PREPARATION OF ALBUMIN NANOPARTICLES USING AN IONIC LIQUID BASED MICROEMULSION-LIKE METHOD

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

in Materials Science and Engineering

by
Begüm DEMİRKURT

June 2018
İZMİR

We approve the thesis of **Begüm DEMİRKURT**

Examining Committee Members:

Assoc. Prof. Dr. Yaşar AKDOĞAN
Department of Materials Science and Engineering, İzmir Institute of Technology

---

Assoc. Prof. Dr. Mustafa EMRULLAHOĞLU
Department of Chemistry, İzmir Institute of Technology

Assoc. Prof. Dr. Murat IŞIK
Department of Food Engineering, Bingöl University

21 June 2018

Assoc. Prof. Dr. Yaşar AKDOĞAN
Supervisor, Department of Materials Science and Engineering, İzmir Institute of Technology

---

Asst. Prof. Dr. Umut ADEM
Co – Advisor, Department of Materials Science and Engineering, İzmir Institute of Technology

---

Prof. Dr. Mustafa M. DEMİR
Head of Department of Materials Science and Engineering

Prof. Dr. Aysun SOFUOĞLU
Dean of the Graduate School of Engineering and Sciences

ACKNOWLEDGEMENTS

I would like to acknowledge the help of many people during my thesis study. Firstly, I would like to thank to my advisor Assoc. Prof. Dr. Yaşar AKDOĞAN for his guidance, care and freedom that he provided me throughout this journey.

I also would like to thank to other members of the thesis committee, Asst. Prof. Dr. Umut ADEM, Assoc. Prof. Dr. Mustafa EMRULLAHOĞLU, Assoc. Prof. Dr. Murat IŞIK and also Prof. Dr. Mustafa M. DEMİR for their valuable comments and suggestions.

I would like to extend my sincere thanks to the research scientists at the Center for Materials Research (IZTECH) for their help on SEM analyses and also, to Dane RUSÇUKLU from Biotechnology and Bioengineering Application and Research Center (IZTECH) for her gentle help. I would like to thank the TUBITAK (114Z318) and IZTECH Scientific Research Project (2015_İYTE_01) for supporting me during the project.

Thanks to my colleagues and lab mates for their support and positive input.

And last, I wish to thank all of my family and friends for their supports. I dedicate this thesis to my grandfather who is my angel.

ABSTRACT

PREPARATION OF ALBUMIN NANOPARTICLES USING AN IONIC LIQUID BASED MICROEMULSION-LIKE METHOD

Rich drug transportation ability of serum albumin protein has inspired scientists to obtain drug nanocarriers from albumin. In the literature, different methods have been developed to prepare albumin nanoparticles and their drug delivery properties have been studied. Here, this study aims to obtain albumin nanoparticles for a first time using ionic liquid (IL) included systems. Goal of this project is using imidazolium based ionic liquids (green solvent) to prepare albumin nanoparticles as alternative solvents for the commonly used organic solvents.

The use of volatile, toxic and flammable organic solvents in the albumin nanoparticle production has various negative effects on both human health and environment. Ionic liquids as non-flammable, non-volatile and non-toxic solvent candidates have attracted considerable attention in recent years both in the literature and in industry. Their ability to solve different types of solutes, designability, special mixing ability with water in IL/water binary systems and environmentally friendly properties cause ILs to overtake traditional organic solvents.

This thesis study proposed a novel and environmentally friendly microemulsion-like method for producing albumin nanoparticles in IL/water binary systems. Various experimental parameters such as pH effects, albumin concentrations, water amount, surfactant effects, glutaraldehyde effects, homogenizer effects, etc. were investigated to obtain uniform albumin nanoparticles. As a result, we achieved to synthesize uniformly distributed 200 nm average size albumin nanoparticles at pH 9.0 using 1.5% (w/w) of bovine serum albumin (BSA) in 1-butyl-3-methylimidazolium tetrafluoroborate using TX-100/n-butanol surfactant mixture.

ÖZET

ALBÜMİN NANOPARÇACIKLARININ İYONİK SIVI TEMELLİ MİKROEMÜLSİYON BENZERİ METOT İLE HAZIRLANMASI

Serum albümin proteininin zengin ilaç taşıma kapasitesine sahip olması, ilaç nanotaşıyıcıları yapılmasında bilim insanlarına ilham kaynağı olmuştur. Albumin nanoparçacıklarının üretilmesi ve ilaç taşıyıcısı olarak değerlendirilmesinde farklı yöntemler geliştirilmiştir. Biz de burada, ilk defa iyonik sıvıların kullanıldığı sistemler içerisinde albümin nanoparçacıklarını elde etmeyi hedefliyoruz. Projemizin amacı ilaç yüklü albümin nanoparçacığı hazırlanmasında şimdiye kadar kullanılan organik çözgenlere alternatif olarak imidazolyum tabanlı iyonik sıvıların (yeşil çözgenler) kullanılmasıdır.

