Characterization of Novel Chitosan/Polyelectrolyte Nanoparticles

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Abstract

Chitosan-based nanoparticles are under investigation as delivery vehicles for drugs, genes, and proteins. Due to the biocompatibility, mucoadhesive nature, and biodegradable nature of chitosan and binding qualities, anionic nature, and biocompatible qualities of heparin, chitosan/heparin nanoparticles (CHNPs) are a promising platform for the delivery of labile proteins. However, the literature lacks a definitive process to control the size, efficiency, and yield of CHNPs at higher concentrations of reagents. Additionally there is a need to improve loading efficiency of CHNPs. In this study CHNPs have been synthesized and characterized for size, yield, protein loading efficiency, and release. The CHNPs were prepared through ionotropic gelation of chitosan with heparin. Chitosan concentration (0.5mg/ml, 1mg/ml and 2mg/ml), amount of heparin added (0.004mg, 0.010mg, 0.016mg, 0.080mg, and 0.160mg), and number of sonications (0, 1, 2, 3, and 4) are influential factors in determining particle size, yield, and loading efficiency. The optimized loaded particles had an average size of 740nm, loading efficiency of 99% using Bovine serum albumin-fluorescein isothiocyanate conjugate (FITC-BSA) and 40% using Interleukin-12 (IL-12), and average mass yield of 9mg of nanoparticles. This study provides important methodology to control size, yield, and loading efficiency of chitosan/heparin nanoparticles. These particles, because they can be loaded in mild aqueous conditions, represent a promising delivery platform for cytokines and other labile proteins. Our future studies will determine if CHNPs loaded with the anti-tumor cytokine interleukin-12 (IL-12) is capable of controlling established tumors.
1. Introduction

With the advent of nanotechnology, there has been a movement to create novel drug delivery systems utilizing this technology. Nanoparticulate systems have shown promise in the field of drug delivery systems because of their abilities to create controllable and targeted release systems, increased retention, increased mobility through the body, and increased effectiveness of therapeutics, increased stability of the therapeutic agent, as well as potential to reduce toxicity and off target side effects\textsuperscript{1, 2, 3, 4}. Two materials have shown particular promise in creating an effective drug delivery system: chitosan and heparin.

Chitosan is a naturally occurring positively charged polydisperse biopolymer made of N-acetylglucosamine and glucosamine repeating units. This material is biocompatible, biodegradable, low cost, and mucoadhesive\textsuperscript{1, 4, 5}. Due to its properties, chitosan has shown to be effective in wound healing applications, tissue engineering, and drug delivery systems\textsuperscript{6}. In nanoparticle form, it has demonstrated controlled release of genes, drugs, and proteins, enhanced permeation, improved retention capability and mucoadhesion\textsuperscript{4, 5}. Heparin is a naturally occurring polydisperse biopolymer made of repeating units of 1, 4 linked uronic acid and D-glucosamine\textsuperscript{4, 7}. It is best known as a powerful anticoagulant, however, it has also been found to have a part in inflammatory responses, cell growth and adhesion, antivirus action, angiogenesis, and tumor cell metastasis\textsuperscript{1, 4, 5, 7}.

Studies done by Liu et al investigated the creation and characterization of CHNPs and determined their effectiveness as compound carriers\textsuperscript{4}. In two studies by Shahbazi et al, these compounds have been successfully created into nanohydrogels and nanoparticle systems that use heparin as a chitosan cross-linker for the systemic delivery of heparin\textsuperscript{1, 2}. Other studies have been
done that are similar to Shahbazi’s in that they have also used heparin in chitosan based particles as the drug to be delivered systemically\textsuperscript{3,6}. Studies conducted by Lai et al and Skop et al have shown these particles are capable of encapsulating cytoethal distending toxin for gastric cancer therapy and delivering neural stem cells and growth factors for central nervous system repair respectively\textsuperscript{7,8}. These studies have shown that chitosan/heparin nanoparticles (CHNPs) are viable options for delivery of labile proteins.

However, there is a lack of a definitive process to create particles at a given size, loading efficiency, and yield. The studies conducted by Shahbazi et al used only low concentrations of chitosan and heparin, which may result in low yields and forces researchers to use large volumes of reagents in the given protocol\textsuperscript{1,2}. Additionally the size of the particles in previously mentioned studies have been between 50nm and 600nm, however, variable’s effect on particle size and efficiency can not be deduced due to the changing of multiple variables in each experiment\textsuperscript{1,2}. Loading efficiency of optimized particles in previous studies have been as high as 75%, however these were using heparin as the encapsulated drug, not a secondary growth factor or protein\textsuperscript{1,2}. In the study by Liu et al, FITC-BSA was loaded onto the particles and showed a maximum loading efficiency of 55\%\textsuperscript{4}. Not much research has been done using these nanoparticles to encapsulate other proteins and cytokines such as IL-12. Also, the Liu et al study was the only study to quantify nanoparticle yield (in mg), further studies should be done to support their results\textsuperscript{4}.

