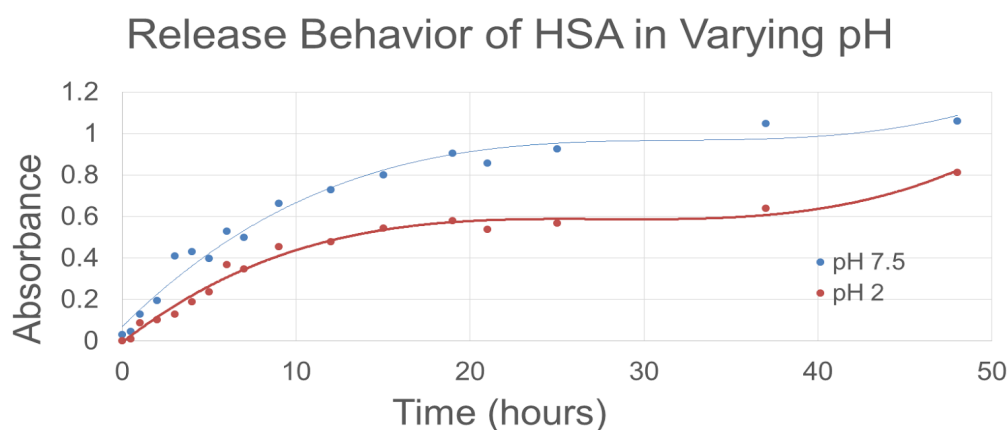
(pH 2) after 48 hours. The average size of those treated in pH 2 was 6.8  $\mu\text{m}$ . Before treatment the average size of microspheres was 4  $\mu\text{m}$ , but after treatment the average overall size was 8  $\mu\text{m}$ .

### **HSA Release from Microspheres in Different Environments**

The average absorbance of two trials was graphed. The microspheres demonstrated an extended release of protein throughout its treatment in varying pH (Figure 4). As time went on the increase of protein concentration also increased. Both also showed a quick initial release since the absorbance quickly increased, but slowed down near the end of the time course. Microspheres treated in pH 7.5 had a higher overall absorbance at 280 nm compared to those treated in pH 2. Both treatments resulted in a plateau in protein concentration increase at 20 hours. After 20 hours, microspheres in pH 2 demonstrated a greater increase in HSA absorbance over the 20

hour to 48 hour time period.

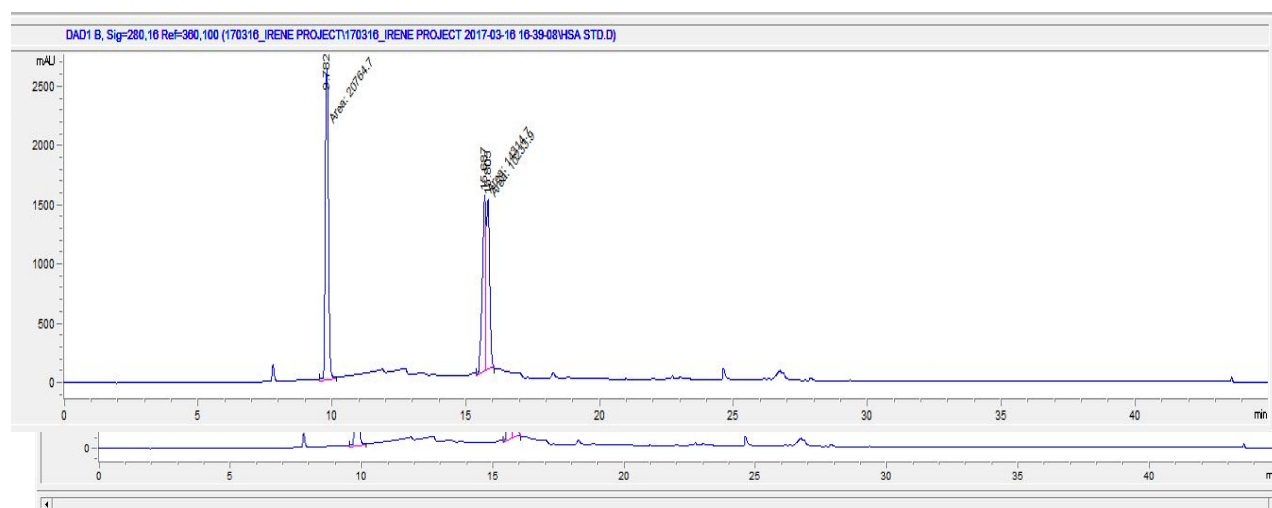
Assuming that when the absorbance was above 1.0 all the HSA had been released, the release curve shows that most of the HSA was observed to have a quick release. In the microspheres treated in pH 7.5, about 60% of the protein was released within the first 10 hours. In comparison, those treated in pH 2, only about 40% of the protein was released.