Uçucu, toksik ve yanıcı organik çözgenlerin albumin nanoparçacık üretiminde kullanılmasının insan sağlığı ve çevre üzerine pek çok olumsuz etkisi bulunmaktadır. Uçucu, toksik ve yanıcı olmayan çözgen adayları olan iyonik sıvılar son yıllarda hem literatürde hem de endüstride çokça ilgi çekmişlerdir. Farklı tipte malzemeleri çözebilme yetenekleri, tasarlanabilirlikleri, iyonik sıvı/su ikili sistemlerinde su ile özel bir şekilde karışma özelliği ve ayrıca çevre dostu özellikleri iyonik sıvıların geleneksel organik çözgenleri geçmesine ve de malzeme üretim çalışmalarında yeni bir ufuk açılmasına sebep olmuştur.

Bu tez çalışması albumin nanoparçacık üretimi için yeni ve çevre dostu mikroemülsiyon benzeri bir metot önermektedir. pH, albümin konsantrasyonu, su miktarı, yüzey aktif madde konsantrasyonu, glutaraldehit etkisi, homojenizatör etkisi vb. gibi pek çok deneysel parametre incelenmiş olup pH 9.0'da %1.5 (w/w) bovin serum albumin kullanılarak düzenli dağılım gösteren ortalama 200 nm boyutlu nanoparçacıklar 1-bütil-3-metilimidazolyum tetrafloroborat içinde TX-100/n-bütanol yüzey aktif madde karışımı kullanılarak sentezlenmiştir.

TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

Table **Page**

CHAPTER 1

INTRODUCTION

1.1. Bionanomaterials

Biomaterials are the materials which interact with biological systems and can be natural and/or synthetic or semi-synthetic, alive and/or lifeless. Bionanomaterials are acquiring an increasing trend in both industrial and scientific area over the past four decades. These materials are very promising for many applications, especially biotechnology applications. They are used in biological systems and fitted for biomedical applications in safe, low-cost and biocompatible manner. [1] Many different types of bionanomaterials are already in the pharmaceutical market, some of them are represented in Table 1.1., and considerable number of them are approved. For example, Doxil™ consists doxorubicin drug encapsulated liposomes which got approval from FDA in 1995. In the other example, paclitaxel drug containing albumin nanoparticles for delivering the drug without any chemical additives called Abraxane™ was approved in 2005 by FDA and in 2008 by the European Medicines Agency (EMA). [2]

Table 1.1. Examples of some bionanomaterials used in the pharmaceutical market. [2]

Product Name	**Drug**	**Type of Nanocarrier**	**Company**
Daunoxome	Daunorubicin citrate	Liposome	Gilead Science, Cambridge, UK
Doxil	Doxorubicin HCl	Liposome	Johnson and Johnson, NJ, USA
Myocet	Doxorubicin	Liposome	Sopherion Therapeutics, NJ, USA
Caelyx	Doxorubicin HCl	Pegylated Liposome	Johnson and Johnson, NJ, USA
Transdrug	Doxorubicin	Poly(alkylcyanoacrylate) nanoparticles	BioAllience, Paris, France
Genexol-PM	Paclitaxel	Methoxy-PEG-polylactide nanoparticles	Samyang, South Korea
Oncaspar	Pegaspargase	PEG-asparaginase nanoparticles	Enzon, NJ, USA
Abraxane	Paclitaxel	Albumin-bound nanoparticles	American Bioscience, CA, USA

Nanoparticle based systems have been broadly investigated for drug delivery purposes. Nanoparticles are colloidal particles whose sizes are in between 10 – 1000 nm. The use of nanoparticles for drug delivery purposes has several following advantages: (1) size, morphology and surface properties of these nanocarriers can be easily controlled, (2) nanocarriers can carry and/or deliver lots of different therapeutic agents like small hydrophobic and/or hydrophilic molecules, proteins, peptides, nucleic acids etc., (3) these nanoparticulate systems are able to improve the stability and solubility of carried drugs which causes reevaluation of the drugs that have poor pharmacokinetics, (4) targeted delivery can be succeeded by engineering of nanoparticles. Superficially, nanoparticles as nanocarriers are able to protect drug deterioration, increase drug absorption and/or improve pharmacokinetic and drug distribution profile. Also, the desired release and biodistribution of drug may be achieved by modifying the composition, milieu and surface of the nanocarriers.[2]

Nanocarriers have different types, such as solid lipid nanoparticles, polymeric nanoparticles, polymeric micelles, dendrimers, drug-polymer conjugates, protein-based nanoparticles, ceramic NPs, magnetic NPs, nanowires, nanotubes, nanocages, etc. (Figure 1.1.) Among all of them, protein-based nanoparticles have acquired prominent interest in both the literature and pharmaceutical industry because of their biodegradability, enhanced storage stability and low toxicity properties.[3] Proteins are natural molecules which have distinctive properties for the applications in materials sciences and biomedical purposes. They are frequently used for nanoparticle fabrication because of their desirable solvent and drug interactions. Due to their natural structure, protein-based nanoparticles are metabolizable, biodegradable and have modifiable surfaces for drugs and/or targeting ligands. Hitherto, different proteins such as albumin, gelatin, elastin, gliadin, legumin, zein, soy, milk and whey proteins etc. have been used to produce nanoparticles successively.[2]

