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Synthesis of Drug Encapsulated Chitosan-Heparin Based Nanoparticles and Release Study

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Honor's Project 4200:497

I affirm that this report represents work performed by me and I assume full responsibility for originality, comprehension, and accuracy of all aspects of the report.

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Contents

Abstract

The main objective of this honor's project is to encapsulate drugs with different molecular weights inside of chitosan-heparin based nanoparticles (Cs-Hep NPs). Three different drugs with low (NNC), medium (IFN-γ), and large (BSA) molecular weights were chosen in order to compare all types of drugs and how they react with the nanoparticles. Analysis of particle size, zeta potential, and a release study was performed. The release took place over a 168-hour time period and liquid samples were taken throughout. The hypothesis was that the molecular weight of the drug will correlate to the physical properties of the nanoparticles.

The results of the particle size analysis show that the largest particle size was determined to be interferon gamma (IFN-γ, ~2500nm), NNC-042090 (NNC, ~450nm), Nanoparticles (~350nm), and bovine serum albumin (BSA, ~50nm). The results of the zeta potential show that the encapsulated particles have a higher electro potential than empty nanoparticles. NNC released 80% of the encapsulated drug, BSA released 70% of the encapsulated drug, while IFN-γ only released about 50% of the encapsulated drug. Therefore, judging from these results, the hypothesis partially holds true for all drugs.

Executive Summary

Problem Statement

Drug delivery systems were first developed in the 1950s to help control the release of drugs ingested by the human body. This allows for a constant release rate of the drug that would otherwise be toxic with an uncontrolled release. Nanoparticles (NPs) are one of the best solutions to this problem because of their great compatibility with the human body, the amount of flexibility the nanoparticles present, and because of their ability to control the release of the drugs. Chitosan and heparin were chosen to be the main components of the nanoparticles because of chitosan's ability to deliver drugs through various applications and also because of its positive surface charge. Heparin was chosen because of its coagulative properties and because of its overall negative surface charge. The synthesis of the nanoparticles involves an excess of heparin compared to the chitosan; therefore, it can be assumed that the nanoparticles have a net negative surface charge.

Three different drugs with low, medium, and large molecular weights were chosen in order to study the encapsulation of each drug in the nanoparticles. Using different molecular weights will give a good understanding of all types of drugs and how they react with the nanoparticles. In order to quantify the encapsulated nanoparticles, a particle size comparison, zeta potential comparison, and a release study was performed. The particle size comparison determines the average radius of each of the drugs that are encapsulated in the nanoparticles. The zeta potential comparison shows the difference in the electro potential between the surface of the nanoparticles and the surrounding fluid. This study helps show the stability and the degree of electrostatic repulsion between the encapsulated nanoparticles. The release study was performed three times for each of the three drugs in order to increase accuracy. Franz cells were placed inside of an incubator at 37°C to carry out the release. The release took place over a 168-hour time period and

liquid samples were taken every hour for the first eight hours, then every four hours until hour 32, then every eight hours for until hour 56, and finally every twelve hours for until hour 72.

Results/Discussion

The results of the particle size analysis show that the largest particle size was determined to be interferon gamma (IFN-γ, ~2500nm), NNC-042090 (NNC, ~450nm), NPs (~350nm), and finally bovine serum albumin (BSA, ~50nm). These results are not what was expected from the initial hypothesis because NNC has the lowest molecular weight but has the second highest particle size and BSA has the highest molecular weight yet has the smallest particle size. These results could be due to coagulation of the particles which cause improper readings and results during analysis. The results of the zeta potential show that the encapsulated particles have a higher electro potential than empty nanoparticles. These results were expected according to the initial hypothesis.

The results from the release study show that all the drugs followed a general logarithmic trend for the release from the encapsulated nanoparticles. However, at the end of the 168-hour trial, NNC released 80% of the encapsulated drug, BSA released 70% of the encapsulated drug, while IFN-γ only released about 50% of the encapsulated drug. These results do not support the initial hypothesis of the dependency on the molecular weight for the released amount and release time.

Conclusions

The initial hypothesis for this study was that there was a constant relationship between the release amount and release time of the encapsulated nanoparticles depending on the molecular weight of the chosen drug. For some instances in this study the hypothesis holds true, however for other instances it does not. For example, the hypothesis doesn't hold true for the particle size because the drug with the lowest molecular weight has the largest particle size while the largest

molecular weight has the smallest particle size. Therefore, the particle size has an inverse relationship to the molecular weight. This phenomenon could be caused by the clumping of the smaller sized drugs during the analysis of the particle radius; therefore, obscuring the results. The zeta potential experiment proved that the encapsulated nanoparticles have higher electro potential than empty nanoparticles which was the expected outcome at the beginning of the project.

The release study showed an overall release of 80% for NNC particles, 70% for BSA particles, and 50% for the IFN-γ particles. The results of the analysis of the IFN-γ samples should be considered unreliable because of possible issues from the ELISA assay. Therefore, if only the results from the BSA and NNC release studies are considered, the results align with the initial hypothesis. This is because NNC has the lowest molecular weight and should therefore release more volume quicker than a higher molecular weight drug. However, the release rate of the NNC from the NPs were slower than expected which could be due to NPs clumping together during the release. The results of the release studies were compared to standard curves for each of the drugs which can be seen in **Figures 4-9**. These results do not fully correlate to any trend with the molecular weights of the drugs. Therefore, more analysis should be considered.