This study investigates the characterization and use of CHNPs for the delivery of Interleukin-12 (IL-12) to localized bladder tumors. According to the National Cancer Institute, bladder cancer was the sixth most prevalent cancer type in 2014 with \textasciitilde75,000 new cases and \textasciitilde15,500 deaths\textsuperscript{9}. This cancer also has the highest lifetime treatment costs per patient with costs between \$89,000 and \$202,000 per patient\textsuperscript{10}. This particular type of cancer also has a high rate of recurrence
Thus finding more effective treatments and therapies for bladder cancer has been of interest to researchers. One potential therapy that has been under investigation is Interleukin-12 (IL-12). IL-12 is a naturally occurring cytokine that influences cell differentiation, cell proliferation, and cell immunity. It has also been shown to elicit antitumor effects and stimulate immunologic memory. Alone, intravenous injections of IL-12 can be lethal: the need for an efficient delivery system is vital. Previous work has indicated that use of chitosan to mediate IL-12 delivery in bladder cancer was effective at eliminating localized tumors and protected against tumor rechallenge. With this system, four doses were needed to cure 88-100% of mice. In order to reduce the number of doses, a highly efficient and controlled drug delivery system for IL-12 needs to be investigated. CHNPs are promising drug delivery platform that could be highly efficient and controllable because IL-12 is a heparin binding cytokine. Theoretically IL-12 should bind strongly to heparin, thus creating a high efficiency of encapsulation and give a prolonged delivery.

Due to the high cost of IL-12, there has been little research done investigating the encapsulation in nanoparticle systems including CHNPs. In this study, we propose a better characterization and the novel use of CHNPs for the delivery of IL-12 to localized bladder tumors and other solid tumors.

2. Materials and Methods

2.1 Reagents. Low molecular weight chitosan (90-190kDa, viscosity 20cps) was purchased from Sigma Aldrich (St. Louis, MO). Heparin sodium salt (100mg/ml) was purchased from Sigma Aldrich (St. Louis, MO). Bovine serum albumin-fluorescein isothiocyanate conjugate (FITC-BSA) (A9771-250MG) was purchased from Sigma Aldrich (St. Louis, MO). Glacial acetic acid
(A35-500) was purchased from Fisher Chemicals (Fair Lawn, NJ). The surfactant Pluronic F-68 (P1300-500G) was purchased from Sigma Aldrich (St. Louis, MO). Phosphate buffered solution (PBS, 1X, .0067M) was purchased from Hyclone Labs (Logan, UT). IL-12 was acquired from the University Of Arkansas Biologics Center (UABC).

2.2 Creation of CHNPS

2.2.1 CHNPs/FITC-BSA. 10mg of chitosan was dissolved in 2% acetic acid (10ml total volume) and allowed to stir at 800RPM for 1hr. 1mg of FITC-BSA was added to the chitosan solution and stirred at 800RPM for 5 minutes. A heparin solution was created by adding varying amounts of heparin (0.004mg, 0.010mg, 0.016mg, 0.080mg, and 0.160mg) in 1ml of DiH$_2$O. The heparin solution was added dropwise at 8ml/min to the chitosan/FITC-BSA solution via injection pump. The nanoparticle solution was stirred for an additional 5 minutes at 800RPM. 50mg of Pluronic-68 was added to the solution and stirred for 5 minutes at 800RPM. The nanoparticle solution was sonicated at 20W for 45 sec. The solution stirred at 800RPM for 15 minutes. Sonication and stirring was repeated for varying amounts (0, 1, 2, 3, and 4 sonications). After size quantification (see **Size Characterization**), the nanoparticle solution was centrifuged for 10min at 18,000RPM. The supernatant was stored for later testing. The pellet was resuspended in 10ml DiH$_2$O and centrifuged at 18,000RPM for 10 min. The supernatant was discarded and the pellet was resuspended in 5ml DiH$_2$O. The solution was then briefly sonicated (10s at 20W) and stored for 24hr at -80˚C. The nanoparticles were then lyophilized and stored at -24˚C.

