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Enzymatic degradation of bovine serum Albumin nanoparticles for drug delivery

by

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ABSTRACT

Coacervation is a mild process for developing protein NPs. Bovine serum albumin (BSA) NPs formed *via* this technique were stabilized using poly-L-Lysine (PLL); short interfering ribonucleic acid (siRNA) was used as a model drug for encapsulation. Specific and non-specific degradation of these coated and uncoated BSA NPs were carried using matrix metalloproteinase-2 (MMP-2) and trypsin, respectively. The particles were characterized with atomic force microscopy, zeta-potential, and photon correlation spectroscopy measurements. There was a significant increase in the zeta potential of BSA NPs upon coating. Trypsin digested the uncoated and coated BSA NPs and resulted in higher BSA release from the particles. However, MMP-2 treatment did not result in higher release of BSA from coated NPs despite the cleavability of coated polymer by MMP-2. This study described a method for obtaining BSA NPs in a controllable size range. Such particles showed degradability in the presence of trypsin and could be promising for targeted drug delivery applications.

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LIST OF ABBREVIATIONS

AFM; Atomic Force Microscopy

BSA; Bovine Serum Albumin

FWHM; Full width at half maximum

FITC; Fluorescein isothiocyanate

HCPT; Hydroxycampothecin

HSA; Human Serum Albumin

K_6MK_6; KKKKKKGPQGIASQKKKKKK

MMP-2; Matrixmetalloproteinase-2

MW; Molecular weight

NPs; Nanoparticles

PEI; Polyethylenimine

PLGA; Poly (Lactide-co-glycolide) acid

PLA; Polylactic acid

PLL; poly-L-lysine

PEG; Polyethylene Glycol

PCS; Photon Correlation Spectroscopy

PDADMAC; Poly (diallylmethylammonium chloride)

siRNA; short interfering ribonucleic acid

CHAPTER 1

INTRODUCTION

1.1) Need and importance of Nanoparticles (NPs) for drug delivery

Materials that break down naturally (biodegradable), without initiating any adverse host responses (such as immune, inflammatory, and coagulation), are highly sought after for multiple biomedical applications. In particular, significant effort on developing these materials to form biodegradable nanoparticles (NPs) to deliver therapeutic molecules: low molecular weight drugs, peptides, proteins, and plasmids have been undertaken.[1] For example, several synthetic polymers such as poly (lactide-co-glycolide) acid [PLGA] and polylactic acid (PLA) have been extensively investigated for drug delivery purposes as they have been shown to be both biodegradable and reasonably 'biocompatible'.[2] It has

[1] Jayanth Panyam, Vinod Labhasetwar, "Biodegradable nanoparticles for drug and gene delivery to cells and tissue", Advanced drug delivery reviews, Vol. 55 (2003), pp 329-247

[2] R.A.Jain, "The manufacturing techniques of various drug loaded biodegradable poly (lactide-co-glycolide) devices", Biomaterials, Vol 21 (2000), pp 2475-2490

been shown that the release rates of encapsulated drug/agent from NPs generated from these polymers can be controlled through manipulating many aspects of their physicochemical properties, including copolymer concentration, polymer MW, etc. In addition to a plethora of other synthetic materials, biomolecules such as lipids,[3] albumin,[4] gelatin,[5] etc. have also been investigated as potential candidates for forming NP delivery systems.

Crucial parameters controlling the distribution of NPs throughout the host organism include their size and surface charge. Post administration of NPs into the blood stream, a rapid adsorption of plasma proteins occurs at the blood-material interface that is responsible for initiating a multitude of cellular events. This adsorbed protein layer facilitates NP clearance, as macrophages or monocytes attach to the NPs via the adsorbed protein layer. Given that the adsorption of proteins from solution is strongly influenced by both NP size and surface charge, it is

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[3] Weijun Li, Francis C. Szoka Jr., "Lipid based nanoparticles for nucleic acid delivery", Pharmaceutical Research, Vol. 24 (2007), pp 438-449

[4] K. Langer, S. Balthasar, V. Vogel, N. Dinauer, H. Von briesen and D. Schubert, "Optimization of the preparation process for human serum albumin (HSA) nanoparticles", International journal of pharmaceutics, Vol. 257 (2003), pp 169-180

[5] Truong- Le VL, August JT, Leong KW, "Controlled gene delivery by DNA-gelatin nanospheres", Human gene therapy, Vol. 9 (1998), pp 1709-1717

thought that modulating these parameters will allow for the control of the NP circulation life. It has been suggested that longer circulation times can be achieved through controlling NP size, where the particle should not exceed a diameter of 200 nm as protein adsorption is dependent on the curvature of the surface.[6] Longer circulating particles therefore have better possibility of both localizing to the site of interest and achieving extended drug release kinetics, allowing for an improved therapeutic outcome. The surface charge of the NPs is other important parameter for the circulation of these particles *in-vivo*. Particles with neutral charge are considered to have longer circulation time as compared to their charged counterparts as neutral charged particles are less likely to activate the complement system, which actuates the opsonization of particles.[7]

Many attributes of NPs make them an attractive platform for drug delivery applications: increased therapeutic efficiency for lower amounts of drug, decreased side effects, protection of drug molecules from harsh environmental milieu, etc. An example of how encapsulation of drugs within NPs may lead to a reduction in deleterious side effects includes the

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[6] S.M. Moghimi, A. Christy hunter, J. Clifford Murray, " Long-circulating and target specific nanoparticles: Theory to practice", Pharmacological reviews, Vol. 53 (2001); pp 283-318

[7] D.V. Devine, A.J. Bradley," The complement system in liposome clearance: Can complement deposition be inhibited? ", Advanced drug delivery reviews, Vol. 32(1998), pp 19-29

use of antineoplastic drugs like Doxorubicin. Doxorubicin use is limited on some patients (e.g., cancer patients) because of its high toxicity and numerous side effects.[8] However, their incorporation or binding to the nanoparticles can prove to be a promising means of lowering the potential for side effects, as the entrapped drug does not come into direct contact with healthy cells. For example, the anti-cancer effects of doxorubicin were shown to increase upon being loaded into human serum albumin (HSA) NPs, when compared to drug in solution alone.[9] In addition, encapsulation within the NPs can improve the circulation half-life of peptides, proteins, and nucleic acids used as therapeutic agents; NPs provide protection from enzymatic degradation. For example, the use of short interfering ribonucleic acid (siRNA) as a therapeutic molecule is limited because it is quickly degraded in plasma, resulting in a short

[8] P.K. Singal, T. Li, D. Kumar, I. Danelisen, N. Iliskovic, "Adriamycin induced heart failure: mechanism and modulation", Molecular and Cellular Biochemistry, Vol. 207 (2000), pp 77-86

[9] S. Dries, F.Rothweiler, M.Michaelis, J. Cinatl Jr., J. Kreuter, K. Langer, "Preparation, characterization and maintenance of drug efficacy of doxorubicin-loaded human serum albumin (HSA) nanoparticles", International Journal of Pharmaceutics, Vol 341(2007), pp207-214

residence time *in-vivo*.[10] The use of NPs however can help in alleviating this limitation; in an earlier work,[11] chitosan was used to complex with siRNA to form NPs for its delivery to N1H 3T3 (mouse fibroblast cell line) cells for RNA-mediated therapy. Moreover, the controlled degradation of NPs provides another strategy for controlling the release of encapsulated therapeutic molecules; a strategy employed with many polymeric based NPs, including PLGA. Other advantages to using NPs for drug delivery purposes is their high stability, high carrier capacity, possibility of various routes of administration such as oral, intravenous, etc.[12]

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[10] Yogesh Patil, Jayanth Panyam, "Polymeric nanoparticles for siRNA delivery and gene silencing", International journal of pharmaceutics, Vol 362(2009), pp 195-203

[11] Kenneth A. Howard, Ulrik L. Rahbek, Xiudong Liu, Christian K. Damgaard, Sys Zoffman Glud, Morten Φ. Anderson, Mads B. Hovgaard, Alexander Schmitz, Jens R. Nyengaard, Flemming Besenbacher, JΦrgen Kjems, "RNA interference *in vitro* and *in vivo* using a chitosan/siRNA nanoparticulate system", Molecular Therapy, Vol 14 (2006), pp 476-484

[12] Svetlana Gelperina, Kevin Kisich, Michael D. Iseman, Leonid Heifets, "The potential advantages of nanoparticle drug delivery systems in chemotherapy of Tuberculosis", American journal of respiratory and critical care medicine, Vol 172 (2005), pp1487-1490

1.1.1) Suitability of Bovine Serum Albumin (BSA) for drug delivery

BSA (66 kDa, pI=4.8[13]) is metabolized *in-vivo* to produce innocuous end products,[14] is relatively easy to fabricate into NPs with the range of diameters being somewhat controlled by varying the NP preparation conditions.[15] Also, BSA and its degradation products have been shown to be non-toxic and non-antigenic.[16] NPs generated from this protein can be used to encapsulate other macromolecules, such as proteins or peptides, and can be fabricated to have properties that can modify the

[13] Feng Qian Li, Hua Su, Jing Wang, Ji-yong Liu, Quan-Gang zhu, Yi-bo fei, Yong-Hua Pan, Jin-Hong Hu, "Preparation and characterization of sodium ferulate entrapped bovine serum albumin nanoparticles for liver targeting", International journal of pharmaceutics, Vol. 349 (2008), pp 274-282

[14] Rahimnejad M., M. Jahanshahi, G.D. Najafpour, "Production of biological nanoparticles from bovine serum albumin for drug delivery", African Journal of biotechnology, Vol 5(2006), pp 1918-1923

[15] Guilin Wang, Kevin Siggers, Sufeng Zhang, Hongxing Jiang, Zhenge Xu, Ronald F. Zernicke, John Matyas, and Hasan Uludag, " Preparation of Bovine Serum Albumin (BSA) Nanoparticles stabilized by Polymer Coating", Pharmaceutical Research, 25(12),pp. 2896- 2909 (2008)

[16] Bernhard G. Muller, Hans Leuenberger, Thomas Kissel, "Albumin nanospheres as carriers for passive drug targeting: An optimized manufacturing technique", Pharmaceutical Research, Vol.13 (1996), pp 32-37

release rates of encapsulated agents. For example, BMP-2 was successfully encapsulated into BSA NPs coated with PEI, where it was shown that the coating affected the BMP-2 release rate while un-affecting its bioactivity.[17]

1.2) Nanoparticle formation: coacervation technique

Two researchers, Bungenberg de Jong and Kruyt originally coined the term coacervation in 1930 for a process that involved the separation of an aqueous colloidal solution into two liquid phases: a colloid rich phase, commonly known as the coacervate, and colloid poor phase.[18] Coacervation techniques have been employed for many applications, including protein purification, encapsulation of vitamins, flavours, and drugs.[19]

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[17] Sufeng Zhang, Guilin Wang, Xiaoyue Lin, Maria Chatzinikolaidou, Herbert P. Jennssen, Marcus Laub, Hasan Uludag, "Polyethylenimine-coated Albumin Nanoparticles for BMP-2 delivery", Biotechnology Progress, Vol 24 (2008), pp 945-956

[18] Fredric M. Menger, Bridget M. Sykes, "Anatomy of a coacervate", Langmuir, Vol. 14 (1998), pp 4131-4137

[19] B. Mohanty, H.B. Bohidar, "Systematic of Alcohol-Induced Simple Coacervation in Aqueous Gelatin Solutions", Biomacromolecules, Vol 4(2003), issue 4, pp 1080-1086

Coacervation is a simple and mild process for fabricating protein based nanoparticles.[15] It is a colloidal phenomenon where an aqueous solution of a charged molecule, such as BSA, undergoes liquid-liquid phase separation upon addition of an ionic salt or alcohol to the system (simple coacervation) or upon the addition of oppositely charged molecules (complex coacervation). The resultant coacervate phase remains in equilibrium with the colloid poor supernatant, with both phases being immiscible and incompatible.[18]

Coacervation induced by the addition of a non-solvent can be explained in terms of Flory Huggin's theory. Flory described polymer solutions in terms of lattice model and introduced the term, interaction parameter, χ. This interaction parameter takes into account the energy of interdispersing polymer and solvent molecules or describes the solvent power to dissolve a polymer in simple terms. A thermodynamically good solvent will have lower values for χ.[20]The value of χ increases for poor solvent for polymers.

Thermodynamically, Gibb's free energy change for mixing or dissolution at a constant temperature and pressure is given as:

$$\Delta G_m = \Delta H_m - T\Delta S_m$$

[20] Claudio Thomson, Ho Nam-Tran, Hans P. Merkle, Bruno Gander, "Drug encapsulation by PLA/PLGA coacervation in the light of thermodynamics. 1. Overview and theoretical conditions", Journal of Pharmaceutical Sciences, Vol 87 (1998); pp 259-268

where,

ΔG_m is the Gibb's free energy change accompanying mixing

ΔH_m is enthalpy change accompanying mixing

ΔS_m is entropy of mixing

Flory--Huggin's theory predicted ΔG_m as:

$$\Delta G_m = kT \{n_1 \ln\Phi_1 + n_2 \ln\Phi_2 + n_1 \Phi_2 \chi_1\}$$ [21]

ΔG_m therefore is a function of number of moles of solvent, n, volume fraction, Φ, and interaction parameter, χ, with subscript 1 and 2 standing for solvent and dissolving component, respectively. k is the Boltzmann's constant and T, temperature of the system with χ_1 as the interaction parameter of the dissolving component into the solvent. The first two terms are negative since natural logarithmic value of any number less than 1 is negative, the third term deciding the sign for ΔG. In terms of our system, it can be said that BSA is soluble in water making the value of interaction parameter low, resulting in overall negative value of ΔG. As ethanol is added, the hydrogen bonds between water molecules and the polyion (BSA) rupture because of greater affinity of ethanol to water molecules. The resultant mixture therefore, becomes a poor solvent for BSA. Consequently, the interaction parameter apparently increases causing a shift in the value of ΔG. Therefore, complementary charges of the polyion chain come closer to each other leading to self-charge

[21] Krister Holmberg, "Handbook of Applied Surface and Colloid Chemistry", Vol.1, pp 230

neutralization. This leads to smaller particles, present mainly in the supernatant. At the same time, electrostatic interactions between charged segments of different BSA molecules lead to aggregation i.e. NPs formation. These aggregates may attract other molecules to fully neutralize the charge and grow in size.[19]

Dielectric constant is the other factor, which changes with the addition of ethanol to the aqueous system. It may be defined as a parameter measuring the ability of the solvent to reduce the strength of the electric field surrounding the charge particle in the solvent and hence affects the electrostatic interaction forces between the charged molecules. As per Debye Huckel theory, the polyions are treated as single charges and total interaction free energy is given as:[22]

$F_{electrostatic} = - N_r kT\alpha [\Sigma_i \sigma_i \Phi_i]^{3/2}$,

where,

i is the given particle

N_r is the total number of lattice sites in the system

k is the Boltzmann's constant

T is the temperature of the system

Φ_i is the volume fraction of the given particle

σ_i is the charge density of the particle

[22] Arthur Veis, Catherine Aranyi, "Phase separation in polyelectrolyte systems. I. Complex coacervates of gelatin" Journal of physical chemistry, Vol 64 (1960), pp 1203

α is the electrical interaction constant; A $(\varepsilon T)^{-3/2}$

Therefore, at a given temperature, the strength of electrostatic interaction between the oppositely charged particles increases as $\varepsilon^{-3/2}$, ε being the dielectric constant of the medium. As shown in the earlier work,[23] the dielectric constant of the solution decreases with the increase in the concentration of the ethanol in the system. At 25°C, the value of ε for pure water is 78.8. Our NP formulation uses an ethanol: water ratio of 6:1 (v: v), therefore ε reduces to ~28. Such a reduction in the dielectric value results in a greater propensity for electrostatic interactions to occur intramolecularly or intermolecularly for BSA in solution.

1.3) Role of coating onto NPs surfaces

Protein NPs are generally prone to aggregation and are usually kinetically unstable.[24] Various coating strategies have been employed, using polymers or peptides, to stabilize the colloidal dispersion through neutralizing the surface charges of the BSA NPs, thus preventing NP

[23] Sengwa, R.J., Madhvi, Sankhla, S. Sharma, Journal of solution chemistry, Vol 35 (2006), pp 1037

[24] Wim H De Jong, Paul JA Borm, "Drug delivery and nanoparticles: Applications and Hazards", International journal of Nanomedicine, 3(2008), pp 133-149

aggregation. Strategies for forming stable coatings include covalent conjugation or physical adsorption (usually via electrostatic mechanisms). Given that BSA is negatively charged at pH>pI, it can easily adsorb cationic polymers *via* electrostatic interactions; poly L Lysine, PLL or peptide KKKKKKGPQGIASQKKKKKK, K_6MK_6 used for our study purposes have lysine residues having free amines making them positively charged in distilled deionized water (dd H_2O).

In addition to stabilizing the colloidal dispersion for longer times, coatings can be designed to facilitate drug encapsulation or increase encapsulation efficiency. For example, it has been shown that siRNA encapsulation into the PLGA NPs increased two-fold upon the introduction of the cationic polymer PEI (25 kDa).[25] Moreover, coatings can be designed in terms of their proteolytic sensitivity or their own degradability so as to regulate the release pattern of encapsulated drug over a period of time. For example, in a previous work,[26] clonazepam was loaded into PLGA grafted dextran nanoparticles so as to target localized release in the colon; dextran is specifically degraded by the enzyme,

[25] Yogesh Patil, Jayanth Panyam, "Polymeric nanoparticles for siRNA delivery and gene silencing", International journal of pharmaceutics, Vol 362(2009), pp 195-203

[26] Sun-Woong Jung, Young-Il Jeong, Young-Hoon Kim, Ki-Choon Choi, Sung-Ho Kim, "Drug release from core shell type nanoparticles of poly($_{DL}$-lactide-co-glycolide)-grafted dextran", Journal of microencapsulation, 2005, Vol 22; pp 901

dextranase, which is present in a high concentration in the colon. It was observed that the drug release rate was directly related to the-presence of dextranase, showing that the use of tissue resident enzymes for specific degradation of the NP vehicle is a valid method for controlling the release of encapsulated molecules.

When administered intravenously, NPs are prone to opsonization via the adsorption of plasma proteins. This is the main limitation for developing targeted drug delivery vehicles for localized delivery to various sites in the human body; few coatings have been used to modify the surfaces of the NPs to avoid or reduce opsonization of the NPs. For example, polyethylene glycol (PEG) has been used widely for reducing the protein adsorption. In a previous work,[27] PEG (2000 MW) was used to covalently attach to polylactic acid (PLA) based NPs. It was found that upon PEGylating the surface of the PLA NPs, the adsorbed amount of proteins was reduced by up 57%. Furthermore, increasing the MW of PEG from 2 to 5 kDa further reduced the amount of protein adsorbed onto these NPs by 50%.

[27] R. Gref, M. Luck, P. Quellec, M. Marchand, E. Dellacherie, S. Harnisch, T. Blunk, R.H. Muller, "Stealth corona-core nanoparticles surface modified by polyethylene glycol (PEG): influences of corona (PEG chain length and surface density) and of core composition on phagocytic uptake and plasma protein adsorption", Colloids and surfaces B: Biointerfaces, Vol 18(2000), pp 301-313

1.4) Enzymes for digestion of BSA NPs

Enzymes are proteins with remarkable catalytic activity and high substrate specificity.[28] These biological catalysts operate in aqueous solutions, under physiologically relevant temperatures and pH. Enzymes facilitate chemical reactions by lowering the activation energy of very specific reactions, leading to a dramatic increase in the overall rate of the reaction.

Matrix metalloproteinase 2 (MMP-2) is a member of the matrix metalloproteinase family, which is involved in the breakdown of the extracellular matrix (ECM). MMP-2 has been shown to have a role in angiogenesis,[29] a process where new blood vessels are formed, due to its ability to effectively cleave Type IV collagen,[30] a major structural component of the basement membrane. Considering the fact that MMP-2

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[28] David L. Nelson, Michael M. Cox, "Principles of Biochemistry", 4th edition, pp 190

[29] G.Klein, E. Vellenga, M.W. Fraaije, W.A. Kamps, E.S.J.M. de Bont, "The possible role of matrix metalloproteinase MMP-2 and MMP-9in cancer, e.g. acute leukaemia", Critical reviews in Oncology/Haematology, Vol 50 (2004), pp 87-100

[30] S. Curran, G.I. Murray, "Matrix metalloproteinase: molecular aspects of their roles in tumor invasion and metastasis", European Journal of Cancer, Vol 36 (2000), pp 1621-1630

is over expressed in active tumor sites,[31] and is responsible for tumor growth, we hypothesized that coating BSA NPs with MMP-2 sensitive peptide may result in the breakup of these particle at the sites of high MMP-2 activity (e.g., tumor sites), resulting in the site-specific release of encapsulated drug from these coated NPs. The sequence GPQGIASQ, derived from collagen, has been shown to be highly sensitive to hydrolysis by MMP-2,[32] therefore, the peptide K_6MK_6, incorporating the sequence GPQGIASQ was used for coating onto BSA NPs to investigate the specific degradation of these particles for targeted drug delivery purposes.

