Effect of Cationic Grafted Copolymer Structure on the Encapsulation of Bovine Serum

Albumin

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20 **Abstract**

21 The aim of the present study was to evaluate a library of poly-L-lysine (PLL)-graft (g)-
22 polyethylene glycol (PEG) copolymers for the ability to encapsulate effectively a model protein,
23 bovine serum albumin (BSA), and to characterize the stability and protein function of the
24 resulting nanoparticle. A library of nine grafted copolymers was produced by varying PLL
25 molecular weight and PEG grafting ratio. Electrostatic self-assembly of the protein and the
26 grafted copolymer drove encapsulation.

27 The formation of protein/polymer nanoparticles with a core/shell structure was confirmed using
28 PAGE, dynamic light scattering, and electron microscopy. Encapsulation of the BSA into
29 nanoparticles was strongly dependent on the copolymer-to-protein mass ratio, PEG grafting
30 ratio, and PLL molecular weight. A copolymer-to-protein mass ratio of 7:1 and higher was
31 generally required for high levels of encapsulation, and under these conditions, no loss of protein
32 activity was observed. Copolymer characteristics also influenced nanoparticle resistance to
33 polyanions and protease degradation. The results indicate a copolymer of 15-30 kDa PLL, with a
34 PEG grafting ratio of 10:1, is most promising for protein delivery.

1. INTRODUCTION

One of the most promising methods to improve the controlled delivery of proteins is nano-encapsulation within biocompatible polymers. Although many methods of encapsulating proteins and biomolecules have been discovered, encapsulation using electrostatic interactions is one of the most promising. Encapsulation using electrostatics avoids the use of harsh solvents or direct modification of the protein, which are common with other protein encapsulation techniques and may lead to decreased protein function [1]. The formation of polyion complexes from oppositely charged polymers has been studied extensively, especially for gene delivery [2-5]. In particular, cationic block copolymers of poly-L-lysine (PLL) and polyethylene glycol (PEG) have been widely studied as gene delivery vectors. PLL is a cationic polymer that has been used in many applications in which the polymer associates with negatively charged surfaces and molecules including nucleic acids and proteins [2, 6, 7]. PEG is another widely used polymer which has been found to improve the circulation characteristics and biocompatibility of many materials introduced *in vivo* [8]. Proteins and other particles coated with PEG are generally less immunogenic, less prone to renal clearance, and more resistant to proteolysis [9]. Additionally, coupling PEG onto cationic polymers has been found to reduce the susceptibility of the resulting complex to aggregation [3, 10]. Charged polymers and copolymers have also found growing applicability in encapsulation of protein-based therapeutics [6, 11-15]. Protein polyion complexation has already been demonstrated to be an effective method to encapsulate proteins [12, 16-23]. Additionally, several enzymes complexed with polyions have been shown to retain catalytic activity following encapsulation [12, 16, 24, 25].

Although the characteristics of charged block copolymers that lead to encapsulation of proteins into micelles have been well studied before, there have been relatively few investigations into

what design parameters are important for the encapsulation of proteins by charged grafted copolymers. The purpose of the present work was to investigate how variations in grafted cationic copolymer architecture affect protein encapsulation and stability. First, nine PLL-g-PEG copolymers were synthesized to investigate the effect of PEG:PLL grafting ratio and PLL molecular weight on protein encapsulation. Using BSA as a model protein, we saw that the properties of the copolymer affected protein encapsulation and nanoparticle stability while having no detrimental impact on protein function. Moreover, the protein remained functional toward small molecules as a NP, demonstrating that release of the protein was not necessary for protein-small molecule interactions. Together, the results of this study provide insight on how grafted copolymer architecture affects the properties of self-assembled protein NPs.

2. MATERIALS AND METHODS

Bovine serum albumin (BSA), poly-L-lysine-HBr (PLL) with molecular weights 4-15 kDa, 15-30 kDa, and 30-70 kDa, p-nitrophenyl caprylate, and chymotrypsin from bovine pancreas were purchased from Sigma Aldrich (St. Louis, MO). Polyethylene glycol with 2 and 5 kDa molecular weights and functionalized with a carboxymethyl succinimidyl ester (mPEG-NHS) were purchased from Creative PEGworks. Glutaraldehyde (50%), acrylamide/bisacrylamide (37.5:1) and other polyacrylamide gel casting and running materials were purchased from Fisher Scientific (Pittsburgh, PA). DQ Green BSA was purchased from Life Technologies (Grand Island, NY).