2.2.2 CHNPs/IL-12. Chitosan/Heparin nanoparticles loaded with IL-12 were produced in a similar manner. 5mg of chitosan was dissolved in 2% acetic acid (4.5ml total volume) and allowed to stir at 800RPM for 1hr. 500µg IL-12 (500µl of 100mg/ml) was added to the chitosan
solution and stirred for 5 minutes at 800RPM. 0.080mg of heparin was diluted in 0.5ml DiH₂O and added to the chitosan/IL-12 solution dropwise at 8ml/min via injection pump. The resulting solution was stirred for 5 minutes at 800RPM. 25mg of Pluronic-68 was added to the solution and stirred for 5 minutes at 800RPM. The nanoparticle solution was sonicated at 20W for 45 sec. The solution was then stirred for 15 min at 800RPM. The process of sonication and stirring was repeated (total of 2 sonications). The size of the particles was measured (see Size Characterization). Then the nanoparticle solution was centrifuged for 10min at 18,000RPM. The supernatant was stored for later testing. The pellet was resuspended in 10ml DiH₂O and centrifuged at 18,000RPM for 10 min. This supernatant was also saved and the pellet was resuspended in .5ml DiH₂O. The solution was then briefly sonicated (10s at 20W) and stored at -80°C.

2.3 Creation of CNPs

2.3.1 CNPs/IL-12. Chitosan nanoparticles were created to serve as a comparison for later release studies. Similar to CHNPs, 5mg chitosan was dissolved in 2% acetic acid (4.5ml total volume) and stirred at 800RPM for 1hr. 500µl IL-12 (500µl of 100mg/ml) was added to the chitosan solution and stirred for 5 min at 800RPM. 500µl of 10% Sodium Sulfate solution was added dropwise to the solution at 8mg/ml via injection pump. The resulting solution was stirred for 5 minutes at 800RPM. 25mg of Pluronic-68 was added to the solution and stirred for 5 minutes at 800RPM. The nanoparticle solution was sonicated at 20W for 45 sec. The solution was then stirred for 15 min at 800RPM. The process of sonication and stirring was repeated (total of 2 sonications). Then the nanoparticle solution was centrifuged for 10min at 18,000RPM. The supernatant was stored for later testing. The pellet was resuspended in 10ml DiH₂O and centrifuged at 18,000RPM for 10 min. This supernatant was also saved and the pellet was
resuspended in .5ml DiH₂O. The solution was then briefly sonicated (10s at 20W) and stored at -80°C.

2.4 Size Characterization

2.4.1 All Nanoparticles. 1ml of nanoparticle solution was loaded into a cuvette and placed into a Zetasizer Nano. The size was then measured using dynamic light scattering (DLS) and recorded. The solution used was then placed back into the bulk solution for further processing.

2.5 Efficiency Characterization

2.5.1 CHNP/FITC-BSA. A standard curve was created using known concentrations of FITC-BSA. The fluorescence of the supernatant and standard curve solutions were measured via a plate reader. Concentration of the supernatants was determined and loading efficiency subsequently calculated using the equation of the line. The concentration was then multiplied by the total volume (11ml) to find the amount of protein left in the supernatant. Equation 1 was then used to find the efficiency.

\[
\text{loading efficiency} = \left(\frac{1000 - \mu g \text{ protein in supernatant}}{1000}\right) \times 100\% \quad (\text{Equation 1})
\]

2.5.2 CHNP/IL-12 and CNP/IL-12. Since IL-12 is not fluorescent, an ELISA microarray assay was run to determine IL-12 concentration in both saved supernatants. Much like CHNP/FITC-BSA, the loading efficiency was calculated from the concentration using Equation 1.
2.6 Yield Characterization

2.6.1 CHNP/FITC-BSA. For these particles, the yield was measured by weighing the mass of the particles from the tubes. Some yields were not able to be measured due to an extremely low mass. The yield for CHNP/IL-12 and CNP/IL-12 was not measured.

2.7 Imaging

2.7.1 CHNP/FITC-BSA. Particles were imaged using a FEI Nova Nanolab 200 SEM (1nm resolution at 15kV). Imaging was completed under the supervision of the Arkansas Nano-Bio Materials Characterization Facility staff.

2.8 Release Studies

2.8.1 CHNP/IL-12 and CNP/IL-12. For release studies, the release profiles of CHNP/IL-12 and CNP/IL-12 were compared (n=3). For both samples, 50µg of IL-12 was added to microcentrifuge tubes in a final total volume of 1ml (166µl of CHNP solution in 834µl of PBS and 59µl CHNP solution in 941µl). The tubes were then allowed to stir (125RPM) over the course of the experiment at 37°C. To collect samples, the samples were centrifuged at 13,000RPM for 5 minutes at 4°C. 500µl of supernatant was collected and stored for later. Then 500µl of fresh PBS was added and the pellets resuspended (pipetting and sonication). Supernatant was collected at 0, 1, 2, 3, 4, 8, 12, 24, 36, 48, 72, 96, 110, 134, and 148 hours and was labeled and stored at -80°C. ELISA microarray assay was used to determine amount of IL-12 in the supernatant at each time point. Release profiles were then constructed.
3. Results

3.1 Creation of CHNPs. Chitosan/Heparin nanoparticles were successfully created using the ionotropic gelation method. During the creation process, three main factors were identified as having the greatest effect on particle size, yield, and efficiency. These factors are chitosan concentration, heparin concentration, and number of sonications. To observe the effect of these factors, the chitosan concentration varied from .5mg/ml-2mg/ml, the heparin added varied from 0.004mg-0.160mg, and the number of sonications was varied from 0-4.