Albumin protein is an attractive candidate for manufacturing of nanocarriers because albumin has several following advantages:[3]

- Biodegradable
- Robust, stable between pH 4-9 and at 60°C up to 10 h
- Biocompatible, used in clinical trials for 30 years

- Non-toxic
- Non-antigenic
- Harmless products via in vivo metabolization
- Easy to access
- Easy to purify
- Water-soluble

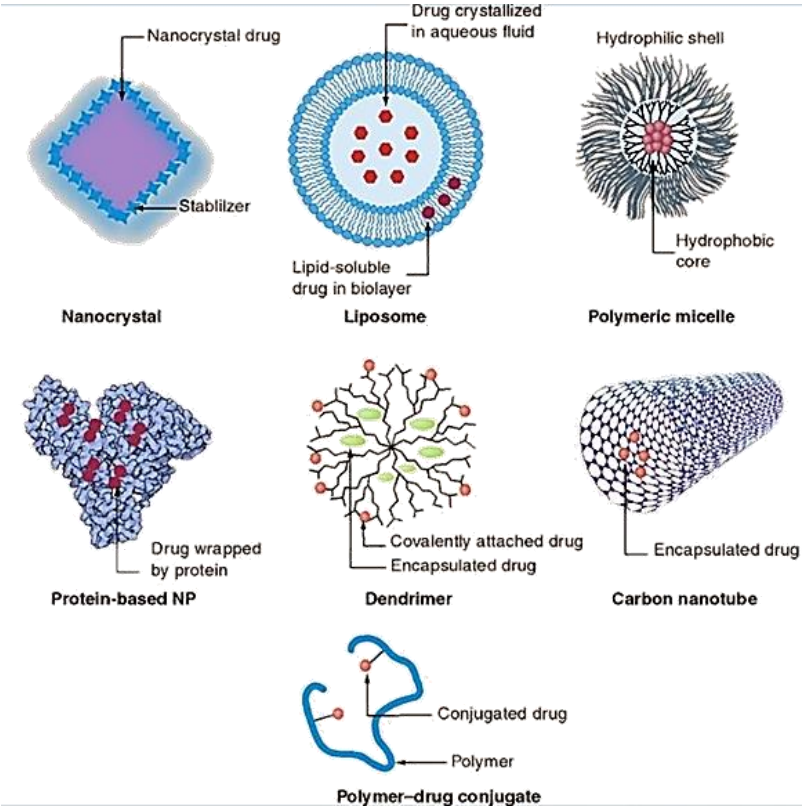


Figure 1.1. Different types of nanocarriers.[4]

In addition to all of these advantages, different binding sites of albumin (Figure 1.2) can incorporate a prominent amount of drug into the nanoparticle matrix. [5-6] By virtue of its well-defined sequence and complete structure, high number of its charged and functional amino acids (e.g. lysine), albumin-based nanocarriers can provide the desirable electrostatic interactions for adsorption of charged molecules without any other additives.[3] Furthermore, they can be prepared via various soft methods which will be mentioned in subheading 1.3.

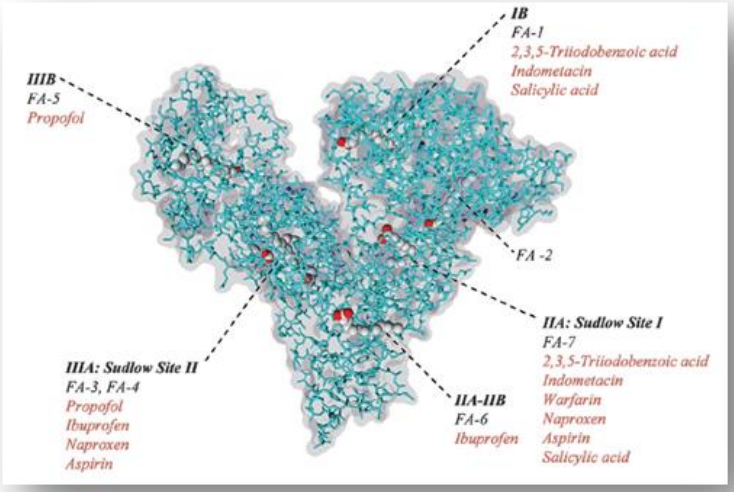


Figure 1.2. Structure and drug binding sites of albumin.[7]

