PREPARATION AND CHARACTERIZATION OF SERUM ALBUMIN NANOPARTICLES OBTAINED FROM MODIFIED BOVINE SERUM ALBUMIN

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ABSTRACT

PREPARATION AND CHARACTERIZATION OF SERUM ALBUMIN NANOPARTICLES OBTAINED FROM MODIFIED BOVINE SERUM ALBUMIN

The serum albumin has been used as a drug nanocarrier for a long time due to its rich drug transportation ability. Here, modified bovine serum albumin (BSA) proteins were obtained by conjugation with ethylenediamine and dopamine molecules, separately. Using these modified proteins, new BSA nanoparticles were obtained by a desolvation method.

Native BSA has a net negative charge at the physiological condition. However, ethylenediamine conjugation yields a positive charge on it, and thus produces cationic BSA (cBSA) protein. On the other hand, dopamine functionalization (D-BSA) makes BSA eager to coordinate with transition metals. After preparation of modified proteins (cBSA and D-BSA), their nanoparticles were prepared with desolvation method but using different crosslinking mechanisms. For cBSA NPs preparation, a traditional crosslinking agent of glutaraldehyde was used. However, for D-BSA NPs preparation, Fe(III) ions were added to the system to achieve the stable nanoparticle formation.

In order to obtain cBSA NPs, several organic solvents were used as desolvating agents. cBSA NPs with an average size around 200 nm were obtained in a high formation yield (54.8%) only through addition of acetonitrile to the cBSA aqueous solution. Similarly, different desolvating agents were studied to obtain D-BSA NPs. The promising results were obtained upon addition of 1:5 (v/v) of water/acetone mixture. After addition of the desolvating agent, Fe(III) ions were added to the solution to interconnect D-BSA with each other. This connection is pH sensitive therefore albumin nanoparticles were stable at basic pH values but not at acidic pH values. By this way, pH sensitive D-BSA NPs around 300 nm particle sizes were obtained.

ÖZET

MODİFİKASYONA UĞRAMIŞ SIĞIR SERUM ALBÜMİN'DEN ELDE EDİLMİŞ SERUM ALBÜMİN NANOPARÇACIKLARIN HAZIRLANMASI VE KARAKTERİZASYONU

Serum albüminin zengin ilaç taşıma yeteneğinden dolayı uzun süredir ilaç nanotaşıyıcısı olarak kullanılmaktadır. Burada, etilendiamin ve dopamin moleküllerinin ayrı ayrı bağlanması ile modifikasyona uğramış sığır serum albümin (BSA) proteinleri elde edilmiştir. Bu modifikasyona uğramış proteinler kullanılarak, yeni BSA nanoparçacıkları çözgenin uzaklaştırılması yöntemiyle üretilmiştir.

Bu çalışmanın amacı, serum albümin proteinine etilendiamin ve dopamin molekülü bağlayarak farklı özellikler kazandırmak ve bu iki albümin proteininden nanoparçacıklar hazırlayarak nanotaşıyıcı malzeme üretmektir.

BSA fizyolojik ortamda eksi yüzey yüküne sahiptir. katyonik sığır serum albümin (cBSA) için kullanılan etilendiamin molekülü, albümin yüzeyindeki karboksil grubuna sahip aminoasitlere bağlanarak protein yüzey yükünü artıya dönüştürmüştür. Dopamin bağlanmış sığır serum albümin (D-BSA) için kullanılan dopamin molekülünde bulunan amin grubu ile albümin yüzeyindeki karboksil grubu ile amid bağının kurulması gerçekleştirilmiştir. Sonuç olarak, cBSA ve D-BSA elde edilmiştir. Modifikasyona uğramış bu proteinler çözgenin uzaklaştırılması yöntemi kullanılarak cBSA ve D-BSA nanoparçacıkları (cBSA NPs ve D-BSA NPs) fakat farklı çapraz bağlayıcı mekanizmaları uygulanarak hazırlanmıştır. cBSA NPs hazırlanırken geleneksel çapraz bağlayıcı olarak gluteraldehit kullanılırken, D-BSA NPs hazırlanırken ise Fe(III) iyonu sisteme eklenerek kararlı nanoparçacık oluşumu sağlanmıştır.

Kullanılan organik çözgenin türüne göre farklı boyutlarda ve farklı verimlerde nanoparçacıklar elde edilmiştir. Elde edilen cBSA nanoparçacıkların verimi asetonitril kullanıldığı zaman %55 ve ortalama büyüklükleri ise 244 nm olmaktadır. Organik çözgen olarak 1:5 (v/v) su/aseton karışımı kullanıldıktan sonra, Fe(III) iyonu eklenmiştir ve pH'a duyarlı yaklaşık 300 nm boyutunda D-BSA nanoparçacıkları %45 verim ile elde edilmiştir. Bu bağlanma pH a duyarlıdır ve albümin nanoparçacıkları bazik pH değerlerinde kararlıdır.

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CHAPTER 1

INTRODUCTION

1.1. Nanocarriers

Nanocarriers (NCs) are colloidal patterns having structures underneath 500 nm size (Neubert 2011). Nanocarriers have been involved in the delivery of drugs and biologically active materials (SiRNA, plasmid, antibodies, bilirubin, ions, peptides, vitamins, fatty acids, etc.) to the target area for a long time (Y. Zhang, Sun, and Jiang 2018). Production of nano-sized carriers with nanotechnology has made controlled drug delivery possible. Both drug transportation to the target point and release mechanisms of nanoparticles are very important for treatment of many diseases. By releasing in a controlled manner, the side effects of drugs can be reduced and treatment can be faster. Nanocarriers are designed using different materials, which can successfully deliver hydrophobic drugs or hydrophilic drugs (Bilia et al. 2018). Compared to the traditional applications, smart drug delivery systems (SDDS) have appeared extraordinary potential in cancer treatment by improving the performance of medicines so that they have many advantageous features. SDDS enhance bioavailability and storage stability, high biocompatibility, minimize systemic side impacts, provide active target to specific cell/tissue, drug loading capacity, and sustained-controlled drug release (Karewicz 2014).

Nanocarriers are classified into mainly three groups, organic-based NCs, inorganic-based NCs and hybrid NCs. Organic NCs are again divided into two parts as polymeric and lipid-based nanocarriers. Polymeric NCs are subdivided into seven carriers as dendrimers, micelles, polymer-conjugate drug/protein, nanoparticles, nanogels, hydrogels, nanocapsules. Lipid-based NCs are liposomes, solid-lipid NPs, nanostructured lipid carriers, and nanoemulsions. Inorganic-based NCs are quantum dots, magnetic NCs (iron oxide NPs), gold NPs, carbon nanotubes, and mesoporous silica NPs. Hybrid ones are polymer-lipid hybrid NPs (PLN), monolithic matrix, and core-shell systems as shown in Figure 1. 1. (R. X. Zhang et al. 2017).

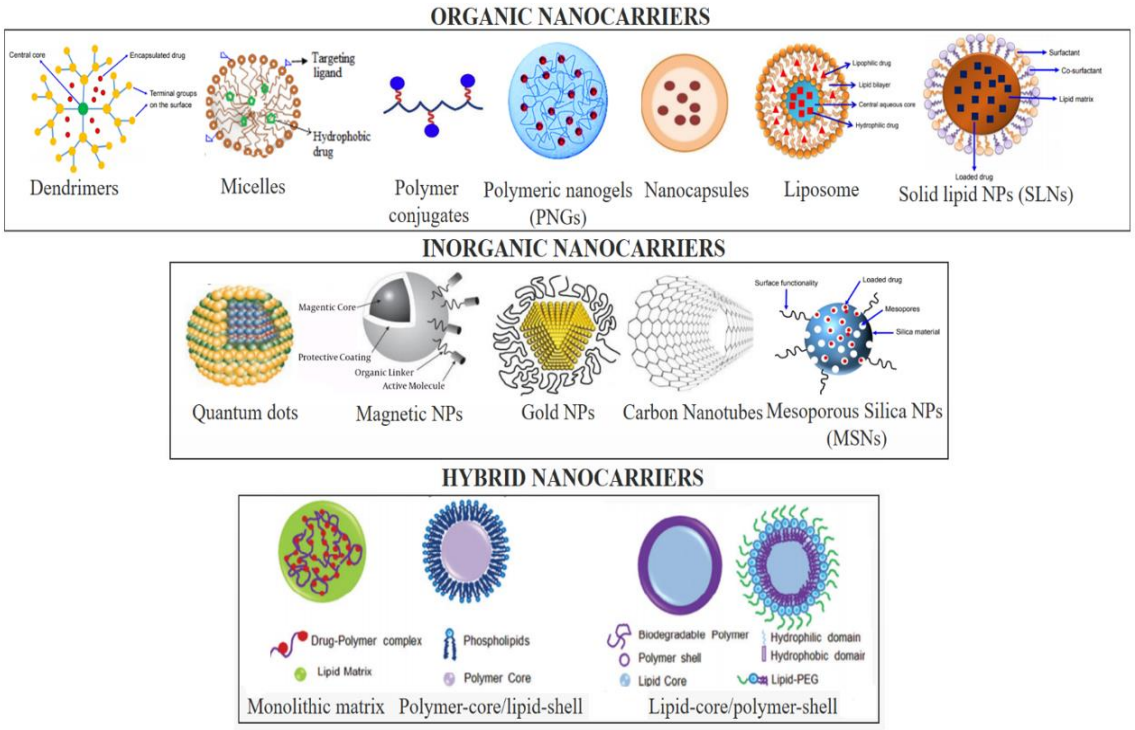

Figure 1. 1. Illustrative structure of the different types of nanocarriers (Din et al. 2017)(Kumar et al. 2020)(Srivastava et al. 2021)(Arora and Jaglan 2016).