3.2 Effect of Chitosan Concentration on CHNPs. Sizes of CHNPs created using .5mg/ml, 1mg/ml, and 2 mg/ml chitosan and 0.004mg, 0.010mg, 0.016mg, 0.080mg, and 0.160mg of heparin were quantified using DLS (see Materials and Methods). Figure 1 shows the effect of chitosan concentration on the particle size.

At 0.5mg/ml chitosan, the average size over all heparin concentrations was 465.55nm ±147.33. When the concentration increases to 1mg/ml, the average size increases to 760.74nm ±46.931.
The average particle size was 1643.2nm ±610.43 for 2mg/ml chitosan. Overall, as chitosan concentration increases, the size of the particles also increases. All particles created with 1mg/ml chitosan were the most uniform (standard deviation for each run was between 31.37nm and 123.97nm). When the chitosan concentration was increased, it caused the particles to be more disperse (standard deviation for each run varied between 65.5nm and 759.49nm) and heparin played more of a role in determining the size of the particle.

The loading efficiency of these particles was then measured as described previously. Figure 2 shows the average loading efficiency for each particle combination.

Figure 2. This graph shows the effect of chitosan concentration on particle loading efficiency (CHNP/ FITC-BSA) when different amounts of heparin were added.

There is an overall inverse relationship between chitosan concentration and loading efficiency. For all samples, the loading efficiency generally decreased as chitosan concentration increased. For 3 samples (0.004mg, 0.016mg, 0.160mg) the peak loading efficiencies were at 1mg/ml chitosan. For 2 samples (0.010mg, and 0.080mg) the loading efficiencies were at 0.5mg/ml
chitosan. With these samples, the loading efficiency leveled out between 1mg/ml and 2mg/ml chitosan. At lower heparin concentrations, the chitosan had less of an effect on the loading efficiency (dominated by heparin concentration).

After lyophilization, particles were removed from the tubing and weighed. The effect of chitosan concentration on yield is shown in Figure 3.

![Figure 3](image)

Figure 3. This figure shows the effect of chitosan concentration on particle (CHNP/FITC-BSA) yield. Yields for heparin concentrations less than 0.016mg were not included due to an extremely low yield. These values were not able to be measured. The values for yield for 0.016mg heparin are estimated, again due to an extremely low yield.

For this measurement, the particles created with the lower heparin amounts (0.004mg, 0.010mg, and 0.016mg), were not quantified. This is due to the extremely low yield, the particles could not be successfully removed from the tubing and the amount was below the sensitivity of the scale and the difference in weight between separate empty tubes. For the other samples, when the chitosan concentration increased, the yield also increased. This could be due to more particles (thus more mass) or due to the increase in particle size. This measurement may not be representative of number of particles due to the larger diameter particles weighing slightly more.
3.3 Effect of Heparin Concentration on CHNPs. The effect of heparin concentration was also investigated in this study. Varying amounts of heparin were added in the creation process. Figure 4 shows the effect of heparin on the particle diameter.

![Figure 4. This figure shows the effect of heparin concentration on particle size (CHNP/FITC-BSA) at different chitosan concentrations.](image)

From this figure, it can be seen that at lower chitosan concentrations, heparin concentration plays less of a role on particle size (as compared to 2mg/ml chitosan and the effect of chitosan on the particle size). The samples tested using 2mg/ml chitosan resulted in heparin having a greater effect on the particle size despite being at a low concentrations. For all samples, as heparin amounts neared 0.160mg, the samples all converged. At this higher heparin concentration, the heparin played a larger role in determining size and resulted in a more uniform size across all chitosan concentrations.
Figure 5 shows the effect of heparin concentration on particle loading efficiency. Overall, as the heparin concentration increases, the loading efficiency increases. For the higher concentrations of chitosan however, there is an initial decrease in efficiency before increasing. A loading efficiency of 99.8% (±.104% and ±.0513%) was achieved with two combinations of heparin and chitosan: 1mg/ml chitosan with 0.160mg heparin and 0.5mg/ml chitosan with 0.080mg heparin. This ratio of chitosan to heparin (1mg/ml: 0.160mg) produced the most efficient particles.

Similar to the effect of chitosan concentration on particle yield measured earlier, the effect of heparin concentration on particle yield was also measured. Figure 6 shows the results of the yield quantification.
Regardless of chitosan concentration, as the heparin amount increased, the yield increased. At higher concentrations of chitosan, the relationship between yield and heparin amount is linear. As the concentration of chitosan decreases, the relationship between heparin and yield becomes less linear and the yield begins to level out. This leveling out observation also occurs after the particles achieve an efficiency of over 99%. A better conclusion could be drawn if the yield was quantifiable at the extremely low heparin concentrations.