1.2. Albumin Nanoparticles and Drug Interactions

Albumin is the most abundant protein in the plasma (35-50 g/L in human serum). It is synthesized 10-15 g/L in the liver daily. It regulates colloid osmotic pressure and carries several nutrients (fatty acids, hemin, etc.) and hydrophobic molecules such as steroid hormones via non-specific binding. Albumin has several functions and the primary ones are maintenance of osmotic pressure, transportation of endogenous and exogenous ligands of the circulatory system, protection of biological system from harmful free radicals as an antioxidant to reduce the oxidative stress.[1]

Albumin is acidic and soluble protein at pH 7.4 (up to 40% w/v), stable in between pH 4 – 9 and also up to 60°C. Commercially, significant amounts of albumins can be reached from different sources containing human serum (HSA), bovine serum (BSA), egg white (ovalbumin), milk, grains and soybeans.[1]

Widely, both HSA and BSA have been studied for production of albumin-based nanoparticles as drug delivery nanocarriers. The studies showed that HSA and BSA are homologous (76% sequence identity).[8] Hence, their many important residues are common and they provide similar interactions with ligands and/or molecules. (Figure 1.3) As homologous proteins, using BSA instead of HSA is advantageous because of its easy

obtaining, low cost, wide acceptance and medical importance in the pharmaceutical field. BSA is a globular protein with 66 kDa molecular weight, its isoelectric point is in between 4.8 – 5.6 with respect to reported values and 585 amino acids which provided approximately 67% α-helical secondary structure of the protein in physiological circumstances. BSA has 17 intramolecular disulfide interactions and free sulf-hydryl Cysteine-34 residue which is shown in Figure 1.3. These sulfur-based interactions are crucial structural elements and ensure the whole structure by regulating the distances and angular constraints in the folded state.[1]

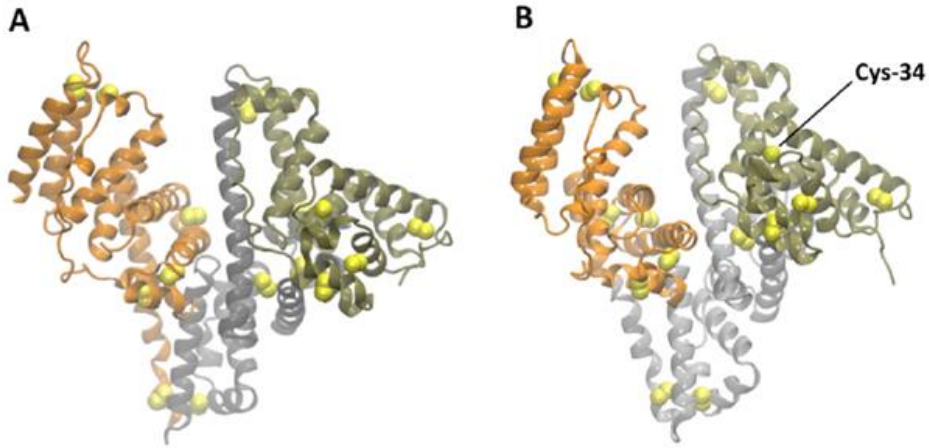


Figure 1.3. Structures of HSA (A) and BSA (B).[1]

Albumin nanoparticles have been prepared easily in defined sizes and also they have reactive functional groups (-SH, -NH_2, -COOH) on their surfaces which causes modifiable surface properties via conjugations with various drugs and ligands. In addition, the charged amino acids in its structure (such as aspartate, glutamate, arginine, lysine), albumin – based nanoparticles allow the electrostatic interactions of charged ligands and/or drugs. Natural origin of albumin, proper delivery properties, occurrence of binding sites for molecules and drugs (Figure 1.2), easily modifiable surface properties allow the solubilization and stabilization of drugs. Table 1.2 shows various albumin and albumin nanoparticle based pharmaceutical products in the market.[1]

Table 1.2. Albumin and albumin nanoparticle based drugs and their status in the pharmaceutical studies.[9]

Product	Drug	Indication	Current Status
ABI-007 (Abraxane®)	Albumin-paclitaxel nanoparticle	Oncology	Marketed
^{99m}Tc-Albures	^{99m}Tc-aggregated albumin	Oncology	Marketed
^{99m}Tc-Nanocoll	^{99m}Tc-aggregated albumin	Oncology	Marketed
Vasovist®	Albumin-binding Gadolinium (III) Complex	Oncology	Marketed
B-22956/1	Albumin-binding Gadolinium (III) Complex	Oncology	Marketed
Levenir®	Albumin-binding fatty acid derivative of insulin	Diabetes	Marketed
Liraglutide (Victoza®)	Albumin-binding fatty acid derivative of GLP-1	Diabetes	Marketed
Albuferon®	Albumin-fusion protein of interferon-α-2b	Hepatitis C	Phase III
AT-103 (Ozoralizumab)	Albumin-binding nanobody directed against human TNF-α	Rheumatology	Phase II
INNO-206	Albumin binding prodrug of doxorubicin	Oncology	Phase II
ABI-008	Albumin-docetaxel nanoparticle	Oncology	Phase II
MTX-HSA	Methotrexate albumin conjugate	Oncology	Phase I/II
MM-111	Albumin fusion protein directed against ErbB2 and ErbB3	Oncology	Phase I/II
AFL-HSA	Albumin conjugate of aminofluorescein	Oncology	Phase I/II

(**Cont. on next page**)

Table 1.2. (cont.)