Trypsin is a robust digestive enzyme, which effectively cleaves most proteins or peptides. Trypsin is produced in the pancreas and released into the duodenum, where it hydrolyses peptides to their smaller building blocks[33]. Considering trypsin to be present in various parts of human body, degradation of coated and uncoated BSA NPs by trypsin

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[31] Holle L, Song W, Holle E, Wei Y, Wagner T, YU X, "A matrix metalloproteinase 2 cleavable melittin/avidin conjugate specifically targets tumor cells in vitro and in vivo", International journal of oncology (2003), Vol 22, pp 93-98

[32] Sarah Netzel- Arnett, Qing-Xiang Sang, William G.I. Moore, Marc Navre, Henning Birkedal-Hansen, Harold E. Van Wart, " Comparative Sequence specificities of Human 72- and 92-kDa gelatinases (Type IV collagenases) and PUMP (Matrilysin)", Biochemistry (1993), Vol 32, pp 6427-6432

[33] http://en.wikipedia.org/wiki/Trypsin

becomes important to understand the effect of coating upon reducing the nonspecific degradation. The purpose of this study was to examine the coating imparting the least release of the BSA or the encapsulated drug in the presence of trypsin at neutral pH of 7.4, and to develop control for MMP-2 mediated degradation studies.

1.5) Characterization techniques for Nanoparticles

1.5.1) Atomic Force Microscopy (AFM)

AFM is one of the primary tools being used today for imaging, measuring and manipulating the matter at the nano scale. The information obtained from AFM varies from the simple physical topography to as diverse as physical properties of the materials. It comprises of a cantilever (low spring constant) with a pointed tip (made usually of silicon or Si_3N_4) at the end to scan the surface of interest.[34] As the tip runs over the sample surface, it experiences a force; which produces a deflection in the cantilever in accordance to Hooke's law (F=-kx, F is the force, k is the spring constant and x is the displacement). A laser beam aimed at the rear surface of the cantilever reflects off onto a four-quadrant photodiode system to measure this deflection produced and record the deflections as a function of time. The software acts upon this recording then and forms images. The most common mode of operation of AFM is the contact mode

[34] http://www.springerlink.com/content/w170xt1n45p1701p/fulltext.pdf

where a constant separation distance is maintained between the surface and the tip. Other modes of operation of AFM include tapping mode and lift mode.

1.5.2) Multiwell Plate Reader

This instrument was used to read the fluorescence generated by fluorescent agents such as (fluorescein isothiocyanate) FITC in the sample solution (excitation wavelength, λ_{ex}=485 nm, emission wavelength, λ_{em} = 527 nm). The instrument excites the sample by a light source using a specific wavelength. Because of the excitation of the sample, it emits light, which is collected by the emission system of the instrument. A light detector measures the emission.

1.5.3) Electrophoresis

Polyacrylamide gels were run to look after the degradation of BSA and MMP-2 cleavable peptide, K_6MK_6 by Trypsin and MMP-2. Electrophoresis is a technique where proteins of different molecular weights are separated with the help of potential difference applied to a gel matrix. This is made possible by the use of a detergent known as sodium dodecyl sulfate (SDS), which imparts a uniform negative charge to the proteins and under the influence of an electric field, all the proteins move towards the positive pole. The protein mixture is run through a gel made

of polyacrylamide. Based on the molecule/molecules to be analyzed, the composition of acrylamide and cross linker can be decided for the porosity of the gel. Proteins move under the influence of an electric current and give a band depending on their mass/charge ratio onto the gel. After running SDS-PAGE, bromophenol blue solution is used for staining protein bands in order to make these bands visible.

1.5.4) Zeta-Potential and Photon Correlation Spectroscopy (PCS) measurements

The Zeta-Potential and size distribution of the NPs were determined using Zetasizer. This instrument works on the principle of dynamic light scattering (DLS) and laser Doppler electrophoresis (LDE) method for determining particle size and zeta potential, respectively.

DLS is a technique used to measure the size distribution of particles in suspension. If particles in the colloidal dispersion are smaller than the wavelength of the incident light used, the intensity of the scattered light is uniform in all directions (Rayleigh scattering); for larger particles however, the intensity is angle dependent (Mie scattering). If a monochromatic light is used, such as laser, it becomes feasible to note the fluctuations in the scattered intensity as a function of time using a detector. The reason for fluctuations is the random Brownian motion of the particles, which results in constructive or destructive interference of light scattered by neighbouring particles. Analysis of time dependent

intensity fluctuations gives us diffusion coefficient of the particles and hence *via* Stokes-Einstein relation equation, diameter of the particles can be calculated.[35] The Stokes-Einstein equation is given as:[36]

$$D = k_B T/6\Pi\eta r$$

where,

D is the diffusion constant

k_B is the Boltzmann's constant

T is the absolute temperature

r is the particle radius

η is the viscosity of the medium

Zeta potential, for particles dispersed in the medium is defined as the difference in the potential between the medium and the stationary layer of fluid around the dispersed particle.[37] The value of zeta potential can be measured by applying an electric field across the suspension. Electric field applied across the colloidal dispersion will result in the migration of the charged particles towards oppositely charged electrode. The movement of the particles is opposed by the viscous forces of the medium; at equilibrium, the particles move with a constant velocity. This velocity is

[35] http://en.wikipedia.org/wiki/Dynamic_light_scattering

[36] http://en.wikipedia.org/wiki/Stokes-Einstein

[37] http://en.wikipedia.org/wiki/Zeta_potential

measured by the instrument and can be used to obtain zeta potential as per Smoluchowski approximations.[38] The velocity is given as:

$$U_E = \varepsilon\zeta/\eta$$

where,

U_E is the velocity

ε is the dielectric constant

ζ is the zeta potential

η is the viscosity of the medium

1.6) Research Hypotheses

The following hypotheses laid the foundation of this research work:

1) BSA coacervates can be stabilized *via* the adsorption of PLL. It is hypothesized that the incorporation of the MMP2 cleavage domain, GPQGIASQ into the PLL peptides can facilitate the breakup of these BSA particles, thus allowing for specific drug delivery to cancer sites.
2) Trypsin is a robust enzyme that is present in various parts of human body. NPs must pass through these areas prior to reaching its target; it is thought that coated and uncoated BSA NPs can be digested by Trypsin. Therefore, we want to understand the effects of coating upon

[38] Manual Number MAN0150, "Malvern Instruments Ltd., U.K.", Issue 2.0 (May 2000)

reducing the non-specific degradation of these NPs, thus increasing the probability of payload reaching the site of interest.

CHAPTER 2

LITERATURE REVIEW

2.1) Use of nanoparticles for drug delivery purposes

The advent of nanotechnology has begun to radically alter the diagnosis, prevention, and treatment of multiple diseases. In particular, several unique attributes of nanoparticles (NPs) are thought to make them ideal for drug delivery applications. To name a few major advantages for use of NPs as drug carriers are their high stability, high carrier capacity (drug molecules can be encapsulated into the matrix leading to high surface area/volume ratio), and possibility of various routes of administration.[1] The NPs can also be designed so as to control their degradation rate to enable the release of the drug in a controlled manner.

A significant effort on manipulating the size and surface properties of NPs has been expended, two basic factors responsible for the advantages of NPs and their *in-vivo* distribution. Controlling the particle size is of paramount importance for the application of NPs in drug

[1] Svetlana Gelperina, Kevin Kisich, Michael D. Iseman, Leonid Heifets, "The Potential Advantages of Nanoparticle Drug Delivery Systems in Chemotherapy of Tuberculosis", American Journal of Respiratory and Critical Care Medicine, Vol 172 (2005), pp 1487-1490

delivery, as particle size is thought to largely dictate *in-vivo* distribution, biological fate and, thus, the ultimate ability of NPs to target specific tissues.[2] For instance, NPs tend to adsorb proteins upon administration. Because of this adsorption, monocytes or macrophages recognizes the adsorbed proteins and clears NPs; this process is known as opsonization. It has been shown that particles less than 200 nm have an increased residence time within the circulatory system.[3] Therefore, extensive studies have been conducted for the express purpose of identifying major factors that ultimately control both the particle size as well as the particle size distribution. In the coacervation technique utilized to form NPs based on human serum albumin (HSA),[4] several process parameters affecting the particle size of the resultant NPs were studied systematically. For instance, the pH of the HSA solution prior to coacervation step was determined as the major factor affecting the particle size. It was found that increasing the

[2] Rajesh Singh, James W. Lillard Jr., "Nanoparticle-based targeted drug delivery", Experimental and Molecular Pathology, Vol 86 (2009), pp215-223

[3] S.M. Moghimi, A. Christy hunter, J. Clifford Murray, " Long-circulating and target specific nanoparticles: Theory to practice", Pharmacological reviews, Vol. 53 (2001); pp 283-318

[4] K. Langer, S. Balthasar, V. Vogel, N. Dinauer, H. Von Briesen, D. Schubert, "Optimization of the preparation process for human serum albumin (HSA) nanoparticles", International Journal of Pharmaceutics, Vol 257 (2003), pp 169-180

pH of the HSA solution resulted in smaller particle size; at higher pH, HSA is more ionized because of which there is repulsion among the molecules of HSA and aggregates. Moreover, HSA concentration also affected the particle size. The decrease in the size of resultant particle size with increasing HSA concentration is probably due to increased nucleation of HSA particles upon acetone addition.[5] This study describes a method for obtaining HSA NPs in the 150-280 nm range by adjusting the process parameters. In another work using the same coacervation technique, [5] BSA NPs were formed upon the dropwise addition of acetone or ethanol to the solution of BSA. This study was carried out to study the process parameters affecting the size of BSA NPs. For instance, the use of acetone as a non-desolvation agent resulted in smaller BSA NPs compared to use of ethanol; because of the likelihood that acetone is a better non-solvent for BSA than ethanol. Also, the non-solvent to water ratio influenced the BSA NPs size. The higher ratio resulted in smaller BSA NPs size because of better diffusion of particles in the non-solvent phase.

NP surface properties are another crucial parameter that determines the fate of NPs *in-vivo.* The success of drug targeting largely depends upon the necessity of reducing the opsonization of NPs to a minimum after

[5] Guilin Wang, Kevin Siggers, Sufeng Zhang, Hongxing Jiang, Zhenge Xu, Ronald F. Zernicke, John Matyas, Hasan Uludag, "Preparation of BMP-2 Containing Bovine Serum Albumin (BSA) Nanoparticles Stabilized by Polymer Coating", Pharmaceutical Research, Vol 25 (2008), pp 2896-2906

intravenous administered. The hydrophobicity of NPs enhances the adsorption of blood serum proteins onto the surface of NPs compared to hydrophilic surfaces.[6] Coating the surface of NPs with a suitable hydrophilic polymer to lower the degree of opsonization has been highly researched. In a previous work,[7] NPs developed from poly (methylmethacrylate) (PMMA) coated with hydrophilic polymer, poloxamer 338 or poloxamine 908, showed a reduction in opsonization. In another work,[8] NPs developed from HSA and coated with methoxy-polyethylene glycol (mPEG) showed a reduction in the adsorption of plasma proteins onto the surface of NPs compared to unmodified NPs.

The surface charge of NPs is another important surface property, it reflects the electric potential of the particles at an interface and depends on the medium of dispersion, and surface composition of NPs. Zeta potential is a commonly used parameter for characterizing the surface charge of

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[6] Donald E. Owens, Nicholas A. Peppas, "Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles", International Journal of Pharmaceutics, Vol 307 (2006), pp 93-102

[7] R.H. Muller, K.H. Wallis, S.D. Troster, J. Kreuter, " In vitro characterization of poly (methyl-methacrylate) nanoparticles and correlation to their in vivo fate", Vol 20(1992), pp 237-246

[8] Wu Lin, Martin C. Garnett, Etienne Schacht, Stanley S. Davis, Lisbeth Illum, "Preparation and in vitro characterization of HSA-mPEG nanoparticles", International Journal of Pharmaceutics, Vol 189 (1999), pp 161-170

NPs. Particles with zeta potential above ±30 mV have been shown to be stable in suspensions, where high charge results in repulsion among the particles and prevent the process of agglomeration.[2] Also, a correlation has been attempted between protein adsorption and surface charge of NPs. For example, in a previous study, NPs prepared from polymer blend of poly (lactic-co-glycolic) acid (PLGA) and poly (styrene-co-4-styrene-sulfonate) (PSS) showed increased adsorption of Lysozyme with the increased surface charge density.[9] Modifying the surface of NPs by some specific ligands/peptide for achieving targeted drug delivery is quite worked upon. For instance, Sodium ferulate was encapsulated into the mannose-6-phosphate modified BSA NPs using desolvation technique. The study showed higher drug concentration in the liver compared to other organs such as kidney and specific uptake by hepatic stellate cells.[10]

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[9] Cuifang Cai, Udo Bakowsky, Erik Rytting, Andreas K. Schaper, Thomas Kissel, "Charged nanoparticles as preotein delivery systems: A feasibility study using Lysozyme as model protein", European Journal of Pharmaceutics and Biopharmaceutics, Vol 69 (2008), pp 31-42

[10] Feng-Qian Li, Hua Su, Chen Xu, Qin Xian-Ju, Ji-Yong Liu, Quan-Gang Zhu, Jin-Hong, "Mannose 6-phosphate-modified bovine serum albumin nanoparticles for controlled and targeted delivery of sodium ferulate for treatment of hepatic fibrosis", Journal of Pharmacy and Pharmacology, Vol 61 (2009), pp 1155-1161

2.2) Potential of protein NPs

There has been a considerable interest in developing NPs from synthetic and natural materials, as carriers of small and large molecules for drug delivery; especially the development of protein based NPs. There are several reasons for this prolonged interest as proteins are biodegradable, easy to metabolize, non-toxic, and non-antigenic, making them suitable candidates as drug delivery vehicles.[11] In addition, owing to large number of functional groups present in the proteins, it is relatively easy to modify their surfaces for targeted drug delivery applications.[11]

Numerous groups have reported techniques for the preparation of NPs from proteins (i.e. gelatin, gliadin, albumin).[11] For instance, as early as 1978 NPs were formed from gelatin using a desolvation technique.[12] Gelatin was of particular interest as it is inexpensive, and sterilizable.[11,13] However, this method was not very successful; its tedious and resulting particles are prone to aggregation. A two-step desolvation method was

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[11] Mohsen Jahanshahi, Zahra Babaei, "Protein nanoparticle: A unique system as drug delivery vehicles ", African Journal of Biotechnology, Vol 7 (2008), pp 4926-4934

[12] J.J. Marty, R.C. Oppenheim, P Speiser, "Nanoparticles-a new colloidal drug delivery system", Pharmaceutica Acta Helvetiae, Vol 53(1978), pp 17-23

[13] M. Jahanshahi, M.H. Sanati, S. Hajizadeh, Z. Babaei, "Gelatin nanoparticle fabrication and optimization of the particle size", Physica Status Solidi (a), Vol 205 (2008), pp 2892-2902

further used to develop gelatin NPs, resulting in a stable and homogeneous suspension.[14] Albumin, as a drug carrier, is highly popular. It is the most abundant protein found in the body, and thus is both cheap and easily available. Moreover, it is expected that NPs derived from albumin should be biodegradable, non-antigenic, and non-immunogenic. It is for these reasons that albumin based NPs have been extensively studied for drug delivery purposes.[11] Moreover, these NPs are easy to prepare using mild techniques (i.e. coacervation) and it is possible to obtain these NPs in a defined size range by manipulating certain process parameters such as protein concentration, pH of the protein solution, amount of non-solvent added. For example, a previous study described a method for obtaining Bovine Serum Albumin (BSA) NPs with controllable size range.[5]

To stabilise protein derived NPs, glutaraldehyde is commonly used. [15,16] The use of glutaraldehyde as a cross-linker for imparting

[14] C.J. Coester, K. Langer, H. Von Briessen, J. Kreuter, "Gelatin nanoparticles by two step desolvation -a new preparation method", Journal of Microencapsulation, Vol 17 (2000), pp 187-193

[15] Saikat Das, Rinti Banerjee, Jayesh Bellare, "Aspirin Loaded Albumin Nanoparticles by Coacervation: Implications in Drug Delivery", Trends in Biomaterials and Artificial Organs. Vol 18 (2005), pp 203-212

[16] M. Merodio, A. Arnedo, M.J. Renedo, J.M. Irache, "Ganciclovir-loaded albumin nanoparticles: Characterization and in vitro release properties", European Journal of Pharmaceutical Sciences, Vol 12 (2001), pp 251-259

stability to BSA NPs is questionable because of its toxicity and its probability of reacting with encapsulated peptide/protein drugs, thereby affecting the bioactivity of the agent.[5] As an alternative, use of poly L-Lysine (PLL) [5] and Polyethylenimine (PEI)[17] as coating materials to increase stability of BSA NPs has been in use. A number of agents such as BMP-2, [5] aspirin [15] and ganciclovir [16], have successfully been encapsulated into BSA NPs for drug delivery applications.

2.3) Coacervation

Coacervation can be defined as a process where a homogeneous solution of charged macromolecules, such as proteins, undergo phase separation (liquid-liquid) and result in a polymer macromolecule-rich phase. The number of molecules involved in this process dictates the class of coacervation as being either simple or complex. The resulting coacervates are highly concentrated with the macromolecule.

Bungenberg de Jong, considered the father of coacervate chemistry, systematically studied coacervation phenomena on mixing an aqueous solution of gelatin and gum arabic in an organic solvent; in 1949, Jong gave conditions and differences between two classes of coacervation.

[17] Sufeng Zhang, M.R. Doschak, H.Uludag, "Pharmacokinetics and bone formation by BMP-2 entrapped in polyethylenimine-coated albumin nanoparticles", Biomaterials, Vol 30(2009), pp 5143-5155

[18] He postulated that when pH is higher than the isoelectric point (pI) of gelatin, these macromolecules will be negatively charged and result in simple coacervation. On the other hand, when pH is lower than the pI of gelatin, the two molecules will be oppositely charged and result in complex coacervation phenomena. [2]

The interesting properties of coacervates spurred a significant effort in the development of coacervation techniques for drug delivery applications. Using coacervation phenomena, researchers have tried to develop NPs from various molecules such as chitosan[19], gelatin[20], HSA[21],

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[18] C.J. Van Oss, "COACERVATION, COMPLEX-COACERVATION AND FLOCCULATION", Journal of dispersion science and technology, Vol 9 (1988), pp 561-573

[19] Lee DW, Yun KS, Ban HS, Choe W, Lee SK, Lee KY, "Preparation and characterization of chitosan/polyguluronate nanoparticles for siRNA delivery", Journal of Controlled Release, Vol 139 (2009), pp 146-152

[20] Truong-Le VL, August JT, Leong KW, "Controlled gene delivery by DNA-gelatin nanospheres", Human Gene Therapy, Vol 9 (1998), pp 1709-1717

[21] Marion G. Anhorn, Sylvia Wagner, Jorg Kreuter, Klaus Langer, Hagen Von Briesen, "Specific Targeting of HER2 Overexpressing Breast Cancer Cells with Doxorubicin-Loaded Trastuzumab-Modified Human Serum Albumin Nanoparticles", Bioconjugate Chemistry, Vol 19 (2008), pp2321-2331

and BSA[22] as well as tried to encapsulate various therapeutic agents and drugs. Previously,[19] chitosan NPs were developed using coacervation techniques and proved to be an effective carrier for siRNA delivery. Because of the formation of coacervate, the negative charges of siRNA were neutralized due to excess chitosan present on the surface. As positive charge is critical for delivery purposes, NPs increased the stability of siRNA against degradation in the serum compared to siRNA alone and helped to transport siRNA efficiently to cells. Similarily,[3] NPs from coacervated HSA were explored for developing procedures to obtain NPs in the 100-300 nm range with narrower size distribution. Enzymatic degradation of HSA NPs stabilized by glutaraldehyde in another study was undertaken[23]. The purpose of this study was to investigate the degradability of these NPs in the presence of enzymes such as cathepsin B and trypsin, for the release of encapsulated drugs and see if particle stabilization affected the degradability kinetics.

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[22] Saikat Das, Rinti Banerjee, Jayesh Bellare, "Aspirin Loaded Albumin Nanoparticles by Coacervation: Implications in drug delivery", Trends in biomaterials and Artificial Organs, Vol 18 (2005), pp 203-212

[23] K. Langer, M.G. Anhorn, I. Steinhauser, S. Dries, D. Celebi, N. Schrickel, S. Faust, V. Vogel, "Human Serum Albumin (HSA) nanoparticles: Reproducibility of preparation process and kinetics of enzymatic degradation", International Journal of Pharmaceutics, Vol 347 (2008), pp 109-117

In addition to widespread applications of coacervates in drug delivery, coacervation techniques have been applied to areas such as food and cosmetic formulations[24], and protein purification[25]. Considering protein purification as an example, usually a polyelectrolyte is used which is selectively made to form a complex with the protein of interest to produce coacervate by varying the process parameters for separating it out. Subsequently polyelectrolyte in the coacervate can be removed by ultra-filtration or selective precipitation[26]. In a previous work[26], poly(diallylmethylammonium chloride) (PDADMAC) was used as a polyelectrolyte to separate individual protein from a mixture comprising of BSA, β-lactoglobulin, γ-globulin and ribonuclease A. The yield for each protein in the coacervate was determined with polymer molecular weight (MW) used, ionic strength and pH as the variables.