Many different types of nanocarriers are currently used for cancer therapy or treatment of many diseases. Some types of nanocarriers, nanoparticle formulations, used drugs, their specificity for therapeutic applications with commercial name are indicated as a list in Table 1.1. Some of these nano drug formulations and smart drug delivery nanocarriers are approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA) in below-mentioned. For instance, the first nano-drug for cancer treatment was formulated with PEGylated liposomes of doxorubicin encapsulation (Doxil®, Caelyx®). Doxil® is less than 120 nm particle size and sterically stabilized liposomes composed of phospholipids, cholesterol, and a lipopolymer (PEG). It is used in treatment of breast and ovarian cancers and was approved by FDA in 1995 (Werner et al. 2013). The other example is Abraxane® which is the albumin-conjugated nanoparticle form of the anti-cancer drug paclitaxel. This is an early example of an albumin based nano drug. Albumin NPs (130 nm) conjugated with paclitaxel are used for treatment of non-small lung cancer and metastatic breast cancer. Abraxane® was approved in 2005 by FDA and in 2008 by EMA (Karami, Behdani, and Kazemi-Lomedasht 2020).

Table 1.1. Different type of drug nanocarriers and used in various treatments (Hong et al. 2020).

Type of nanocarrier	Product name	Nanoparticle Formulation	Drug	Indication(s)	Approval
Liposomes	Marqibo®	Sphingomyelin and cholesterol	Vincristine sulfate	Acute lymphoid leukemia	FDA 2012
	Onivyde®	Nanoliposomes	Irinotecan	Pancreatic cancer, Colorectal cancer	FDA 2015, EMA 2016
	Doxil®, Caelyx®	Pegylated Liposomes	Doxorubicin HCl	Breast cancer, Ovarian cancer	FDA 1995
Protein-drug conjugates	Abraxane®	Albumin	Paclitaxel	Metastatic breast cancer, Non-small lung cancer, Pancreatic cancer	FDA 2005, EMA 2008
	Rebinyn®	Glycopegylated coagulation Factor IX	Factor IX	Hemophilia	FDA 2017
Metalic nanoparticles	Feraheme®	Superparamagnetic iron oxide nanoparticle (SPION) covered with dextran	-	Anemia in chronic kidney disease	FDA 2009, EMA 2012
	NanoTherm®	Nanoparticles of superparamagnetic iron oxide coated with amino silane	-	Glioblastoma, prostate, pancreatic cancer	EMA 2009

1.2. pH-Sensitive Smart Nanocarriers

In recent decades, stimulus-sensitive nanocarriers have been developed to trigger drug release in drug delivery systems. These stimuli are external stimuli (such as light, ultrasound, electric field, magnetic field, and heating) or internal stimuli (such as change in pH or concentrations of ions, small molecules, and enzymes) (Pan et al. 2019). Among

them, pH-sensitive systems can be suitable candidates for the delivery of therapeutic agents to cancerous cells and inflammatory tissues. The reason for selecting a pH-sensitive nanocarrier is that the pH value in extracellular areas where cancerous tissues, as well as inflamed or wound tissue are found is in the range of 6.4 – 6.8 and shows acidic properties compared to blood and normal tissues. The pH values of intracellular endosomes (6.5) and lysosomes (<5.0) exhibit even lower pH values than the blood circulation pH values (7.4) (Yoshida et al. 2013). The reason for this is the large amounts of lactic acid released by cancerous cells after their glucose metabolism to the extracellular matrix. Nanocarriers can be obtained from pH-sensitive polymers to improve great release of drugs in cancerous tissues (acidic environments).

In the literature, three different mechanisms have been used in drug delivery studies using pH-sensitive polymers:

1) Changes in the charge and/or hydrophilic properties of the polymer (Liang and Kitts 2014) (J. O. Kim, Kabanov, and Bronich 2009),

2) Breaking of chemical bonds between drug and nanocarrier (Binauld and Stenzel 2013) (Pang et al. 2016),

3) Breaking the cross-links within the nanocarrier (Liu et al. 2015) (Yang et al. 2020).

Liu et al. obtained a stable nanocapsule from polymers obtained from N-(3-aminopropyl) methacrylamide (APM) and acrylamide (AAM) using ethylene glycol dimethacrylide (EGDMA) crosslinker (Liu et al. 2015) . The obtained nanocapsule was used to transport the miR-21 oligonucleotide. The pH sensitive ester bonds made by the EDGMA crosslinker can be broken by hydrolysis. Due to the degradation of the nanocapsule at pH 5.4, the drug was released more than in pH 7.4. Another example is pH sensitive BSA nanoparticles derived from bovine serum albumin (BSA) protein (Yang et al. 2020). The aldehyde groups of glutaraldehydes used as crosslinkers combine with the amine groups in BSA to form Schiff base bonds. Since Schiff base bonds are sensitive to pH, they break at acidic pH and open the nanoparticle. In this way, the drug loaded on the BSA nanoparticle was released in 60% at pH 6.5 within 24 hours, but only 30% at pH 7.4.

1.3. Protein Based Carrier Systems

Nanoparticles can provide therapeutic efficacy and functionality in promising treatments. They can be designed to have optimal size and surface properties to progress their biodistribution and increase the time in the bloodstream without harming other cells. Therefore, nanoparticles especially protein-based nanoparticles are of great interest in experimental studies.

Biomacromolecules have been used as carriers including proteins (albumin, lipoproteins, silk fibroin, collagen, keratin) and polysaccharides (chitosan, cyclodextrin, hyaluronic acid, heparin, pectin, etc.) and they are suitable for forming fibers, nanoparticles, hydrogels, drug conjugates, or different nanocarriers (Y. Zhang, Sun, and Jiang 2018).

With increasing interest in recent years, compared to potential colloidal drug delivery systems using synthetic polymers, protein-based nanocarriers are of particular interest because of their safety. Natural biomacromolecules, proteins have certain unique functionalities and potential applications such as drug transport in both biomedical and materials science. The use of nanoparticles for both pharmaceutical and nutraceutical delivery is common, and these nanoparticles can be prepared from a variety of materials such as protein, polysaccharides, and synthetic polymers. It is known that protein nanocarriers are advantageous compared to conventional treatment methods by changing the surface using various ligands and providing site-specific drug conjugation and active targeting. The most prominent features of protein-based nanocarriers are as follows;

- renewability,
- non-toxicity,
- less immunogenicity,
- biocompatibility,
- biodegradability,
- stability,
- high nutritional value,
- high drug loading content (if essential)
- long blood circulation time,
- natural biochemical and biophysical properties,
- surface modification

The protein-based nanocarriers are generally in the form of nanoparticles and a variety of conceivable drug loading mechanisms can be applied, including electrostatic attraction, hydrophilic, hydrophobic interactions, and covalent bonding (Jahanshahi and Babaei 2008). Furthermore, these nanoparticles can also be effectively conformable for surface modification and covalent binding of drugs and targeting ligands so that these properties making them of great significance in biomedical fields.

1.4. Serum Albumin Proteins

Serum albumin (SA) protein is a small globular protein of about 5 nm with a molecular weight of 66.5 kDa. It is found in blood plasma with a concentration of 35-50 g/L in human serum., SA has a negative charge at neutral pH and a positive charge at acidic pH, with an isoelectric point (pI) of 4.7 in water at 25 ^{0}C (Von Storp et al. 2012). SA has the ability to bind both positively and negatively charged molecules. At normal pH = 7.4, albumin has a negative charge of around -20.3 mV on its surface (Jachimska and Pajor 2012). Besides this, albumin is significant for numerous physiological processes like maintenance of colloidal osmotic pressure, immune modulating, endothelial stabilization, regulating the blood pH, binding, and transporting nutrients to cells (Zhang et al. 2017). SA proteins are soluble in water, moderately soluble in concentrated salt solutions and stable at different pHs in the range of 4–9 (Hassanin and Elzoghby 2020).

SA has different binding sites so that it can act as a potent drug carrier and also the other substances that can be transported by albumin includes bilirubin, biliary acids, hormones, ligands, metals, anions, long-chain fatty acids, nitric oxide, and endotoxins (Rahimizadeh, Yang, and Lim 2020)(Akdogan, Junk, and Hinderberger 2011)(Y. Akdogan et al. 2016)(Tatlidil, Ucuncu, and Akdogan 2015).

Human Serum Albumin (HSA) and Bovine Serum Albumin (BSA) are among the most important candidates for manufacturing albumin-based nanoparticles as drug delivery nanocarrier systems. BSA's chemical composition, spatial structure, and biological properties are quite similar to those of HSA (Akdogan, Reichenwallner, and Hinderberger 2012). BSA and HSA are very similar in their amino acid composition (76%) as indicated in Figure 1.2. BSA is greatly preferred globular protein instead of HSA because of its abundance, low cost, ease of purification, and wide acceptance in pharmaceutical fields (Li et al. 2016).

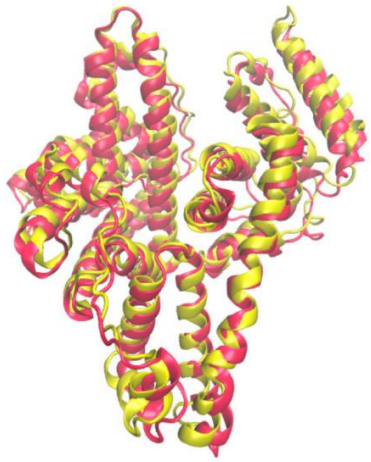

Figure 1.2. The spatial structures of bovine (pink) and human (yellow) serum albumin proteins (Khaibrakhmanova, Nikiforova, and Sedov 2020).

Furthermore, there are different ways for loading drugs to albumin (for instance, covalent bonds, surface coating, and electrostatic absorption). These approaches can be used due to the presence of various functional groups (-SH, -NH_2, -COOH) in the basic structure of albumin on their surfaces which causes modifiable surface properties via conjugations with various drugs and ligands as shown in Figure 1.3.

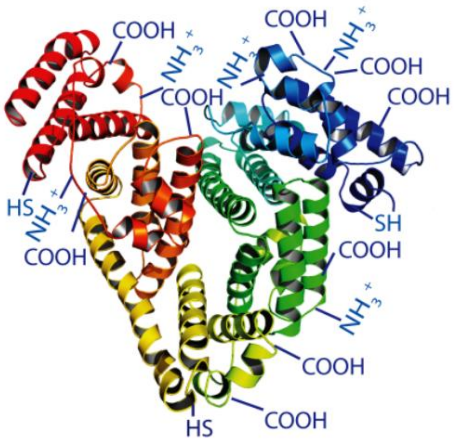

Figure 1.3. The structure of bovine serum albumin (BSA) protein and its active surface groups (Saha et al. 2019).