From these results, the combination of 1mg/ml chitosan and 0.160mg heparin was chosen as the optimal combination because it produced highly efficient particles (>99%) and produced a larger amount of particles. For later IL-12 loading experiments, 1mg/ml chitosan/ 0.160mg heparin particles were used.

3.4 Effect of Sonication on CHNPs. Sonication was identified as a crucial step in formation of CHNPs. The physical breaking up of aggregated particles could influence overall particle size and loading efficiency. Figures 7 and 8 show the effect of number of sonications on particle size and loading efficiency respectively.
Figure 7. This figure shows the effect of number of sonications on particle size (CHNP/FITC-BSA) at 0.016mg heparin.

From this figure it can be seen that as sonication is increased, the particle size decreases. Also, the standard deviation of the samples decreases meaning sonications help improve uniformity of the CHNPs. 2 sonications was found to have a particle size of 753.967nm ±31.37nm and resulted in the most uniform particles.

Figure 8. This figure shows the effect of number of sonications on particle loading efficiency (CHNP/FITC-BSA) at 0.016mg heparin.

When the loading efficiency was measured, a similar trend emerged. As sonication increased, the efficiency decreased. Unlike the particle size, the efficiency became more varied as a result of more sonications. For all experiments, two sonications was chosen as the optimal number of sonications. Using two allowed for very uniform particles that still retained a high efficiency. It also allowed for the time to perform the protocol to be drastically reduced.
3.5 **Effect of Loading Process on CHNP Efficiency.** The above experiments incorporated the protein FITC-BSA during the creation process. An additional experiment was performed to determine whether adsorption of a protein onto the particles was a viable loading option. CHNPs were created using 0.5mg/ml chitosan and 0.080mg heparin. FITC-BSA was not added before heparin in this case. 1mg FITC-BSA was added to the particle solution after sonications and was allowed to stir at 37°C overnight. The next day the sample was centrifuged down and the supernatant collected for efficiency testing. The average efficiency was 99.23% ±.18 which was comparable to the encapsulation method.

3.6 **Particle Imaging and Resuspension Studies.** Scanning electron microscopy was also performed on these particles. Figure 9(a, b) shows two images captured during SEM investigation. In these pictures, two separate areas were imaged. Both of these areas showed aggregates of particles into larger structures. Individual particles around the estimated particle size (via DLS earlier) were not found; instead these aggregate structures were prevalent.

![Figure 9(a, b). These images show the surface of the CHNP aggregates that had formed after lyophilization. These images did not match particle size estimated by DLS earlier and lead to the belief that the freeze-drying process leads to CHNP aggregate formation.](image)
After finding the formation of aggregates of particles (from Figure 9(a, b)), studies were performed to resuspend the particles and measure its effect on particle size and efficiency. When the freeze dried samples were simply resuspended in diH₂O (0 sonications), it resulted in a DLS reading with a peak at 982nm and 5560nm. This showed that after lyophilization, the particles were aggregating. In this form the particles still had a loading efficiency of 99.4%. After 1 sonication, the average particle size decreased to 716.3nm and had an efficiency of 99.94%. After a second sonication the size decreased to the range of the original particles (~561.9nm) and had an efficiency of 99.84%.

3.7 Loading of IL-12. Chitosan-heparin nanoparticles (CHNPs) and chitosan nanoparticles (CNPs) were fabricated and loaded with IL-12. CHNPs were created using the combination of 1mg/ml chitosan, 0.160mg heparin, and 2 sonications. These conditions were determined to be the optimal conditions to create a large amount of highly efficient particles. CNPs were also loaded to serve as a comparison to CHNPs. Particle size was measured after loading and processing. CNPs had a diameter of 348.4nm and the CHNPs had a diameter of 897.7nm. The loading efficiency of CNPs and CHNPs were also compared. When measured, CNPs had a loading efficiency of 95.64% while the CHNPs had a loading efficiency of 40.82%. The CHNP loading efficiency for the actual IL-12 loaded particles was drastically lower than the FITC-BSA particles.

3.8 Release Studies. The release profiles of CHNPs and CNPs were measured and compared. Figure 10 shows the release profile of IL-12 from these particles.
From this figure, it was shown that the CHNPs have a faster release of IL-12 while the CNPs have a more sustained release. The CHNPs had released 100% of encapsulated IL-12 within two hours. At this same time the CNPs had only released 18.7% of the IL-12.