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[24] Manli Deng, Meiwan Cao, Yilin Wang, "Coacervation of Cationic Surfactant with weakly Charged Anionic Polyacrylamide", Journal of Physical Chemistry B, Vol 113 (2009), pp 9436-9440

[25] P.L. Dubin, J. Gao, K. Mattison, "Protein Purification by Selective Phase Separation with Polyelectrolytes", Separation & Purification Reviews, Vol 23 (1994), pp 1-16

[26] Ying-fan Wang, Jeff Y. Gao, Paul L. Dubin, "Protein Separation via Polyelectrolyte Coacervation: Selectivity and Efficiency", Biotechnology Progress, Vol 12 (1996), pp 356-362

2.4) Design of enzymaticaly cleavable systems for drug delivery

Enzymatic degradation is a crucial mechanism for controlling the release of encapsulated drugs within a nanoparticle system.[27] Moreover, the uncontrolled degradation of particles taken orally, prior to reaching their target of interest, is a serious problem. Therefore, understanding particle degradation in the presence of different enzymes is important. The degradation of HSA NPs by enzymes such as trypsin, pepsin, cathepsin B, were undertaken under simulated physiological conditions. [23] Trypsin degraded particles efficiently at neutral pH of 7.4 and was a function of particle stabilization. In another study, the release of 10-hydroxycamptothecin (HCPT) encapsulated into BSA NPs in the presence and absence of trypsin was undertaken in the medium of pH of 7.4. Trypsin accelerated the release of HCPT in its presence, indicating the degradability of BSA NPs (reference).

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[27] K. Langer, M.G. Anhorn, I. Steinhauser, S. Dries, D. Celebi, N. Schrickel, S. Faust, V. Vogel, "Human Serum Albumin (HSA) nanoparticles: Reproducibility of preparation process and kinetics of enzymatic degradation", International Journal of Pharmaceutics, Vol 347 (2008), pp 109-117

There are certain enzymes such as matrix metalloproteinase 2 (MMP-2)[28] and CD10[29] that are highly expressed in tumours. Researchers are actively pursuing a lot of work to use the systems that can result in an extended release of encapsulated agent in the presence of these enzymes, to realize the targeted drug delivery applications. For example, in a previous work,[30] collagen mimetic peptides were developed. Conjugation of this peptide to stearic acid carried out in such a manner that generated

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[28] Holle L, Song W, Holle E, Wei Y, Wagner T, Yu X, "A matrix metalloproteinase 2 cleavable melittin/avidin conjugate specifically targets tumor cells in vitro and in vivo", International journal of oncology (2003), Vol 22, pp 93-98

[29] Chin Pan, Pina M. Cardarelli, Matthew H. Nieder, Lesley B. Pickford, Sanjeev Gangwar, David J. King, Geoffrey T. Yarranton, Dana Buckman, William Roscoe, Fengmin Zhou, Adam Salles, Tseng-Hui Chen, Killian Horgan, Yi-Hong Wang, Thi Nguyen, Christopher R. Bebbington, "CD10 Is a Key Enzyme Involved in the Activation of Tumor-activated Peptide Prodrug CPI-0004Na and Novel Analogues: Implications for the Design of Novel Peptide Prodrugs for the therapy of $CD10^+$ Tumors", Cancer Research, Vol 63(2003), pp 5526-5531

[30] Nihar Sarkar, Jayati Banerjee, Andrea J. Hanston, Adekunle I. Elegbede, Theresa Rosendahl, Aaron B. Krueger, Abir L. Banerjee, Shakila Tobwala, Rongying Wang, Xiaoning Lu, Sanku Mallik, D.K. Srivastava, "Matrix Metalloproteinase- Assisted Triggered Release of Liposomal Contents", Bioconjugate Chemistry, Vol 19(2008), pp 57-64

liposomes result in peptide pointing outwards from the surface for easy access of this peptide to cleavage by MMP-9, an endopeptidase expressed in tumors[28]. In this study, carboxyfluorescein used as a dye for incorporation into liposomes released at a faster rate in the presence of MMP-9 than observed in the presence of trypsin or no enzyme. In another work[31], NPs were designed to self assemble in the presence of a protease. Superparamagnetic magnetite (Fe_3O_4) NPs functionalized with biotin or neutravidin. The PEG chains linked to protease (MMP-2) substrate were coated on the surface of NPs to assure the mixture of NPs remain disperse in the solution. The introduction of MMP-2 to the system resulted in the shedding of its substrate, thereby removing the PEG coat. Because of shedding of the coat, the NPs self assembled because of high affinity between biotin and neutravidin and result in NPs clumps at tumor sites. So, it appears that NPs can be designed to assemble or dis-assemble in the presence of environmental stimulus.

[31] Paula Gould, "Tumor protease triggers self assembly: Nanoparticles", Nanotoday, Vol 1 (2006), pp 15

CHAPTER 3

MATERIALS & METHODS

3.1) Materials

Bovine serum albumin (BSA) and the endopeptidase, Matrixmetalloproteinase-2 (MMP-2) and doxorubicin hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). FAM-labelled short interfering ribonucleic acid was purchased from Ambion Inc. (Austin, TX, USA). EDTA-Trypsin was purchased from Invitrogen (Carlsbad, CA, USA) and diluted 1:10 with Hank's Buffered Salt Solution (HBSS) before use. Hydro bromide (HBr) salt of Poly-L-Lysine (PLL) of different Molecular Weights (MWs) (0.9, 4.2, 13.8 and 24 kDa) were purchased form Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. The peptide with a sequence of KKKKKKGPQGIASQKKKKKK (K_6MK_6) was purchased from SynBioSci (Livermore, CA, USA) and used without further purification. Fluorescence isothiocyanate (FITC) was obtained from Pierce (Rockford, IL, USA). Gradient gels of 4-20 % acrylamide concentration were used for our study purposes and obtained from Bio-Rad Laboratories (Woodinville, WA, USA). Prestained protein ladder was purchased from Bio-Rad Laboratories (Woodinville, WA, USA) and used as a marker for gels study. Sodium phosphate (Na_2HPO_4), sodium phosphate, monobasic,

monohydrate [Na $(HPO_4)_2$], Sodium Chloride (NaCl) lab grade reagents were purchased from EMD Chemical Inc. (Darmstadt, Germany). Tris-HCl, $CaCl_2$, Brij-35, NaN_3, Trisma Base, Glycine, SDS, Glycerol, Bromophenol Blue, Tris-HCl and β-Mercaptoethanol were purchased from Sigma Aldrich (St. Louis, MO, USA) and used without further purification. Ethanol and methanol were purchased from Fischer Scientific (Ottawa, Ontario, Canada). Ethanol was used as a desolvation agent for coacervation method. Acetic acid was obtained from Caledon Laboratories limited (Georgetown, Ontario, Canada). Acetic acid and methanol was used in the preparation of staining and destaining solution for gels.

3.1.1) Buffers and Solutions

3.1.1.1) Phosphate Buffer

Phosphate buffer was prepared from the aqueous stock solutions of 0.5 M Na_2HPO_4 and 0.5 M Na $(HPO_4)_2$. Solutions were mixed to obtain appropriate pH (7.4) and diluted with deionized water (di-H_2O) to a final ionic concentration of 0.1 M.

3.1.1.2) Tris Buffer

Tris buffer was formed using 50 mM Tris- HCl, 150mM NaCl, 10mM $CaCl_2$, 0.05% Brij-35 and 0.02 % NaN_3[32].

3.1.1.3) Glycine Running Buffer (10X)

Glycine running buffer was prepared using 29 grams of Trisma base, 144 grams of Glycine and 10 grams of SDS. These lab reagents were dissolved in di-H_2O to 1 liter of final volume. [33] This buffer was diluted with 1:10 with di-H_2O before using it for electrophoresis purposes.

3.1.1.4) SDS Glycine Sample Buffer (2X)

SDS Glycine sample buffer was prepared using 4mL of 10% (w/v) aqueous Sodium Dodecyl Sulfate (SDS), 2 mL of Glycerol, 1 mL of 0.1% (w/v) Bromophenol Blue, 2.5 mL of 0.5 M Tris-HCl, pH=6.8, 0.2 mL of β-Mercaptoethanol and 0.3 mL of dd H_2O.[2]

3.1.1.5) Coomassie Blue Stain Solution

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[32] Janelle L. Lauer-Fields, Kathleen A. Tuzinski, Ken-ichi Shimokawa, Hideaki Nagase and Gregg B. Fields, "Hydrolysis of Triple-helical Collagen peptide Models by Matrix Metalloproteianses", The Journal of Biological Chemistry, 2000, Vol 275 (18), pp 13282-13290

[33] Handbook from Gradipore (North Ryde, NSM, Australia)

To 75 mL of acetic acid, 500 mL of methanol was added followed by addition of 0.25 grams of Coomassie to this binary mixture, and diluted to 1 L total volume using di-H_2O.

3.1.3) Destaining Solution

Total volume of 1 L destaining solution was comprised of 100 mL of acetic acid, 400 mL of methanol, and 500 mL of di-H_2O.

3.2) Methods

3.2.1) Fabrication of coated BSA NPs

BSA NPs were formed *via* a coacervation technique.[34] Aqueous BSA (10 mg/mL) solution (250 µL) was added to equal volume of 10 mM aqueous NaCl solution in glass vials obtained from Kimble Glass Incorporation (Vineland, NJ, USA) under stirring (600 rpm). Stirring was done using a magnetic stirrer over a magnetic plate obtained from Corning Incorporation (Corning, NY, USA). Fresh flask was used for making NPs

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[34] Guilin Wang, Kevin Siggers, Sufeng Zhang, Hongxing Jiang, Zhenge Xu, Ronald F. Zernicke, John Matyas, and Hasan Uludag, " Preparation of Bovine Serum Albumin (BSA) Nanoparticles stabilized by Polymer Coating", Pharmaceutical Research, 25(12),pp. 2896- 2909 (2008)

each time. For making large batches of BSA NPs suspension, 50 mL of conical glass flask were used using a large magnetic stirrer. The flask used was cleaned with hot water, followed by washing with Sparkleen1 detergent (Fisherbrand, Pittsburgh, PA, USA) and rinsed with copious amounts of di-H_2O. After 15 minutes of stirring, ethanol (final volume ratio of ethanol to starting BSA binary solution = 6) was added dropwise using Fisher brand pipettes (San Diego, CA, USA) to the above aqueous system under stirring (600 rpm) to form nanoparticles. The stirring was continued for 3 hours after the complete addition of ethanol at room temperature. To stabilize these protein NPs, different MWs (0.9, 4.2, 13.8 and 24 kDa) of PLL and peptide K_6MK_6, were introduced to the system. K_6MK_6 is highly sensitive to hydrolysis by MMP-2[35] **[4]**. Different concentrations (0.1, 0.3 and 1 mg/mL) of PLL and K_6MK_6 were employed for coating purposes; 500 μL of PLL or K_6MK_6 in di-H_2O was added dropwise to BSA NPs (with ethanol) suspension under constant shaking of 500 rpm using a shaker, obtained from Labnet (Woodbridge, NJ, USA). The shaking was continued for 1 hour at room temperature to allow coating. Experiments were performed in triplicates.

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[35] Stephane G. Levesque, Molly S. Shoichet "Synthesis of Enzyme-Degradable, Peptide-Cross-Linked Dextran Hydrogels", Bioconjugate Chemistry, 2007, Vol 18, pp 874-885

3.2.2) Coating Efficiency of PLL onto BSA NPs

3.2.2.1) FITC labeling of PLL (FITC-PLL)

PLL used for stabilizing BSA NPs was labeled with FITC to determine the overall coating efficiency. FITC-PLL was obtained by incubating 10 µL of 0.1 mM FITC solution (in DMSO) with 1 mL of PLL (2 mg/mL in 100 mM phosphate buffer, pH=7.4) for 1 hr at room temperature. Ethanol (9 mL) was then added to this solution, centrifuged at 3000 rpm for 15 minutes, and the supernatant was removed. The pellet was washed with 5 mL of ethanol and centrifuged for 15 minutes, at 3000 rpm. The solids obtained were dried under vacuum for 5 hours and stored in the dark at 4^{o}C until used.

3.2.2.2) FITC-PLL coating efficiency measurements

The NP coating efficiency of FITC-PLL was determined using fluorescence measurements, where FITC-PLL (0.9, 4.2 and 24 kDa) at concentrations defined previously were employed. Phosphate buffer (300 µL, pH= 7.4) was added to 300 µL of coated NPs suspension and vortexed using a vortex genie 2 (Scientific Industries, Bohemia, NY, USA). The suspensions were then centrifuged at 15,000 rpm for 1 hour. A 200 µL of the supernatant in duplicate was added to 96-well plates (NUNC, Rochester, NY, USA) and fluorescence (λ_{ex}: 485nm; λ_{em}: 527 nm) was

read using a multiwell plate reader (Thermo Labsystems, Franklin, MA, USA). The amount of FITC-PLL (uncoated FITC-PLL) in the supernatant was quantified by the calibration curve developed by using FITC-PLL in di-H_2O. Three sets of independent experiments were performed. The coating efficiency was calculated as: {1- (FITC-$PLL_{supernatant}$/ FITC-$PLL_{initial}$)} x 100%.

3.2.3) Stability of PLL coated BSA NPs based on a Fluorescence Technique

3.2.3.1) FITC labelling of BSA (FITC-BSA):

The procedure adopted for labelling BSA was same as that used for labelling PLL, with the exception that BSA concentration was 10 mg/mL. Dried FITC-BSA was stored in dark at 4^{o}C for future use.

3.2.3.2) Stability tests

In order to stabilize BSA NPs, four different MWs (0.9, 4.2, 13.8 & 24 kDa) of PLL were used. PLL concentrations used for coating were 0.1, 0.3 and 1.0 mg/mL. FITC-BSA was added back to the BSA solution used for NP formation, to a total concentration of 5%. Subsequently formed BSA NPs were then coated with PLL's (as discussed previously), diluted by 100% with phosphate buffer (pH=7.4, sodium azide=0.02%), and incubated at 37^{o}C under shaking (500 rpm). At 0, 1, 3 and 7 days, 500

μL of aliquots were recovered from this solution and centrifuged at 15,000 rpm for an hour. A 200 μL of the supernatant in duplicate was added to 96-well plates (NUNC, Rochester, NY, USA) and fluorescence (λ_{ex}: 485 nm; λ_{em}: 527 nm) was determined using a multiwell plate reader (Thermo Labsystems, Franklin, MA, USA). The amount of FITC-BSA in the supernatant was calculated based on the calibration curve produced with FITC-BSA in deionized water. Three sets of independent experiments were performed. The release of FITC-BSA was calculated as: [1- (FITC-BSA $_{supernatant}$/ FITC-BSA $_{initial}$)] x 100%.

3.2.4) Enzymatic degradation studies

3.2.4.1) Trypsin and MMP2 Degradation of free BSA and K_6MK_6

Gel electrophoresis was used to characterize the degradation of free BSA and K_6MK_6 by Trypsin and MMP-2. We also tried digestion of PLL by Trypsin or MMP-2 using the same technique. BSA solution (2 mg/mL) in phosphate buffer (pH=7.4, sodium azide=0.02%) was incubated at 37^{o}C on a shaker at 500 rpm with and without Trypsin or MMP-2. PLL (MWs: 0.9, 4.2 and 24 kDa) of concentrations 0.1, 0.3 and 1.0 mg/mL in phosphate buffer (pH=7.4, sodium azide=0.02%) was added to BSA solution in the volume ratio of 1:1 and incubated at 37^{o}C on a shaker at 500 rpm. The purpose of adding PLL was to see if MW and concentrations of PLL used is making any delay in the formation of

Tryptic digests of BSA with time. The ratio of BSA:enzyme was kept at 250 (w:w) in both the cases. At 6, 24 and 72 hours aliquots were taken out, and run onto gradient gels. Gels were run using the Mini-Trans Blot Cell (Bio-Rad laboratories, USA). Samples were mixed with SDS Glycine sample buffer in the volume ratio of 1:1. Mixtures were heated at 90-95^{o} C for 3-5 minutes, followed by centrifugation at 3000 rpm for 3 minutes before loading onto gels. The gel was run at 50 V for 3 hours, removed from the cassette, washed with di-H_2O, immersed in Coomassie Blue staining solution, and shook for 12 hours at 60 rpm at room temperature. Gels were destained by immersion in the destaining solution for 6-8 hours and used for image capturing.

K_6MK_6 digestion *via* Trypsin, or MMP2 was characterized using electrophoresis. Tris buffer was used instead of Phosphate buffer for making solution of K_6MK_6 and MMP-2 as MMP-2 activity is optimal in Tris-Buffer[1]. The solution of K_6MK_6 (1 mg/mL) was incubated with and without Trypsin or MMP-2 at 37 ^{o}C on a shaker at 500 rpm. The ratio of peptide: enzyme was kept at 250 (w:w). At 1, 3 and 5 days, aliquots from this solution were withdrawn and looked for digests using same gels and procedure as described above. Gels were run for 30 minutes under 50 V potential differences.

3.2.4.2) Trypsin or MMP2 Degradation of PLL or K_6MK_6 coated BSA NPs

Fluorescence test was used to characterize degradation of coated BSA NPs in the presence of Trypsin or MMP-2. To stabilize BSA NPs, PLL of MWs 0.9, 4.2 and 24 kDa were used at concentrations 0.1, 0.3 and 1.0 mg/mL. A 0.1 mg/mL of K_6MK_6 was also used for BSA NPs coating. The resultant PLL and K_6MK_6 coated BSA NPs suspensions were diluted by 100% with phosphate buffer and tris buffer respectively. Finally, these suspensions were incubated with and without trypsin or MMP-2 at room temperature over the shaker at 500 rpm. MMP-2 was not used for PLL coated BSA NPs as electrophoresis studies showed no digestion of BSA by MMP-2. The ratio of BSA: enzyme and $K_6MK_{6:}$ enzyme was kept at 250 (w: w) for PLL and K_6MK_6 coated BSA NPs respectively. The amount of FITC-BSA in the supernatant was calculated based on the calibration curve produced with FITC-BSA in deionized water. A stock solution of BSA NPs was formulated and three sets of experiments were performed. The release of FITC-BSA was calculated as: [1- (FITC-BSA $_{supernatant}$/ FITC-BSA $_{initial}$)] x 100%.

3.2.4) siRNA encapsulation and enzymatic degradation

3.2.4.2) siRNA encapsulation into uncoated and coated BSA NPs

Short interfering ribonucleic acid (siRNA) encapsulated into BSA NPs using the coacervation technique, was used as a model drug. Aqueous NaCl (125 μL) was added to equal volume of aqueous BSA solution under

stirring at 600 rpm for 15 minutes. siRNA solution (10 µL of 0.15 mg/mL) was added to this binary mixture, followed by 1 hour of incubation under stirring at 600 rpm. Ethanol (final volume ratio of ethanol to starting BSA binary solution = 6) was then added dropwise to the system to induce coacervation followed by 3 hours of incubation under stirring. The siRNA loaded BSA NPs suspensions were then incubated with equal volume of PLL or K_6MK_6 to coat these polymers onto uncoated BSA particles under the shaker at 500 rpm for 1 hour. The same procedure was adopted for encapsulating doxorubicin (10 µL of 0.5mg/mL) and actenomycin (5 µL of 2mg/mL) into the BSA NPs.

3.2.4.3) Enzymatic degradation of siRNA loaded coated and uncoated BSA NPs

FAM labelled siRNA was used to study the release kinetics of siRNA from BSA NPs in the absence and presence of Trypsin *in-vitro*. To coat these BSA NPs, PLL of MWs 0.9, 4.2 and 24 kDa, and K_6MK_6 were used at concentration of 1.0 mg/mL. The resultant PLL and K_6MK_6 coated BSA NPs suspensions were dialyzed against 1 mM NaCl three times and subsequently, diluted by 100% with phosphate buffer. Finally, these suspensions were incubated with and without trypsin at room temperature over the shaker at 500 rpm. The ratio of BSA: enzyme was kept at 250 (w: w). The amount of siRNA in the supernatant was calculated based on the calibration curve produced with siRNA in dd-H_2O. The release of siRNA was calculated as [1- (FITC-BSA $_{supernatant}$/ FITC-BSA $_{initial}$)] x 100%.