1.5. Albumin Nanoparticles Preparation Methods

The rich drug transportation ability of the serum albumin protein has inspired scientists to synthesize drug nanocarriers from serum albumin proteins. In the literature, various albumin nanoparticle synthesis methods exist e.g. emulsification, complex coacervation (the chemical methods), nano-spray drying, electrospray (the physical methods), self-assembly, and desolvation methods (the self-assembly method) (Sozer et al. 2020)(Demirkurt and Akdogan 2018)(Demirkurt, Cakan-Akdogan, and Akdogan 2019)(Hassanin and Elzoghby 2020)(Tarhini et al. 2018)(Hong et al. 2020). The commonly used one is the desolvation method.

Desolvation is a self-assembly method (Figure 1.4.) used for the preparation of albumin nanoparticles that takes place with a continuous mixing at a certain speed by adding a desolvating agent (ethanol, methanol, acetone, etc.) to a well-dissolved albumin solution in water (Kianfar 2021).

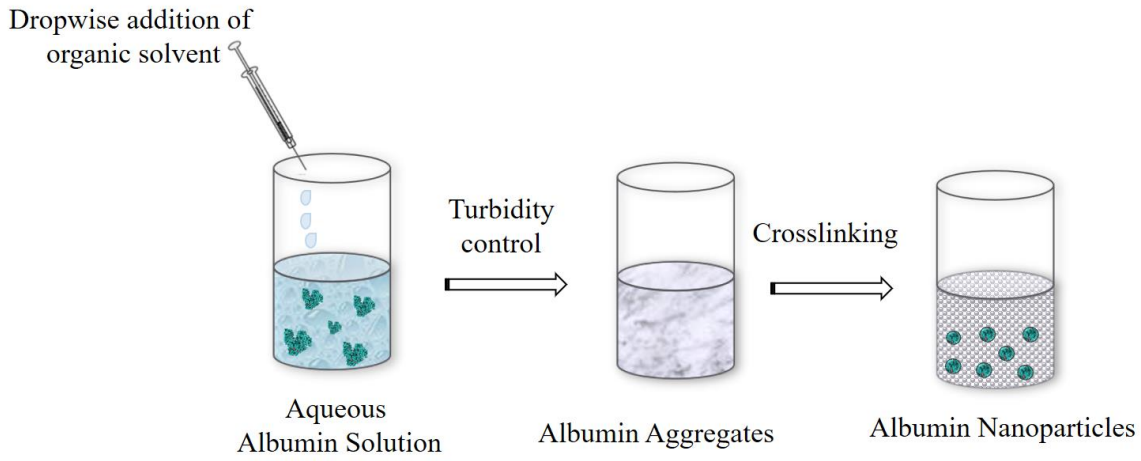

Figure 1.4. Schematic representation of the desolvation method for preparation of albumin nanoparticles.

During the desolvating agent addition, protein begins changing its structure gradually and the water solubility of albumin decreases. Finally, the addition of glutaraldehyde causes particle crosslinking (Figure 1.5) (Akdoğan et al. 2016). There are several factors that affect the particle size and size distribution of albumin NPs. These parameters include pH, temperature, amount of glutaraldehyde, stirring rate (rpm), drop rate of desolvating agent, buffer type, desolvating agent type, solvent:water ratio, and protein concentration(Langer et al. 2003)(Von Storp et al. 2012).

The obtained native albumin nanoparticles have a negative surface charge due to the anionic feature of albumin at pH 7.4. On the other hand, in the literature, different methods have been developed to prepare also cationic albumin NPs. Cationic albumin nanocarriers have better binding ability to anionic drugs and they can penetrate membrane of cells more compared to the anionic albumin nanocarriers (Lu et al. 2005). These cationic nanocarriers are used for drug delivery to treatment of brain tumors (Byeon et al. 2016), lung cancers (Han et al. 2014), and DNA/siRNA delivery for gene therapy (Eisele et al. 2010).

Kesharwani et al. prepared cationic bovine serum albumin (cBSA) conjugated biocompatible polyester polymers such as poly (D,L-lactide-co-glycolide) (PLGA) nanoparticles for targeted drug delivery to treat brain tumors (Kesharwani et al. 2016).

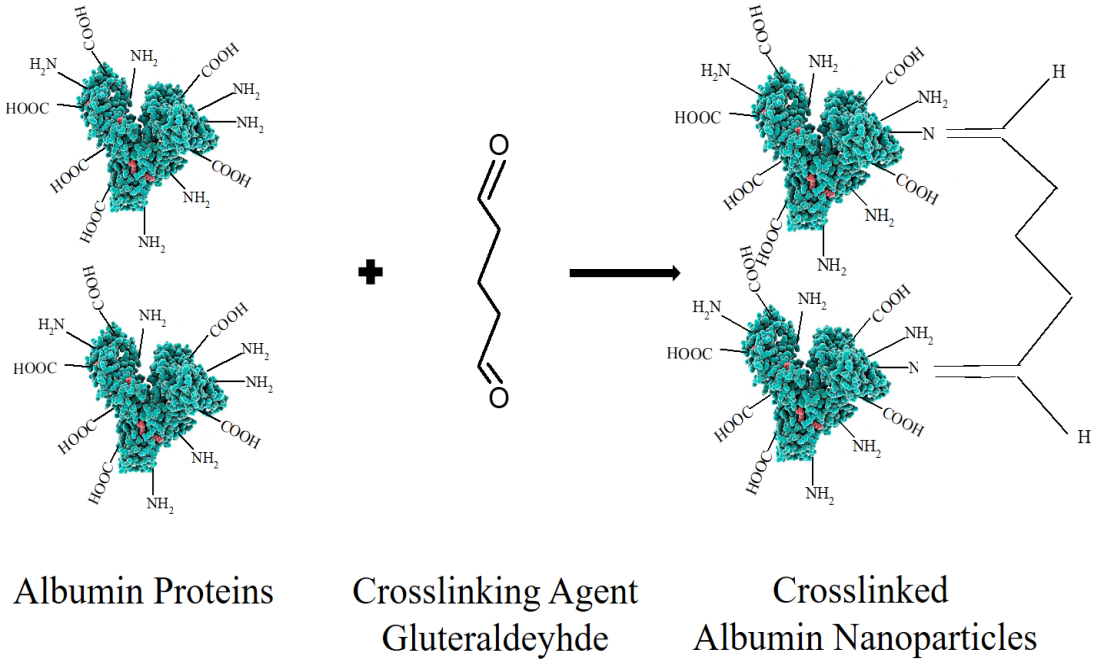

Figure 1.5. Albumin nanoparticles crosslinking mechanism.

Thöle et al. prepared cBSA and liposomes coated with polyethylene glycol (PEG-liposomes), separately and then cBSA can be covalently linked to liposomes in order to achieve brain capillary endothelial cell-directed drug delivery (Thöle et al. 2002). In another study, Saha groups reported fatty-amine-conjugated cBSA nanoparticles by conjugating laurylamines to the BSA protein for hydrophobic drug delivery (Saha et al. 2019). Abbasi et al. prepared cHSA NPs by coating anionic HSA NPs with positively charged polyethylenimine (PEI) through the electrostatic binding (Abbasi et al. 2012). Han et al. prepared cBSA proteins via ethylenediamine conjugation, and prepared self-assembled cBSA NPs by mixing cBSA with siRNA which interact with each other electrostatically (Han et al. 2014). Byeon et al. prepared cHSA NPs with an emulsification method using a high-pressure homogenizer after surface modification of HSA with ethylenediamine (Byeon et al. 2016).

1.6. Dopamine Conjugated BSA Nanocarrier

Dopamine molecule offers a combination of biological and chemical properties that could be useful in biochemical materials. It is used in surface attachment, self-polymerization, crosslinking, self-healing and underwater reactivity (Kaushik et al. 2015). As far as we know, just a few studies on dopamine-based drug delivery hydrogels have been published but there are no studies about pH-sensitive dopamine-based albumin nanoparticles synthesis via desolvation method (Ren et al. 2017)(Gao et al. 2019)(Yan et al. 2019).

Dopamine (3,4-dihydroxyphenylethylamine (DA)) and related catecholic molecules (levodopa, adrenaline, noradrenaline, etc.) have been considered as anchoring molecules and highly reactive molecules. Dopamine molecule is separated into two forms, non-oxidized and oxidized as represented systematically in Figure 1.6. Catechol oxidation occurs easily in alkaline conditions or in the presence of oxidizing agents thus reactive o-quinones are formed (Göksel and Akdogan 2019). Catechols, which is non-oxidized form, are responsible for non-covalent and adhesive bonds with inorganic materials (metal ions or metal oxide surfaces), hydrogen bonding between catechols, and reversible complexation with metal ions (Yildiz et al. 2020)(Kırpat et al. 2017)(Wang et al. 2013).

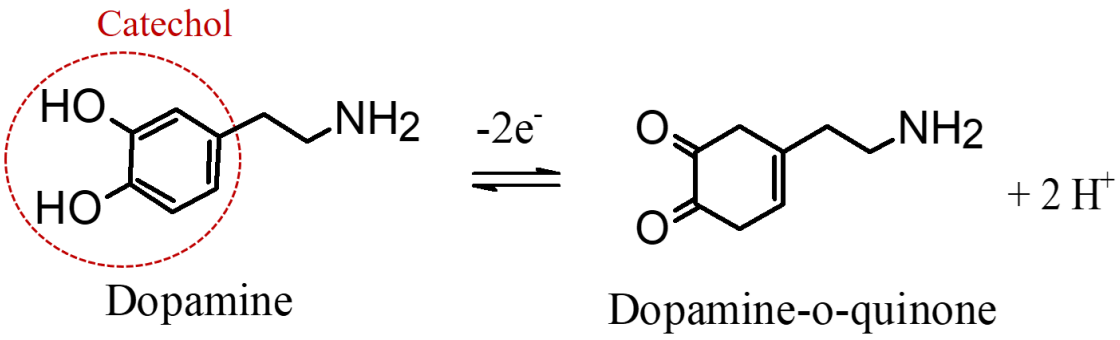

Figure 1.6. Non-oxidized dopamine molecule and its' oxidized form of quinone molecule.