4. Discussion

4.1 Creation of CHNPs. This protocol for creation of CHNPs via ionotropic gelation was successful. In this process the chitosan and heparin are held together electrostatically. In the encapsulation of FITC-BSA/IL-12, there are three main options for the method of protein attachment. One method would be that the proteins can bind to the chitosan initially and the heparin then binds to the chitosan after addition and encapsulates the protein. Another method would be that the protein is freely floating in solution and with the addition of heparin; protein is caught between the heparin and chitosan binding therefor encapsulating the protein. The final method would be that the protein is freely floating in solution when the particles form and then adhere to the chitosan or heparin post creation. After investigating adsorption of the protein onto a particle, we found that the loading efficiency was not affected by protein loading process. This finding led to the belief that the protein loading process may be adsorption of the protein.
regardless of addition of protein before or after particle formation. Further studies should be completed to discern the method of protein encapsulation/adherence. A study quantifying their release rate would provide vital information as to how the protein is bound and will help make a more definitive conclusion as to which method the protein attaches to the particle. Regardless of the protein adherence method, this protocol helped identify key factors that influenced the particle size, yield, and efficiency.

4.2 Effect of Chitosan Concentration on CHNPs. To measure the effect of chitosan concentration on CHNP size, yield, and efficiency nanoparticles were fabricated with 0.5mg/ml, 1mg/ml, and 2mg/ml chitosan. Overall there is a direct relationship between particle size and chitosan concentration. At lower heparin concentrations, the chitosan concentration plays a more prominent role in determining size. It is believed that in these cases, it is the determining factor because chitosan is the dominant reagent species.

Investigation into different chitosan concentrations varies between studies and highly impacts the particle size. In two studies by Shahbazi et al, chitosan concentration varied between 0.1mg/ml and 1mg/ml and had particles that ranged from 63nm to 610nm\(^1,2\). Variance of size in their study was partially due to chitosan concentration tested. A study by Lai et al used 1.2 mg/ml chitosan to create particles (did not characterize) and had particle sizes between 312nm and 983nm (source of change strictly protein concentration)\(^7\). Only one study (Liu et al) investigated larger chitosan concentrations (1mg/ml-10mg/ml) during their characterization phase\(^4\). The average particle size measured in Liu’s study was between 200nm and 800nm, which is more comparable to the values found in the investigation, presented here, but was still considerably smaller than measured values\(^4\). The trends observed regarding effect of chitosan concentration on particle size of Liu’s study and this study are shared\(^4\). The protocol provided in this study provides a method
to create larger particles. Larger size particles can be useful for drug delivery applications that require a slower rate of clearance from the body.

Loading efficiency of CHNPs was measured from the fluorescence of FITC-BSA left in the supernatant of the solution. Other than a general decrease in efficiency, a single trend between the chitosan concentration and the efficiency did not exist. Instead, two main patterns arose. Half of the samples peaked at 1mg/ml chitosan and the other half peaked at 0.5mg/ml. Since there isn’t a trend, it leads to the belief that chitosan concentration does not determine the loading efficiency as much as heparin concentration does. In the Liu et al study, they also used FITC-BSA to quantify loading efficiency. Their results show that efficiency increases to a peak then begins to decrease. Three of the heparin concentration curves from the study presented here also follow this trend. If lower concentrations of chitosan were investigated for 0.010mg and 0.080mg heparin, it may result in a similar curve and would give a cohesive overall trend that would be consistent through multiple studies. The particles created in Liu’s study had a peak efficiency of 50%. The results shown here show an efficiency that is nearly double that of Liu et al.

When the yield was quantified, it revealed a direct relationship between particle yield and chitosan concentration. This leads to the determination that heparin is the limiting reactant. In the creation process, chitosan reacts with all of the available heparin. When this heparin runs out, no more particles are formed. This would explain why the yield keeps increasing as heparin amount increases. The increase in yield could have been due to a larger size particle that weighs more per particle. In that case, the actual number of particles may not have increased; rather the weight of each particle could have increased. More studies should be done to quantify the actual number of particles. Then a more accurate yield could be calculated and determine if yield actually increases or if the mass of each particle increases.
From what was found here, lower concentrations of chitosan (and heparin) result in vastly less yield. Based on this, I would suspect that some of the other studies previously mentioned that did not quantify yield, such as Shahbazi et al, have a very small yield\textsuperscript{1,2}. This in turn would make the process of creating these particles more inefficient. Liu et al were the only research group to investigate the mass yield of CHNPs\textsuperscript{4}. The yield in their chitosan concentration study was 9mg which was achieved at 4mg/ml chitosan. At 1mg/ml and 2 mg/ml a yield of 7mg was reported. Thus this protocol provides a method to create more particle mass (14mg at 2mg/ml) at a much higher efficiency.