Introduction

The modern-day drug delivery systems are only 60 years and have be developed and advanced for the human body.[1] However, there are still multiple discoveries to be made to improve the drug delivery inside the human body, specifically in the CNS (central nervous system). The main objective of this honor's project is to encapsulate drugs with different molecular weights inside of chitosan-heparin based nanoparticles (Cs-Hep NPs) that are beneficial for various applications in the human body. For example, healing and regeneration in the CNS is one such application. The characterization of Cs-Hep NPs such as particle size, zeta potential, etc. were conducted when these drugs/proteins are encapsulated. Multiple release studies were performed to record the rate at which the drugs/proteins will be released from the nanoparticles. After the testing is complete, all the data were assessed in order to compare and summarize the differences between the drugs'/proteins' release rate from the NPs with respect to molecular weight. The two specific objectives of this project as described below:

1. Synthesis and characterization of the drug encapsulated Cs-Hep NPs

The first step for completing this project was to synthesize and characterize the Cs-Hep NPs system. The lab had already synthesized these nanoparticles successfully in previous procedures.[2] Next, the encapsulation process of the drugs was completed. Three different drugs were used which were, interferon-γ (IFN-γ), Bovine Serum Albumin (BSA), and NNC-042090. The main hypothesis for this objective is that synthesized Cs-Hep NPs have the capability to encapsulate the previously mentioned drug molecules. Part of this process included synthesis of NPs, encapsulation of NPs, separation of NPs by sequential centrifugation, and freeze-drying the solution of the drug/protein encapsulated NPs. This produced a dry sample that made it easy to handle and test.

2. The release study of the drug encapsulated Cs-Hep NPs

The synthesized system was assessed for a drug/protein release study using standard release protocol available in the lab. The drug samples from the release study were analyzed by using an UV spectrometer and ELISA (enzyme-linked immunosorbent assay) depending upon the drug/protein. The samples were drawn after an equal time interval to analyze the stepwise release. The hypothesis for this objective is that the whole system will provide controlled drug release.

Three different drugs were chosen to be analyzed during this experiment: bovine serum albumin (BSA), interferon gamma (IFN-γ), and NNC (NNC 05-2090). These three drugs were chosen because of their wide range in molecular weights; NNC having the lowest molecular weight, IFN-γ having the next largest, and BSA having the largest overall molecular weight. During this study, the correlation between the different molecular weights were assessed with the particle sizes, the zeta potentials, and the release study results. The hypothesis was that the trends will follow the correlation between the different molecular weights.

Background

The beginning of the modern-day drug delivery systems began in 1950 and have been very successful for creating several oral and transdermal controlled chemical release mechanisms.[3] However, as of late (1980s-present), there have been no successful clinical trials for advanced chemical release systems. These failures are caused by two different types of barriers within the human body, biological barriers, and physicochemical barriers.[1] The physical-chemical barriers have been the poor water solubility of the drugs, the large molecular weight of the drugs, and the difficulty of controlling the drug release kinetics. The main biological barrier is getting the drug to be dispersed throughout the entire body instead of being localized around the release center.

Better control over the dispersion of the drug will give more control of the dosage and concentrations of drugs being delivered.[1]

Chitosan, which is a biodegradable, biocompatible polymer, is considered safe for wound dressing applications and human consumption. Chitosan-based nanoparticles are being heavily investigated because of its ability to deliver drugs through various applications and because of its chemical functional groups that can be easily modified. Another benefit of the chitosan polymer is that nanoparticles that are formed by chitosan typically had a positive surface charge.[4]

Heparin is a naturally occurring compound produced by the liver which has become the most common anticoagulant drug used in the world today. Heparin is paired with the chitosan to be the main ingredients in the synthesis of the nanoparticles in this experiment. The heparin is used in an excess quantity than chitosan and hence it is assumed that the NPs surface has a negative charge.[5]

Experimental Methods

Materials

Chitosan (Mycodev, 90 kDa, 90% DDA), heparin sodium salt from porcine intestinal mucosa (Sigma), glacial acetic acid (Fisher Scientific), sodium acetate trihydrate, nanowater was used to synthesize NPs. Drug NNC04-2090 (Torcis Bioscience) and proteins, BSA and IFN- ɣ are used for the experiments. Pierce 660 nm Protein Assay Reagent (450mL) and pre-diluted protein assay standard sets were used to analyze the BSA and NNC from the release study. ELISA kit (Peprotech) is used to analyze IFN-ɣ.

Methods

The procedure for this project was broken down into three main parts. First, it was necessary to synthesize the nanoparticles by following a procedure previously established by the senior student in the Leipzig Bioengineering lab.[2] The procedure entailed creating an acetate buffer (pH~5) and then mixing the buffer with chitosan and heparin. The chitosan mixture should have a concentration of 0.90 mg/mL and the heparin mixture should have a concentration of 0.95 mg/mL. Next, the heparin and chitosan were mixed together at a ratio of 15 mL heparin to 5 mL chitosan. The mixture was then mixed at 800 RPM for 3 hours and left to sit overnight. The solution was then decanted, placed into a 50 mL C-tube, and centrifuged at 4300 RPM for 20 minutes with rotor JS-5.3. After the solution was centrifuged, the top 15 mL of the supernatant was removed and replaced by 15 mL of filtered nanowater. The solution was vortexed for 5 minutes and then placed back into rotor JS-5.3. The C-tubes with solution were centrifuged for 15 minutes at 4300 RPM. The C-tubes were removed from the centrifuge and 15 mL of the supernatant was removed from the tubes. The mixture was vortexed for 5 minutes and was finally stored in a -20°C freezer.