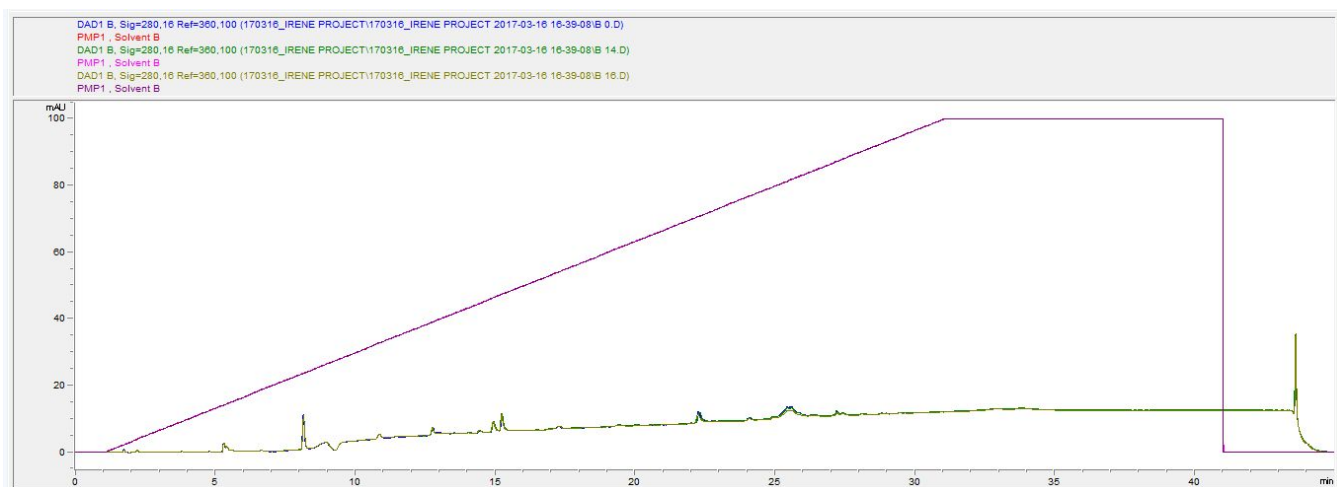
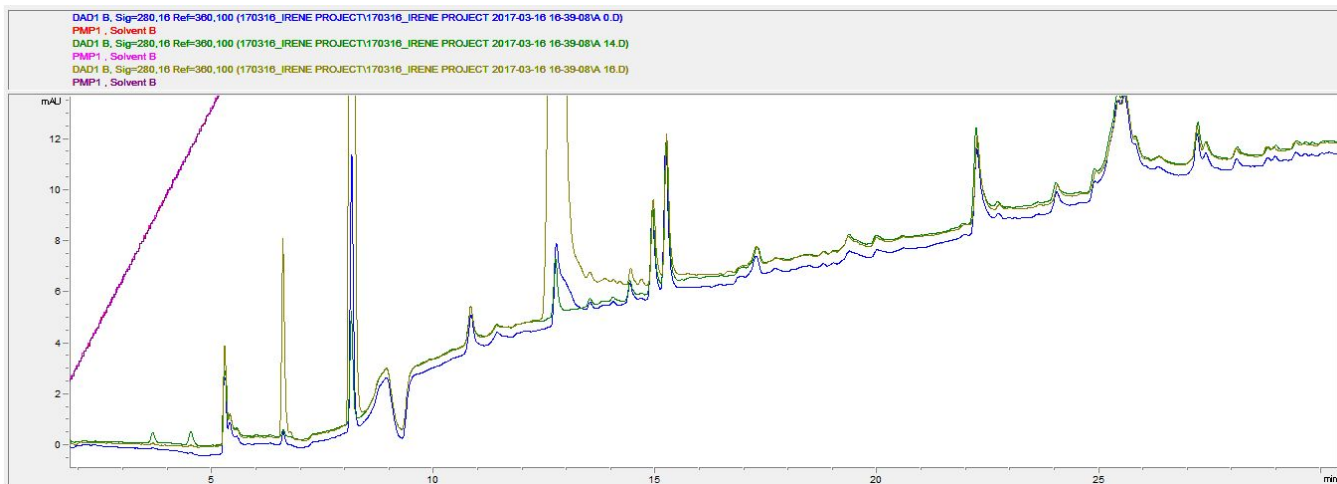


### HPLC Analysis of Structural Integrity

To determine whether HSA had maintained its structural integrity through the making of microspheres and the treatment in varying pH, the last part of the experiment was designed to determine whether HSA had been broken down. The wavelength used was 280 nm, which detects the tryptophan groups of HSA. A standard solution of HSA with the calculated expected concentration (1 mg/mL) was run and it showed two peaks with the height of 2600 and 1480 mAU (Figure 5). The treated samples were compared to the standard to analyze integrity. Figure 6 shows a chromatogram with data from 0,