2.1 PLL-g-PEG Grafted Copolymer Synthesis

PLL-g-PEG was synthesized using the succinimidyl ester functional group on the PEG to modify primary amine groups of the PLL. Various masses of 2 kDa and 5 kDa mPEG-NHS (a 50/50

mixture) were added to 15 mg of 4-15, 15-30, and 30-70 kDa PLL-HBr dissolved in 200 μ L of phosphate buffered saline (PBS). The amounts of PEG required to form each copolymer were pre-determined experimentally by trial and error (data not shown). For 4-15 kDa PLL (defined as “low molecular weight”), 28, 40, and 48 mg of a 50/50 mixture of 2 and 5 kDa PEG were added to the dissolved PLL to produce the grafting ratios 2, 10, and 20, respectively. For 15-30 kDa PLL (defined as “medium molecular weight”), 4, 40, and 48 mg of a 50/50 mixture of 2 and 5 kDa PEG were added to the dissolved PLL to produce the grafting ratios 2, 10, and 20. For 30-70 kDa PLL (defined as “high molecular weight”), 2, 10, and 18 mg of a 50/50 mixture of 2 and 5 kDa PEG were added to the dissolved PLL to produce the grafting ratios 2, 10, and 20, respectively. The mixtures were allowed to react for 2 hours before being washed with ultrapure water in a 10 kDa centrifugal concentrator. After 3 washes, each grafted copolymer was lyophilized and stored at -20°C.

The grafting ratio of each PLL-g-PEG was determined using proton nuclear magnetic resonance spectroscopy (^1H NMR). Each grafting ratio is an approximation as a distribution of PLL molecular weights was used to synthesize the grafted copolymer. Lyophilized polymer was dissolved in D_2O and ^1H NMR spectroscopy was performed using a Bruker Avance INOVA 400 MHz spectrometer. The grafting ratio, or PEG chains per PLL chain, was determined by integrating the PLL and PEG peaks at 4.3 ppm and 3.7 ppm, respectively. A summary of the copolymers produced is shown in Table 1.

2.2 BSA Nanoparticle Synthesis

BSA nanoparticles (NPs) were formed through the electrostatic interaction of the cationic backbone of the PLL-g-PEG copolymer and negatively charged BSA. BSA was prepared by

dissolving lyophilized BSA in PBS to a concentration of 1 mg/mL. The BSA was then filtered through a 0.2 μ m syringe filter to remove larger aggregates. Then the filtered protein concentration was determined by measuring the absorbance of the sample at 280 nm. The filtered BSA solution was further diluted in PBS to produce a 0.266 mg/mL BSA solution.

PLL-g-PEG copolymer was dissolved in PBS at varying concentrations in order to add the same volume of polymer solution to BSA while varying the copolymer-to-protein mass ratio (C:P ratio) when forming nanoparticles. Copolymer-to-protein mass ratios of 1:1, 2:1, 3:1, 7:1, 10:1, and 14:1 were produced to study the effect of copolymer concentration on protein encapsulation. NPs were formed by adding 7.5 μ L of copolymer solution to 25 μ L of BSA at 0.266 mg/mL under gentle vortexing and allowing the solution to incubate for 1 hour at room temperature, similar to the approach of Gaydoss and coworkers [24].

Following NP formation, NPs were further stabilized using glutaraldehyde to cross-link amino groups [27]. From a 50% stock solution, glutaraldehyde was diluted in PBS to produce a 0.025% glutaraldehyde solution. After 1 hour of NP formation, 5 μ L of 0.025% glutaraldehyde was added to the NP samples followed by incubation for 3 hours at room temperature. The cross-linked NPs were either used immediately or stored at 4°C.

2.3 SEM analysis of BSA Nanoparticles

The morphology and size of the BSA NPs were analyzed with an FEI Quanta 600 scanning electron microscope (SEM) at an accelerating voltage of 20 kV. Samples of BSA were encapsulated using a PLL-g-PEG (15-30 kDa), grafting ratio 10, with a C:P ratio of 7:1, and were deposited onto aluminum SEM stubs using a drop-casting method and allowed to air-dry at room temperature for 24 hours. Dried samples were sputter-coated with gold using a Cressington

108 sputter coater. Average sample size and size distribution of the NPs were determined by analyzing 117 particles using ImageJ image analysis software.