CjC-1134-PC	Albumin conjugate of exendin-4	Diabetes	Phase I/II
ABI-009	Albumin-rapamycin nanoparticle	Oncology	Phase I
ABI-010	Albumin nanoparticle with a HSP90 inhibitor	Oncology	Phase I

Besides the advantageous modifiable properties of albumin, natural binding sites of albumin for drugs and/or molecules are responsible for various desirable interactions for drug delivery purposes. According to the Sudlow's classification, special sites in albumin are specified (Figure 1.2). Subdomain IIA and IIIA are two major binding sites. The site IIa is called "warfarin binding site" and has flexible, large, multichamber cavity within the subdomain IIA. As amantadine, azidothymidin and azapropazone negatively charged, heterocyclic compounds are bound to this site where hydrophobic interactions are pronounced. Site IIIA, that has been remarked as the "benzodiazepine and indole binding site", is structurally similar to the site IIA however more negatively charged compounds such as halothane, propofol, diazepam, ibuprofen and nonsteroidal anti-inflammatory drugs (NSAIDs) are bound to this site. Electrostatic, hydrophobic interactions and hydrogen bonding are predominant at site IIIA. Subdomain IB is a secondary site for binding of some compounds with different properties such as iophenoxic acid, myristic acid, lidocaine, indomethacin, naproxen, bilirubin and warfarin. The structural flexibility of albumin and so, produced albumin nanoparticles allow binding of various molecules to the nanocarrier reversibly.[10]

1.3. Albumin Nanoparticle Preparation Methods

Balance of attractive and repulsive forces in the albumin is essential for the production of albumin nanoparticles. Generally, albumin nanoparticle preparation methods stand following two actions: (1) decreasing intramolecular interactions of the protein and (2) increasing the protein unfolding. Pending the nanoparticle formation, conformational changes occur via unfolding of its native structure. This causes

conformational changes and interactive sites exposure from interior to exterior. Protein concentration, solution conditions such as solvent type, pH, ionic strength, preparation conditions (low or high energy methods) and cross-linking type can affect the albumin nanoparticle preparation. Intermolecular cross-linking increased and hydrophobic interactions decreased upon changing the conformation of protein and also experimental conditions. The chemical or thermal cross-linking causes a network formation and leads to durable albumin nanoparticles.[1]

In the literature, various albumin nanoparticle production methods exist like desolvation, emulsification, thermal gelation, nano-spray drying, nanoparticle albumin-bound technology (nab-technology) and self-assembly. The most used ones are desolvation and emulsification methods.

Desolvation method (Figure 1.4) is commonly used for producing albumin nanoparticles. It is based on self-assembly phenomenon, which is driven thermodynamically. In general, organic solvent (ethanol, acetone, methanol or ethyl acetate) as a desolvating agent is added dropwise to the aqueous albumin solution until the solution gains turbidity. Turbidity tells that homogenous solution turns into following two phases: (1) pure solvent phase, and (2) albumin-rich phase. Decreasing the solubility of the protein is sourced from conformational changes of the protein, which induces formation of albumin coacervates in albumin-rich phase. Frequently, formed coacervates are hardened via chemical cross-linking with the use of glutaraldehyde for obtaining stable nanoparticles. Using cross-linking agent promotes solidification of lysine and arginine residues via condensation reactions. Concentration, ionic strength, pH and stirring speed of aqueous albumin solution, type, amount and drop rate of desolvating agent affect the properties of obtained nanoparticles.[1]

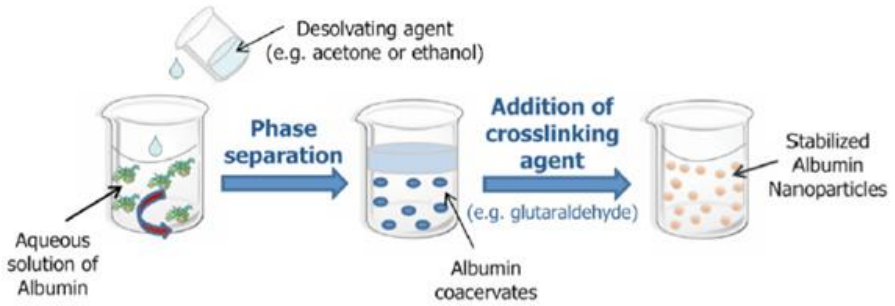


Figure 1.4. Schematic representation of the desolvation method.[1]