In this study we relied upon low molecular weight chitosan (~90-190kDa) with a viscosity of 20cps and a deacetylation degree of 74%. Discrepancies between sizes, efficiencies, and yield between different studies could be linked to chitosan properties. Molecular weights, deacetylation degrees, and viscosity of chitosan could also impact particle size and efficiency. For example, in both studies by Shahbazi et al, 85% deacetylated chitosan was used (molecular weight never specified)\textsuperscript{1,2}. This value of 85% DDA is fairly common between multiple studies\textsuperscript{4,6}. However, in studies by Skop et al, 50kDa chitosan with a deacetylation degree of 75-85% was used\textsuperscript{8}. Viscosity is occasionally mentioned in chitosan specification. Yang et al simply used low molecular weight chitosan (exact size not given) with a range of viscosities between 20cps and 300cps\textsuperscript{3}. In the study by Liu et al, a higher viscosity (900cps) and higher deacetylation degree (90.6%) was used\textsuperscript{4}. Most of these studies make no specific reference to molecular weight, DDAs, and viscosity, usually only one or two are described\textsuperscript{1,2,3, and 4,6,7,8}. These slight differences in chitosan properties such as molecular weight, viscosity, and deacetylation degree may be accountable to the differences between size and efficiency of CHNPs between these studies. This idea is supported by a study conducted by Liu et al\textsuperscript{4}. They had shown that molecular weight did
in fact play a role in particle size and loading efficiency\textsuperscript{4}. However, more studies should be conducted to support their claim as well as investigate the potential of viscosity and deacetylation degree to change particle size and efficiency.

4.3 Effect of Heparin Concentration on CHNPs. To measure the effect of heparin concentration on particle size, efficiency, and yield, particles were created using 0.004mg, 0.010mg, 0.016mg, 0.080mg, 0.160mg of heparin. With the exception of 2mg/ml, there is a direct relationship between size and heparin concentration, however, it is not as drastic as it was for chitosan concentration. At lower heparin concentrations, heparin has less of an effect on the size. As the concentration increases, size is not as dependent on chitosan concentration and the sizes converge.

Analysis of loading efficiency also showed an overall direct relationship between heparin concentration and loading efficiency. Additionally, at higher overall concentrations of heparin, the efficiency is more uniform and predictable. This analysis also reveals a ratio at which the particles become over 99% efficient. 1mg/ml chitosan/0.160mg heparin and 0.5mg/ml chitosan/0.080mg heparin resulted in the peak efficiency. This ratio was determined to be the ideal conditions for the creation of a drug delivery system.

The effect of heparin concentration on yield of nanoparticles was also quantified and analyzed. As the concentration of heparin increases at higher chitosan concentrations, the yield follows a more linear relationship. Due to this, the yield is more predictable at higher concentrations of chitosan. At lower concentrations of chitosan, results showed that as heparin concentration increased then the yield hits a point where it stabilizes. The point at which the yield stabilizes is also the point at which the maximum efficiency occurs. Once it surpasses the ideal ratio (ex.
1mg/ml chitosan/ 0.160mg heparin) no additional particles are made and each of the reagents are completely used by each other. It is suspected that the higher concentrations of chitosan would yield similar curves once the ideal ratio is surpassed. In general this ratio creates an ultra efficient particulate system with a good amount of nanoparticle yield.

In the creation of CHNPs, there is some discrepancy between units used for the quantification of heparin used. In studies, such as Shahbazi et al, international units (IU) were used to describe the amount of heparin used\(^1\)\(^,\)\(^2\). Typically IU is used when heparin is used as the therapeutic for anticoagulation. In studies that are not using heparin as the drug, Lai et al and Liu et al, use mg/ml to describe heparin amount\(^4\)\(^,\)\(^7\). The use of these different units has made comparing heparin amounts more difficult than the chitosan concentrations. This may lend to the reason why particle properties vary so much from study to study. When compared to this study, Lai et al and Liu et al used much more heparin than used in this study. In their studies a base of 1mg heparin was used\(^4\)\(^,\)\(^7\). In the Liu et al study, this amount was varied between .4mg and 2mg\(^4\). In the present study, a maximum of .16mg heparin was used. The use of much lower heparin amounts may be the key to gaining a higher efficiency for the particles.

**4.4 Effect of Sonication on CHNPs.** Though other research groups have not investigated sonication as a key variable, this study identified sonication as a key factor in determining particle size and efficiency. By altering the amount of sonication, the particle size and efficiency can be fine tuned. Overall sonication decreases the variance and size of the particles. This is due to the physical agitation that breaks apart any clusters of particles. Initially these clusters can be a variety of sizes, then after sonication, the uniformity increases and the size decreases. Increasing the amount of sonication can also decrease the loading efficiency. This is also believed to be due to the physical agitation of the particles. The breaking apart of clusters of nanoparticles can
dislodge the protein. After dislodging, the protein may not be able to reattached/become re-encapsulated by the particles and therefore floats freely in solution.