2.4 TEM analysis of BSA Nanoparticles

The internal structure of BSA NPs was analyzed using transmission electron microscopy (TEM). BSA was encapsulated using PLL-g-PEG (15-30 kDa) with a grafting ratio of 10 and a C:P ratio of 7:1 as described in the nanoparticle synthesis section. Following nanoparticle synthesis, 30 μ L of nanoparticles were placed on a formvar-carbon coated grid and allowed to air-dry at room temperature overnight. The sample was then negatively stained with 30 μ L of phosphotungstic acid (pH 7.4), which was wicked off the grid after 1 minute using filter paper. The sample was allowed to dry for 2 hours before observation using a JEOL JEM-2100 electron microscope.

2.5 Gel Migration Assay and Encapsulation Efficiency

The extent of BSA encapsulation into NPs was determined using a gel retardation assay. Samples containing approximately 2 μ g of BSA, BSA exposed to glutaraldehyde, or BSA NPs were loaded onto 8% SDS-PAGE gels. The samples were not boiled and sample buffer (61% (v/v) DI water, 13% (v/v) 0.5 M Tris-HCl pH 6.8, 26% (v/v) glycerol, 2.1% (w/v) SDS, and 0.01% (w/v) bromophenol blue) did not include a reducing agent. SDS-PAGE gels were run at 200 V on a Biorad tetracell mini gel electrophoresis apparatus until the dye front reached the bottom of the gel. The running buffer (1.5 g/L Tris Base and 7.2 g/L glycine) did not contain SDS. The SDS-PAGE gels were stained with Coomassie G-250 before imaging. The relative band intensity of the non-encapsulated BSA was compared to the BSA with which copolymer was added to determine the extent of protein encapsulation.

2.6 Nanoparticle Size and ζ -Potential

The hydrodynamic diameter and surface charge of NPs were measured using a ZetaPALS Brookhaven Instruments Corporation ζ -potential analyzer. Prior to the measurements, samples were diluted in PBS to a total volume of 750 μ L or 1.5 mL and passed through a 0.2 μ m syringe filter directly into a disposable cuvette to remove dust from the sample. Dynamic light scattering (DLS) was used to determine the NP hydrodynamic diameter. A total of 5 measurements, lasting 2 minutes per measurement at a 90° angle were used to determine the average size of the NPs by intensity. The ζ -potential, determined by using phase analysis light scattering (PALS), was calculated from the electrophoretic mobility using Smoluchowski's equation [26]. A total of 10 measurements, each lasting 30 seconds, were used to determine the average ζ -potential of the NPs.

2.7 Retention of Protein Esterolytic Activity

The effect of encapsulation on protein function was determined by measuring the cleavage of a p-nitrophenyl ester by BSA [27]. Based on the method used by Córdova et al., the ester binding and hydrolysis of encapsulated BSA was measured by monitoring the release of p-nitrophenol spectrophotometrically [28]. To measure esterolytic activity, 5 μ L of 6 mM p-nitrophenyl caprylate in isopropanol was added to 100 μ L of NPs, and the mixture was allowed to incubate 20 hours at 37°C before absorbance measurements were taken at 410 nm on a Packard Spectracount plate reader.

2.8 Stability of NPs Against Polyanions

The stability of NPs against heparin, a polyanion with a high charge density, was evaluated using the gel retardation assay described above. NPs were produced from low, medium, and high molecular weight (all 10:1 grafting ratio) copolymers at 1:1, 7:1, and 14:1 C:P ratios. The NPs

were then incubated with PBS (control) or heparin in PBS at 0.1 mg/mL and 1 mg/mL for 24 hours at 37°C before PAGE.

2.9 Stability of NPs Against Protease Degradation

NPs were prepared following the method described above to encapsulate DQ Green BSA, a form of BSA that is so heavily labeled with BODIPY dye that the fluorescence is self-quenched. When degraded by protease, this quenching is relieved as fragments are released, and thus the protein degradation can be measured fluorometrically [29]. For these assays, DQ Green BSA NPs were prepared from LMW 10, MMW 10, and HMW 10 copolymers and encapsulated at 1:1, 7:1, and 14:1 C:P ratios. The NPs were incubated with chymotrypsin at 0.25 mg/mL at 37°C, and the fluorescence was measured over time (Exc: 485 nm Em: 535 nm) using a Beckman Coulter DTX 880 Multimode Detector.