In the extensively used emulsification method (Figure 1.5), aqueous albumin solution as a polar phase is mixed with an apolar phase (such as oils and/or a water-immiscible organic solvents). Microemulsion is an emulsion type in which the dispersed phase is in the form of very small droplets usually produced and maintained with the aid of surfactants by high- or low- energy processes. (ref) High - energy methods include particular mechanical devices like high-pressure homogenizers, high-speed homogenizers and/or ultrasonicator. High-energy application to the system causes drop creation to generating the final nano-sized emulsions. Created macrometric drops are deformed and disrupted by application of high-energy, then surfactant(s) molecules align via adsorption at the interface of formed nanoparticles and provide steric hindrance for stabilization of them. Low-energy methods use the chemical energy to produce nano-sized droplets which is stored in the chemical system with the help of physicochemical properties of the excipients like surfactant(s), co-surfactant, polar and apolar phase. For consolidation of produced nanoparticles, chemical (e.g. glutaraldehyde, formaldehyde or diacid chloride) and/or thermal cross-linking (heating over 120 °C) are used.[1]

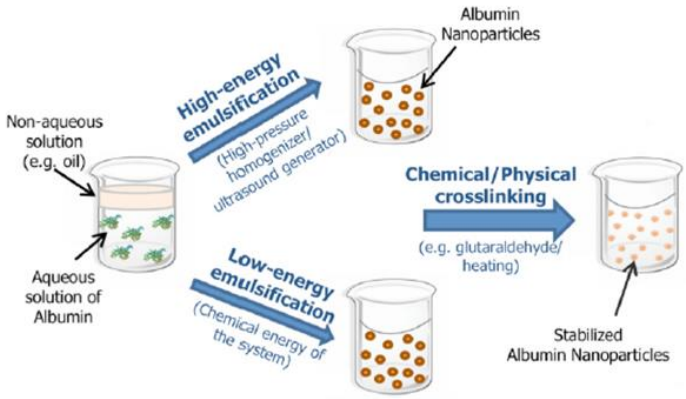


Figure 1.5. Schematic representation of the emulsification method.[1]

1.4. Ionic Liquids (ILs)

Ionic Liquids (ILs) have been called "green" solvents recently. Hitherto, they are used in organic synthesis, electrochemistry, catalysis, biological and various nanotechnology applications. (Figure 1.6) They are purely ionic, salt-like materials

composed of weakly coordinated cations and anions and hence, they are liquid (generally, m.p.<100°C). Low melting point values are sourced from the asymmetric combination of large organic cations and relatively small organic and/or inorganic anions. This asymmetry reduces the lattice energy and hence, melting point. The cation selection has a strong effect on the IL properties. The functionality and chemistry of ILs are strongly depended on the choice of anion. The combination of available cations and anions causes theoretically 10^{18} ILs. In reality, 1000 ILs are defined in the literature and ~300 of them are available in the market. Figure 1.7 shows the most commonly used cations and anions for the design of ILs.[11]

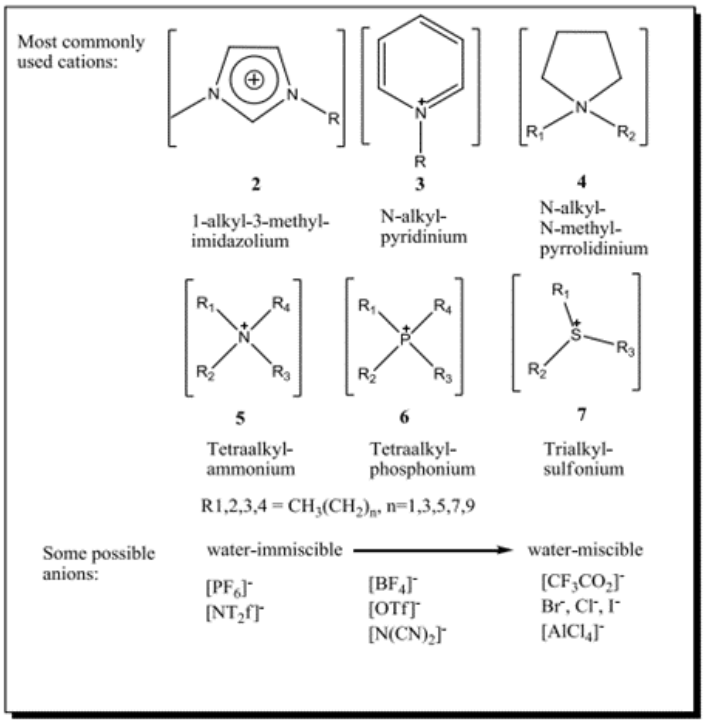


Figure 1.6. Commonly used cations and anions for the synthesis of ILs.[12]