Next, the encapsulation of the drugs in the nanoparticles took place. The diluted drug (in nanowater) was then added to the nanoparticle solution by using a syringe while mixing the nanoparticle solution at 800 RPM. This ensured maximum encapsulation of the drug in the nanoparticles. After being mixed for 1 hour, the solution was transferred to a 50 mL C-tube. The tube was then transferred to the centrifuge with the JA-25.5 rotor. The solution was centrifuged at 8612 RPM (9000 x g) for 15 minutes. Then, the C-tube was taken out of the centrifuge, and 15 mL of the top supernatant was removed and replaced by 15 mL of filtered nanowater and then vortexed for 5 minutes. The C-tube was placed back into the centrifuge and ran again at 8612 RPM for 15 minutes. After complete, the top 15 mL of supernatant was removed and then the remaining

solution was vortexed and then stored in a -80°C freezer to be prepared for freeze drying. Freeze drying occurred once the solution was completely frozen.

The final part of the procedure consisted of a time release study of the three different types of drugs in the nanoparticle system. The release study took place in a Franz cell and was conducted over the time period of a week. Below, in **Figure 1**: Setup of a Franz Cell, the setup of the Franz cell for the release study is shown.

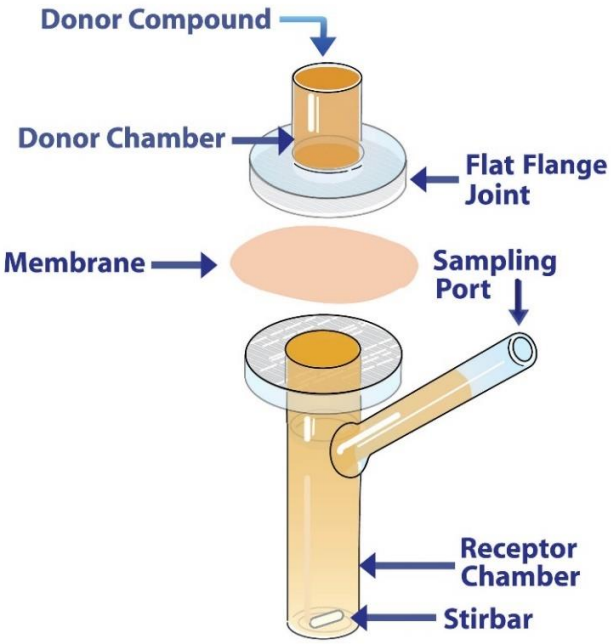

Figure 1: Setup of a Franz Cell

The receptor chamber was first filled with 15 mL of Phosphate Buffered Saline (PBS) in order to control the pH of the solution and also act as a drying agent for the proteins to encourage denaturation. Next, the stir-bar was added along with the membrane, flat flange joint, and a clamp to secure the connection between the donor and receptor chambers. Once the connection was secured, 3.5 mL of the drug/PBS mixture was added to the donor chamber. Nine total release studies (3 drugs x 3 runs) were performed, which included three studies of each encapsulated drug

and a standard study for each nonencapsulated drug. For the encapsulated drug samples, the ratio of PBS to the encapsulated drug was 1 mL to 2.5mL in the donor chamber.

To start the release study, the Franz cells were placed onto a stirring plate in an incubator in order to simulate internal human body conditions. The stirring plate was set to 200 RPM in order to keep the solution moving and to prevent stagnation in the receptor chamber. A sample (~0.2mL) was taken from the Franz cell through the sampling port every hour, for eight hours, then every four hours for 32 hours, then every eight hours for 56 hours, and then finally every twelve hours for 72 hours. After the sample was taken, the Franz cell was replenished with an equal amount of fresh PBS that was added through the sampling port. Samples were taken with needles attached to 1 mL syringes. Each sample was clearly labeled and stored in 2 mL tubes which were placed in a -20°C freezer until analyzed.

Analysis of the BSA samples collected after the release study was conducted using Pierce 660 nm assay (the protocol used was published by Thermo Scientific).[6] While the NNC samples were analyzed using the UV spectrometer at 290 nm to determine the concentration of each drug as time increased through each sample. First, the standard curve was determined using the known concentration of the drug/protein using the same respective protocols.

To analyze the IFN-γ samples, an ELISA protocol was followed which was published by PeproTech.[7] After all data was collected, it was analyzed and summarized.

Data and Results

The figures below show the results of the experiments performed throughout this study. **Figure 2** relates to the particle size of the NPs. Error bars have been added to the figure to show the variance of all samples tested. The letters A, B, and C represent the difference between the

samples. If they are the same letters then there is no significance between the groups, and if the letters are different then the groups are significantly different. The particle size was calculated for the empty nanoparticles, the BSA encapsulated NPs, the NNC encapsulated NPs, and the IFN-γ encapsulated NPs. The particle diameter (nm) is the parameter being compared in **Figure 2**.

Figure 3 relates to the zeta potential of the different types of NPs. The zeta potential was used to express the electro-kinetic charge that developed between the surface of the nanoparticles and the liquid surrounding the nanoparticle.[8] Error bars have been added to the figure to show the variance of all samples tested for zeta potential. The letters A, B, and C represent the difference between the samples. If they are the same letters then there is no significance between the groups, and if the letters are different then the groups are significantly different. **Figure 3** shows the zeta potential in millivolts for each encapsulated nanoparticle.