3.3) Characterization

3.3.1) Physical characterization of Nanoparticles

The desired BSA NPs (uncoated and coated) were analyzed using Atomic Force Microscopy (AFM), Photon Correlation Spectroscopy (PCS) and Zeta Potential techniques. Coated and uncoated BSA NPs were analyzed with AFM to determine particle size and the presence of aggregate formation. PCS and zeta potential measurements were carried out to determine the size distribution and surface charge of the particles, respectively. Electrophoresis and multiwell plate reader were also used to look after the degradation of BSA or K_6MK_6 by Trypsin or MMP-2 and for BSA release experiments respectively.

3.3.1.1) Atomic Force Microscopy (AFM)

An MFP-3D AFM (Asylum Research, Santa Barbara, CA, USA) using AC240TS cantilever, was used throughout this work to analyze the NP properties in tapping mode. Phosphate buffer (45 µL) was added to 5 µL of sample of interest and sonicated for 3 minutes to reduce aggregate formation. Samples of about 5 µL were air dried onto PELCO^R Mica discs (TED PELLA, Inc.; Redding, CA, USA). The mica disc was then fixed onto the microscopic glass slides. The cantilever was fixed onto a holder and inserted into a MFP-3D head of the AFM. Using Igor Pro imaging

software (version 5.04 B), video mode was opened and camera was focused onto the tip. Using the knobs of MFP-3D head, light source was adjusted and angle of photo detector mirror was set to zero the deflection. Using sum and deflection meter window, alignment was completed. The sum is the total amount of light collected by photo detector in volts and was set in between 5-7 and deflection was set to zero or very close to it. The vertical adjustment of the MFP-3D head over the sample was adjusted using the knobs on the side of the head. The system was auto tuned and the various parameters were set before starting the scan of the sample. The scan size was set to 2 µm with scan rate adjusted to 1 Hz. At 90^{o} of scan angle scan points and scan lines were set to 256 each. With remaining parameters untouched, scanning of the sample was done and images were saved using this same software.

3.3.1.2) Zeta Potential and Photon Correlation Spectroscopy (PCS) measurements

The mean particle size of the coated and uncoated BSA NPs were determined by dynamic light scattering (Zetasizer 3000 HS, Malvern Instruments Ltd., UK) using a 633 nm He-Ne laser at a scattering angle of 90^{o}. Uncoated BSA NPs were used directly for the measurements. PLL or K_6MK_6 coated BSA NPs were diluted 1:2 with 1 mM NaCl before the measurements. BSA NPs coated with 0.1 mg/mL of 0.9 and 4.2 kDa PLL coated BSA NPs were used without dilution as there were inaccurate

readings observed after dilution of these coated NPs suspensions. The surface charge of these NPs was determined by measuring the electrophoretic mobility of the particles using the same instrument at 25^{o}C. The coated BSA NPs suspensions were diluted 1:4 with 1 mM NaCl before measurements.

CHAPTER 4

RESULTS & DISCUSSIONS

4.1) Formation of uncoated and coated bovine serum albumin (BSA) nanoparticles (NPs)

Coacervation methods have been widely studied as a mild method for fabricating NPs from proteins such as HSA,[1] BSA,[2] and gelatin.[3] This research focused on determining the coacervation conditions for controlling the formation of BSA based NPs, so as to tune NP size, surface

[1] K. Langer, S. Balthasar, V. Vogel, N. Dinauer, H. Von Briesen, D. Schubert, "Optimization of the preparation process for human serum albumin (HSA) nanoparticles", International journal of pharmaceutics, Vol 257 (2003), Issue 1-2, pp 169-180

[2] Guilin Wang, Kevin Siggers, Sufeng Zhang, Hongxing Jiang, Zhenge Xu, Ronald F. Zernicke, John Matyas, and Hasan Uludag, " Preparation of Bovine Serum Albumin (BSA) Nanoparticles stabilized by Polymer Coating", Pharmaceutical Research, 25(12),pp. 2896- 2909 (2008)

[3] B. Mohanty, H.B. Bohidar, "Systematic of Alcohol-Induced Simple Coacervation in Aqueous Gelatin Solutions", Biomacromolecules, Vol 4(2003), issue 4, pp 1080-1086

charge, stability, and drug release characteristics. Overall stability of NPs was increased through coating the formed NPs with cationic polymer, poly-L-lysine (PLL) and the peptide, KKKKKKGPQGIASQKKKKKK (K_6MK_6). The physicochemical properties of the generated NPs were characterized using zeta potential, photon correlation spectroscopy (PCS) and atomic force microscopy (AFM) techniques.

The peptide sequence GPQGIASQ is sensitive to hydrolysis by MMP-2;[4] MMP-2 digestion of K_6MK_6 coated BSA NPs was carried out to investigate the feasibility of the coated NPs for targeted drug delivery applications. Non specific degradation of K_6MK_6 and PLL coated BSA NPs by trypsin were also carried out to investigate the release kinetics of BSA so as to determine the coating that can impart a reduction or stability against tryptic degradation. Short interfering ribonucleic acid (siRNA) was used as a model drug to be encapsulated into the BSA NPs for our study purposes. Tryptic digestion of siRNA loaded BSA NPs coated with PLL or K_6MK_6 were investigated.

[4] Stephane G. Levesque, Molly S. Shoichet "Synthesis of Enzyme-Degradable, Peptide-Cross-Linked Dextran Hydrogels", Bioconjugate Chemistry, 2007, Vol 18, pp 874-885

4.2) PLL adsorption onto BSA NPs

Given the pK_a of the ε–NH_2 group in PLL is 10.5, the PLL will be positively charged at the experimental pH of ddH_2O.[5] Thus, it is supposed that the anionic BSA will electrostatically interact with the cationic PLL, leading to its adsorption onto the formed BSA NPs. To examine the amount of PLL adsorbed onto BSA NPs, different MWs and concentrations of PLL were employed.

As shown in **Figure 4.1**, the adsorbed amount of 0.9, 4.2 and 24 kDa PLL onto BSA NPs increased with the PLL concentration. The adsorbed amount for 0.9 and 4.2 kDa were similar and increased from ~50 to ~250 μg PLL/mg BSA for 0.1 and 1 mg/mL PLL concentration, respectively. These systems did not tend to show a plateau in adsorption, indicating the possibility for further adsorption with additional increase in the coating concentration. The results obtained are in agreement with the zeta potential studies of PLL coated BSA NPs; an increase in the zeta potential from 3.1±0.2 to 10.1±1.7 mV and 10.5±2.1 to 12.5±0.26 mV was observed for BSA NPs coated with 0.9 and 4.2 kDa PLL, respectively, as the concentration of PLL was increased from 0.1 to 1.0 mg/mL. The adsorbed amount of 24 kDa PLL increased from 55 to ~140 μg PLL/mg BSA for PLL concentrations of 0.1 and 1.0 mg/mL, respectively. Moreover, unlike the 0.9 and 4.2 kDa PLL's, 24 kDa adsorption

[5] David L. Nelson, Michael M. Cox "Principles of biochemistry", 4th edition, pp-78

approached a plateau value suggesting that the surface was adsorbed with a maximum value of ~140 µg PLL/mg BSA for the 24 kDa PLL. This is the likely reason for a small increase in the zeta potential of 24 kDa PLL coated BSA NPs as the PLL concentration was increased from 0.1 to 1.0 mg/mL. The likely reason for higher adsorbed amounts of 0.9 and 4.2 kDa PLL onto BSA NPs as compared to 24 kDa PLL could be the fact that surface area available for smaller MW polymers is larger than for high molecular weight (MW) polymers. It is also possible that smaller PLL's might display increased penetration into the interior of the NPs for increased adsorption.

4.3) BSA NP characterization

4.3.1) Zeta Potential of uncoated and coated BSA NPs

Uncoated and coated BSA NPS were characterized using the zeta potential technique. Two average zeta potentials were noted for uncoated BSA NPs for two different sets of experiments. The average zeta potential of uncoated BSA NPs coated with K_6MK_6 was observed to be -8.5 mV (**Figure 4.2 A**) and for BSA NPs coated with PLL, the zeta potential was noted to be -11 mV (**Figure 4.2 B**). Previous work, where uncoated BSA NPs were formed using ethanol as the desolvation agent, has shown zeta

potentials of -10 mV[6] and -26 mV[2]**.** The -26 mV result, however, was different due to the use of phosphate buffer (pH=7.4) for making BSA solution, which may have ionized BSA (pI of 4.8) more than the ddH_2O used for our study purposes and resulted in higher zeta potential.

Given the electrostatic interactions between the oppositely charged PLL and BSA, it is likely that the PLL coating will affect the zeta potential of the NPs. To test this hypothesis, the zeta potential of BSA NPs coated with different MWs and concentrations of PLL were investigated (**Figure 4.2 B**). It was observed that the zeta potential of the BSA NPs increased upon being coated with PLL; from -11±1.2 mV for uncoated BSA NPs to 3.1±0.2 to 20.6±0.4 mV for PLL coated BSA NPs. The zeta potential of BSA NPs coated with 0.1, 0.3, and 1.0 mg/mL of 0.9 kDa PLL were found to be 3.1±0.2, 6.7±2.1, and 10.1±1.7 mV, respectively. This was also indicative of increased adsorption of PLL onto BSA NPs with increasing PLL concentration. The zeta potentials for BSA NPs coated with 4.2 kDa PLL were 10.8±2.6, 9.1±1.9, and 12.3±0.26 mV for PLL concentration of 0.1, 0.3, and 1 mg/mL, respectively. The zeta potential values for BSA NPs coated with 24 kDa PLL were 19.3±0.3, and 19.4±0.6 and 20.4±0.2 mV, respectively. This trend was found to be in agreement

[6] Sufeng Zhang, Guilin Wang, Xiaoyue Lin, Maria Chatzinikolaidou, Herbert P. Jennssen, Marcus Laub, Hasan Uludag, "Polyethylenimine-coated Albumin Nanoparticles for BMP-2 delivery", Biotechnology Progress, Vol 24 (2008), pp 945-956

with the adsorption plateau observed for 24 kDa PLL adsorption onto BSA NPs with increasing concentration (**Figure 4.1**). The increase in zeta potential of BSA NPs with increasing coating concentration in an earlier work has been reported.[2] The differences in zeta potential of BSA NPs coated with 24 and 4.2 kDa, and 24 and 0.9 kDa PLL were found to be statistically different ($P<0.05$, ANOVA: Single factor), contrary to BSA NPs coated with 0.9 and 4.2 kDa PLL where these differences were not statistically different ($P>0.05$, ANOVA: Single factor). A continuous increase in the zeta potential was observed for BSA NPs coated with 0.9 kDa, PLL complementing the PLL adsorption experiment, where adsorption plateau for this PLL was not observed. Taken together; the use of 24 kDa PLL significantly affected the zeta potential of PLL coated BSA NPs as compared to increase in the MW of PLL from 0.9 to 4.2 kDa.

K_6MK_6 has lysine residues suitable for BSA NP adsorption and hence different concentrations of this peptide were employed for coating onto the he BSA NPs (**Figure 4.2 A**). It was observed that zeta potential of BSA NPs increased upon being coated; from -8.5±1.3 mV for uncoated BSA NPs to more than 4.5 mV for K_6MK_6 coated BSA NPs. The zeta potential observed to increase from 4.8±0.6, 5.3±0.55 and 7.7±0.8 mV for the K_6MK_6 concentration of 0.1, 0.3, and 1.0 mg/mL, respectively. The corresponding values for 4.2 kDa PLL coated BSA NPs were 6.4±1.6, 7.1±0.15 and 10.2±2.3 mV, respectively.

4.3.2) Size distribution of uncoated and coated BSA NPs

The PCS was used to characterize uncoated and coated BSA NPs (**Figure 4.3**) and full width at half maximum (FWHM) analysis of peaks obtained from PCS technique (**Table 1&2**) was carried out to understand the size distribution of the particles. The mean particle diameter of uncoated BSA NPs was observed to be 306±75 nm (**Figure 4.3 A**) and 247±50 nm (**Figure 4.3 B**). Previous work, where BSA NPs were prepared using ethanol as a desolvation agent, reported size to be around 260±20 nm^2.

The influence of PLL MW, PLL concentration (**Figure 4.3 B**) and K_6MK_6 concentration (**Figure 4.3 A**) on the particle size was studied using the PCS technique. The diameter of uncoated BSA NPs increased from 247±50 nm to 3096±193 and 3847±1556 nm upon coating with 0.1 mg/mL of 0.9 and 4.2 kDa PLL, respectively. This could be attributed to polymer bridging or patch flocculation mechanism among the particles leading to aggregation[7]; former mechanism calls for the fact that the polymer added to colloidal dispersion can anchor simultaneously to different surfaces (**Figure 4 A**), whereas the latter mechanism assumes that cationic polymer adsorbed forms patches of charges on the negatively charged surface; electrostatic interaction during the Brownian motion of the particles may then drive the system to aggregation (**Figure 4 B**). The

[7] Krister Holmberg, "Handbook of applied surface and colloid chemistry", Vol 1 (2002); pp 137

same phenomenon in an earlier work was observed; [2] large aggregates were observed for BSA NPs coated with 0.01 mg/mL of 24 kDa PLL. Also, aggregation was reported for NPs developed from pegylated poly lactic acid (PLA-PEG) and poly (lactic-co-glycolide) acid (PLGA-PEG);[8] NPs developed from these copolymers having lower content of PEG showed aggregation possibly because of incomplete coverage of the surfaces of NPs by PEG chains. The size of the BSA NPs coated with 0.3 and 1.0 mg/mL of 0.9 kDa PLL was 265±22 nm and 170±47 nm, respectively; corresponding values for BSA NPs coated with 4.2 kDa PLL were 370±92 nm and 155±11 nm, respectively. It is likely that, at these concentrations, these polymers were able to cover the surfaces considerably of BSA NPs; to not induce patch-mediated flocculation among the particles. No large particle size for BSA NPs coated with 0.1 mg/mL of 24 kDa PLL was observed, an observation consistent with the earlier work;[2] no large aggregates were observed for BSA NPs coated with 0.1 mg/mL of 24 kDa PLL. Particle size of the coated BSA NPs was observed to be 321±40 nm, 306±47 nm and 313±54 nm for 24 kDa PLL concentrations of 0.1, 0.3 and 1.0 mg/mL, respectively.

[8] Konstantinos Avgoustakis, "Pegylated Poly(lactic) and Poly (lactic-co-glycolide) nanoparticles: Preparation, properties and possible applications in drug delivery", Current drug delivery, Vol 1(2004), pp321-333

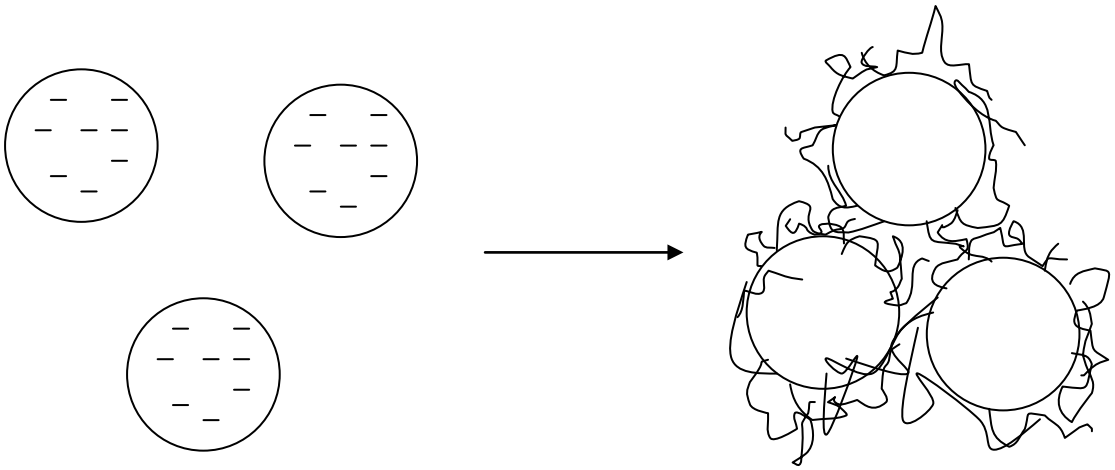

Figure 4 A Particles flocculating by bridging mechanism

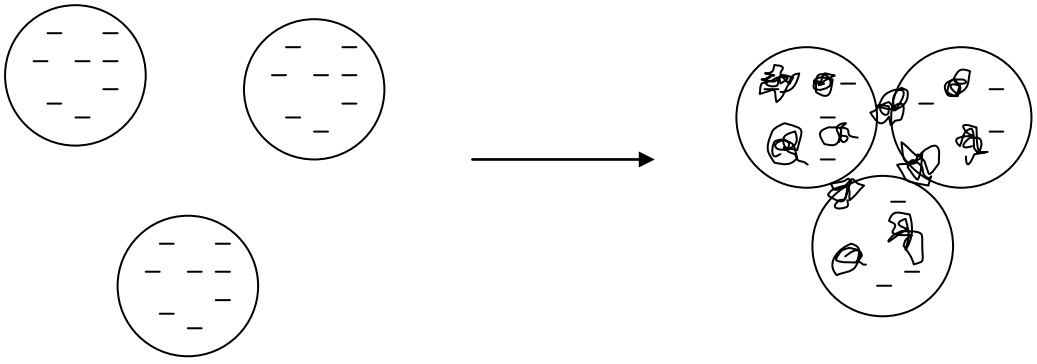

Figure 4 B Particles aggregating by patch flocculation mechanism

The average particle size of BSA NPs coated with K_6MK_6 (2.3 kDa) was observed to vary from 306±75 nm for uncoated BSA NPs to 514±162 nm, 284±35 nm and 133±60 nm, respectively, for K_6MK_6 concentrations of 0.1, 0.3, and 1.0 mg/mL. Corresponding values for BSA NPs coated with 4.2 kDa PLL were 2284±162 nm, 316±35 nm and 180±60 nm, respectively. An interesting observation to be noted was the absence of very large particles for BSA NPs coated with 0.1 mg/mL of

K_6MK_6, as has been observed for BSA NPs coated with 0.1 mg/mL of 0.9 and 4.2 kDa PLL.

The FWHM analysis of peaks revealed a significant increase in the FWHM values (**Table 1**) for coated BSA NPs than uncoated BSA NPs. An increase in the FWHM values was noted for all the BSA NPs coated with 0.1 mg/mL of 0.9, 4.2 and 24 kDa PLL, indicating their broad size distribution. There appeared to be a noteworthy decrease in the FWHM values with the increase in PLL concentration from 0.1 to 0.3 mg/mL for BSA NPs coated with 0.9 and 4.2 kDa PLL, respectively. There was not much decrease in the FWHM value for BSA NPs coated with 0.1 and 0.3 mg/mL of 24 kDa PLL. Followed by this decrease, an increase in the FWHM value was noted again for BSA NPs coated with 1.0 mg/mL of PLL (0.9, 4.2 and 24 kDa) concentration; indicating a shift from a comparative narrower size distribution to a broader size distribution at this concentration.

An increase in the FWHM values for BSA NPs coated with K_6MK_6 was also noted, indicating broader size distribution. A reduction in the FWHM values (**Table 2**) with the increase in concentration of K_6MK_6 from 0.1 to 0.3 mg/mL was noted, followed by an increase at 1.0 mg/mL of K_6MK_6 concentration; indicating a shift from a relatively narrower size distribution to a broader size distribution.

Fabricated BSA NPs were analyzed under AFM in the height and phase mode to confirm the size of the particles measured by PCS

technique and to investigate the surface properties of the sample surface as well. Despite the appearance of some aggregates, uncoated BSA NPs appeared to be spherical particles (**Figure 4.4 A**) that were ~150 nm in diameter. These values appeared smaller than recorded by PCS technique, probably because of shrinkage of the particles during the drying process necessary for AFM imaging.

Coating the BSA NPs with 0.3 mg/mL of 0.9, 4.2 and 24 kDa PLL resulted in average particle diameters of ~200 nm, ~180 nm and ~250, respectively under AFM (**Figure 4.4 B, C and D**). Moreover, it was apparent that the particle size and shape were less uniform for 0.9 and 4.2 kDa PLLcoatings. Compared to PCS measurements, these particles were smaller in size (typically in the 100-250 nm range), probably due to shrinkage of particles during drying process for AFM imaging. However, BSA NPs coated with 0.3 mg/mL of 24 kDa PLL had a more uniform size distribution and were more spherical. The phase images of all the PLL coated BSA NPs revealed darker regions (**Figure 4.5 B, C and D**), which is probably because of adsorbed PLL onto the BSA surfaces. All the particles did not show the same bright colour; brighter regions on the particles possibly indicate an uneven distribution of PLL.

For BSA NPs coated with 0.1, 0.3 and 1.0 mg/mL of K_6MK_6, the particle size was observed to be ~300 nm, ~250 nm and ~120 nm, respectively (**Figure 4.4 E, F and G**). It was apparent that as the concentration of K_6MK_6 increased, the average size decreased complying

with the observation obtained from PCS technique. Also, particles were observed to be more spherical and uniform in size as the concentration of K_6MK_6 was increased. The percentage of dark particles (**Figure 4.5 E, F and G**) were observed to increase with the increasing concentration of K_6MK_6 indicating towards its increased adsorption.