2.10 Statistical Analysis

Statistical analysis was performed in Microsoft Excel using one-way analysis of variance (ANOVA) and pair-wise comparisons by Tukey's *post hoc* test. Data from the protease degradation study were fitted using a one site saturation model in SigmaPlot.

3. RESULTS AND DISCUSSION

3.1 PLL-g-PEG Synthesis

A library of nine PLL-g-PEG copolymers was synthesized to study the effect of PLL molecular weight and PEG grafting ratio on BSA encapsulation. A mixture of PEG molecular weights were used for the copolymers because a future aim of our work, outside the scope of the present study, is to develop copolymers functionalized with two different types of ligands at different distances

from the PLL backbone. The synthesis of PLL-g-PEG was performed as illustrated in Figure 1, with increasing quantities of PEG added to the PLL to produce higher grafting ratios. The approximate number of PEG chains grafted onto each PLL chain was determined using ^1H NMR. An example of the grafting ratio calculation and ^1H NMR spectrum may be found as supplementary material (Section S1). A summary of the library used and the results obtained is presented in Table 1.

3.2 BSA Encapsulation Efficiency

To determine the extent of BSA encapsulation, NPs assembled from the copolymer library were characterized by gel retardation assays. Results showed that the BSA exposed to glutaraldehyde migrated slightly faster compared to unmodified BSA (Fig. 2, lane 2 of each panel). At low glutaraldehyde and protein concentrations, intramolecular cross-linking of the protein is more likely to occur than intermolecular cross-linking [30]. The results of Figure 2 indicate that glutaraldehyde did not cause intermolecular cross-linking of BSA, as no additional higher molecular weight aggregates were observed following treatment. The slightly faster migration of BSA through the gel is likely a result of intramolecular cross-linking of the BSA that hindered the ability sodium dodecyl sulfate (SDS) to fully denature the protein. Upon the addition of copolymer, a combination of cross-linking occurred between copolymer chains and perhaps between the copolymer and protein. At the 1:1 C:P ratio, some heterogeneous retardation of BSA mobility (“smearing”) was apparent on the gel, with a concomitant decrease in band intensity or spreading of the band for the free protein (Fig. 2, lane 3 of each panel). In cases at which the free protein band widened at a 1:1 C:P ratio, this appears to be due to a portion of the BSA becoming intramolecularly crosslinked by glutaraldehyde, producing a free protein band that appears to be a mixture of what is observed in lanes 1 and 2. The apparent association of the grafted copolymer

212 with BSA increased as the C:P ratio was increased above 1:1, with markedly improved
213 encapsulation of BSA being apparent at higher C:P ratios for all of the copolymers. At C:P ratios
214 between 3:1 and 7:1, high molecular weight copolymers retarded the mobility of effectively all
215 of the BSA molecules (Fig. 2, row 1, lane 6), indicating that encapsulation or loading of the
216 protein into the NPs was 100%. When the C:P ratio was increased even further, all of the protein
217 was retained in the stacking gel, which also suggested complete loading of the protein into NPs.
218 These results demonstrated a direct relationship between C:P ratios and BSA encapsulation.

219 The molecular weight of the copolymer PLL chain also had a significant effect on protein
220 encapsulation. For most of the copolymers, higher C:P ratios were required to encapsulate BSA
221 effectively with the low and medium molecular weight copolymers compared to the high
222 molecular weight copolymers. For the medium molecular weight copolymers, some retardation
223 was observed for all C:P ratios, but a C:P ratio of at least 7:1 was required for protein migration
224 to be fully retarded. Similarly, although encapsulation of BSA with low molecular weight
225 copolymers resulted in inhibited migration of the protein, the low molecular weight copolymers
226 were generally the least effective in retarding migration. The LMW 2 copolymer was one
227 exception. Due to a higher number of copolymer chains relative to the MMW 2 and HMW 2, the
228 LMW 2 copolymer may have been able to crosslink between copolymer chains better than the
229 other copolymers, leading to more efficient encapsulation. This indicates that the higher the
230 molecular weight of the copolymer, the more efficiently the copolymer encapsulates the protein.