Figures 4, **5** relate to the BSA trials. **Figure 4** shows the standard curve of the BSA encapsulated NPs. **Figure 4** was used as a reference for the different absorbance values captured from the release study when being evaluated. **Figure 5** shows the data collected from the BSA release study trials. Error bars are included to show the amount of variation between the data collected and the standard curve from **Figure 4**. **Figure 4** shows the percentage of cumulative release of the BSA per hour.

Figures 6, **7** relate to the NNC trials. **Figure 6** shows the standard curve of the NNC encapsulated NPs. **Figure 6** was used as a reference for the different absorbance values captured from the release study when being evaluated. **Figure 7** shows the data collected from the NNC release study trials. Error bars are included to show the amount of variation between the data

collected and the standard curve from **Figure 6**. **Figure 7** shows the percentage of cumulative release of the NNC per hour.

Figures 8, **9** relate to the IFN-γ trials. **Figure 8** shows the standard curve of the IFN-γ encapsulated NPs. **Figure 8** was used as a reference for the different absorbance values captured from the release study when being evaluated. **Figure 9** shows the data collected from the IFN-γ release study trials. Error bars are included to show the amount of variation between the data collected and the standard curve from **Figure 8**. **Figure 9** shows the percentage of cumulative release of the IFN-γ per hour.

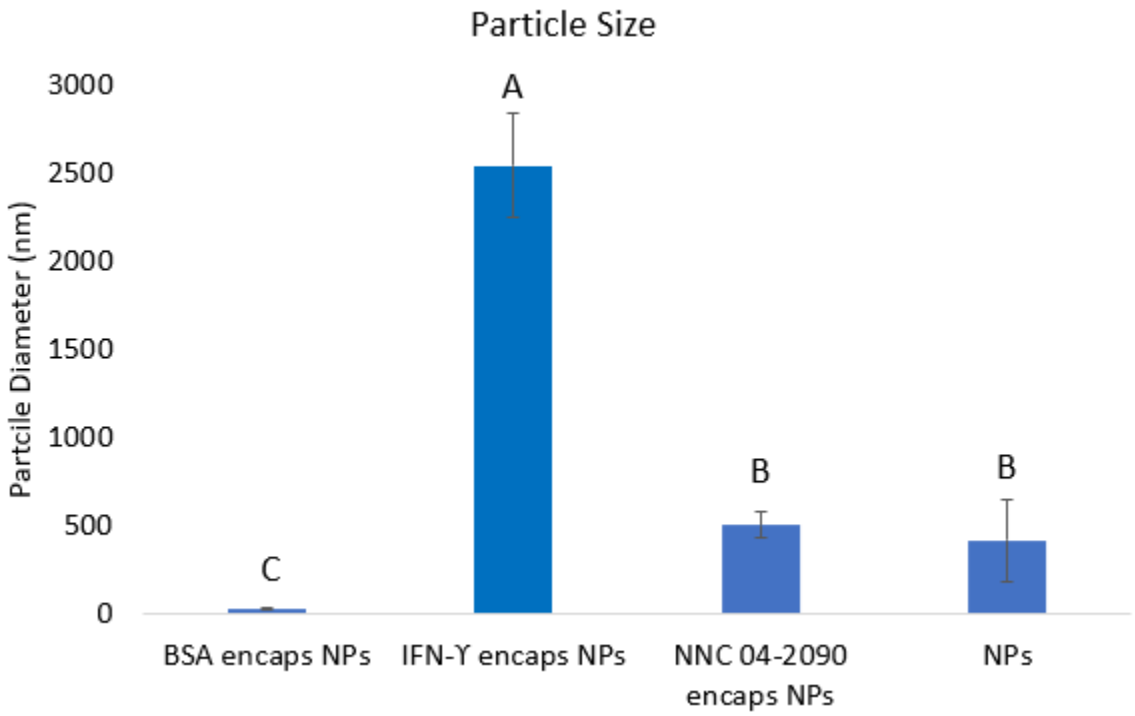

Figure 2: Particle size of the nanoparticles only (NPs) and drug/protein encapsulated NPs. Results are presented as a mean of 3 different trials with error bars shown as standard error. The letters A, B, and C relates data within 20% of each other.