Differently, catechols play role in the cohesive interactions. In the presence of Fe(III) ions, catechol groups are interconnected and form a strong network (Saiz-Poseu et al. 2019). This coordination is pH sensitive. Above pH 9.0 tris coordination is observed but at lower pH values bis and mono coordinations are obtained (Figure 1.7.).

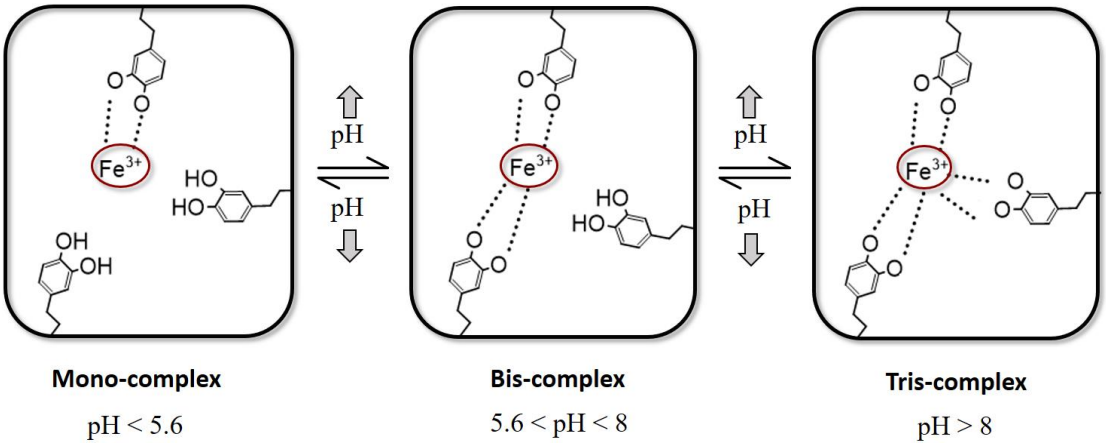

Figure 1.7. The pH-dependent of coordination complexes of Fe(III)–catechol group of dopamine as a form of mono-bis-tris.

In literature, Kim et al., designed novel drug-loaded NPs for pH-responsive drug delivery through the co-electro-spraying method, which was achieved via metal catechol coordination complexes between Fe(III) and 3,4-dihydroxyphenylalanine (DOPA) (B. J. Kim et al. 2015). DOPA is found in recombinant mussel foot protein type-1 (Mfp1) proteins, which are inspired by the Mfp1 proteins found in the filaments of mussels. Doxorubicin drug-loaded nanoparticles have been synthesized with the Fe(III)–DOPA complexes. The release rate and loading amount of doxorubicin are different from conventional doxorubicin parameters due to the pH-sensitive Fe(III)-DOPA NPs. In the first 3 hours, the amount of drug released in pH 6.0 buffer medium was determined as 4 times the amount of drug released in pH 7.4 buffer medium (B. J. Kim et al. 2015).

The other example is that Krogsgaard et al. designed and fabricated self-healing hydrogels with catechol-functionalized cationic polymers consisting of DOPA-polyallylamine (DOPA–PAA) and DOPA-chitosan hydrogels (Krogsgaard, Hansen, and Birkedal 2014). DOPA–PAA make the coordination complex with aluminium (Al(III)), gallium (Ga(III)), indium (In(III))) and DOPA–chitosan hydrogels were formed a complex with iron (Fe(III)). Among others, Fe(III):DOPA–chitosan hydrogels are the strongest one around pH 8, its storage moduli of up to ~30 kPa. This value shows that the mechanical properties can be adjusted by selecting the polymer. They also observed self-healing ability of hydrogels using different metal ions. For self-recovery investigations, the tris-catecholato–Al(III) and tris-catecholato–Fe(III) hydrogels at pH 8 fully recovered

their original moduli due to the presence of tris-crosslinks (Krogsgaard, Hansen, and Birkedal 2014).

The final study is about Fe(III):Dopamine-BSA hydrogel. Zhu et al. have produced adhesive biomaterials for use in medical applications by utilizing Fe(III)–dopamine coordination. Hydrogels designed for use in tissue adhesion were obtained from dopamine conjugated bovine serum albumin (BSA) protein. The BSA-dopamine complex becomes a good adhesive gel form with the addition of Fe(III) in basic media and uses to help close up the surgical incisions, but also can help with wound healing after surgery (Zhu et al. 2017).

1.7. Motivation of the Thesis

Albumin nanoparticles are principally used in biological, medical, and bionanotechnologic applications like smart drug delivery systems due to their various advantages. For albumin-based nanoparticles, surface charge and modifications are important factors for their drug loading capacity, transportation to the target cell/tissue and drug release mechanisms.

Here, we synthesized cationic BSA nanoparticles from cationic BSA proteins via a simple desolvation method. Also, an alternative to the glutaraldehyde crosslinking mechanism we studied a pH sensitive albumin nanoparticle synthesis mechanism using dopamine modified BSA and Fe(III) ions. These two modified albumin drug nanocarriers can serve the purpose of overcoming drug delivery challenges from various perspectives.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Bovine serum albumin (BSA, Mw: 66.5 kDa lyophilized powder, >96%), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimidehydrochloride (EDC), *N*-Hydroxysuccinimide (NHS), ethylenediamine (EDA), dopamine hydrochloride (DAH), glutaraldehyde solution (8% (v/v) in H2O), iron (III) chloride hexahydrate, hydrochloric acid (HCl), phosphate-buffered saline (PBS), methanol, ethanol, propanol, isopropanol, acetone, were purchased from Sigma – Aldrich. Acetonitrile was purchased from Merck. All chemicals and solvents were analytical grade and utilized without any purification procedures. The pH of the ethylenediamine and dopamine hydrochloride solutions were adjusted with 6 M and 1 M HCl solutions using an OHAUS STARTER 3100 pH meter, respectively.

2.2. Preparation of Modified BSA Proteins

In this section, two different preparation methods of modified bovine serum albumin (cBSA and D-BSA) will be explained.

2.2.1. Cationic Bovine Serum Albumin Protein (cBSA)

cBSA was prepared as previously described in our publication and elsewhere (Akdogan et al. 2012). 50 mg BSA was dissolved in 500 µL of deionized water. 650 µL ethylenediamine was mixed with 500 µL deionized water, and then the solution pH was adjusted to 4.75 by addition of 9 mL HCl (6 M). The BSA solution was added to the ethylenediamine solution as illustrated in Figure 2.1. 14.5 mg EDC was dissolved in 15 µL deionized water, then it was slowly added to the BSA-ethylenediamine solution. The reaction was stirred at 750 rpm for 2 h. The reaction was stopped by the addition of 4 M

acetate buffer (pH 4.75). cBSA was purified by 0.1 M acetate buffer and deionized water for the elimination of excess amount of EDC and ethylenediamine by 3 cycles of centrifugation using centrifugal filter (molecular cut off: 50 kDa) (13,000 rpm, 3 min). The final cBSA was collected from centrifugal filter, and then it was lyophilized and stored at 4 °C. The cBSA yield was 70%.

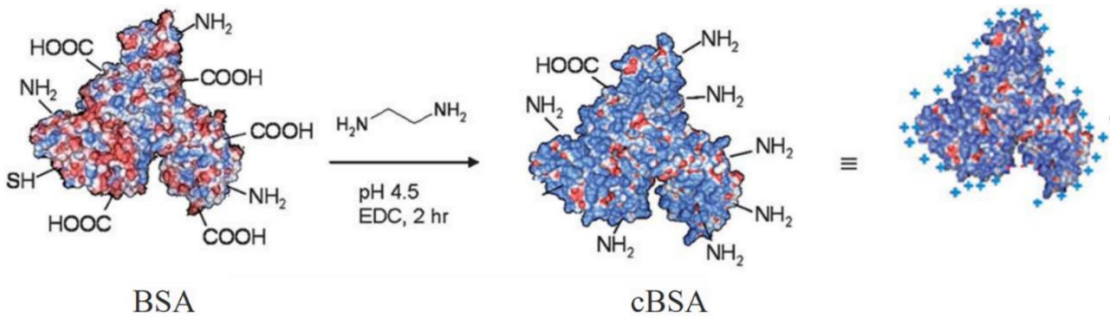

Figure 2.1. Modification of BSA protein with ethylenediamine (Akdogan et al. 2012).

2.2.2. Dopamine Conjugated Bovine Serum Albumin (D-BSA)

50 mg BSA and 24.6 mg dopamine hydrochloride (DAH) were dissolved in 5 mL of PBS (0.01 M, pH: 7.4), separately. Then the BSA solution was added to the dopamine hydrochloride solution (

Figure *2.2*.) and pH was adjusted to 6 by addition of 500 µL HCl (1 M). 7.2 mg EDC and NHS were dissolved in 200 µL deionized water, then it was slowly added to the BSA-DAH solution. The reaction was stirred at 700 rpm, 37 °C, under argon gas and dark conditions for 2 h. D-BSA was purified by deionized water for the elimination of excess amount of EDC/NHS and dopamine hydrochloride by 3 cycles of centrifugation using centrifugal filter (molecular cut off: 50 kDa) (12,000 rpm, 1 min). The final D-BSA was collected from centrifugal filter, and then it was lyophilized and stored at 4 °C. The D-BSA yield was 68.6%.