231 The larger molecular weight PLL copolymers encapsulated BSA at lower C:P ratios compared to
232 the smaller molecular weight copolymers, which is similar to higher PLL molecular weights
233 improving DNA condensation through improved ion pairing [31, 32]. This decrease in mass of
234 copolymer required for efficient encapsulation was likely a result of the differences in the

electrostatic charge of the copolymers. Because the grafted copolymer was synthesized by conjugating PEG to the amine groups that give the PLL polymer its positive charge, higher grafting ratios result in copolymers with fewer amine groups and a lower charge. Similarly, for a fixed grafting ratio, high molecular weight PLL has a greater number of positively charged amine groups per PLL chain than the medium or low molecular weight PLL. This gives the high molecular weight copolymers more charge overall, and enables them to more easily associate with the BSA. In contrast, medium and low molecular weight copolymers have more PLL amine groups substituted by PEG. The low and medium molecular weight copolymers have had more of the charge neutralized, leading to the observed higher quantities of copolymer needed to effectively associate with the BSA. Although using high amounts of copolymer to encapsulate a protein is not necessarily desirable, using copolymers with a large number of PEG chains is likely to be beneficial *in vivo* [33]. Therefore, there is a need to balance efficient encapsulation with sufficient PEGylation.

Together, the results of Figure 2 indicate that copolymer molecular weight, C:P ratio, and grafting ratio all impact the encapsulation of BSA. For a fixed PLL molecular weight, increasing the amount of grafted PEG generally led to a decrease in the amount of protein encapsulated for a given C:P ratio. PEG grafting onto PLL, however, is important in the formation of micelle-like nanoparticles rather than large aggregates commonly formed from cationic polymers and negatively charged biomolecules [31, 34]. To limit the number of particles further characterized, the PLL molecular weight and the C:P ratio (1:1, 7:1, and 14:1) were varied while the grafting ratio was held constant (10:1) for the remainder of the study.

3.3 NP Structure and Morphology

In addition to the gel retardation assay, electron microscopy was used to confirm the formation of BSA NPs. In Figures 3 and 4, BSA NPs were observed using scanning (SEM) and transmission electron microscopy (TEM). SEM results for the encapsulation of BSA with MMW 10 and a C:P ratio of 7:1 show NPs that were typically ellipsoids but often had irregular morphologies. The size of the BSA NPs based on SEM ranged from about 20 – 100 nm, with an average of 50 nm (Figure 4). Thus, the sizes of our NP were similar to those reported in previous characterization of protein polyion complexes [16, 24].

TEM was used to determine the internal structure of the NPs. The TEM micrograph in Figure 3 (Panel B) shows NPs produced from MMW 10 with a C:P ratio of 7:1 that ranged from approximately 20-100 nm in diameter. The interior of the NPs had a punctate appearance with a bright outline. The white outline surrounding each particle is consistent with the formation of a PEG corona, which was similar to that observed around other PEGylated NPs [35]. This contrasts with BSA that was encapsulated with MMW PLL alone (Panel C), in which only a thin dark outline surrounded these particles that were typically much larger than 100 nm. The layering of the light outer region over the punctate interior suggests that the PEGylated NPs had a core/shell structure. A core-shell structure is consistent with the observations of others that have produced polyion complexes [5, 36]. This is likely due to steric effects of the hydrophilic PEG favoring placement at the outer surface of the particle and the cationic component of the polymer favoring association with negatively charged carboxylic groups on the BSA surface.

3.4 NP Physicochemical Properties

Dynamic light scattering and PALS were used to determine the size and ζ -potential of NPs formed from LMW 10, MMW 10, and HMW 10 copolymers at a 7:1 C:P ratio (Table 2). The

279 particle size, when measured using dynamic light scattering, indicated that NPs were
280 approximately 20 nm in diameter for all three copolymers tested. Light scattering indicated that a
281 distribution of NPs was formed, with some NPs slightly smaller than 20 nm and other NPs up to
282 100 nm in diameter (data not shown). Although this was smaller than what was observed using
283 electron microscopy, all three techniques indicate that a distribution of nanoparticle sizes was
284 formed with a size ranging from approximately 20-100 nm. The low contrast of the NPs when
285 observed under SEM favors larger particles to be more easily observed and differentiated from
286 the background, which may skew the average particle size. Additionally, differences in sample
287 preparation may contribute to the differences observed.