4.4) Digestion of BSA and K_6MK_6 by enzymes: SDS-PAGE

The digestion of BSA and K_6MK_6 peptide by trypsin or MMP-2 was confirmed using SDS-PAGE techniques. Trypsin is known to hydrolyze the peptide bonds where contributed by either a lysine or an arginine residue,[9] and MMP-2 effectively cleaves the glycine-isoleucine (GI) bond in the sequence GPQGIASQ in K_6MK_6.[4]

Upon incubation of BSA with trypsin, low molecular weight protein species were observed (**Figure 4.6)** after 6 hours suggesting BSA degradation. Tryptic digestion of BSA has been confirmed by SDS-PAGE techniques[10] and other techniques[11] that showed HSA and BSA fragments

[9] David L. Nelson, Michael M. Cox "Principles of biochemistry", 4th edition, pp-99

[10] Gabriela A. Eppel, Sandor Nagy, Margaret A. Jenkins, Ronald N. Tudball, Michael Daskalakis, Nicholas D.H. Balazas and Wayne D. Comper, "Variability of Standard Clinical Protein Assays in the analysis of a Model Urine Solution of

were similar in size and charge. Moreover, the decrease in the intensity of the lower MW protein species bands with increasing incubation with trypsin suggests further enzymatic degradation of these species by trypsin. Upon incubation of BSA with MMP-2; no low MW protein species were observed (**Figure 4.7**) even after 3 days of incubation. This was expected as the cleavage domain for MMP-2 is very specific and probably not found in BSA.

K_6MK_6 digestion by trypsin or MMP-2 was also studied. As shown in **Figure 4.8**, trypsin and MMP-2 digested K_6MK_6 based upon disappearance of K_6MK_6 band with time. K_6MK_6 band disappeared completely in the presence of trypsin after 1 day of incubation, whereas there was a faint band visible after Day 1 in the presence of MMP-2, indicating trypsin to be a stronger enzyme compared to MMP-2 in digesting K_6MK_6. The digestion of K_6MK_6 by trypsin and MMP-2 indicated the possibility of using this peptide as a coating material for drug loaded BSA NPs for targeted drug delivery for sites rich in MMP-2 activity.

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fragmented Albumin", Journal of biomaterials science (Polymer edition) Vol 33 (2000), No.6, pp 487-494

[11] Gabor Markus, David K. McClintock, Barbara A. Castellani, "Tryptic hydrolysis of Human Serum Albumin", The Journal of Biological Chemistry, Vol 242 (19676), pp. 4395-4401

4.5) Digestion of K_6MK_6 and PLL coated BSA NPs

4.5.1) Natural release of BSA from coated BSA NPs

The release of BSA from PLL coated NPs is summarized in **Figure 4.9**. The purpose of this study was to investigate the release of BSA from PLL coated BSA NPs as a measure of stability in the basic nanoparticulate system. BSA release was found to be highest for the 0.9 kDa PLL coated NPs. The cumulative BSA release was observed to be 35±3% from BSA NPs coated with 0.1 mg/mL of 0.9 kDa PLL under the experimental conditions. A decrease in the cumulative BSA release was noted with an increase in the PLL concentration to 28.5±4.5% and 19.5±3.5 % after 7 days of incubation for BSA NPs coated with 0.3 and 1 mg/mL of 0.9 kDa PLL, respectively. PLL of MW 4.2, 13.8 and 24 kDa were also employed for coating BSA NPs. The cumulative BSA release was 22±7%, 19.3±3.8% and 17.6±2.8% for BSA NPs coated with 0.1, 0.3 and 1.0 mg/mL of 4.2 kDa PLL, respectively. The corresponding values for BSA NPs coated with 13.8 and 24 kDa PLL were 25.6±1.6%, 18.9±5% and 17.2±2%, and 21±3%, 18.2±5% and 15.5±3.5%, respectively after 7 days of incubation. The cumulative BSA release was decreased with increasing concentration of PLL for all the MW used for coating BSA NPs; however the increase in MW of PLL after 4.2 kDa did not much change the BSA release profile. Albumin release from drug

delivery systems in the form of nanoparticles, microspheres and films has been reported previously. For example, in a previous study,[12] BSA release from degradable polymer matrices, composed of poly (L-lactic acid), PLA and pluronic; coating of this polymer matrix with PEI resulted in a decrease in BSA release with the increasing concentration of PEI used for coating.

The stability of BSA NPs coated with 0.3 mg/mL of 0.9 and 24 kDa PLL (**Figure 4.10),** over 7 days of incubation in phosphate buffer (pH=7.4), was also studied using the AFM. It was observed that 0.9 kDa PLL coated BSA NPs increased in size from ~200 nm to over 500 nm is size over the study period of 7 days. Also, an increase in aggregation was observed with time. In contrast, BSA NPs coated with 24 kDa PLL under similar conditions showed a minimal increase in particle size from ~200 nm to ~300 nm over the study period of 7 days. It may be that the low average zeta potential of 6.7 mV for BSA NPs coated with 0.3 mg/mL of 0.9 kDa PLL was not large enough to prevent aggregation, whereas BSA NPs coated with 0.3 mg/mL of 24 kDa PLL system showed a large average zeta potential of 19.5 mV that may have inhibited NP aggregation.

[12] Tae Gwan Park, Smadar Cohen, Robert Langer, "Controlled Protein Release from Polyethylenimine- Coated Poly(L-Lactic Acid)/Pluronic Blend matrices, Pharmaceutical Research, Vol. 9(1992), pp 37-39

4.5.2) Release profile of BSA from coated BSA NPs in the presence of enzymes

The release profile of BSA from PLL or K_6MK_6 coated BSA NPs in the presence of trypsin or MMP-2 was studied using FITC-labelled BSA. As shown in **Figure 4.11**, the degree of cumulative BSA release in the presence of trypsin was observed to be higher under neutral pH. The cumulative BSA release was observed to be 31.5±1.2%, 26.5±6% and 23±2% from BSA NPs coated with 0.1, 0.3 and 1.0 mg/mL of 0.9 kDa PLL in the absence of trypsin (**Figure 4.11 C)**. The corresponding values in the presence of trypsin were 58.5±2.6%, 44±2.5% and 30±3%, respectively after 7 days of study period. The cumulative BSA release was noted to be 22.5±0.6%, 17.4±6% and 17.2±4.5% for BSA NPs coated with 4.2 kDa PLL (**Figure 4.11 B**), and 19±1%, 18.5±1.9% and 22.2 ±0.2 % for BSA NPs coated with 24 kDa PLL (**Figure 4.11 A**), for PLL concentrations of 0.1, 0.3 and 1.0 mg/mL, respectively in the absence of trypsin after 7 days. The corresponding values in the presence of trypsin were 57.2±1.9%, 49.5±3.2% and 18.3±0.8% and 60.6±2.2%, 54.2±0.65% and 47.5±5.5%. The differences in the cumulative BSA release from PLL coated BSA NPs in the presence and absence of trypsin from Day 1 was found to be statistically significant for all the coating concentrations ($P<0.05$, ANOVA: Single factor). An exception to this was observed for 0.9 and 4.2 kDa PLL for coating concentration of 1 mg/mL. Therefore, we conclude that trypsin is digesting the NPs effectively. An interesting

observation is the decrease in the cumulative release of BSA with the increasing concentration of PLL (for all the MWs) used for coating BSA NPs for all the MWs with time. This could be likely because of thick coat of PLL onto BSA NPs with increasing concentration of PLL, which resulted in a decrease in the relatively quick release of BSA. The formation of thick layer of PEI onto BSA loaded PLA/Pluronic polymer blend matrix with increasing concentration of PEI has been reported before[12].

The cumulative BSA release from K_6MK_6 coated NPs in the presence of trypsin was observed to be approximately 3 folds higher after 5 days of study period (**Figure 4.12**). The cumulative BSA release was observed to be 16.8±1.5%, 50.5±3% and 12.6±0.6% from 0.3 mg/mL of K_6MK_6 coated BSA NPs in the absence of any enzyme, in the presence of trypsin and MMP-2, respectively. An anomaly was observed in the presence of MMP-2, where despite cleavability of peptide K_6MK_6 by MMP-2, no extended or greater release of BSA was observed. We believe this is likely because of only one cleavable point present in the peptide; probably MMP-2 does not cleave enough to result in the disintegration of the particles.

4.6) siRNA encapsulation into uncoated and coated BSA NPs

Doxorubicin is widely used as an antineoplastic agent and this drug was incorporated into the matrices of nanoparticles to limit its toxicity and side effects to normal tissues.[13] We tried to encapsulate this drug into PLL coated BSA NPs *via* a coacervation technique; the maximum encapsulation efficiency achieved was 3% in our hands. Previous work, where doxorubicin was loaded onto HSA and gelatin NPs achieved an encapsulation efficiency of 70-95%[14] and ~42%,[14] respectively. The reason for this could be the use of glutaraldehyde as a cross linker; it can form a bridge between the doxorubicin and a protein molecule.[14] This is not desirable so that we did not want to use this cross linker in our formulations. Also, we tried incorporating actenomycin into the PLL

[13] S. Dreis, F. Rothweiler, M. Michaelis, J. Cinatl Jr., J. Kreuter, K. Langer, "Preparation, Characterization and maintenance of drug efficacy of doxorubicin-loaded Human Serum Albumin nanoparticles", Pharmaceutical Nanotechnology, Vol 341(2007) pp. 207-214

[14] Eliano Leo, M.A. Vandelli, R. Cameroni and F. Forni, "Doxorubicin-loaded gelatin nanoparticles stabilized by Glutaraldehyde: involvement of the drug in the cross linking process", International Journal of Pharmaceutics, 155 (1997), pp 75-82

coated BSA NPs, but maximum encapsulation efficiency of 3% was again achieved. Since these are very low encapsulation efficiencies, a short interfering RNA (siRNA) was used as an alternative model drug and incorporated into the matrix thereafter.

The siRNA was encapsulated into the BSA NPs *via* a coacervation technique and encapsulation efficiency was assessed using FAM labelled siRNA. As shown in **Table 3**, the encapsulation efficiency of siRNA encapsulated into the NPs was found to increase from 12±3% for uncoated BSA NPs to greater than 50% for coated BSA NPs. Also, an increase in the encapsulation efficiency was noted with the increase in the MW of PLL. Encapsulation efficiency achieved was 58±5%, 66±6%, 83±6% and 52±3% respectively for BSA NPs coated with 1.0 mg/mL of 0.9, 4.2, 24 kDa PLL and K_6MK_6 respectively. siRNA being highly anionic and polar; an increase in the encapsulation efficiency with the use of PLL is may be due to positive charges in the polymer (PLL or K_6MK_6) matrix that helped retain siRNA in the NPs. Similar observation was reported in the earlier work,[15] where encapsulation of siRNA in the PLGA nanoparticles was tried; the introduction of 25 kDa PEI into the 40 kDa PLGA matrix increased the encapsulation efficiency to 80% compared to 43% attained with 40 kDa PLGA alone. Also, the same approach was employed to

[15] Yogesh Patil, Jayanth Panyam, "Polymeric nanoparticles for siRNA delivery and gene silencing", International journal of pharmaceutics, Vol 362(2009), pp 195-203

encapsulate the siRNA with a PEG-polycation diblock copolymer,[16] PEG-poly (3-[(3-aminopropyl) amino] propyl aspartamide. The presence of diamine side chain in this diblock polymer interacted with siRNA to form polyplex micelles and encapsulate the siRNA. The increase in the number of positive moieties in the PLL with its increasing MW might be the likely reason for the increase in the encapsulation efficiency with the use of higher MW PLL.

4.7) Tryptic digestion of siRNA loaded uncoated and coated BSA NPs

4.7.1) siRNA release profile from uncoated and coated BSA NPs in the presence of Trypsin: Using phosphate buffer

The siRNA encapsulated into the uncoated and coated BSA NPs was investigated for release profile in the absence and presence of trypsin with phosphate buffer (pH=7.4) as the release medium. The values were

[16] Kazunori Kataoka, Keiji Itaka, Nobuhiro Nishiyama, Yuichi Yamasaki, Motoi Oishi and Yukoi Nagasaki, "Smart polymeric micelles as nanocarriers for oligonucleotides and siRNA delivery", Nucleic Acid Symposium Series, Vol 49(2005), pp 17-18

normalized to 0% for day 0 as shown in **Figure 4.6.1**. The amount of siRNA released from the uncoated BSA NPs was noted to be 2.3 ± 0.2 ng after 7 days of study period. In the presence of trypsin, the corresponding values were 0 ng to 3.3 ± 0.4 ng. The amount of siRNA released was 0.9 ± 0.8 ng, 2.2 ± 0.6 ng, 1.5 ± 0.9 ng and 1.5 ± 0.7 ng for BSA NPs coated with 1.0 mg/mL of K_6MK_6, 0.9 kDa, 4.2 kDa and 24 kDa, respectively. In the presence of trypsin, the corresponding values were 2.5 ± 0.8 ng, 4.3 ± 0.7 ng, 5.0 ± 0.5 ng and 7.0 ± 0.3 ng, respectively after 7 days. Higher MW PLL having a greater charge density can thereby retain larger amounts of siRNA in the nanoparticles; exposure to trypsin (which effectively cleaves the lysine residues) resulted in larger release of siRNA from the NPs and hence highest release of siRNA was noted for 24 kDa PLL coated siRNA loaded BSA NPs.

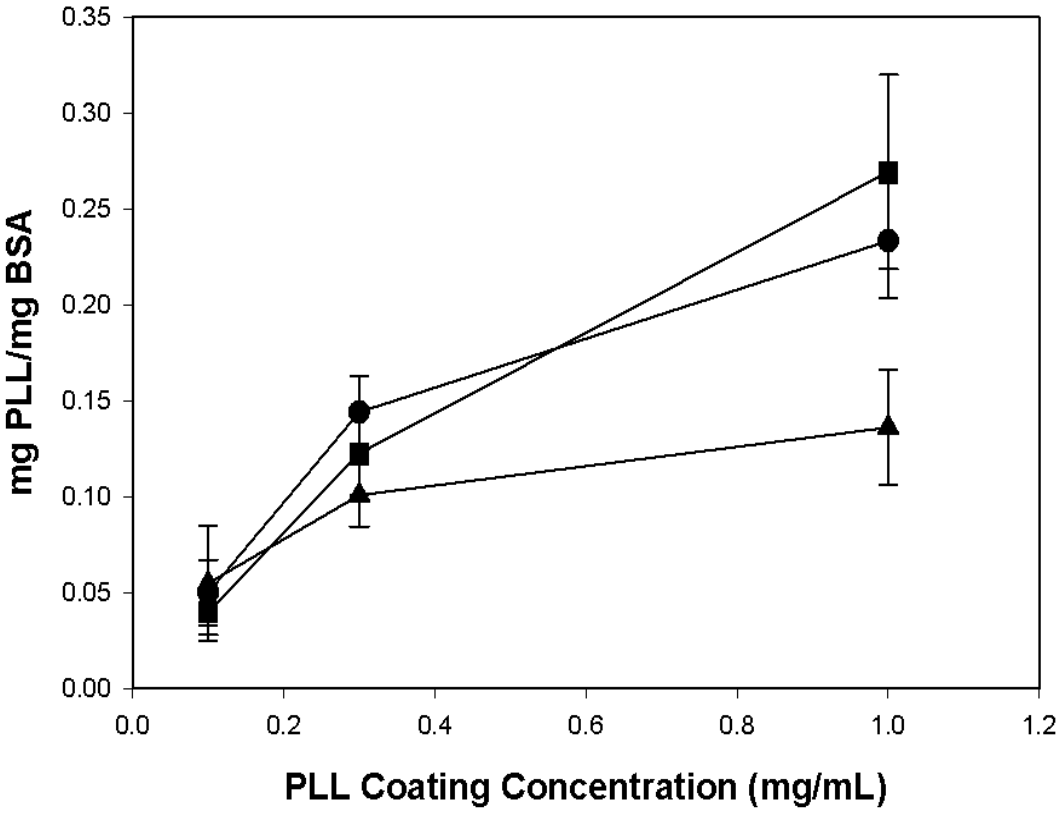


Figure 4. 1. **PLL adsorption onto BSA NPs using FITC labelled PLL of different MWs.** PLL MWs used were 0.9 (●), 4.2(■) and 24 (▲) kDa. The amount of FITC-PLL was calculated in the supernatant using the calibration curve. Trend lines are provided as a guide to the eye. Data points represent average of n = 3, error bars are ± 1SD. The amount of FITC-PLL adsorbed onto BSA NPs increased with the increasing concentration of 0.9 and 4.2 kDa PLL. A plateau was observed for the adsorbed amount of 24 kDa PLL onto BSA NPs.

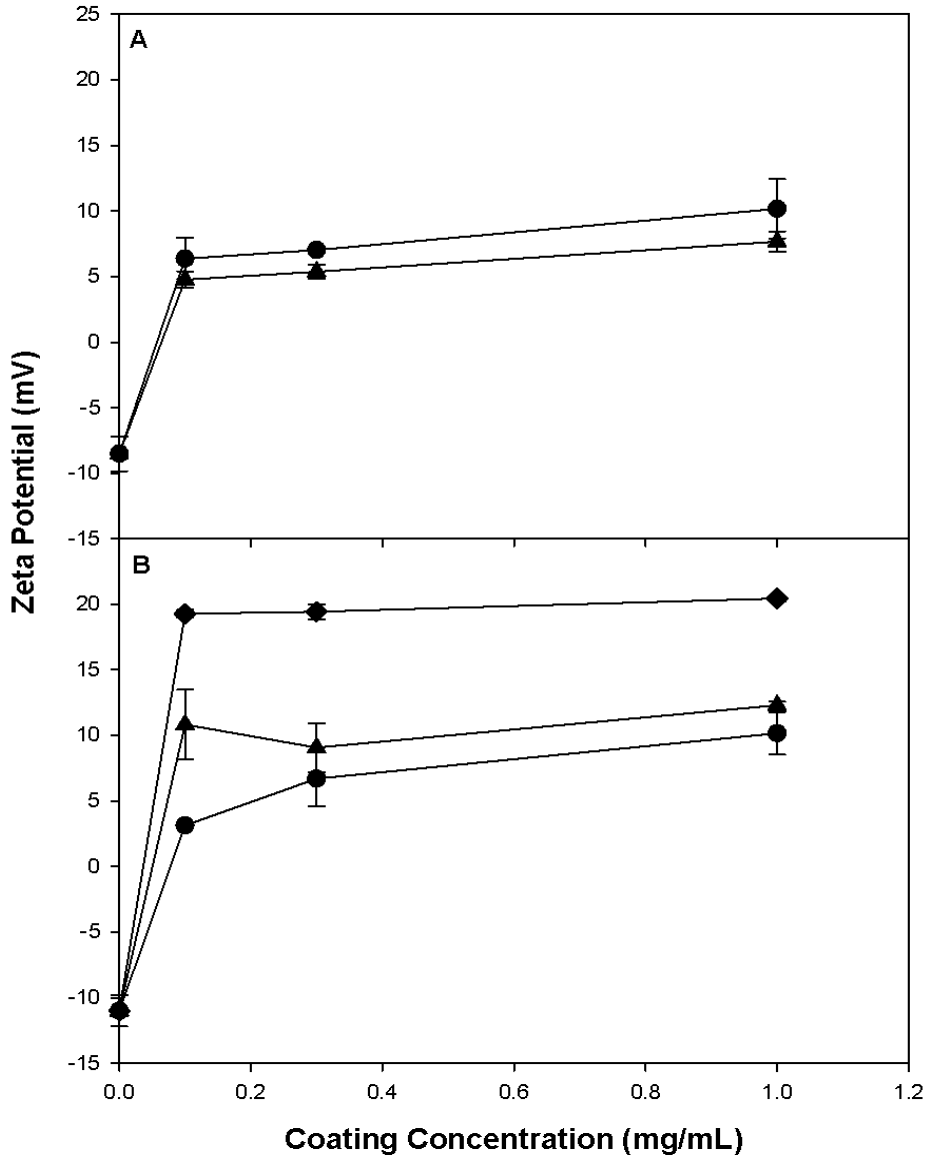


Figure 4. 2 Zeta Potential of BSA NPs coated with PLL or K_6MK_6.
PLL MWs were varied from 0.9 (●), 4.2 (▲) and 24 (◆) kDa (B). PLL MW of 4.2 kDa (▲) and K_6MK_6 (●) were used to coat BSA NPs (A) for zeta potential measurements. Trend lines are provided as a guide to the eye. Data points represent average of $n \geq 3$, error bars are ± 1SD. Zeta potential of coated NPs increased significantly and increased from ~ 3 to ~ 10 mV as concentration was increased from 0.1 to 1.0 mg/mL for 0.9 kDa PLL coated BSA NPs. 24 kDa PLL coated BSA NPs gave the highest zeta potentials of ~20 mV with almost no change with increasing concentration of PLL. Zeta potential of K_6MK_6 coated BSA NPs increased with K_6MK_6 concentration, with increase from -5.5 mV to ~ 7.7 mV for uncoated BSA NPs and BSA NPs coated with 1 mg/mL of K_6MK_6, respectively.