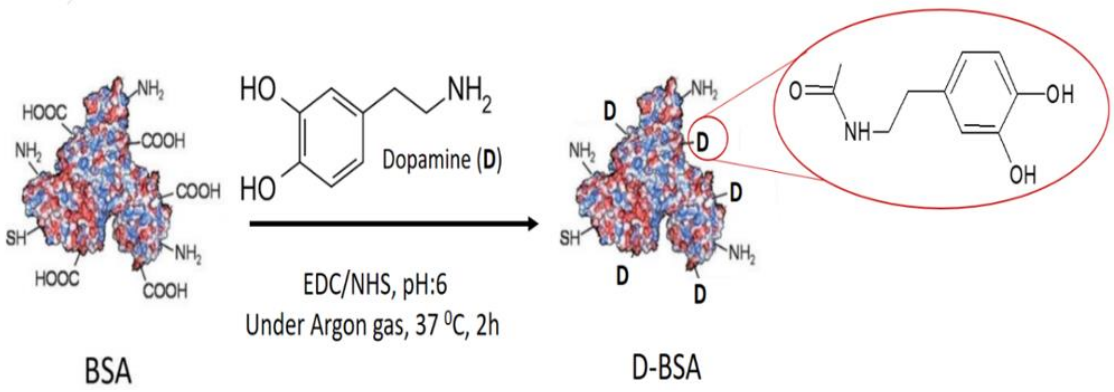

Figure 2.2. Modification of BSA protein with dopamine molecule.

2.3. Preparation of BSA and Modified BSA Nanoparticles

All albumin nanoparticles were obtained by the desolvation method. The size and charge of the synthesized albumin nanoparticles vary according to the type and quantity of the solvent used. Therefore, nanoparticles were synthesized by different parameters such as albumin concentration, water/organic solvent ratio, pH, stirring rate, types of desolvating agents, cross-linker amounts, etc.

2.3.1. cBSA NPs and BSA NPs

cBSA NPs and BSA NPs were synthesized by the desolvation method using different desolvating agents, and cross-linked by glutaraldehyde. Methanol, ethanol, propanol, isopropanol, acetone and acetonitrile were tested as desolvation agents. Briefly, 62.5 mg cBSA (or BSA) was dissolved in 1 mL deionized water for 20 min. The desolvating agent was added dropwise to cBSA (or BSA) solution using a syringe pump with a flow rate of 1.0 mL/min. The cBSA aqueous solution did not turn turbid upon addition of 1:6 (v/v) of methanol or 1:6 (v/v) of ethanol; turned slightly turbid upon addition of 1:5 (v/v) of propanol, 1:5 (v/v) of isopropanol or 1:4 (v/v) of acetone. The most turbid cBSA solution was obtained by adding 1:4 (v/v) of acetonitrile. Then, 18 μL of 8% (v/v) cross-linking agent glutaraldehyde was added to each reaction.

On the other hand, 62.5 mg BSA (1 mL deionized water) aqueous solutions turned turbid quicker by adding the desolvating agents. Yet, 1:6, 1:4, 1:3, 1:3, 1:4 and 1:4 (v/v)

of methanol, ethanol, propanol, isopropanol, acetone and acetonitrile were added to the BSA solution to have similar conditions with cBSA experiments.

The all NPs were stirred overnight at 750 rpm to increase the stability of the NPs by crosslinking entirely. Aliquots NP solutions were precipitated by centrifugation at 14,000 rpm for 45 min. Pellets were washed with distilled water to eliminate desolvating agents, unreacted cBSA, BSA and glutaraldehyde.

2.3.2. D-BSA NPs

52 mg D-BSA was dissolved in 1 mL deionized water for 20 min. The desolvating agent was added dropwise to D-BSA solution using a syringe pump with a flow rate of 1.0 mL/min. The D-BSA aqueous solution did not turn turbid upon addition of 1:4, 1:4 and 1:3 (v/v) of isopropanol, propanol and acetonitrile; turned slightly turbid upon addition of 1:4 (v/v) of ethanol. The most turbid D-BSA solution was obtained by adding 1:5 (v/v) of water/acetone mixture. Then, 31.2 μL of 81 mM $FeCl_3.6H_2O$ cross-linking agent was added to each reaction. In the end, 62.5 μL 0.01 M NaOH was added to make the final solution medium basic. The NPs were stirred overnight at 700 rpm to enhance the stability of NPs with the help of crosslinking (Figure 2.3.). At the purification step; aliquots NP solutions were precipitated by centrifugation at 14,000 rpm for 15 min. Pellets were washed with distilled basic water to eliminate desolvating agents, unreacted D-BSA and $FeCl_3.6H_2O$.

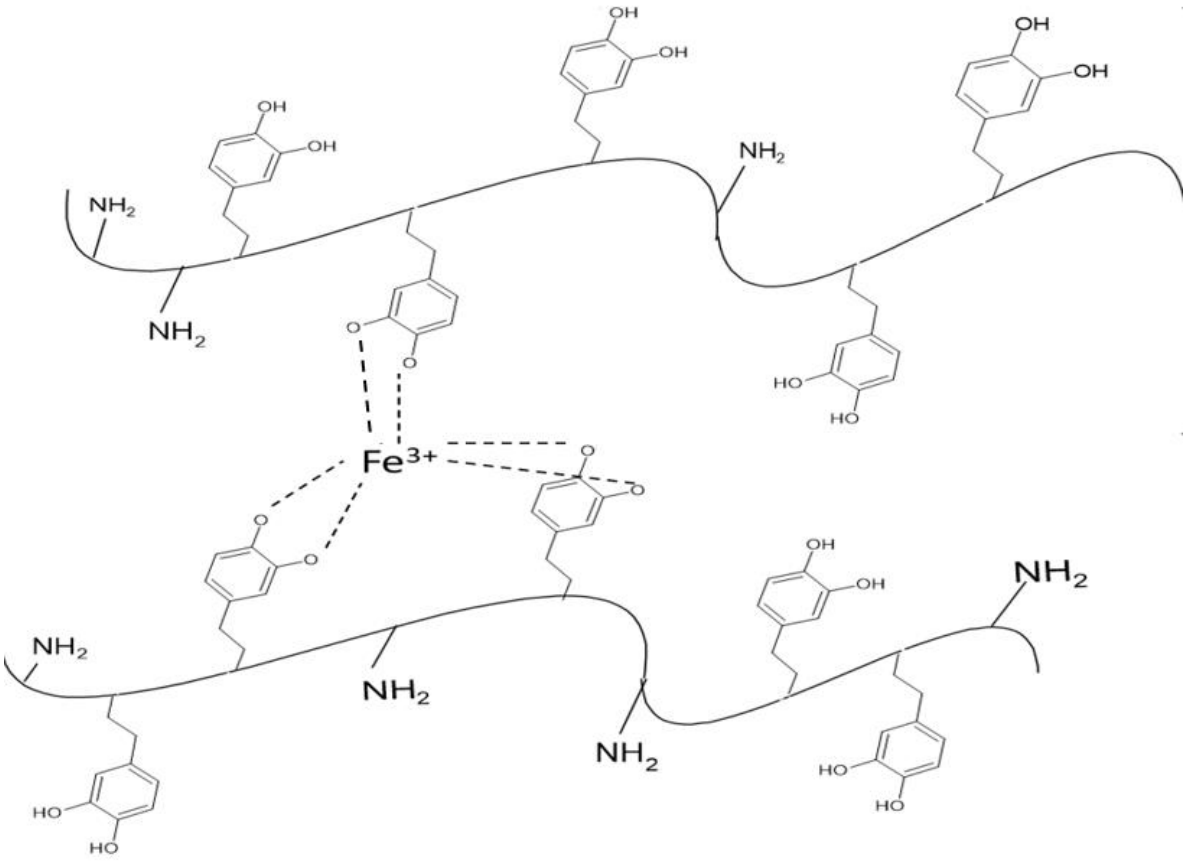

Figure 2.3. Schematic illustration of the formation of dopamine conjugate BSA (D-BSA) NPs by desolvation method in the presence of Fe(III) crosslinker agent.

2.4. Characterization of cBSA, D-BSA, BSA proteins and their NPs

The zeta potential and molecular weight of cBSA, D-BSA, and BSA were characterized in distilled water by a Malvern dynamic light scattering (DLS) Nano-ZS instrument (Worcestershire, UK) and a mass spectrometer using Bruker Autoflex-III (smartbeam) MALDI TOF/TOF system, respectively. Their aqueous solutions were measured by attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectrometer (Thermo Scientific Nicolet iS50).

After the purification of NPs, pellets were lyophilized (FreeZone Freze Dry Systems in 0.04 mbar vacuum and below -51 °C) for the yield calculations. Lyophilized NPs were dissolved in distilled water for the size and zeta potential measurements. The size and shape of NPs were analysed by the scanning electron microscope (SEM, Carl Zeiss 300 VP and FEI QUANTA 250 FEG). Dissolved NPs were diluted 50 times with

distilled water from their dissolved NP solutions. 5 µL solutions were dropped on an aluminum foil and samples were left for the drying for one day. Then, dried samples were coated with 8 nm thick gold for 1 min 20 kV using QUORUM Q150 RES and EMITECH K550X in a vacuum for SEM imagining. The accelerating voltages were 5–7 kV. Also, size measurements of NPs were analysed by using a Malvern dynamic light scattering (DLS) at a wavelength of 632 nm. The scattering angle was set at 173°. The zeta potentials of nanoparticles were determined by a Malvern dynamic light scattering (DLS) Nano-ZS instrument (Worcestershire, UK).

CHAPTER 3

RESULTS AND DISCUSSION

Conjugation of ethylenediamine and dopamine hydrochloride to the BSA protein yielded cBSA and D-BSA proteins, respectively. cBSA and D-BSA nanoparticles were obtained by desolvation methods followed by the addition of different crosslinking agents, glutaraldehyde and Fe(III), respectively.

3.1. Synthesis and Characterization of cBSA and D-BSA Proteins

Cationized BSA (cBSA) was obtained by amination of the surface with ethylenediamine through an amide linkage. Addition of ethylenediamine to the carboxylic acid groups of amino acids including aspartic acids (Asp) and glutamic acids (Glu) were detected by the MALDI TOF mass spectrum (Figure 3.1. (A)). An increase in the average molecular weight of BSA from 66490 g/mol to 68545 g/mol indicated that on average 49 ethylenediamine molecules reacted with the available carboxylic acid side chains of 99 amino acids (Asp and Glu) (Sozer et al. 2020).