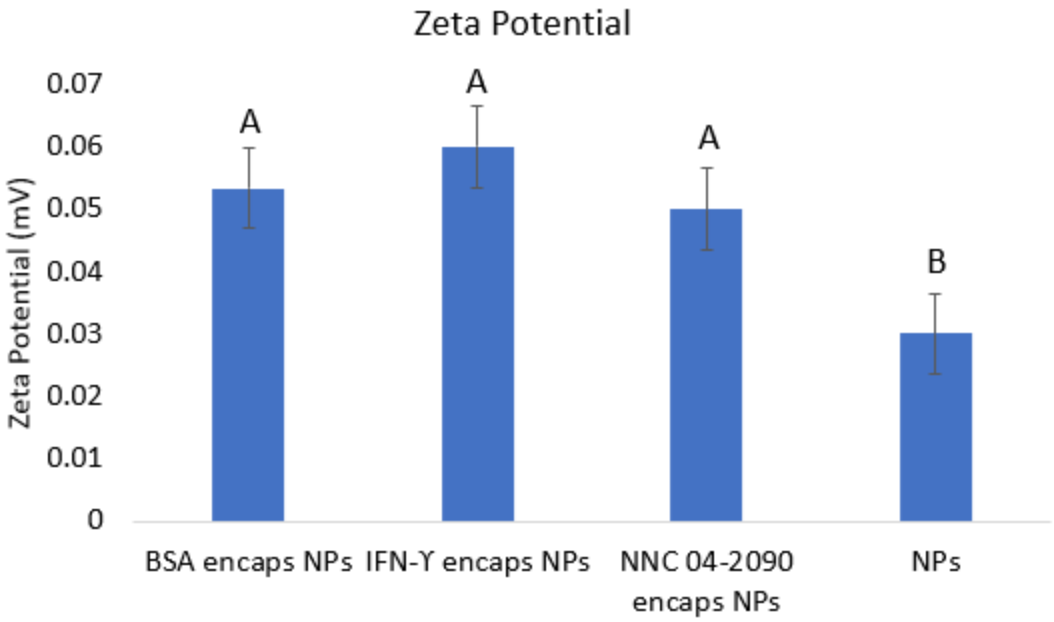

Figure 3: Zeta potential of the nanoparticles only (NPs) and drug/protein encapsulated NPs. Results are presented as a mean of 3 different trials with error bars shown as standard error. The letters A and B relates data within 20% of each other.

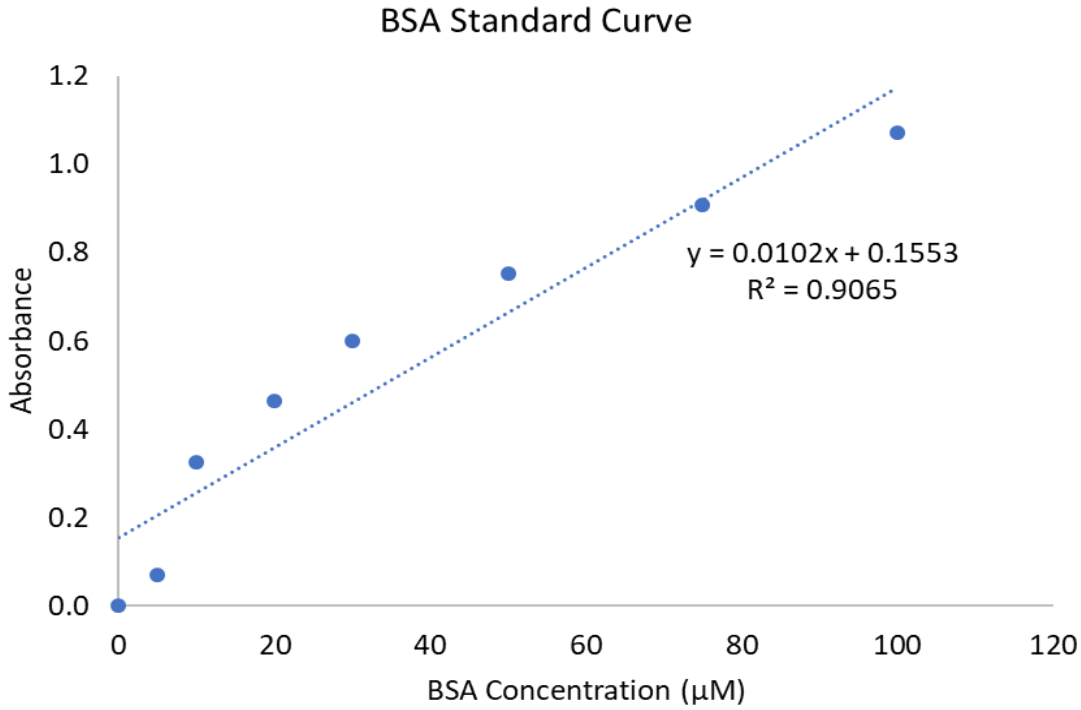

Figure 4: Standard curve BSA at different concentrations

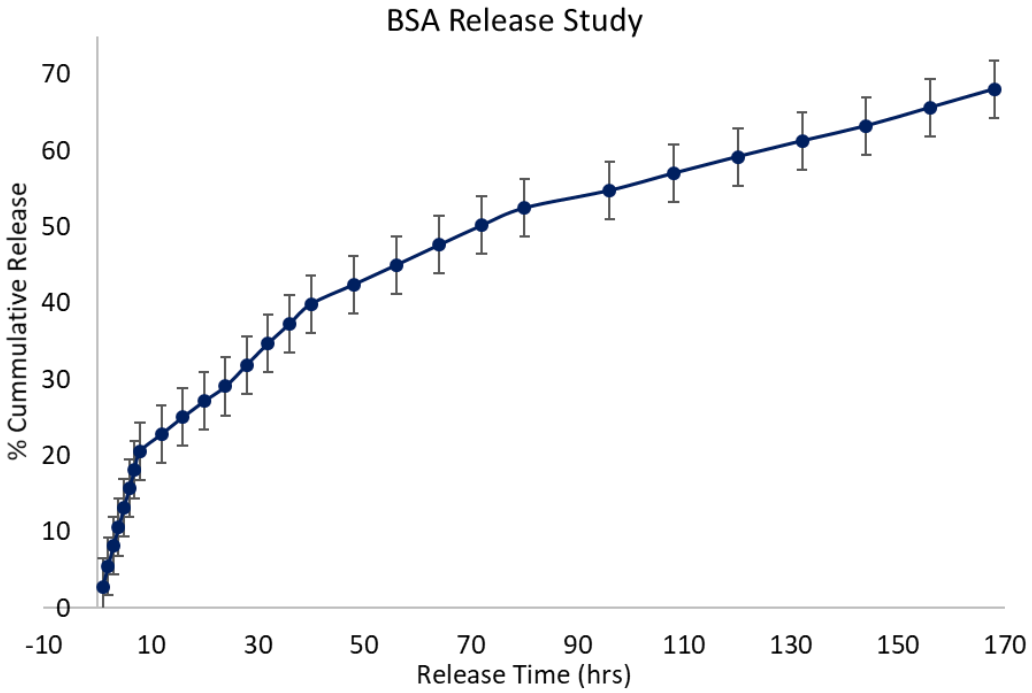

Figure 5: Release study of BSA from NPs, results are presented as a mean of 3 different trials with error bar shown as standard error