24, and 48 hours of treatment in pH 2 overlaid. At all time points within the acidic pH treatment, there were two distinct peaks present, although the baseline contained noise. The presence of peaks signifies the maintenance of protein structure. The largest increase in peak height was between hours 24 and 48. At 48 hours, where protein concentration is the greatest, the peak height was 230 and 80 mAU, which was significantly lower than the standard. In the chromatogram from the basic environment treatment, there were also two distinct peaks, showing that there were tryptophan groups intact. The baseline was also unclear, with many peaks of its own. In the overlaid chromatogram of samples treated in pH 7.5, there is little increase in peak







*Figure 7. Chromatograms of HSA released from microspheres treated in pH 7.5 from 0, 24, and 48 hours detected at wavelength 280 nm. The top shows a complete chromatogram while the bottom shows a zoomed version. The data from hours 0 (blue), 24 (green), and 48 (yellow) are shown.*

## Discussion

### Morphological Analysis

The microspheres maintained their small size due to the high speed spinning in their formation. The pH dependence of swelling is shown in Figure 3a and 3b. The microspheres in the acidic environment had a lower degree of swelling as seen by looking at their shape compared to the microspheres treated in the neutral environment. Therefore, the swelling ability of the microspheres decreased in the 0.1 M HCl solution. Those treated in pH 2 had a lower swelling degree because of the dense structure created by the protonated amino groups of CM-CS (Zhang, Guo, Peng, & Jin, 2003). The degree of dissociation in the chitosan was restricted, resulting in a loss of swelling. Some research shows that this occurred because the ability of CM-CS to swell may have been suppressed and the chitosan may have formed some loops according to previous studies by Zhang et al. (2003). A less dense structure resulted due to loop

formation and enhancement of the electrostatic interaction between –COOH groups of the SA and –NH<sub>2</sub> groups of the CM-CS in low pH (Zhang et al., 2003). Therefore, the microspheres treated in pH 2 tended to have a smaller diameter than those treated in pH 7.5. In comparison, microspheres treated in neutral pH had a higher swelling degree because the biopolymers maintained their structure and swelling properties under a pH similar to that of water. Therefore, microspheres in the Tris-HCl buffer were less dense after treatment.

The encapsulation efficiency observed was low. This may have been because the biopolymers were unable to capture the protein. A contributing source of error for the low encapsulation efficiency to occur is that the ratio of oil to solution may have been too high. Since the HSA is trapped in the ionized lattice of CM-CS/SA when a CaCl<sub>2</sub> solution is added according to Lemoine et al. (1998), the initial concentration of the biopolymers may not have been sufficient if over half of the protein was unable to be captured. The encapsulation efficiency must be improved if the CM-CS/SA microspheres were to be used as a potential drug carrier since many peptide drugs are expensive and the production cost would be high.

### **HSA release from microspheres in different environments**

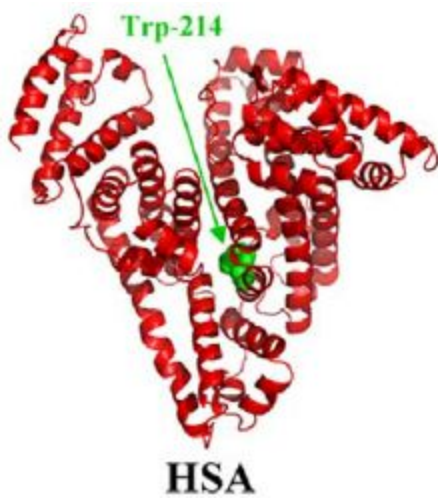
The results agreed with the expected variance in swelling degree between microspheres treated in pH 2 and pH 7.5. HSA was released at a slower rate at pH 2 due to the lower swelling degree. HSA was released at a faster rate at pH 7.5 since CM-CS/SA have higher permeability due to higher swelling ability in neutral conditions.

The process for the drug release was through diffusion across the CM-CS/SA membrane, so the higher the swelling, the faster the release. At pH 2, the drug diffused slower because the protein could not pass through the membrane due to the stronger interaction between CM-CS and SA in the acidic environment (Leach & Schmidt, 2004). In neutral conditions, the swelling was much higher and this resulted in a large amount of HSA being released from the microspheres by diffusion through the less dense membrane.