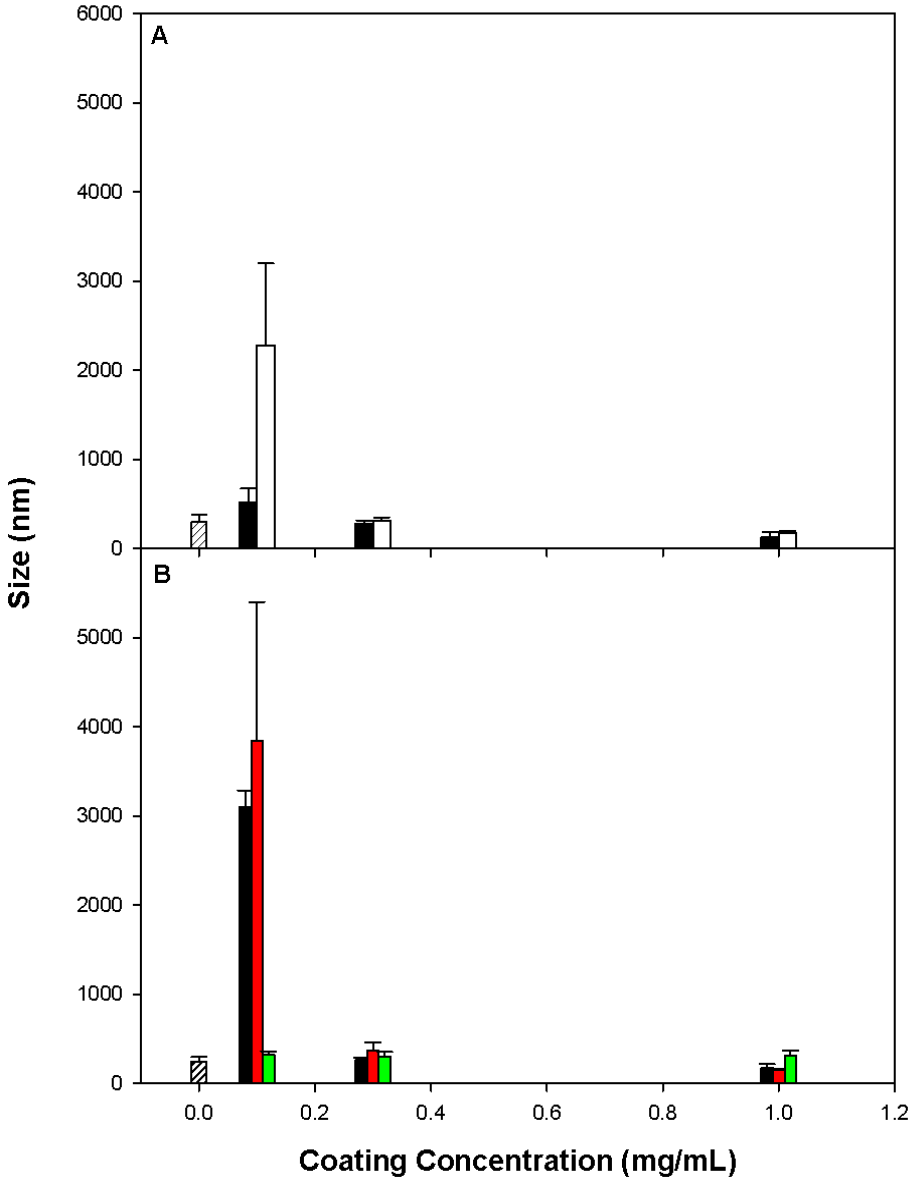


Figure 4. 3 Mean particle diameter of BSA NPs coated with PLL or K_6MK_6.
PLL MWs used were 0.9 (■), 4.2 (■) and 24 (■) kDa (B). PLL MW of 4.2 kDa (□) and K_6MK_6 (■) were used to coat BSA NPs (A) for the measurements. Data points represent average of ≥ 3, error bars are ± 1SD. The mean particle diameter of PLL coated BSA NPs was found to be greater than that of uncoated BSA NPs. Large particle diameter were noted for BSA NPs coated with 0.1 mg/mL of 0.9 and 4.2 kDa PLL, with particle diameter observed to increase from ~250 nm (BSA NPs) to ~3100 nm and ~3850 nm respectively. There was not much variation in the mean particle diameter for BSA NPs coated with 24 kDa PLL at all concentrations used. The mean particle diameter of BSA NPs coated with 0.1 mg/mL of 4.2 kDa PLL was found to be significantly higher as compared to corresponding K_6MK_6 coated BSA NPs.

NPs	Coated Polymer	Coating Conc. (mg/mL)	½ of peak height	FWHM (range)
BSA	None	0	8.85;29.75	6;27
BSA	0.9 kDa PLL	0.1	31.9	252
BSA	0.9 kDa PLL	0.3	14.9	37
BSA	0.9 kDa PLL	1.0	13.8;31.7	8.6.;42
BSA	4.2 kDa PLL	0.1	36.5	990
BSA	4.2 kDa PLL	0.3	7.3	123
BSA	4.2 kDa PLL	1.0	5.3	560
BSA	24 kDa PLL	0.1	7.4	77
BSA	24 kDa PLL	0.3	25	71
BSA	24 kDa PLL	1.0	14.9	209

Table 1 FWHM analyses of peaks obtained from PCS results for uncoated BSA NPs and BSA NPs coated with PLL.

NPs	Coated Polymer	Coating Conc. (mg/mL)	½ of peak height (%)	FWHM (nm)
BSA	None	0	4.4;41.05	39;95
BSA	K_6MK_6	0.1	7.35	173
BSA	K_6MK_6	0.3	33.7	101
BSA	K_6MK_6	1.0	4.65;21.25	30;120

Table 2 FWHM analyses of peaks obtained from PCS results for uncoated BSA NPs and BSA NPs coated with K_6MK_6.

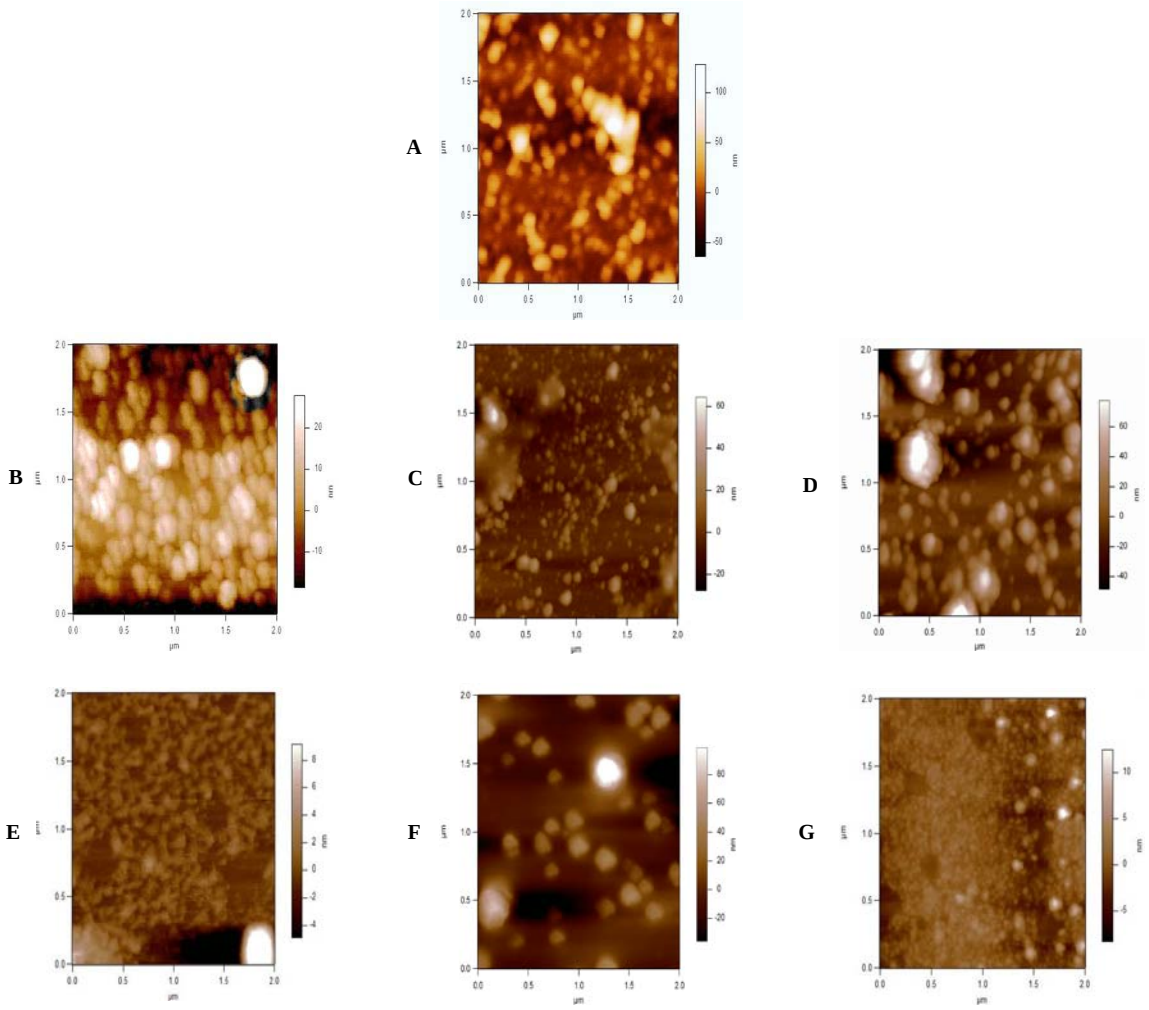


Figure 4. 4 AFM pictures (height image mode) of uncoated and uncoated BSA NPs
Uncoated BSA NPs (A), BSA NPs coated with 0.3 mg/mL of 0.9 (B), 4.2 (C) and 24 (D) kDa PLL, and 0.1 (E), 0.3 (F) and 1.0 (G) mg/mL of K_6MK_6. Larger NPs were observed with coating. Uniform and spherical NPs were observed with increasing MW of PLL and concentration of K_6MK_6.

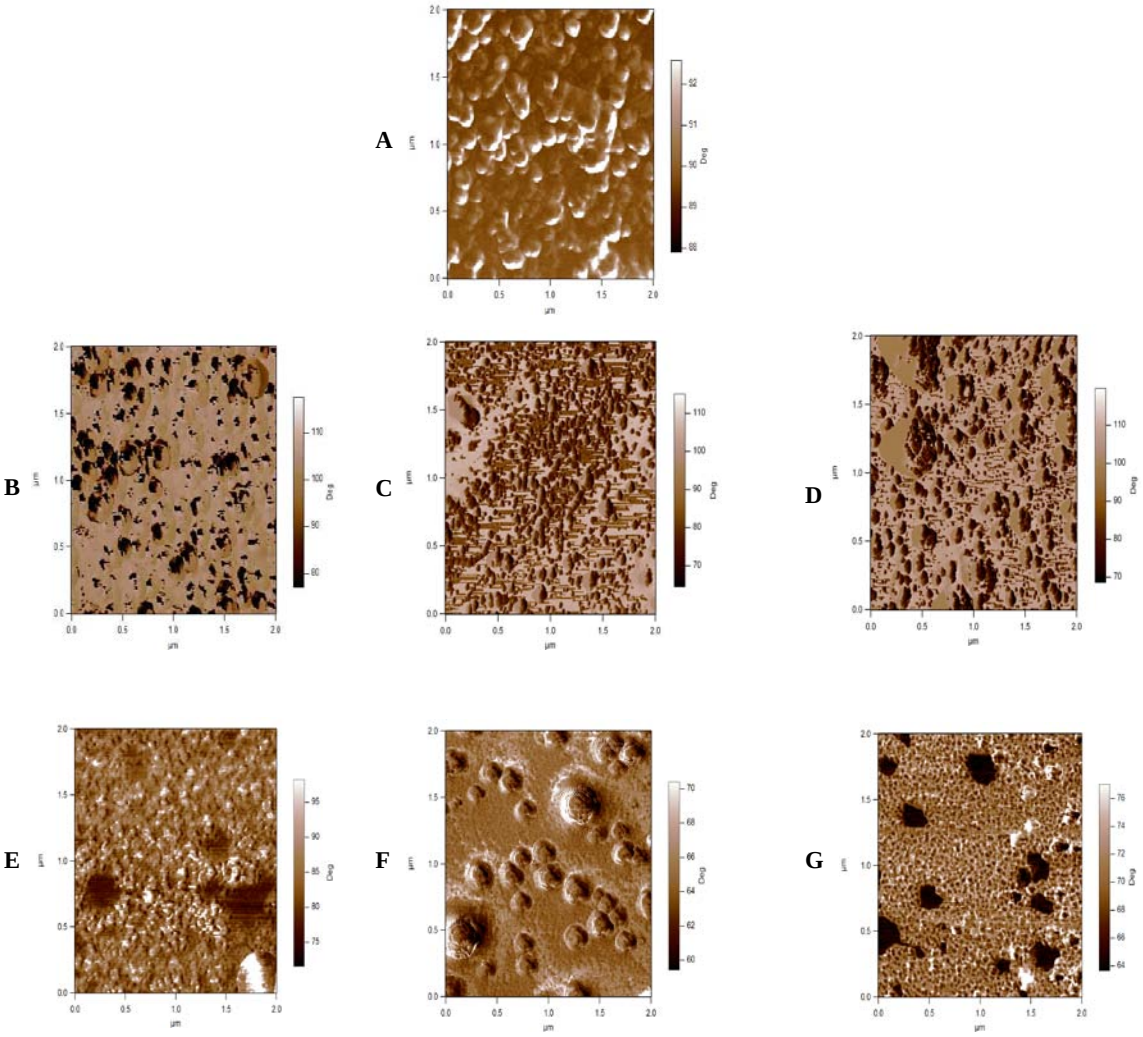


Figure 4. 5 AFM pictures (phase image mode) of uncoated and coated BSA NPs
Uncoated BSA NPs (A), BSA NPs coated with 0.3 mg/mL of 0.9 (B), 4.2 (C) and 24 (D) kDa PLL, and 0.1 (E), 0.3 (F) and 1.0 (G) mg/mL of K_6MK_6. Coating onto the BSA NPs with PLL or K_6MK_6 resulted in darker regions indicating these regions to be soft or more elastic.

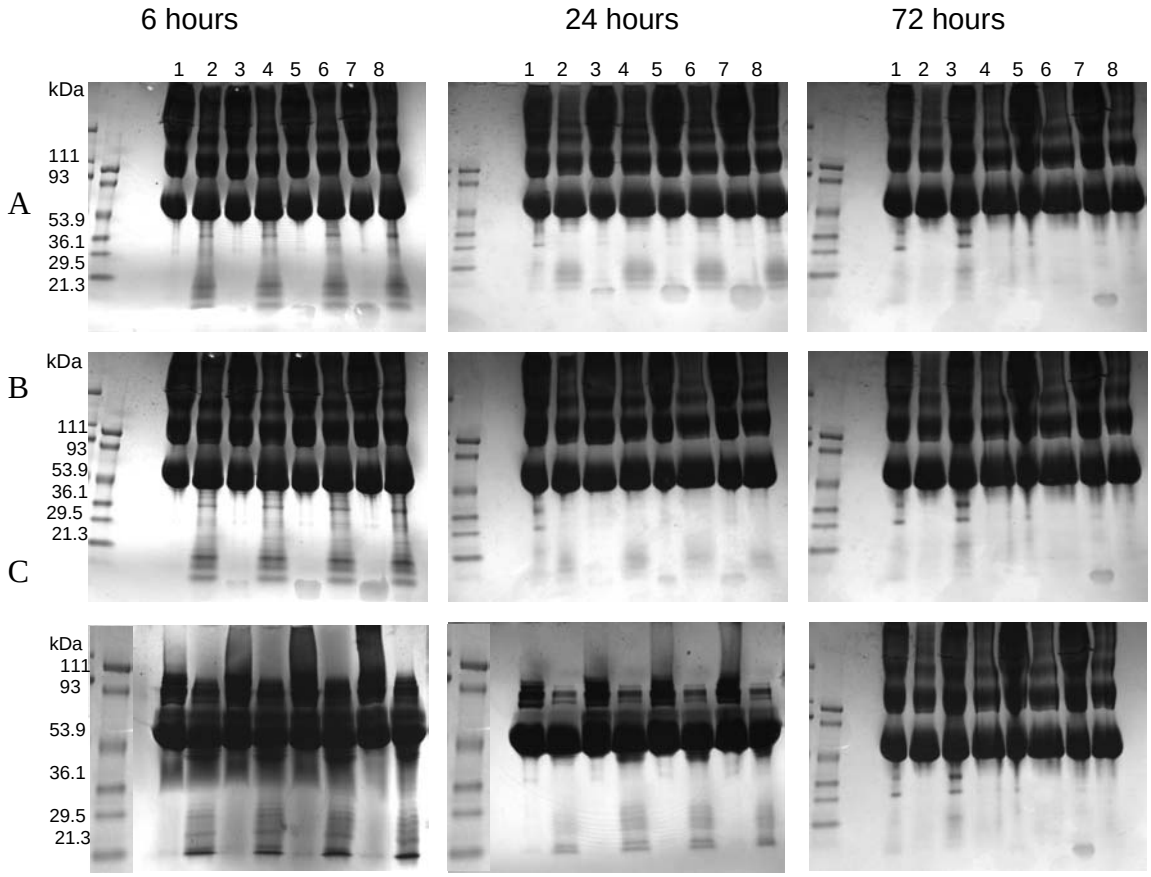


Figure 4. 6 Tryptic digestion of BSA by electrophoresis.

BSA and PLL solutions were prepared in phosphate buffer containing sodium azide (pH=7.4). BSA solution was incubated with (lane 1) and without (lane 2) Trypsin. PLL solution was also added to BSA solutions and incubated with and without trypsin. Lanes 1, 3, 5 and 7 represent PLL and BSA solution mixture, with PLL concentration of 0.1, 0.3 and 1.0 mg/mL respectively. Even number lanes are the corresponding solutions containing Trypsin. PLL MWs used were 0.9 (row A), 4.2 (row B) and 24 (row C) kDa. BSA was overloaded onto the wells to see the Tryptic digests clearly. Three independent experiments were performed. Trypsin was found to digest the BSA based on the appearance of smaller MW protein species.

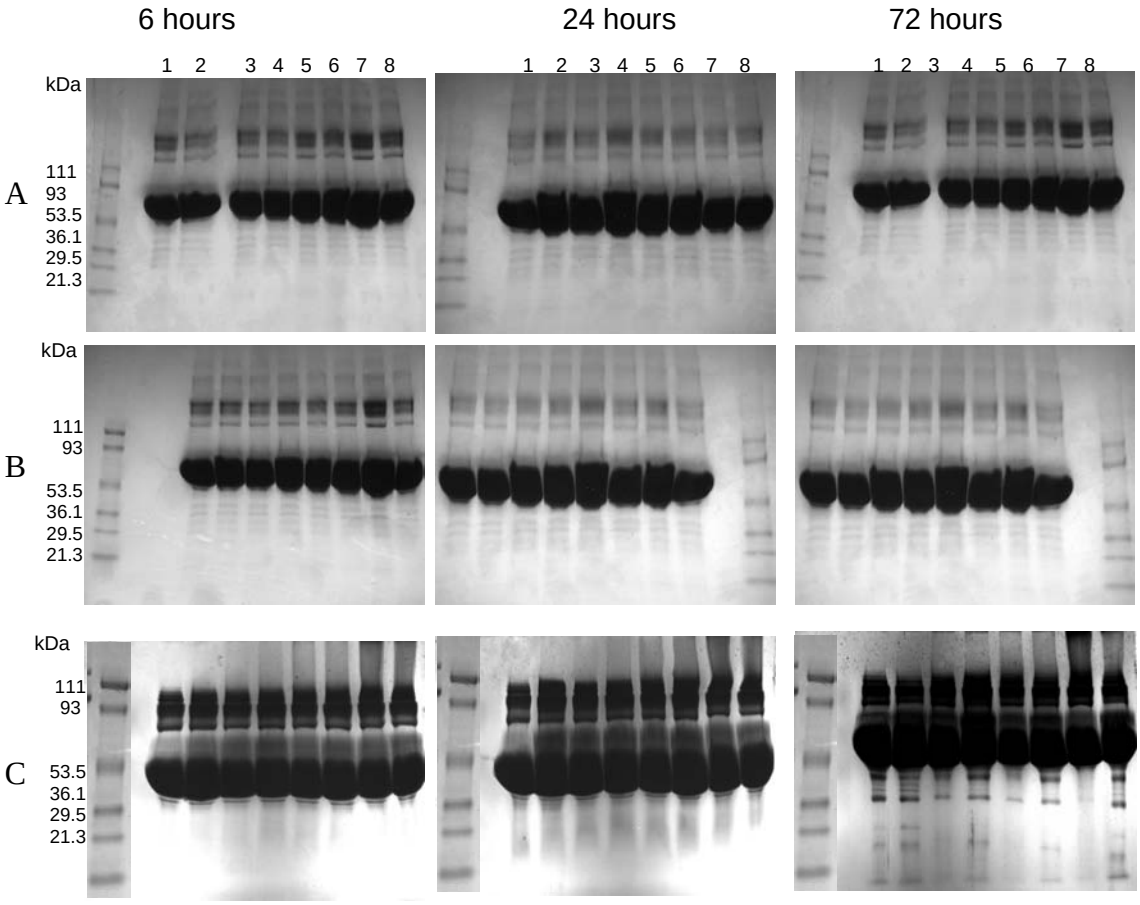


Figure 4. 7 MMP-2 digestion of BSA by electrophoresis.
PLL and BSA solutions were prepared in Tris- buffer. BSA solution was incubated with (lane 1) and without (lane 2) MMP-2. PLL solution was also added to BSA solutions and incubated with and without MMP-2. Lanes 1, 3, 5 and 7 represent PLL and BSA solution mixture, with PLL concentration of 0.1, 0.3 and 1.0 mg/mL respectively. Even number lanes are the corresponding solutions containing MMP-2. PLL MWs used were 0.9 (row A), 4.2 (row B) and 24 (row C) kDa. BSA was overloaded onto the wells to see the MMP-2 digests clearly. Three independent experiments were performed. MMP-2 was seen to not digest the BSA based on no appearance of low MW protein species.