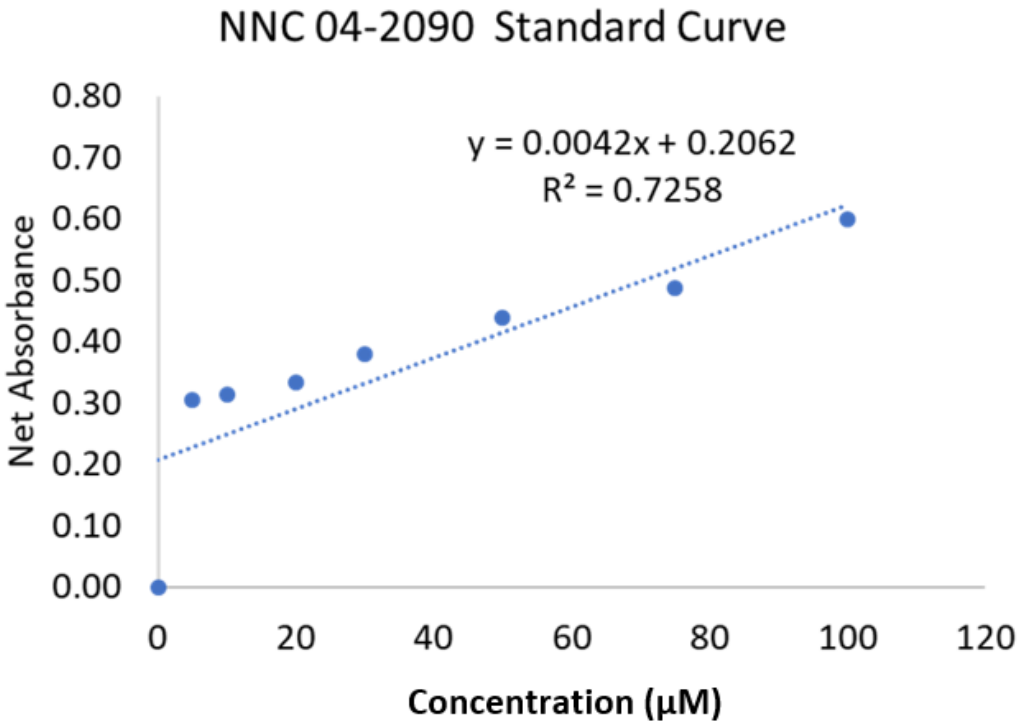

Figure 6: Standard curve of NNC 05-2090 at various concentrations

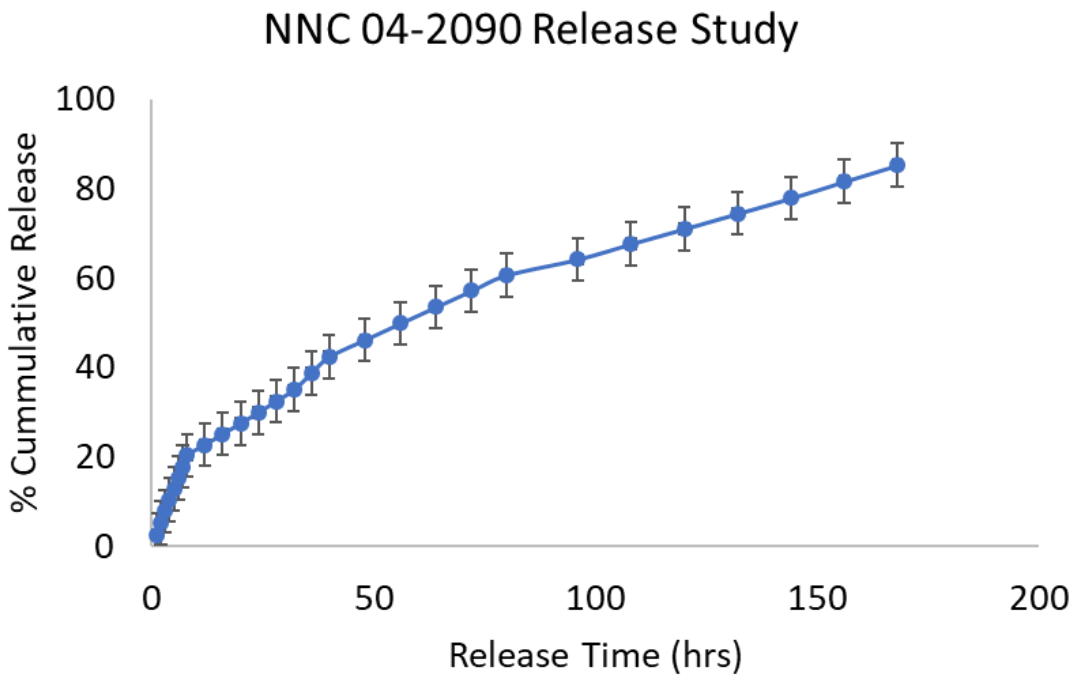

Figure 7: Release study of NNC 05-2090 from NPs, results are presented as a mean of 3 different trials with error bar shown as standard error