4.5 Particle Imaging and Resuspension Studies. From initial SEM images, it was found that lyophilization drastically alters the particle size and morphology. A large aggregated structure was created which did not correspond to the particle size determined prior using DLS. Further investigation supported this claim via DLS measurement of unsonicated lyophilized particles. This could be due to the particles not being coated with enough surfactant. Without the surfactant, the nanoparticles would essentially stick together and form these aggregate structures. Lyophilization may amplify this effect and thus causing the surfaces that were observed. After sonication of the lyophilized particles, the size returned to that prior to lyophilization while still holding their efficiency above 99%. This occurs because the rigorous sonication physically disrupts the aggregate structure and breaks it into the smaller particulate form. To try to elucidate the true morphology and reduce lyophilization effects, studies should be completed measuring the effect of amount of surfactant (Pluronic-68) on particle aggregation and environmental SEM should be conducted on the unlyophilized samples.

4.6 Loading of IL-12. For IL-12 loading studies, CHNPs were created using 1mg/ml chitosan and 0.160mg heparin. This combination of chitosan and heparin was chosen because it resulted in a protocol that produced large amounts of particles that were extremely efficient at loading the dummy protein FITC-BSA. When loaded the size was 897.7nm for CHNP and 348.4nm for CNPs. The CHNP size is slightly larger than those formulated with FITC-BSA (736.97nm). This is due to a different protein attached and variation in size between batches of nanoparticles created using the same conditions. When the efficiency of these particles was measured the results were not what were anticipated. The measured CHNP loading efficiency was 40.82%
which is over two times smaller than the efficiency with FITC-BSA (>99%). Since IL-12 is a heparin binding protein, the particles were expected to be able to capture IL-12 with a high efficiency. Since this is not the case, it is hypothesized that during the synthesis process, critical binding sites on the IL-12 were hidden and prevented the better attachment of heparin and IL-12.

4.7 Release studies. Since the particles were able to encapsulate IL-12, the investigation proceeded into release studies. For initial release studies, particles were placed in PBS solution within dialysis tubing and the concentration of IL-12 outside the membrane was measured. When these samples were tested, it showed that no IL-12 was making it through the membrane. We changed our protocol and tried the release again, which was successful. Figure 10 had showed the release profile of CHNPs and CNPs. Within two hours the CHNPs had released 100% of the encapsulated IL-12. The graph shows this value has slightly higher than 100% because the batch of CHNPs used in the second test were suspected of having a slightly higher loading efficiency which resulted in addition of slightly more than 50µg of IL-12 total. When this release is compared to the CNPs, the differences are very evident. CNPs showed a sustained release that had only released 32% of the IL-12 at the end of 48 hours. As mentioned previous, we believe that during the synthesis process key binding sites on the IL-12 are blocked by chitosan and resulted in the low loading efficiency. This could also be a reason as to why the particles have such a fast release. Due to these sites being blocked, IL-12 is not able to bind as securely to the heparin and will therefore release into solution easier. Since the CHNPs showed a faster release rate than the CNPs, these particles could be used as a fast release component of a multi nanoparticulate drug delivery system where the drug needs to be administered fairly quickly but not a bulk dose.
5. Conclusion

This study proposed the use of chitosan/heparin nanoparticles as a drug delivery system for the bladder cancer therapeutic IL-12. Through this investigation, a protocol was developed that successfully created CHNPs that range from 340.76nm to 2427.5nm and showed how parameters such as chitosan concentration, heparin concentration, and sonication affected the particle size, loading efficiency, and yield. From this investigation, an optimal CHNP was determined to be created using 1mg/ml chitosan and 0.160mg heparin or any scaled amount of these reagents that utilize the same ratio of chitosan to heparin. This combination resulted in a high yield protocol that created highly efficient particles (FITC-BSA). Two sonications was determined the optimal number of sonications because it made the particles more uniform throughout the solution while allowing them to retain their high loading efficiency. It was also found that lyophilization seriously impacts the structure of CHNPs and imaging these particles may prove to be a challenge. When this protocol was used to create IL-12 loaded particles, it resulted in similar sized particles with much lower efficiency. Since IL-12 was still encapsulated, release studies were performed. From these studies it was found that a CHNP/IL-12 delivery system will release 100% of encapsulated IL-12 within two hours. This could be adventitious for applications that require a shorter drug delivery time frame.

There are numerous future studies that should be pursued after this initial investigation. The mass yield of the nanoparticles provides one opportunity. In this study we measured the total mass, not the exact number of particles. This could have resulted in some combinations having a higher mass yield but a lower nanoparticle number. A method to quantify the actual number of nanoparticles needs to be identified. Also, the method of binding of protein (FITC-BSA and IL-12) to the CHNPs created using incorporation and adsorption creation methods should be found.
to better the loading efficiency and release of these particles. A new imaging technique, such as TEM or environmental SEM, should be used to determine the true morphology of CHNPs. Finally, anti-tumor studies should be conducted to conclude whether CHNPs are a successful IL-12 delivery system for bladder cancer.

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7. References