Similarly, modification of the BSA protein with dopamine was carried out by amide linkage. Dopamine has one amine group and binds free carboxylic acid groups of amino acids using EDC/NHS. MALDI TOF mass spectrum shows that a few dopamine molecules were successfully linked to the albumin carboxylic acid groups (Figure 3.2 (A)). Average molecular weight of BSA was increased from 66650 g/mol to 66900 g/mol. These differences pointed out that about 2 dopamine molecules could bind to BSA. These limited binding results were significant to avoid the gelling property of dopamine molecules, and to not disrupt the structure of albumin protein since dopamine molecules have catechol groups and bigger than ethylenediamine molecules.

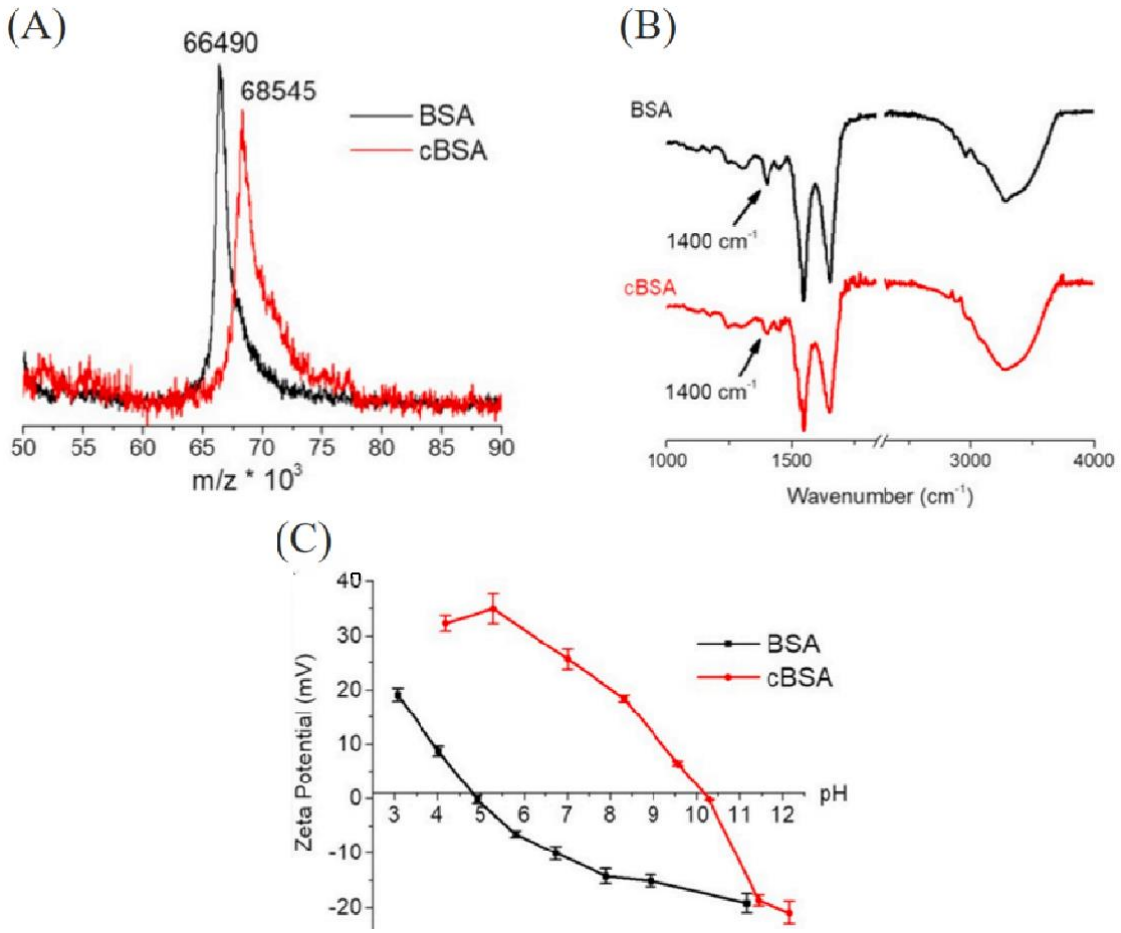

Figure 3.1. (A) MALDI-TOF mass spectra of native BSA (black) and cationic BSA (cBSA) (red) proteins. (B) ATR-FTIR spectra of native BSA (black) and cBSA aqueous solutions (red). (C) The zeta potentials of native BSA (black) and cationic BSA (cBSA) (red) proteins at different pH values from 3.0 to 12.0 (Sozer et al. 2020).

ATR-FTIR spectroscopy also showed the cationization of BSA with ethylenediamine, and dopamine conjugation (Figure 3.1 (B) and Figure 3.2 (B)). The band at 1400 cm^{-1} was attributed to the carbonyl (C=O) bond in the side chain carboxylic groups of Asp and Glu in BSA. Conjugation of ethylenediamine and dopamine with Asp and Glu decreased the signal intensity at this wavenumber, indicating the decrease of carboxyl group in cBSA (Cheraghipour and Javadpour 2013). But a limited amount of dopamine conjugation was not detected by FTIR spectrum.

Ethylenediamine conjugation converted the negative net charge on the BSA (− 11 ± 1) into a positive net charge (+25 ± 2) at pH 7.0 (Figure 3.1. (C)). Also, the zeta

potentials of BSA and cBSA at different pH values (3.0–12.0) were measured (Figure 3.1. (C)). For the native BSA, the positive zeta potential decreased from +20 to 0 when pH was raised from 3.0 to 5.0. Above the isoelectric point (pI = 5.0), the zeta potential of BSA decreased with increasing the pH. On the other hand, the pI value of the cBSA was determined as about 10.1. Thus, a net positive charge on cBSA is maintained over a broad pH range.

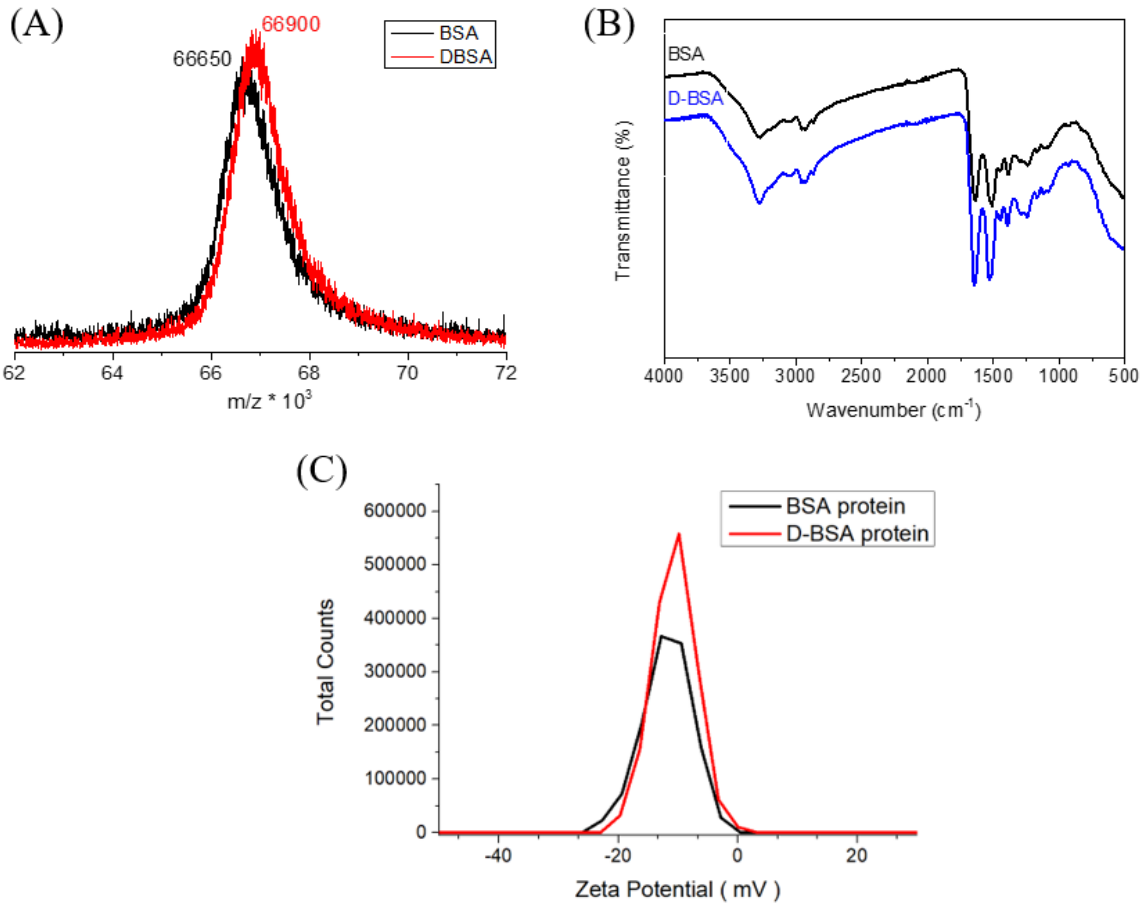

Figure 3.2. (A) MALDI-TOF mass spectra of native BSA (black) and dopamine conjugated BSA (D-BSA) (blue) proteins. (B) ATR-FTIR spectra of native BSA (black) and D-BSA (blue) powders (C) Zeta potentials spectra of BSA (black) and D-BSA (red).

Figure 3.2 (C) shows that the zeta potentials of BSA and D-BSA proteins were approximately -11 mV and -10 mV, respectively. It shows that the surface charge of D-

BSA protein is changed slightly because only 2 dopamine molecules bind to the carboxyl groups of the BSA protein which was confirmed by mass analysis.