288 To learn how the copolymers affected the NP surface charge, the ζ -potential was measured for
289 NPs formed from LMW 10, MMW 10, and HMW 10. In contrast to particle size, each of the
290 three copolymers produced NPs with different ζ -potentials. The NPs produced from the HMW
291 10 copolymer were slightly positive in charge. This was expected since of the three copolymers
292 tested the HMW 10 has the lowest number of positively charged amine groups substituted with
293 PEG, resulting in particles with a positive charge. NPs produced from the MMW 10 copolymer
294 had the most negative charge of the three measured. A negative surface charge may suggest the
295 presence of protein, as well as PEG, near the particle surface. It is possible, given there was a
296 small amount of free BSA at this C:P ratio based on the gel retardation assay, that some free
297 protein was associated with the NP surface which may have resulted in the non-linear
298 relationship between the ζ -potential and the copolymer molecular weight. Even though a small
299 amount of free protein was observed on the PAGE gels for the other two copolymers, it is
300 possible that the charge density of the MMW 10 copolymer and the extent that the polymer was
301 PEGylated made this effect more prevalent compared to the other two copolymers. The lower

charge density of the MMW 10 copolymer, relative to the HMW 10 copolymer, may have allowed for a combination of PEG and small quantities of protein to be located at the particle surface. The more highly PEGylated LMW 10 NPs, however, may prevent this from occurring. The LMW 10 NPs had a near neutral charge, which was expected from NP produced from the most PEGylated of the three copolymers. The higher degree of PEGylation may cause the PEG to become densely packed and prevent any protein binding near the particle surface. Overall, the ζ -potential of the NPs tested was near neutral, which could be a positive characteristic for minimizing non-specific interactions with strongly anionic biomolecules such as serum proteins and cell surface proteoglycans.

3.5 Retention of Protein Esterolytic Activity

To examine the effect of encapsulation on protein function, the esterolytic activity of BSA toward a p-nitrophenyl ester was measured. Albumin can bind to and hydrolyze ester substrates [37]. Results showed that the ability of BSA to bind and cleave a p-nitrophenyl ester was not adversely affected by encapsulation or cross-linking (Fig. 5): esterolytic activity of the encapsulated BSA was similar to that of the non-encapsulated BSA. When BSA was encapsulated with the medium or high molecular weight copolymers, the activity of the protein was slightly higher than the non-encapsulated protein. The C:P ratio had a relatively minimal effect on protein activity, with higher C:P ratio NPs showing slightly higher esterolytic activity.

Our finding that encapsulation slightly enhances BSA esterolytic activity is consistent with previous observations that the catalytic activity of lysozyme toward a p-nitrophenol ester was enhanced following encapsulation with a polyaspartate-PEG copolymer [38]. Those authors suggested that the enhanced activity was likely due to accumulation of the substrate in the

micelle, leading to a higher concentration of substrate near the protein than in the surroundings of the micelle. This explanation is also consistent with the observation that increasing the C:P ratio led to an increase in protein catalytic activity. Others have similarly seen an increase in apparent enzyme activity following encapsulation of catalase into polyion complex micelles [16].

3.6 Stability of NPs Against Polyanions

Many serum proteins and heparin sulfate proteoglycans have an overall negative charge, which may cause dissociation of polyion complexes *in vivo*. To learn how susceptible the copolymer NPs are to negatively charged molecules the stability of LMW 10, MMW 10, and HMW 10 NPs to polyanions was evaluated using heparin (Figure 6). Generally, the NPs were resistant to the effects of heparin as most of the protein remained associated with the NPs after co-incubation. Although very little dissociation of the protein was observed, for all NPs tested, increasing heparin concentration led to protein release. The low molecular weight copolymer had the lowest stability against polyanions, as more BSA was released from the NPs compared to medium and high molecular weight copolymers. This may be due to the low molecular weight copolymer having the lowest charge density among the three copolymers tested. The low molecular weight copolymers have the fewest cationic amine groups per copolymer chain making them more susceptible to disassociation by a polyanion. For all three copolymers tested, the 7:1 C:P ratio showed the greatest stability against heparin at 1 mg/ml compared to the 1:1 and 14:1 ratios. At the 1:1 C:P ratio, the protein is not fully encapsulated, making the amount of free protein detected in the presence of heparin already high. The 14:1 C:P ratio in particular was among the least stable of the NPs. NPs with high C:P ratios (14:1) may possibly be more susceptible to dissociation by polyanions due to less organization between copolymer and BSA layers, reducing the efficiency of glutaraldehyde cross-linking. At the 7:1 C:P ratio, glutaraldehyde

cross-linking of NPs completely encapsulating the protein is most efficient relative to the 1:1 and 14:1 C:P ratios.