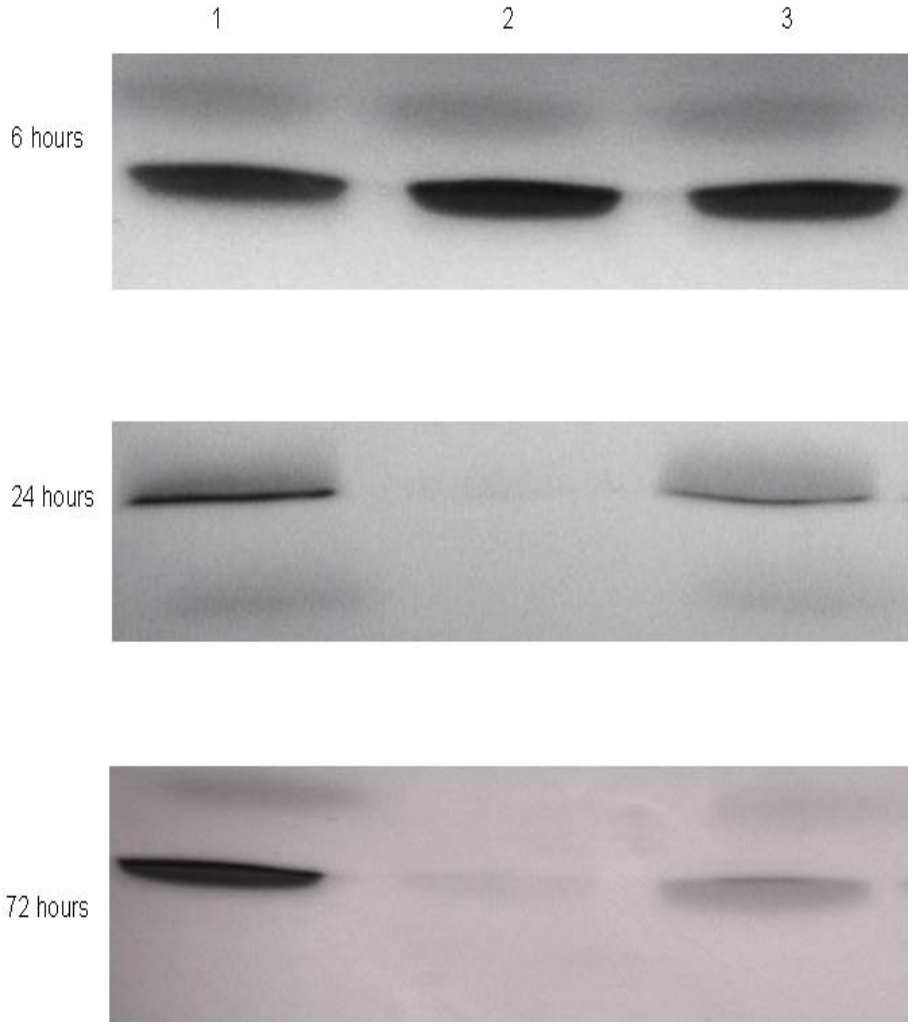


Figure 4. 8 Tryptic or MMP-2 digestion of K_6MK_6 by electrophoresis.
K_6MK_6 in Tris- Buffer was incubated with enzymes Trypsin or MMP-2. Lane 1 represents the peptide band whereas lanes 2 and 3 show the peptide band in the presence of Trypsin and MMP-2 respectively at indicated times. Three independent experiments were performed. Trypsin and MMP-2 were seen to digest K_6MK_6 based upon almost no appearance of K_6MK_6 band. Trypsin was observed to digest K_6MK_6 completely and quickly as compared to MMP-2 on the basis of no appearance of peptide band in the presence of Trypsin.

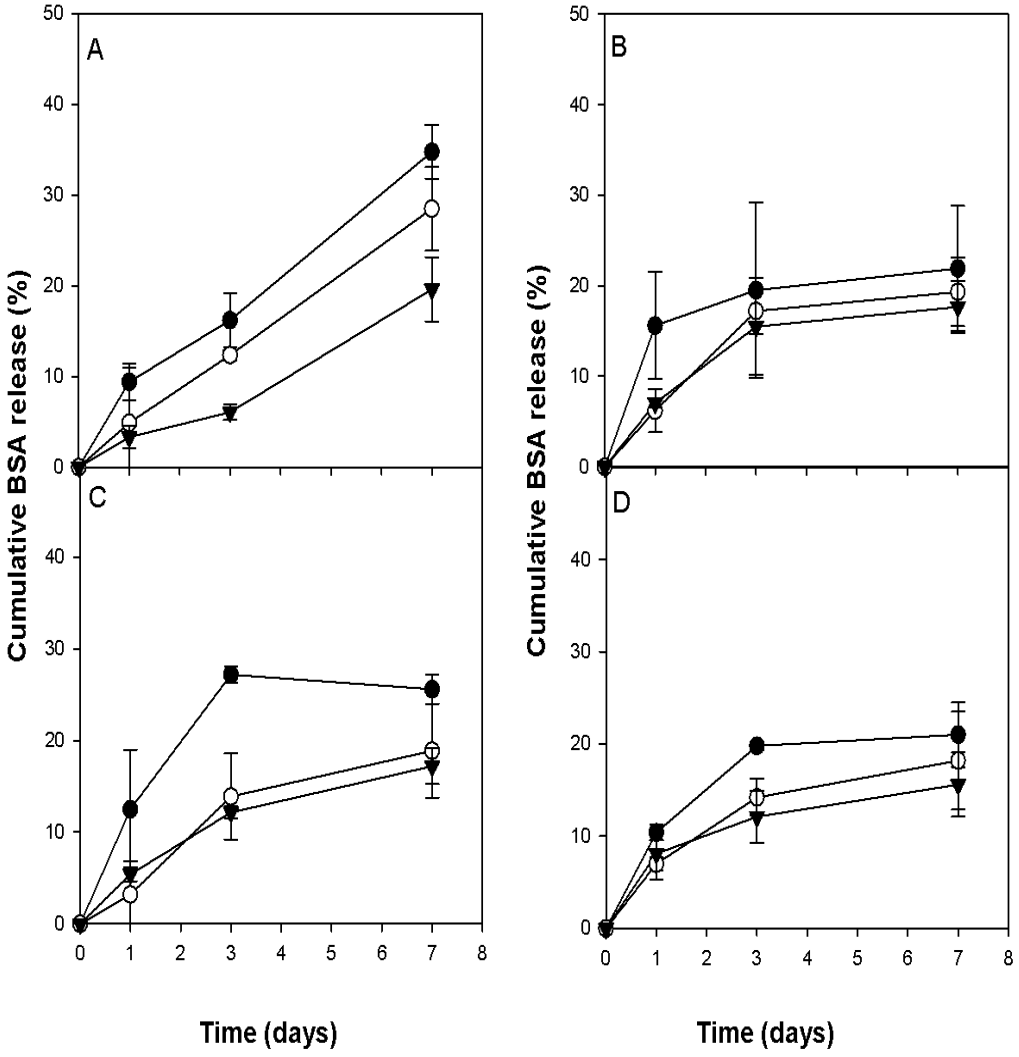


Figure 4. 9 Stability of PLL coated BSA NPs based on release profile of FITC-BSA from coated BSA NPs.

BSA NPs formed *via* coacervation were coated with PLL and release of FITC-BSA was measured in phosphate buffer (pH=7.4). MWs of PLL used for coating onto BSA NPs were 0.9 (A), 4.2 (B), 13.8 (C) and 24 (D) kDa with their concentrations as 0.1(●), 0.3 (○) and 1.0 (▼) mg/mL. Trend lines are provided as a guide to the eye. Data points represent average of $n \geq 3$, error bars are ± 1SD. Continuous release of FITC-BSA from 0.9 kDa PLL coated BSA NPs and almost no release of FITC-BSA from 4.2, 13.8 and 24 kDa PLL coated BSA NPs after day 3 was observed

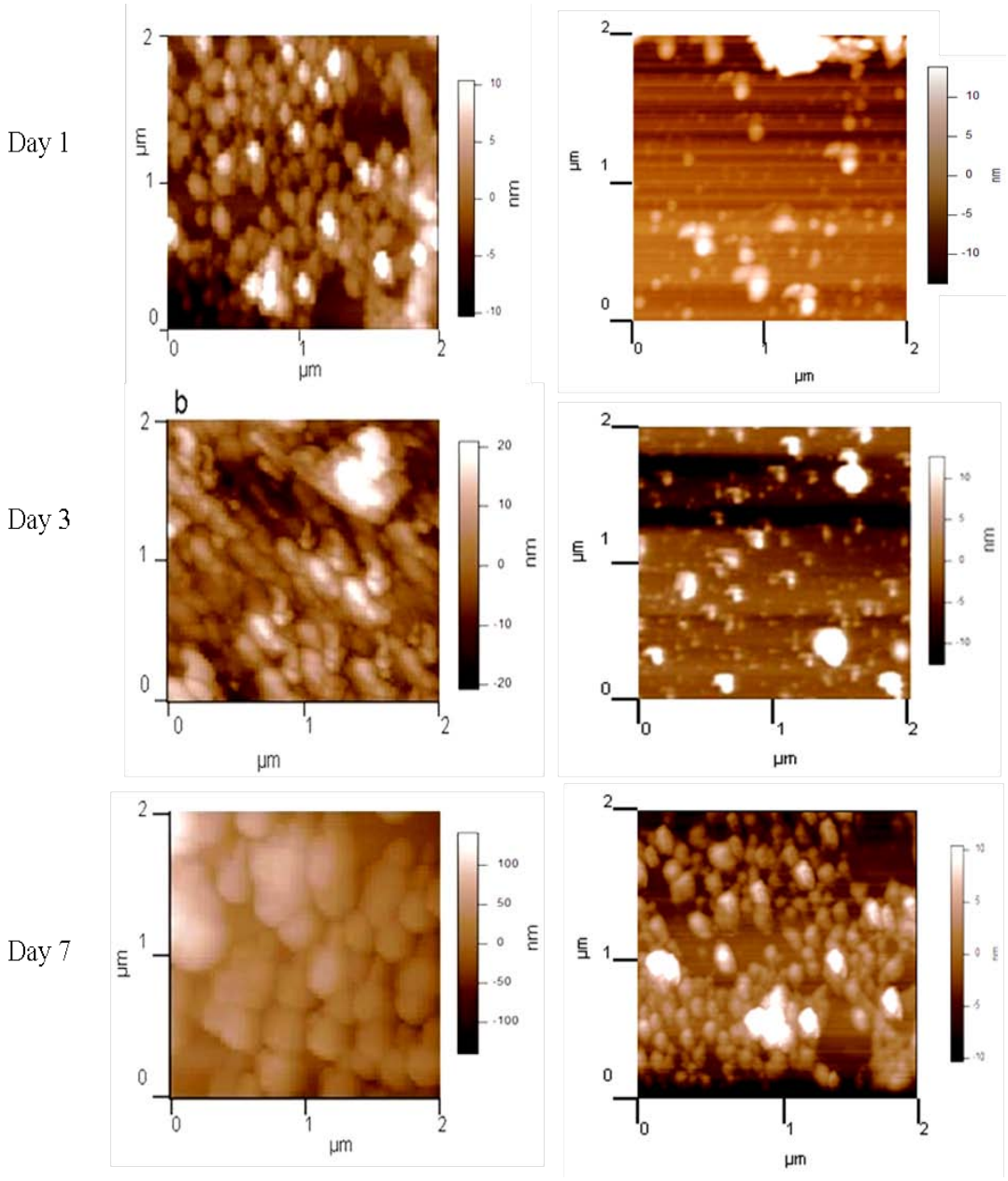


Figure 4. 10 AFM pictures of coated BSA NPs.
BSA NPs coated with 0.3 mg/mL of 0.9 (left column) and 24 (right column) kDa PLL. PLL coated BSA NPs suspensions were diluted with phosphate buffer (pH=7.4) and incubated. Samples were analyzed under AFM at Day 1, 3 and 7. An increase in the particle size as well as aggregation was observed for 0.9 kDa PLL coated BSA NPs. Partial increase in the particle size was noted with time for 24kDa PLL coated BSA NPs.

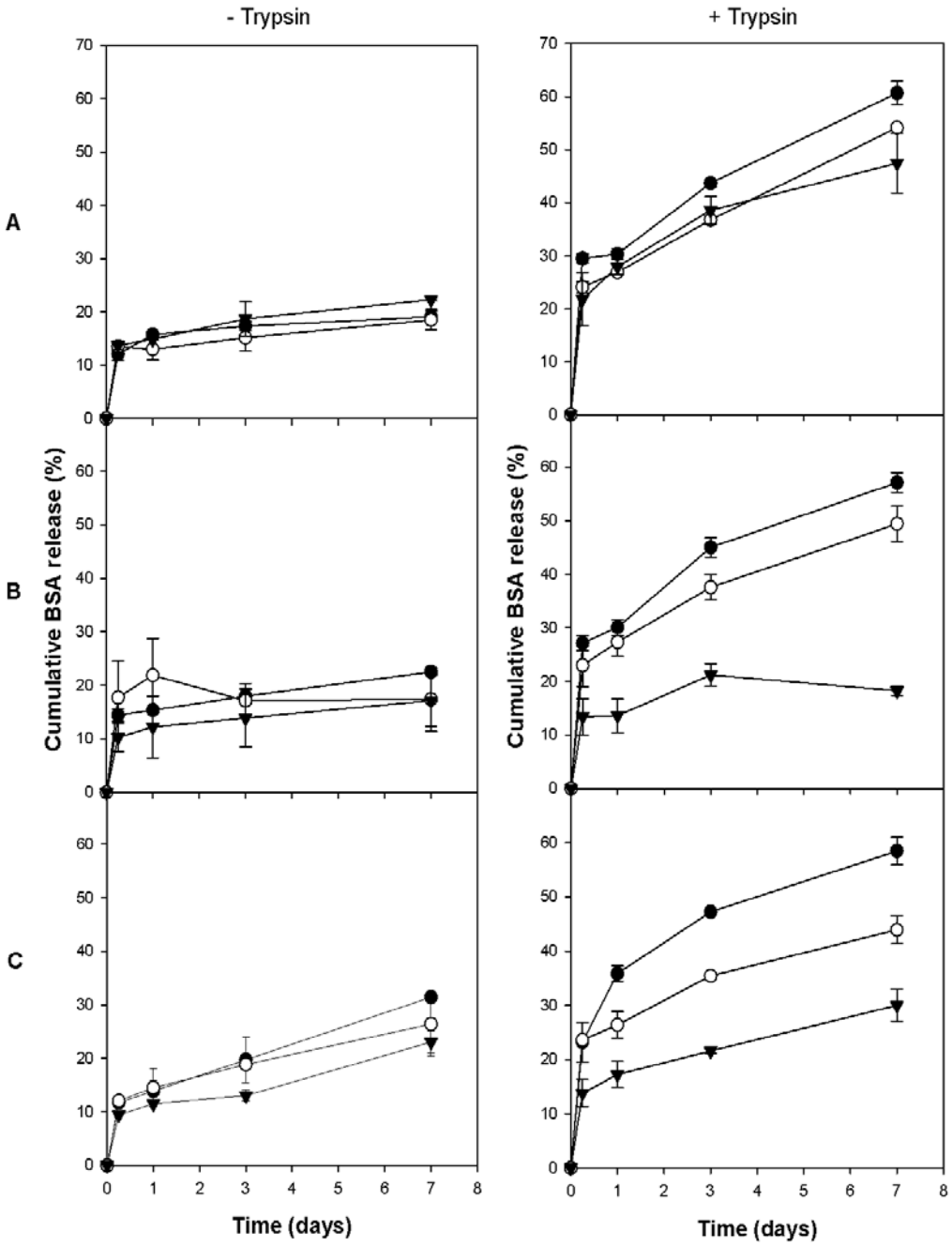


Figure 4. 11 Tryptic digestion of PLL coated FITC-BSA NPs based upon a fluorescence test. Fluorescence value of the supernatant was calculated in the absence and presence of Trypsin. PLL MWs were varied from 0.9 (row C), 4.2 (row B) and 24 (row A) kDa. Symbols (●), (○) and (▼) represent FITC-BSA release from 0.1, 0.3 and 1.0 mg/mL of PLL coated BSA NPs. Trend lines are provided as a guide to the eye. Data points represent average of $n \geq 3$, error bars are ± 1SD. Trypsin was found to digest the coated BSA NPs. The digestion rate was found to decrease with the increasing concentration of 0.9 and 4.2 kDa PLL used for coating onto BSA NPs.

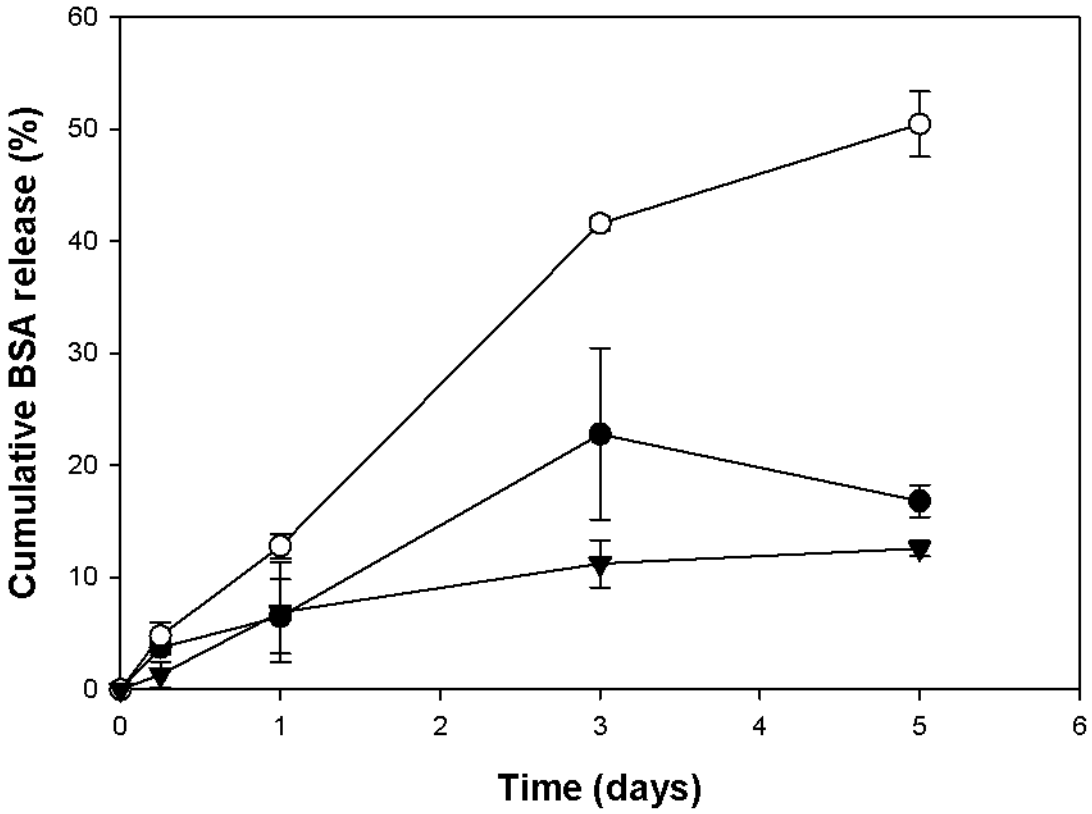


Figure 4. 12 Release profile of BSA from BSA NPs coated with 0.1 mg/mL of K_6MK_6 in the presence and absence of enzymes.