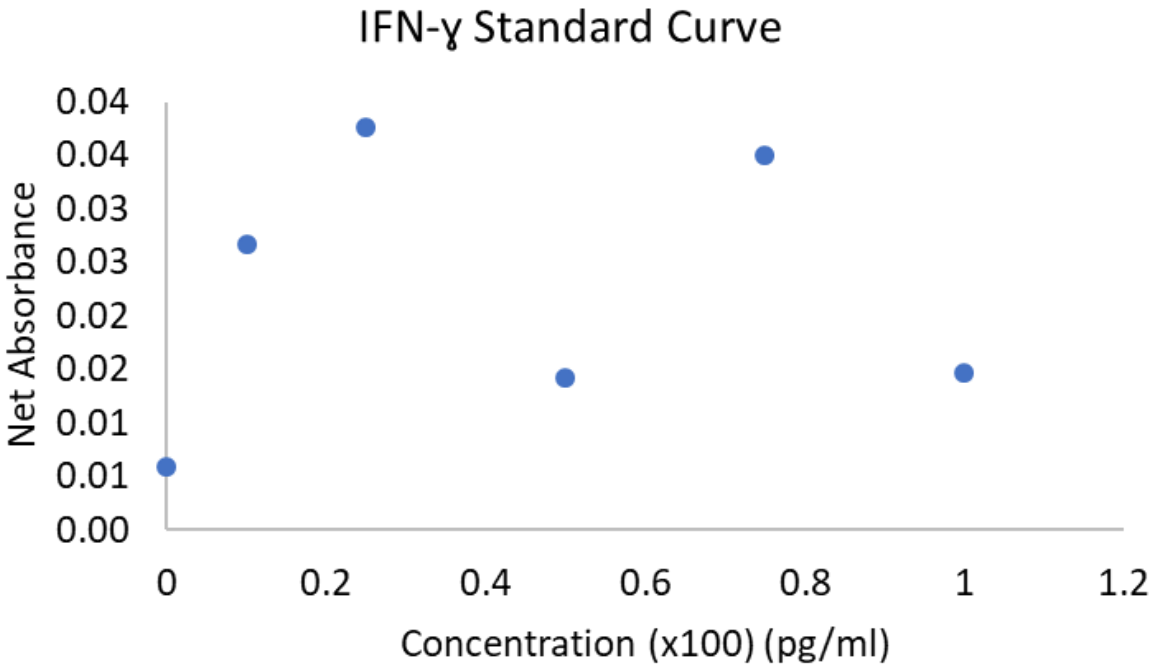

Figure 8: Standard curve of IFN-γ at various concentrations

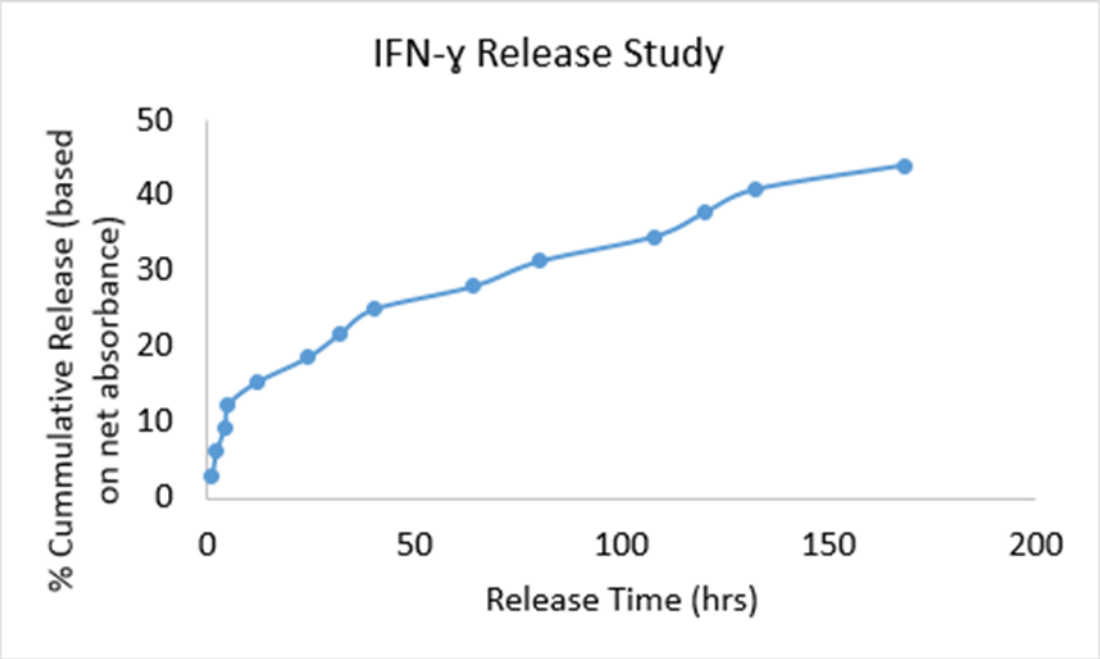

Figure 9: Release study of IFN-γ from NPs, results are presented as a mean of 3 different trials. Error bars were not able to be added because of the inconsistency of the standard curve of IFN-γ

Discussion/Analysis

The initial hypothesis for this study was that NPs have a capability to encapsulate drug/protein molecules within them. Also, it was hypothesized that these encapsulated drug/proteins will be released controlled over the period of time depending upon the particle size. We were anticipating the particle of NNC encapsulated drugs would be smaller than IFN-ϒ and BSA encapsulated NPs would have been the largest since molecular size of BSA is largest then IFN-ϒ and then drug molecule NNC. The exact molecular weights can be found in the Appendix in **Table 1**. Portions of this hypothesis were found to be correct but were not as consistent as previously hypothesized.