3.7 Stability of NPs Against Proteolysis

Proteolysis is a major cause of inactivation of pharmaceutically relevant proteins in clinical settings. In order to determine if our NP could protect BSA from proteolysis, the effect of encapsulation was evaluated using chymotrypsin as a model protease [39, 40]. These assays used BSA that was heavily labeled with a self-quenching BODIPY fluorescent dye, with degradation of the BSA being observed as an increase in fluorescence [29]. Results showed that the non-encapsulated BSA was rapidly degraded by chymotrypsin, as indicated by the increase in fluorescence over the first 24 hours of the study (Fig. 7, filled circles). Cross-linking the BSA with glutaraldehyde reduced the maximal degree of proteolysis (Fig 7, filled inverted triangles). Encapsulation provided additional protection from the protease, with the BSA NPs releasing only 25% of the fluorescence relative to the free protein after 48 hours of exposure to chymotrypsin. Although the extent of chymotrypsin protection was found to be relatively similar for all of the copolymers, the high molecular weight copolymer appeared to offer slightly lesser protection. Additionally, the C:P ratio had little effect on protecting against BSA degradation. These results indicated that glutaraldehyde cross-linking and copolymer complexation can markedly reduce the susceptibility of a protein to protease-mediated degradation.

Glutaraldehyde cross-linking as well as other cross-linking strategies have been commonly used to enhance the stability of proteins and polyion complexes against proteases and other forms of degradation [30, 41]. The increased stability of proteins modified by cross-linkers, such as glutaraldehyde, has been attributed to an increase in protein rigidity due to intramolecular and/or

intermolecular cross-linking between proteins and polymers [42]. Chymotrypsin has preferential activity at aromatic amino acid residues [43]. Intramolecular cross-linking of BSA likely affected conformational changes of the protein and reduced the access of chymotrypsin to the hydrophobic residues the protease preferentially cleaves.

Similarly, the formation of rigid, intermolecular cross-links by glutaraldehyde between copolymer chains as well as between the copolymer and BSA could be expected to further prevent access of the chymotrypsin to the protein. Additionally, PEGylation of the copolymers can sterically hinder interactions between the chymotrypsin and the protein NPs. Similar results were observed by others who found that encapsulation of DNA into polyplexes by PLL-g-PEG reduces the susceptibility of the DNA to degradation by nucleases [44, 45]. Protection as a result of PEGylation is consistent with the observation that the HMW 10 copolymer provided the least protection against proteolysis of the three NPs. The HMW 10 copolymer had the fewest PEG chains protecting the protein compared to the low and medium molecular weight copolymers (at a given C:P ratio).

4. CONCLUSION

Protein encapsulation into polyion complexes is a promising approach to improve upon successful protein delivery. Only limited investigation, however, has been done into how characteristics of grafted cationic copolymers affect the encapsulation and stability of protein polyion complexes. The approach taken in this study found that PLL molecular weight, PEG grafting ratio, and C:P ratio all affect encapsulation of BSA. Protein catalytic activity was retained through the encapsulation process and even showed increased levels of activity at high C:P ratios for the medium and high molecular weight copolymers. Over a constant grafting ratio

391 of 10, the medium and high molecular weight copolymers showed more resistance to polyanions
392 than the low molecular weight copolymer. The low and medium molecular weight copolymer
393 provided more protection for the protein to proteolysis compared to the high molecular weight
394 copolymer. Future studies that focus on protein delivery under physiological conditions will
395 undoubtedly provide additional framework for the design of an efficient protein delivery system.

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Figure 1. Synthesis of PLL-g-PEG Copolymer and Synthesis of BSA NPs. mPEG-NHS (2 and 5 kDa) was grafted onto 4-15 kDa, 15-30 kDa, and 30-70 kDa PLL. Three different grafting ratios (approximately 2, 10, and 20) were produced by varying the mass of PEG added to each

PLL. BSA NPs were produced through self-assembly of the negatively charged BSA protein and the cationic PLL-g-PEG copolymer. After one hour of assembly in phosphate buffered saline, the NPs were cross-linked using 0.003% glutaraldehyde.