3.2. Synthesis and Characterization of cBSA NPs and BSA NPs

Here, cationic BSA nanoparticles (cBSA NPs) were prepared by a simple desolvation process at pH 7.0. Different desolvating agents such as methanol, ethanol, propanol, isopropanol, acetone and acetonitrile were used to produce cBSA NPs. Figure 3.3 (A) shows the yield % of cBSA NPs formation when 62.5 mg of cBSA dissolved in 1 mL of water. Using alcohols e.g. methanol, ethanol, propanol, or isopropanol as desolvating agents produced very low amounts of cBSA NPs with the average yield % of NP formations 3.7 ± 0.1%, 3.2 ± 1.1%, 4.7 ± 1.9%, and 6.3 ± 0.4%, respectively. These results showed that alcohols with short carbon chains have poor desolvation ability for cationic BSA. Increasing the length of alkyl chain of alcohol from one to three slightly improved the yield of cBSA NP formation. Acetone and acetonitrile are polar aprotic solvents which are capable of forming hydrogen bonds with only hydrogen donors. Addition of acetone to cBSA solution produced cBSA NPs with a 12.2 ± 0.6% yield of NP formation which was relatively higher than the results of used alcohols. On the other hand, using acetonitrile as desolvating agent produced a significant amount of cBSA NPs with 54.8 ± 0.2% yield of NP formation. Higher dipole moments of acetonitrile ($\mu = 4.00$) and also acetone ($\mu = 2.90$) could favor the cationic albumin binding compared to the used alcohols with lower dipole moments; methanol ($\mu = 1.70$), ethanol ($\mu = 1.70$), propanol ($\mu = 1.60$), isopropanol ($\mu = 1.63$) (Lee, Park, and Whitesides 2003)(Rizk and Elanwar 1968). Therefore, extensive electrostatic interaction between acetonitrile and cBSA, together with the hydrophobic interactions and hydrogen bonding led to more cBSA NPs formation (Wang et al. 2013).

On the other hand, when non-modified BSA was used to produce NPs, all used desolvation agents yielded a significant amount of NPs, except for methanol with 10.5 ± 0.2% yield of NP formation (Figure 3.3 (B)). Methanol with the lowest carbon chain has a low desolvation ability for the native BSA, too. The addition of ethanol, propanol, isopropanol, acetone, or acetonitrile to an aqueous solution of BSA produced a considerable amount of BSA NPs with 39.1 ± 5.5%, 95 ± 2.4%, 68.6 ± 2.6%, 84.0 ± 3.7%, and 82.0 ± 6.5% yields of NP formations, respectively. Increasing the length of carbon

chain in the used alcohols induced more BSA desolvation due to the larger hydrophobic interactions between BSA and the alcohols. Addition of acetone or acetonitrile enabled the production of significant amounts of BSA NPs, in accord with literature findings (Mohammad-Beigi et al. 2016)(Sadeghi et al. 2014).

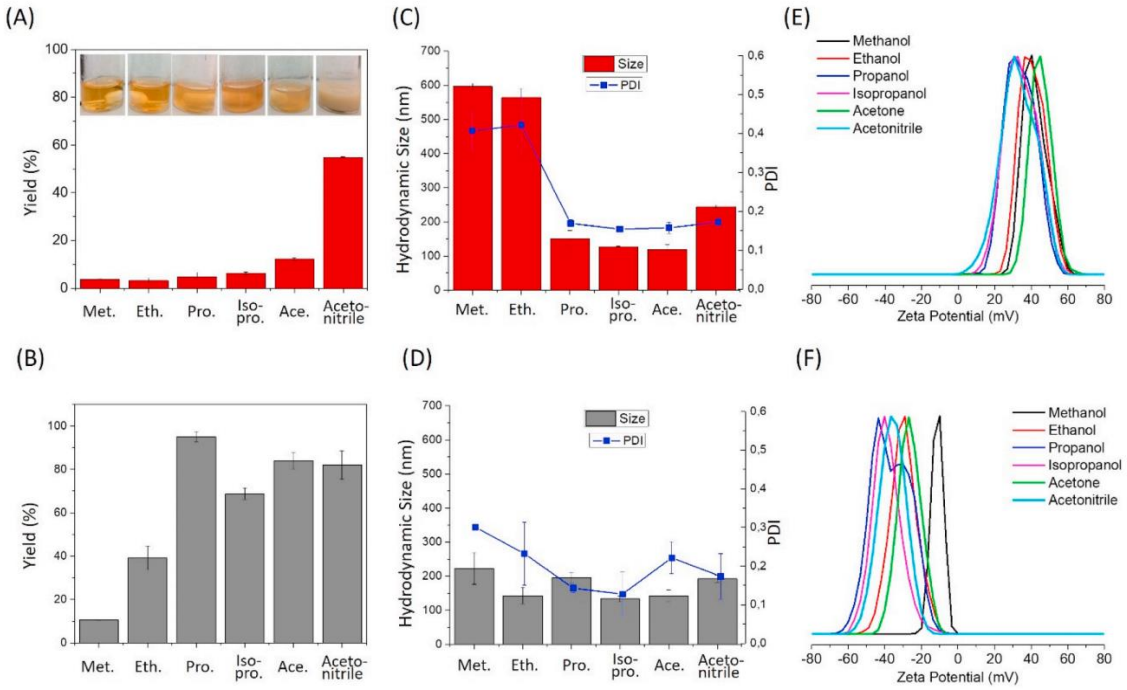

Figure 3.3. The yield percentages of cBSA (A) and BSA (B) NPs formations, the average hydrodynamic sizes and PDI values of cBSA (C) and BSA (D) NPs, and the zeta potentials of (E) cBSA and (F) BSA NPs produced with different desolvating agents including methanol, ethanol, propanol, isopropanol, acetone and acetonitrile (Sozer et al. 2020).

Figure 3.3 (C) and (D) showed the average hydrodynamic sizes of produced cBSA NPs and non-modified BSA NPs using different desolvating agents and the corresponding PDI values, respectively. A limited number of cBSA NPs obtained with methanol and ethanol were analyzed with DLS and SEM techniques (Figure 3.3 (C) and Figure 3.4. (A)). When methanol or ethanol were used, the obtained average hydrodynamic sizes were about 600 nm with the corresponding PDI values of about 0.45. This PDI value showed the presence of cBSA NPs with a wide range of particle sizes.

Actually, their SEM images showed the formation of cBSA NPs aggregates (Figure 3. 4. (A) and (B). On the other hand, using propanol, isopropanol or acetone yielded smaller nanoparticles with average hydrodynamic sizes about 150 ± 24, 127 ± 1 and 119 ± 13 nm, respectively. Their PDI values which were lower than 0.2 showed the presence of cBSA NPs with a relatively uniform size distribution. Addition of acetonitrile to cBSA aqueous solution produced relatively larger cBSA NPs with average hydrodynamic size 244 ± 4 nm compared to the those of cBSA nanoparticles after addition of propanol, isopropanol, or acetone. Also, the corresponding PDI value was about 0.2 indicating a narrow size distribution. Figure 3. 4. (C-E) showed the SEM images of well dispersed spherical cBSA NPs obtained with use of propanol, isopropanol, acetone and acetonitrile. The sizes of cBSA NPs obtained from SEM images smaller than the results of DLS because of dry state.

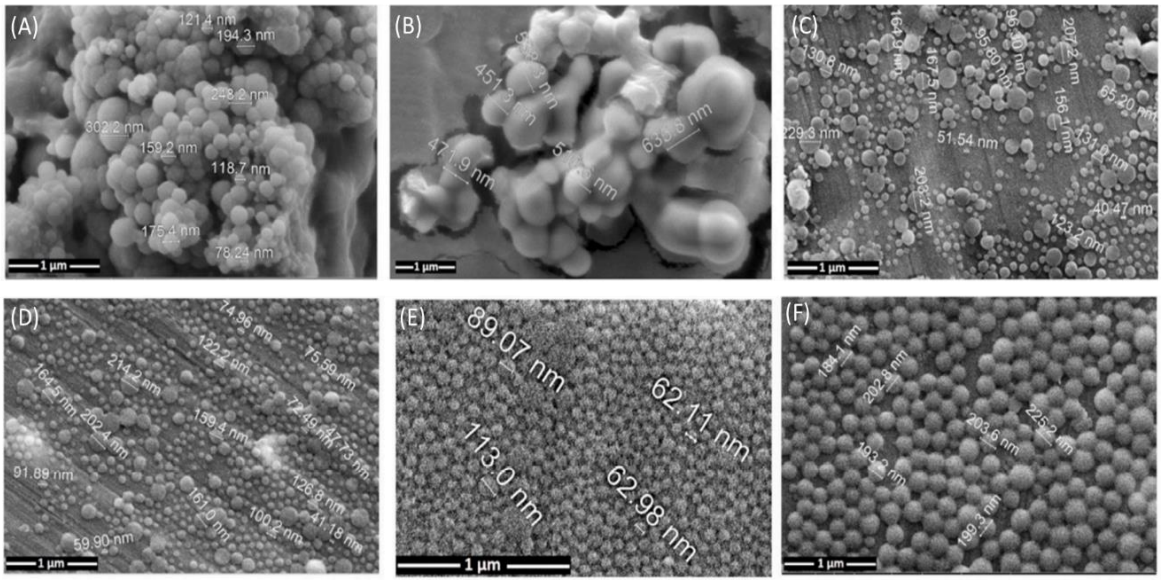

Figure 3.4. SEM images of cBSA NPs produced using different desolvating agents; (A) methanol, (B) ethanol, (C) propanol, (D) isopropanol, (E) acetone, and (F) acetonitrile (Sozer et al. 2020).

On the other hand, the hydrodynamic sizes of non-modified BSA NPs produced using different desolvating agents were very similar to each other (Figure 3.3 (D)). Their average sizes varied between 100 and 200 nm. In addition, the SEM images showed well dispersed spherical BSA NPs with similar average particle sizes below 100 nm (Figure 3.5). Obtaining higher average particle size by DLS due to the formation of hydration shell and swelling of nanoparticles with water.

The synthesized cBSA NPs and BSA NPs had also different zeta potentials. The zeta potentials of the produced cBSA NPs with different desolvation agents were found to be between +38 and + 44 mV (Figure 3.3 (E)). On the other hand, native BSA NPs obtained from non-modified BSA using different desolvation agents had negative potential charges between − 43 and − 27 mV. Only, BSA NPs produced after addition of methanol had a lower net negative charge of − 11 mV (Figure 3.3 (F)).