There are many reasons that ILs are considered as "green" choice for the solvent selections. Their chemical and physical properties such as low vapor pressure, chemical and thermal stability, low toxicity for human health and environment, recyclability, electrical conductivity, non-flammability and high solvating power make them green and strong candidates for use in different application areas. The selection of the cation-anion combination has dramatic effects on the properties of ILs like density, viscosity, polarity and conductivity. Because of their designability and environmentally friendly properties, ILs are used instead of traditional volatile organic solvents in materials preparation,

organic synthesis and/or separation purposes.[13] Furthermore, ILs can generally dissolve (macroscopically and/or microscopically) polar and apolar molecules due to their amphiphilic properties.[14]

There are several interactions between ions in ILs. They have Coulombic forces between their anions and cations and other interactions such as hydrogen bonding, pi-pi stacking and/or dispersion interactions. Because of the combination of these forces, neat ILs have nanoheterogeneous structure. In the literature, this nanoheterogeneity has been widely investigated for imidazolium cation based ILs, which have polar and apolar domains. Also, in neat IL systems, IL framework is populated densely and all ions are surrounded by their counter-ions. This special and useful interactions in neat IL systems caused to start extensive studies about IL/water binary systems to open a greener way for various types of materials preparations.[15]

1.6. Imidazolium Cation based ILs and Their Aggregation Properties in IL/water Binary Systems

Among all ILs, imidazolium cation based ILs (Figure 1.7) are one of the most studied ones. 1-butyl-3-methyl-imidazolium cation ($[C_nMIM]^+$, n=4) with a weakly coordinating BF_4^- ($BMIMBF_4$) were discovered in 1992 and it was one of the first examples of second generation ILs which were found air and moist stable.[16] $[C_nMIM]^+$ based ILs are extensively studied in materials preparation, colloidal and interfacial sciences. Generally, desired properties of this type of ILs are controlled with their carbon-chain length and anion choice as mentioned previously. Through various studies in the literature, self-assembling property of $[C_nMIM]^+$ based ILs are occurred when $n \geq 4$ in water because of the mentioned structural nanoheterogeneity.[15] Imidazolium based ILs with anions such as tetrafluoroborate is widely used for desired applications because of its stability.

Dupont et al. proposed that [CnMIM] cation could be surrounded by at least three BF4 anions and as expected, each anion could be surrounded by at least three [CnMIM] cation. However, coordination numbers for cation and anion may change with respect to type of anion and the length of alkyl side chain. Nonetheless, hydrogen bonded network is valid for imidazolium based ILs in the solid and liquid state. Hence, a 3-D network (Figure 1.8) are formed by (i) columns of cations and anions and (ii) alkyl chain

aggregates. Furthermore, for n≥4 [CnMIM]+, alkyl chain aggregation is observed causing nonpolar domains with nm – scale.[17]

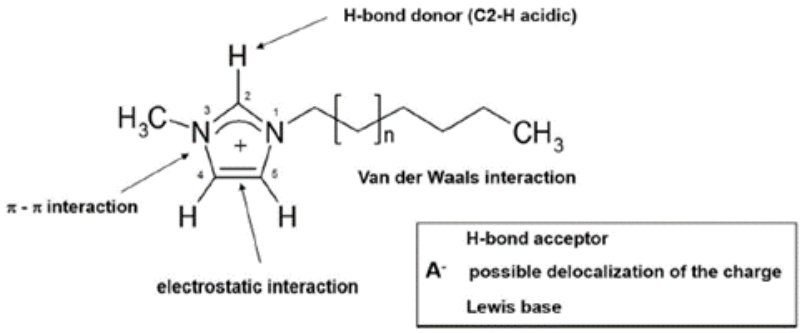


Figure 1.7. Structure of the imidazolium based ILs. [16]

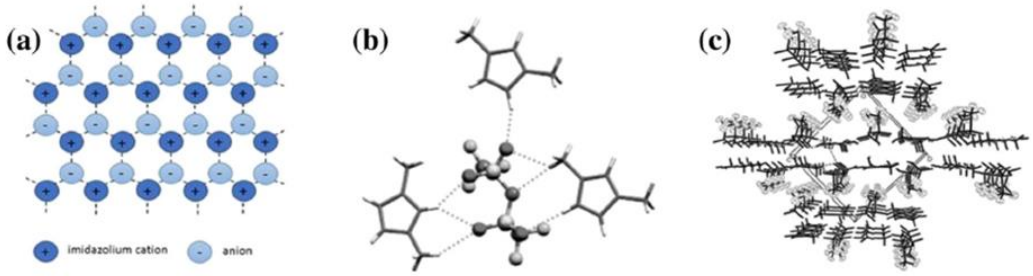


Figure 1.8. (a) 2-D network, (b) cation-anion interactions and (c) 3-D network representation of $BMIMBF_4$. [17]

When water is added to the bulk $BMIMBF_4$, it is known from the literature studies that water and $BMIMBF_4$ mix in all proportions macroscopically. It means no phase separation is observed by direct visual test. Although $BMIMBF_4$ and water is macroscopically miscible at any mole fraction, mixtures of water and $BMIMBF_4$ are micro-heterogenous and have mesostructured aggregates above the critical aggregation concentration (CAC) (C_{IL} ~ 0.3 M) according to literature studies.[18] The structure of these mesostructured aggregates (~120 nm radius) are revealed by dynamic light scattering (DLS) and small – angle neutron scattering (SANS) analyses which are assembled from spherical, polydisperse building blocks with a radius of approximately 12 Å.[19]