Trypsin (○) and MMP-2 (▼) were used as enzymes. Symbol (●) represents the release of FITC-BSA from coated NPs in the absence of any enzyme. Trend lines are provided as a guide to the eye only. Data points represent average of ≥n3, error bars are ± 1SD. There was a burst release of BSA in the presence of Trypsin. MMP-2 was observed to inhibit the release of FITC-BSA from the coated BSA NPs in spite of cleavability of K_6MK_6 by MMP-2

NPs	Coated Polymer	Coating Conc. (mg/mL)	Encapsulation Efficiency (%)
BSA	None	Na	10±3
BSA	0.9 kDa PLL	1	58±5
BSA	4.2 kDa PLL	1	66±6
BSA	24 kDa PLL	1	83±6
BSA	K_6MK_6	1	52±3

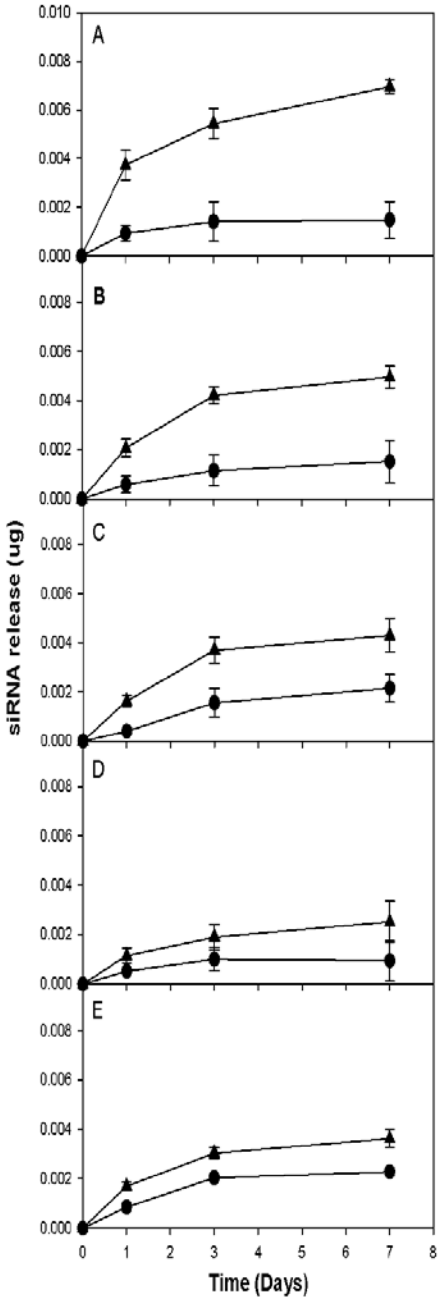


Figure 4. 13 siRNA release profile from uncoated and coated BSA NPs.
siRNA release from uncoated BSA NPs (E) and BSA NPs coated with 1 mg/mL of K_6MK_6 (B), 0.9 (C), 4.2 (D) and 24 (E) kDa PLL in the presence (▲) and absence (●) of Trypsin. Trend lines are provided as a guide to the eye only. Data points represent average of $n \geq 3$, error bars are ± 1SD. 24 kDa PLL coated BSA NPs showed the highest encapsulation efficiency and steep rise in the percent of siRNA released from the NPs in the presence of Trypsin and least in K_6MK_6 coated BSA NPs where encapsulation efficiency was least, among the coated NPs.

CHAPTER 5

CONCLUSIONS AND FUTURE WORK

5.1) Conclusions

On the basis of work carried out during this research project, the following significant conclusions may be drawn:

1. Based on the results from the PCS studies, flocculation among the particles was noted for BSA NPs coated with 0.1 mg/mL of 0.9 and 4.2 kDa PLL. No flocculation was observed for BSA NPs coated with 24 kDa and K_6MK_6 for all coating concentrations used. A decrease in the size for BSA NPs coated with 0.9 and 4.2 kDa PLL and K_6MK_6 was noted with the increasing concentration of these PLL's.
2. Based on the results from the Zeta Potential studies, there was a significant increase in the zeta potential of BSA NPs upon coating with cationic polymers. NPs coated with 24 kDa PLL gave the highest zeta potentials.
3. Based on the results from the AFM studies, uncoated BSA NPs were noted to be less elastic and spherical; upon coating, particles were observed to be irregular in shape and were relatively elastic and less stiff. BSA NPs coated with 0.3 mg/mL of 0.9 kDa PLL showed increase in particle size as well as aggregation among the particles after 7 days of

study period; BSA NPs coated with 24 kDa PLL showed least increase in size and aggregation.

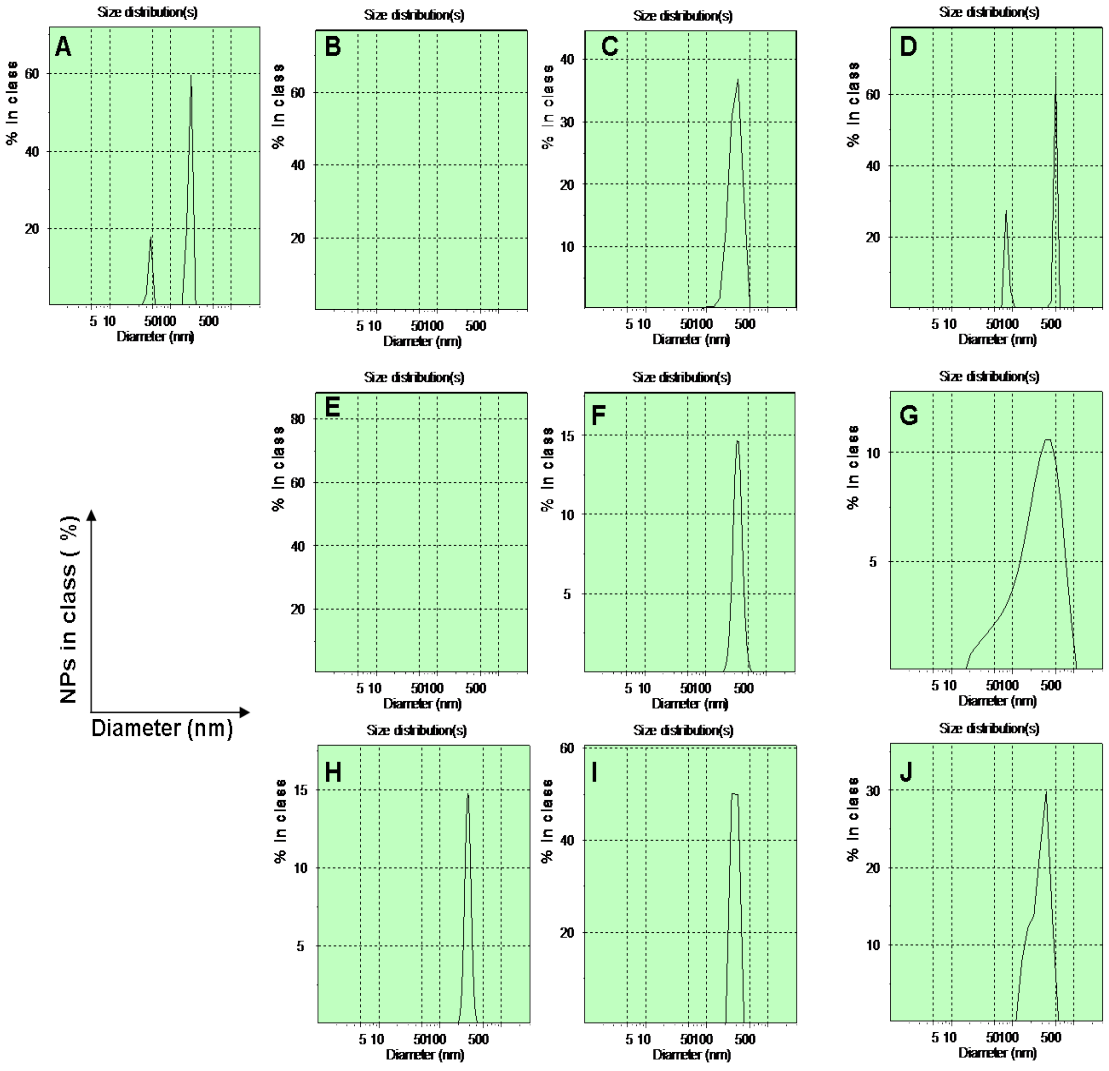
4. The peptide KKKKKKGPQGIASQKKKKKK (K_6MK_6) was successfully coated onto BSA NPs, based upon photon correlation spectroscopy technique.
5. The MMP-2 was observed to digest the peptide K_6MK_6 based upon the SDS-PAGE technique.
6. The proteolytic enzyme trypsin was observed to digest both BSA and K_6MK_6 peptide based upon SDS-PAGE analysis.
7. Incubation of NPs with trypsin resulted in the higher release of BSA from PLL and K_6MK_6 coated NPs. This indicates the possibility of NP degradation in the presence of proteolytic enzymes.
8. Despite cleavability of K_6MK_6 by MMP-2, there was no change in the release of BSA from NPs coated with K_6MK_6 in the presence of MMP-2.
9. There was a significant increase in the encapsulation efficiency of siRNA in BSA NPs upon PLL coating. Increase in the encapsulation efficiency was noted with increasing molecular weight of PLL used for coating the NPs. Coating with the peptide K_6MK_6 also resulted in greater encapsulation efficiency.
10. The siRNA release from BSA NPs was observed to increase with increasing MW of PLL coating in the presence of trypsin; this result is in line with the higher siRNA entrapped in the particles for higher MW PLL.

5.2) Future Work

Following suggestions are proposed as next steps to this research program:

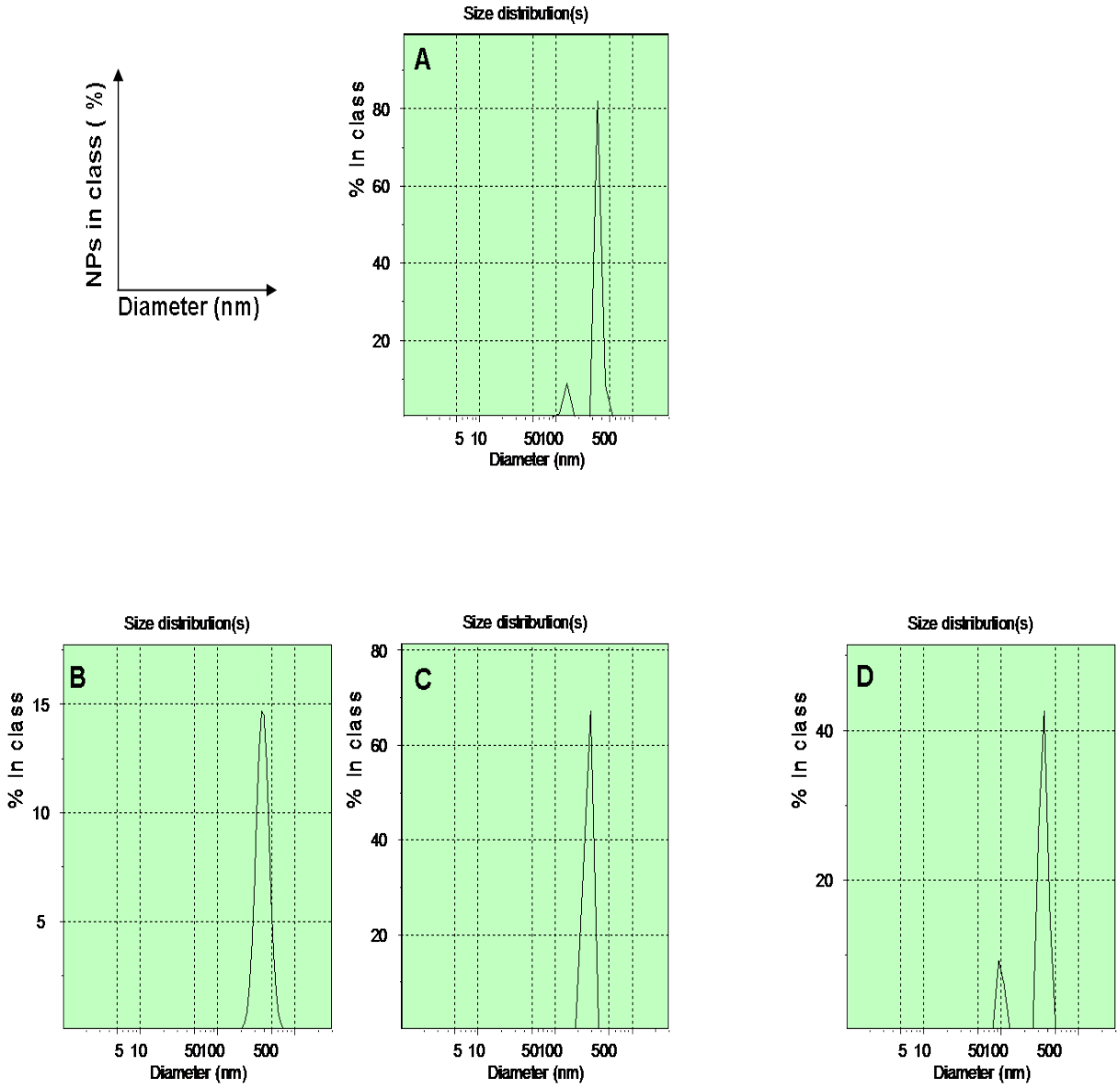
1. It is clear that the peptide K_6MK_6 is cleavable by MMP-2 and successfully coats BSA NPs. There was no significant difference in the BSA release from K_6MK_6 coated BSA NPs in the presence and absence of MMP-2. This is likely due to presence of only one cleavage point in the peptide K_6MK_6. Probably, increasing the number of cleavable points in the peptide might serve as a solution. In order to test this, success of coating such a peptide onto BSA NPs needs to be undertaken first.
2. It is important to study the siRNA release profile from BSA NPs coated with MMP-2 cleavable peptide, especially in the presence of MMP-2; this study will suggest the likely use of these particles for targeted drug delivery applications.
3. The effects of non-specific degradation of the coated and uncoated BSA NPs should be studied. The release of siRNA in the presence of serum may be an important investigation.
4. BSA NPs coated with PLL have displayed varying zeta potentials depending on the molecular weight of the polymer used for coating purposes; adsorption of proteins onto these particles could be an interesting study to predict the possible *in-vivo* fate of these particles.

APPENDIX-A



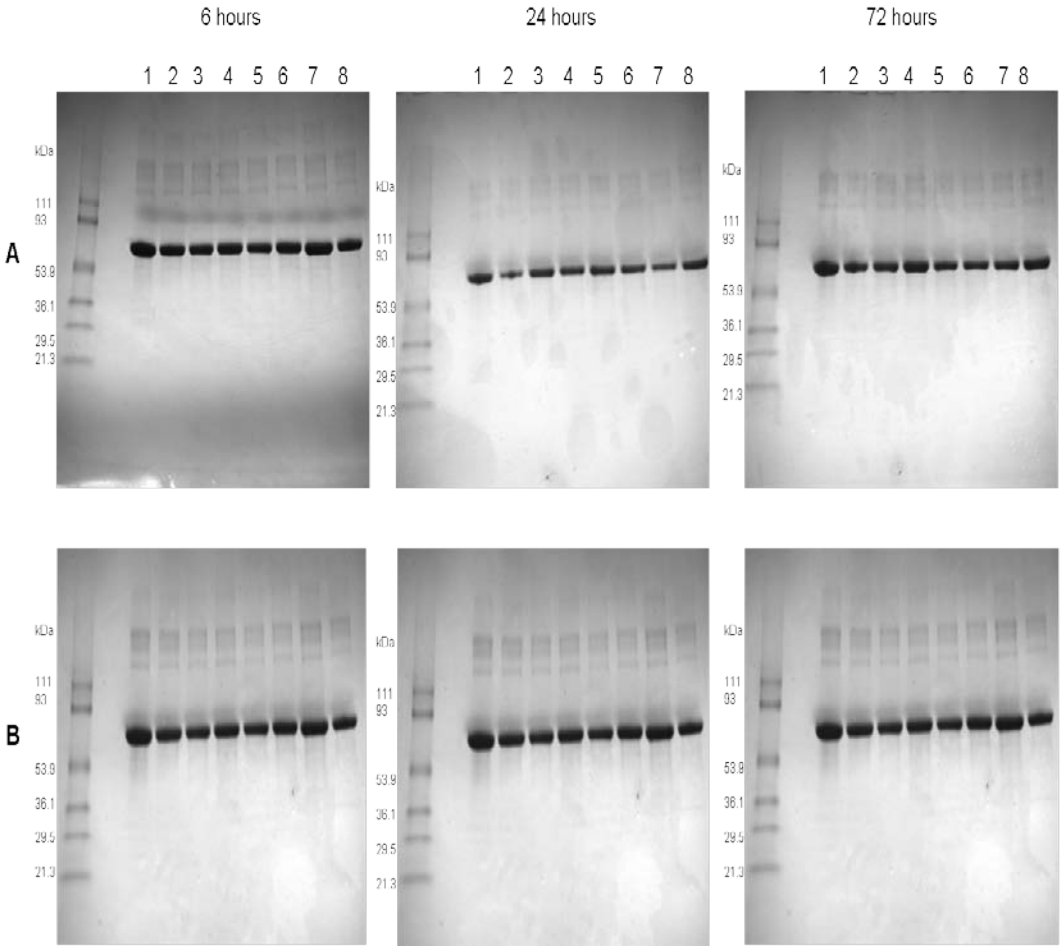
Size distribution of BSA NPs (A) coated with different MWs of PLL, 0.9 (B, C, D), 4.2 (E, F, G) and 24 (H, I, J) kDa. Concentrations of PLL used for coating were 0.1 mg/mL (B, E, H), 0.3mg/mL (C, F, I) and 1.0 mg/mL (D, G, J).

APPENDIX-B



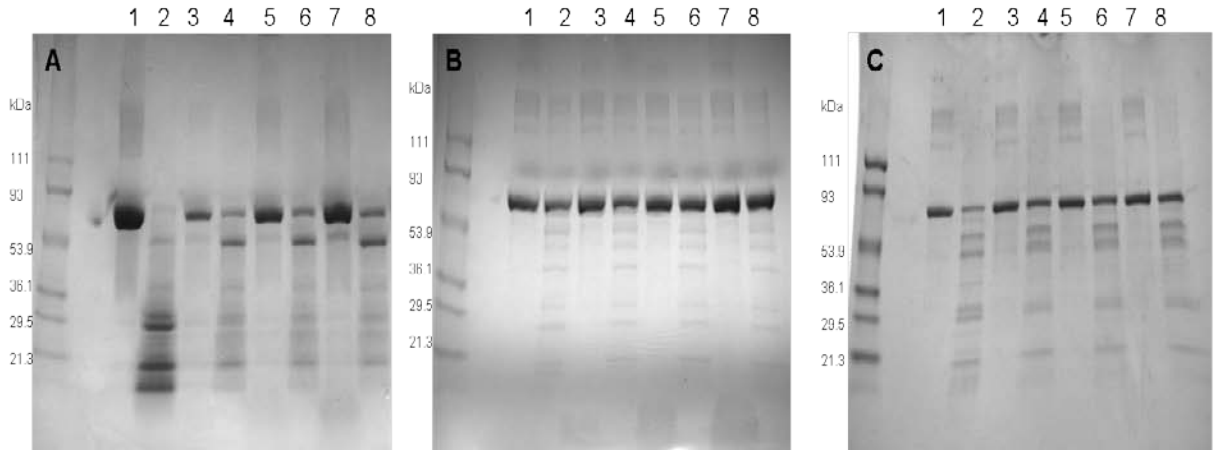
Size distribution of BSA NPs (A) coated with different concentrations of K_6MK_6. Concentrations used for coating were 0.1 (B), 0.3 (C) and 1.0 (D) mg/mL

APPENDIX-C



MMP-2 digestion of BSA by electrophoresis. PLL and BSA solutions were prepared in Tris- buffer. BSA solution was incubated with (lane 1) and without (lane 2) MMP-2. PLL solution was also added to BSA solutions and incubated with and without MMP-2. Lanes 1, 3, 5 and 7 represent PLL and BSA solution mixture, with PLL concentration of 0.1, 0.3 and 1.0 mg/mL respectively. Even number lanes are the corresponding solutions containing MMP-2. PLL MWs used were 0.9 kDa (row A) and 4.2 kDa (row B).

APPENDIX-D



Tryptic digestion of BSA by electrophoresis. PLL and BSA solutions were prepared in phosphate buffer. BSA solution was incubated with (lane 1) and without (lane 2) trypsin. PLL solution was also added to BSA solutions and incubated with and without trypsin. Lanes 1, 3, 5 and 7 represent PLL and BSA solution mixture, with PLL concentration of 0.1, 0.3 and 1.0 mg/mL respectively. Even number lanes are the corresponding solutions containing MMP-2. PLL MWs used were 0.9 kDa (A), 4.2 kDa (B) and 24 kDa (C).