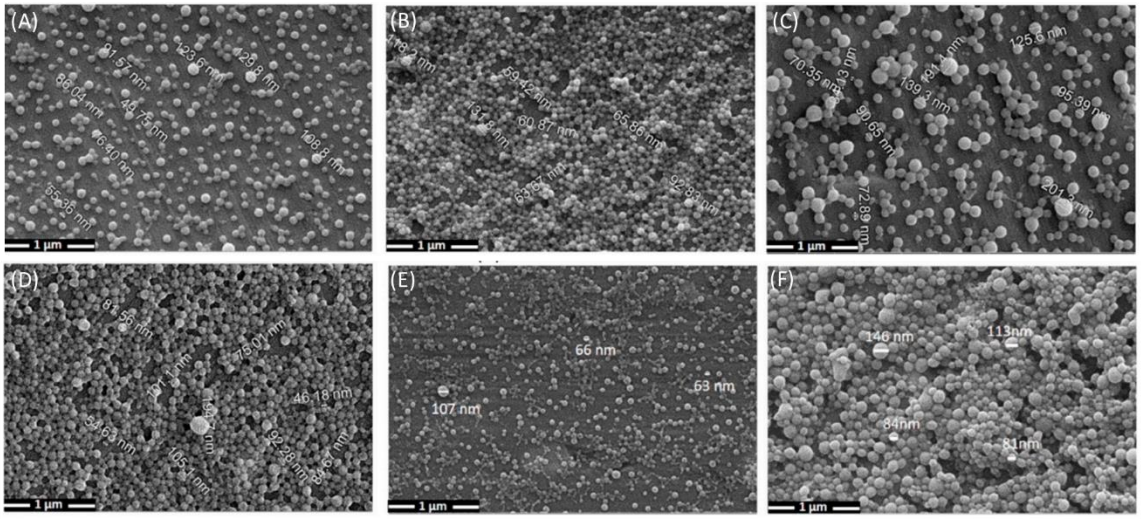

Figure 3.5. SEM images of BSA NPs produced using different desolvating agents; (A) methanol, (B) ethanol, (C) propanol, (D) isopropanol, (E) acetone and (F) acetonitrile (Sozer et al. 2020).

3.3. Synthesis and Characterization of D-BSA NPs

Here, D-BSA NPs were prepared by a simple desolvation process at pH 10. Instead of using glutaraldehyde as a crosslinker, Fe(III) ions were used to stabilize the nanoparticles. Formations of mono, bis, and tris coordinations, which are determinants for the nanoparticle formation, were controlled by the pH value of the mixture. At basic pH, tris coordination between catechol groups of dopamine and Fe(III) can be damaged at lower pH values. Therefore, formation of pH-sensitive nanoparticles could be achieved.

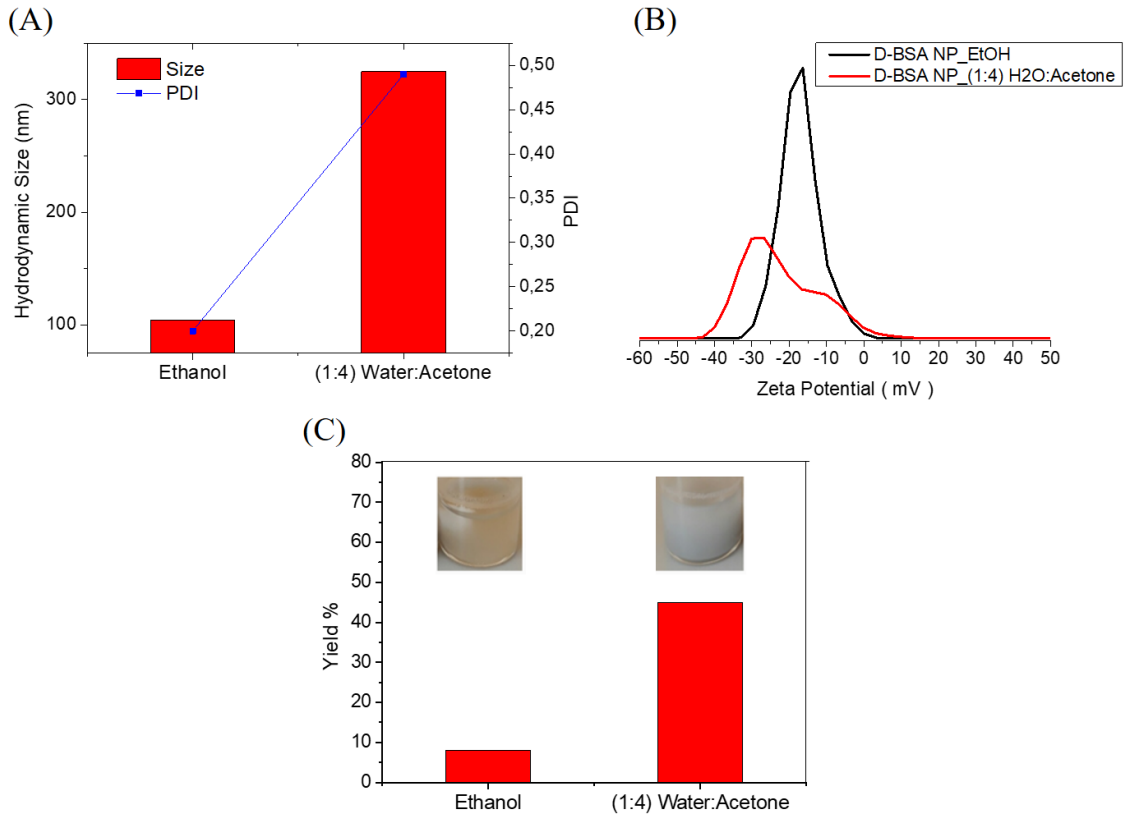

Figure 3.6. The average hydrodynamic sizes and PDI values (A), the zeta potentials (B) and the yield percentages (C) of D-BSA NPs produced with ethanol and (1:4) H_2O:Acetone.

Different desolvating agents (methanol, ethanol, acetone, isopropanol, propanol, 1:5 (v/v) of water/acetone mixture, and acetonitrile) were used to obtain D-BSA nanoparticles. However, only ethanol and acetone yielded an acceptable amount of D-BSA nanoparticles. When ethanol was used as a desolvating agent, a low amount of D-BSA NPs with the average yield of 8% was obtained. On the other hand, when water/acetone mixture was used, a high amount of yield about 45% was obtained. Figure 3.6 (A) indicated the average hydrodynamic sizes and the corresponding PDI values of the produced D-BSA NPs using ethanol and 1:5 (v/v) of water/acetone mixture as desolvating agents. For ethanol, the average hydrodynamic size and PDI values were 104 nm and 0.2, which showed the presence of D-BSA NPs with a relatively uniform size distribution. For 1:5 (v/v) of water/acetone mixture, the obtained average hydrodynamic size was about 325 nm with the corresponding PDI value 0.49. This PDI value showed the presence of D-BSA NPs with a wide range of particle sizes. The average zeta

potentials of D-BSA NPs were -28 mV and -18 mV when water/acetone and ethanol were used as desolvating agents, respectively (Figure 3.6. (B)).

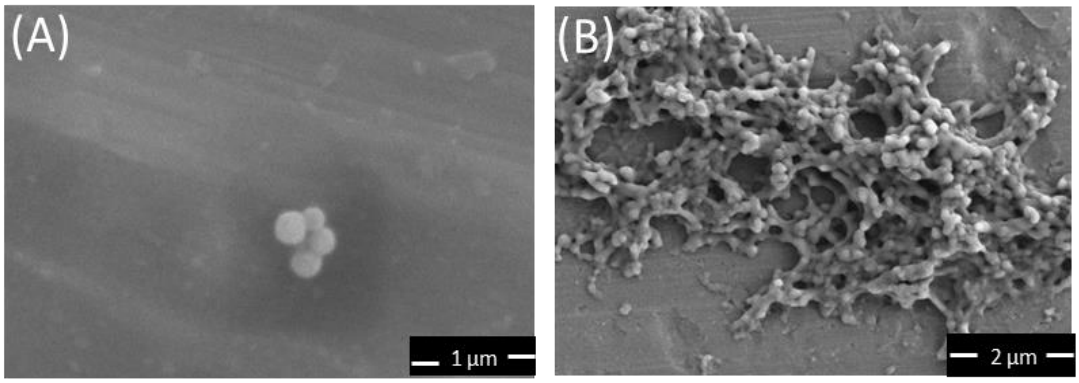

Figure 3.7. SEM images of D-BSA NPs produced using (A) ethanol and (B) 1:5 (v/v) H_2O:Acetone

In addition, the SEM images (Figure 3.7.(A)) of D-BSA NPs obtained when ethanol was used as a desolvating agent indicated spherical D-BSA NPs with average particle sizes of about 100 nm. In Figure 3.7.(B), D-BSA NPs were obtained when 1:5 (v/v) H_2O:Acetone was used as a desolvating mixture, SEM images showed the formation of D-BSA NPs with average particle sizes about 300 nm. It had high yield and also the images looked like more sticky and spherical particles because of the linkage of dopamine molecules and Fe (III) crosslinker.

CHAPTER 4

CONCLUSION

In this work, modified cBSA and D-BSA proteins and their nanoparticles were fabricated using a simple desolvation method, and then compared with anionic native BSA NPs. cBSA and D-BSA were obtained using ethylenediamine and dopamine, respectively in the presence of EDC. They were characterized by MALDI-TOF, ATR-FTIR, and zeta potential measurements.

cBSA NPs were obtained in a high formation yield (54.8%) only through addition of acetonitrile to the cBSA aqueous solution compared to the other desolvating agents such as methanol (3.7 wt%), ethanol (3.2 wt%), propanol (4.7 wt%), isopropanol (6.3 wt%), and acetone (12.2 wt%). Acetonitrile with the highest dipole moment interacts with cBSA proteins much electrostatically which caused more desolvation, in addition to the hydrophobic interaction and hydrogen bonding. The SEM analyses showed that the sizes of cBSA NPs and BSA NPs prepared using acetonitrile were around 200 nm and 100 nm, respectively.

D-BSA NPs were obtained in high formation yield (45%) by addition of 1:5 (v/v) H_2O:Acetone solvent mixture. SEM images indicated that the sizes of D-BSA NPs were about 300 nm and sticky, spherical particles. The pH sensitive coordination complex of biocompatible dopamine molecules with Fe (III) as a crosslinker was enabled us to create a unique method of making modified albumin nanoparticles.

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