Figure 2. Encapsulation Efficiency of BSA with high, medium, and low molecular weight PLL-g-PEG copolymers with grafting ratios 2, 10, and 20. Each column corresponds to the mPEG grafting ratio for the copolymer. The row corresponds to the PLL molecular weight, with high, medium, and low being 30-70 kDa, 15-30 kDa, and 4-15 kDa PLL, respectively. In each panel, the sample in each lane is as follows with the NP lanes differentiated by C:P ratio: Lane 1: BSA; Lane 2: BSA incubated with glutaraldehyde; Lane 3 1:1 NP; Lane 4: 2:1 NP; Lane 5: 3:1 NP; Lane 6: 7:1 NP; Lane 7: 10:1 NP; Lane 8: 14:1 NP. The extent of BSA encapsulation was determined by comparing the relative band density of the BSA monomer for each C:P ratio to the non-encapsulated protein.

Figure 3. Electron Microscopy of BSA NPs. The formation of BSA NPs was confirmed using SEM (A) and TEM (B). A TEM micrograph of BSA complexed with unPEGylated 15-30 kDa PLL was used as a control (C). The SEM and TEM scale bar lengths are 100 nm. The NPs in the above micrographs were produced using BSA and medium molecular weight PLL, PEG grafting ratio 10 with a 7:1 C:P ratio.

Figure 4. NP Size Distribution. The size distribution of MMW 10 7:1 NP was determined from multiple the SEM micrographs with a representative one shown above (A). The scale bar length is 500 nm. To determine the size distribution, NPs were treated as spherical particles and the particle diameter was determined using Image J (B). The size distribution was determined from 117 NPs. The average particle size was approximately 50 nm.

Figure 5. Retention of BSA Esterolytic Activity. BSA catalytic activity was measured by monitoring the hydrolysis of p-nitrophenyl caprylate with absorbance values taken at 410 nm after approximately 20 hours. Activity was normalized to that of the non-encapsulated BSA. The data is the mean and standard deviation of three replicates. * indicates $p < 0.05$ between BSA and the indicated NP.

Figure 6. NP Polyion Stability after Incubation with Heparin. NPs were incubated with heparin at 37°C for 1 day. Lane 1: BSA; Lane 2: 1:1 NP, 0 mg/ml heparin; Lane 3: 1:1 NP 0.1 mg/ml heparin; Lane 4: 1:1 NP 1 mg/ml heparin, Lane 5: 7:1 NP 0 mg/ml heparin; Lane 6: 7:1 NP 0.1 mg/ml heparin; Lane 7: 7:1 NP 1 mg/ml heparin; Lane 8: 14:1 NP 0 mg/ml heparin; Lane 9: 14:1 NP 0.1 mg/ml heparin; Lane 10: 14:1 NP 1 mg/ml heparin.

Figure 7. Stability of NPs to Protease Degradation. DQ Green BSA was encapsulated to form LMW 10, MMW 10, and HMW 10 NPs at 1:1, 7:1, and 14:1 C:P ratios. NPs were exposed to chymotrypsin (0.25 mg/ml) at 37°C over 48 hours. Proteolysis was monitored fluorometrically. Each data point represents the mean of three replicates and the error bars represent the standard deviation.

Figure 1
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Figure 2
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LMW 10

MMW 10

HMW 10

Figure 7
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Table 1. Summary of PLL-g-PEG Library Produced for BSA Encapsulation. The observed grafting ratios were measured using ¹H NMR.

Polymer	PLL Molecular Weight	Targeted PEG Grafting Ratio	Observed PEG Grafting Ratio (±3 of Targeted Grafting Ratio)
LMW 2	Low (4-15 kDa)	2	4.6
LMW 10	Low (4-15 kDa)	10	7.9
LMW 20	Low (4-15 kDa)	20	23.5
MMW 2	Medium (15-30 kDa)	2	4.6
MMW 10	Medium (15-30 kDa)	10	11.0
MMW 20	Medium (15-30 kDa)	20	19.1
HMW 2	High (30-70 kDa)	2	3.6
HMW 10	High (30-70 kDa)	10	11.2
HMW 20	High (30-70 kDa)	20	17.1

Table 2. Physicochemical Characterization of BSA NPs. The average hydrodynamic diameter for LMW 10, MMW 10, and HMW 10 7:1 NPs was determined using dynamic light scattering. The ζ -potential of the NPs was measured using Phase Analysis Light Scattering (PALS). The results shown are the mean and standard deviation of three replicates.

	LMW 10 NP	MMW 10 NP	HMW 10 NP
Diameter (nm)	24 $\pm$ 0.6	18.8 $\pm$ 3.4	23.5 $\pm$ 3.2
ζ-Potential (mV)	-2.3 $\pm$ 4.3	-12.4 $\pm$ 1.2	5.3 $\pm$ 2.5