In application to the microspheres' potential as a drug delivery system, the low swelling and release in low pH is beneficial. In the GI tract, the peptide drug must pass through the acidic environment of the stomach (pH 1-2) and later pass through the intestines (pH 7.4) where absorption occurs. Since the stomach contains many protease enzymes that break down protein, the drug deliverer cannot release the drug into the stomach, or else the protein will be broken down. It instead should be released when in the small intestines, where the peptides can be absorbed into the blood stream. Accordingly, the release kinetics of the CM-CS/SA microspheres show the potential to be useful in drug delivery. There was a lesser release in low pH and a greater one is neutral pH. But, some protein was still released into the stomach, where structural integrity cannot be ensured. Also, the release kinetics showed an extended release which is beneficial to patients. Drugs with an extended release stay in the body longer and result in a more controlled administration of drugs, especially in patients who must repeatedly administer therapy for metabolic disorders such as diabetes (Neutel, Zhao, & Karyekar, 2013).

### HPLC Analysis of Structural Integrity

Even if the CM-CS microspheres have the proper release kinetics to form a drug delivery system, the structural integrity of the protein must be maintained. Since a protein's structure is directly related to its function, the protein must remain intact. HSA structures were detected at 0, 24, and 48 hours in both acidic and neutral environments. The wavelength of 280 nm was used to measure tryptophan groups in HSA.



In both acidic and neutral environments, the structural integrity of the tryptophan group was maintained due to the initial stability of HSA and the biopolymer coating. In all test groups, the tryptophan was able to be detected. The standard for HSA showed much higher peaks than the treatment groups. Additionally, the treatment groups did not have clean baselines, there was a lot of noise. This may have been because portions of the microsphere were broken down and portions of it were detected in the HPLC. It also may have been because a portion of the HSA was broken down, leading to the unclear baseline. This would also account for the difference in concentration between the expected calculations from the UV spectrophotometer and the concentrations shown on the HPLC.

The peak height also shows that the concentration increased in each time period. Therefore, the release of protein also matches the data shown in the readings from the

UV spectrophotometer. The sudden jump in increase in low pH could be attributed to the slow release of protein due to the low swelling factor. In comparison, the peaks of the samples treated in neutral pH did not have as significant change because most of the protein was released within the first 24 hours since its swelling capacity is much larger than the microspheres in the low pH.

### **Conclusion**

The current study showed that CM-CS/SA microspheres prepared by a water in oil emulsion has the potential to be a peptide drug delivery system through the parental route. The slower release of HSA in low pH due to the lower swelling degree is important for the development of oral protein therapeutics because the drug must be released in the small intestine where it is absorbed. Therefore, if there is a slower release, then the drug has a lower chance of being released into the stomach and begin degraded by proteolytic enzymes. The release kinetics of the HSA also showed an extended release, which is a more advantageous drug design. Because the microspheres maintained faster release kinetics in neutral pH, most of the protein would be released in the intestines where it would be absorbed. The CM-CS microspheres were also able to preserve the structural integrity of HSA over a 48 hour period without being degraded in both pH 2 and pH 7.5. Since the structural integrity was maintained, the drug would still be useful and create a response in the human body if the microspheres were used as a drug carrier. Orally administered insulin is still far from a reality, but it is closer than it seems. Since the CM-CS/SA was able to coat HSA, it shows that biopolymer microspheres have release kinetics that align with physiological

conditions and may maintain protein structure in low pH.

### **Further Research**

A source of error that may have factored into the absorbance of the protein while analyzing using UV spectroscopy was that the microspheres residues may have entered the sample. When the treated samples of microspheres were centrifuged to collect the supernatant, the microspheres actually were less dense than the solutions and collected on top of the supernatant. To collect the solution carrying the released protein, a micropipette was used. The tip was submerged under the sample and then the sample was collected. Because of this technique, some of the treated microspheres may have been collected in the samples that were read by the UV spectrophotometer, making the absorbance higher than it actually should be. To combat this flaw, the microspheres should be further hardened using isopropyl alcohol because previous studies have shown that the density increases with the isopropyl alcohol's addition (Zhang et al., 2003). Then, when retrieving the supernatant, only released protein would be collected, providing more accurate results.

Because the experiment did not factor in enzymes that degrade protein, the potential use of the CM-CS/SA microsphere is limited. In the future, I would like to repeat the experiment with treatment of microspheres under enzymatic conditions while adding a protease inhibitor cocktail to microspheres.

Additionally, the encapsulation efficiency was low. Repeating the experiment and trying to improve the efficiency is necessity because much of the protein is going to

waste and would not be resourceful if the microsphere was to be continually studied. A potential solution could be to decrease the amount of paraffin solution added when making the microspheres.

Testing intestinal permeability of HSA after treatment of microspheres for absorption into bloodstream would also show if the microspheres should be continually studied. A possible testing method would be to see if the microspheres can release the protein across a skin like membrane. Then, the sample could be run through HPLC to see if any HSA was detected in the solution that passes through the membrane.

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