The results from the particle sizing can be seen in **Figure 2.** The expected results were that the NPs would be have the smallest radius, then the NNC encapsulated particles, the IFN-γ encapsulated particles, and finally the BSA encapsulated particles. The actual results show that the smallest particles are the BSA encapsulated particles, then the empty nanoparticles, the NNC particles and finally the IFN-γ particles. One explanation for why this phenomenon could've happened are because the nanoparticles stuck together during the analysis. This would result in a skewing of the results greatly and produce the results collected. In order to analyze with more accurate results, a procedure for separating each nanoparticle must be followed to ensure measurement of each individual nanoparticle instead of nanoparticle clusters or coagulations.

The results of the zeta potential can be seen in **Figure 3.** The results from this experiment generally follow the hypothesis for this study. The empty nanoparticles show the lowest zeta potential while the other three drugs show roughly the same values for their zeta potential. These results show that there are negligible differences between the zeta potentials between the different types of drugs. However, there is a significant difference between the zeta potential values of empty nanoparticles versus encapsulated nanoparticles.

A standard curve was constructed for each of the three drugs involved in the study. The results can be seen in **Figures 4, 6, 8.** The BSA and NNC standard curves produced useful results; however, the IFN-γ curve produced inconclusive and varied information. An explanation for the inconclusive results could be poor cleaning during the ELISA protocol which could result in the variating results. Another possibility could be the improper formulation of the rinsing and cleaning agents for the ELISA protocol. Or bad reagent (too old) was supplied for the ELISA procedure. Each possibility could greatly affect and alter the results of the IFN-γ samples.

The standard curves for BSA and NNC showed a general linear trend throughout the graph. BSA shows a R^2 value of 0.9065 and the NNC shows a R^2 value of 0.7258. Both are reasonable values to assume a linear trend. These standard curves were the basis of computing concentration of release samples drawn at different time points.

The average results from the three release studies are shown in **Figures 5, 7, 9.** The release study for BSA resulted in an overall release of about 70% of the encapsulated drug over the 168-hour testing period. The release rate follows a general logarithmic trend, which meant higher initial release and slower release over the rest of the experiment. The release study for NNC resulted in an overall release of about 80% of the encapsulated drug over the 168-hour testing period. Since the NNC molecules are very tiny, burst releases were expected in the first half of the trials; however, none of these properties were observed from the collected data. The release rate also follows a logarithmic trend, similar to the BSA trend. The release study for IFN-γ resulted in an overall release of only 50% of the encapsulated drug over the 168-hour testing period. These results seem skewed, since IFN-γ has the lowest molecular weight of any of the drugs, therefore it should have the most drug released. However, the trend of the release rate follows the same logarithmic trend as the other two drugs. Some error likely occurred with this assay during analysis and unfortunately could not be retested because of time constraints.

Error bars were used in the graphs in order to show the variance between each of the three trials for each of the drugs and each of the standards that were calculated. Due to a lack of time, no additional analysis or trials were able to be completed to verify/expand on the results gathered in this report. To improve accuracy in the future, additional analysis and trials should be considered.

Conclusions/Recommendations

The initial hypothesis for this study was that there was a constant relationship between the release amount and release time of the encapsulated nanoparticles depending on the drug of choice's molecular weight. This theory was not constant for the whole project. As seen in **Figure 2**, the particle size of each encapsulated drug does not correlate to the molecular weight of the drugs. This is believed to be inconsistent because of the common case of nanoparticles sticking together, causing improper measurements of particle radii. The results of the zeta potential for different drugs can be seen in **Figure 3**. The zeta potential followed the trend of the hypothesis and correlates from the lowest molecular weight drug to the highest molecular weight drug.

The data from the release studies were compared to standard curves for each drug. These results can be seen in **Figures 4-9.** Error bars are shown on the BSA and NNC release study graphs to show the variance from the standard curves. The BSA sample released 70% of its drug in the 168-hour time period and the NNC sample released around 80% of its drug in the same amount of time. These results were expected because NNC has the lowest molecular weight; however, the rate at which the NNC was released was a lot slower than anticipated. The IFN-γ release studies were not able to be compared to a standard because of how poorly the standard curve turned out from the ELISA protocol. Therefore, no definitive information could be obtained from this study.

Recommendations for this project include using fresh and sealed assay agent for the ELISA protocol in order to ensure correct analyzation of IFN-γ. For future experiments that are similar to this one, a different selection of drugs could be beneficial. To ensure the accuracy of measuring particle size, the nanoparticles should be vortexed intensely in order to attempt to break up clusters of nanoparticles before analysis. Also, having more than three Franz cells when testing the release studies would help ensure accuracy and would save a lot of time.

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Appendices

Table 1: Molecular weights of all three drugs used in the experiments

Drug Molecular Weights		
BSA	IFN-γ	NNC
Bovine serum albumin	Interferon Gamma	NNC 05-2090
66.5 kDa	16.9 kDa	451 g/mol
Highest		Lowest