Russina et al. revealed the following three main concentration regimes for water and $BMIMBF_4$ mixtures in the light of Raman, FTIR, XRD and neutron diffraction techniques: (1) In $0<X_w<0.25$, water molecules do not show self-aggregation, they form hydration shells around anions up to $Xw\leq0.25$, (2) In $0.25<X_w<0.75$, self-organized, spherical, nm scale capsule - vesicle like aggregates, which contains ample water in it and have swelling behavior in this transient microscopically bicontinous-like regime, form in IL network, (3) In $0.75<X_w<0.99$, microscopic structure of the mixture changes to ionic solutions of the $BMIMBF_4$ in water and disruption of the mesoscopic structures is observed ($X_w\sim 0.98$).[15] Based on self-diffusion measurements for the entire range of water concentration, Murgia et al. concluded mesoscopic domain formation, too.[19] The properties of the confined water in $BMIMBF_4$ were investigated by NMR and Saihara et al. inferred that at 50% mol of water in $BMIMBF_4$, water pools are formed in nanodomain structure.[20] Also, Cascao et al. suggested that water incorporation in pockets of the IL nanodomain maintains most of the Coulombic interactions of neat $BMIMBF_4$ and positions of those water pools are close to the cations.[19]

In summary, pure 1,3-dialkylimidazolium ionic liquids have hydrogen-bonded polymeric supramolecular structure which is $[(DAI)_x(X)_{x-n})]^{n+}$ $[(DAI)_{x-n}(X)_x)]^{n-}$ type network (DAI is the 1,3-dialkylimidazolium cation and X is the anion). This structure is a general trend for the liquid phase. The addition of water and/or macromolecules occurs with a disruption of the hydrogen bond network and generates nano-structures with polar and non-polar regions where inclusion-type compounds can be formed. These inclusion compounds can involve water pools, molecules, ions, macromolecules and nanoparticles and the stabilization of this process is mainly due to the electronic and steric effects provided by the nano-structures of the type $[(DAI)_x(X)_{x-n})]^{n+}$ $[(DAI)_{x-n}(X)_x)]^{n-}$. When they are diluted infinitely with other molecules they can form solvent-separated ion pairs that an increase of the concentration of the imidazolium salt they collapse to form contact ion pairs - through hydrogen bonds involving the cation with the anion – and a further salt concentration increment leads to triple ions, etc. (Fig. 1.9)[21]

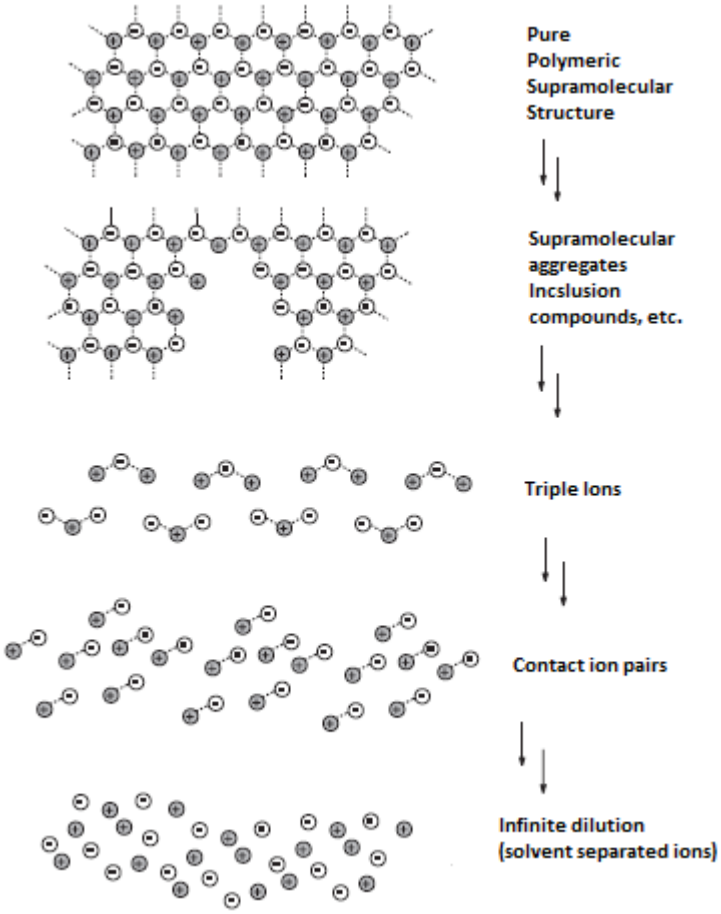


Figure 1.9. Network representation of IM based ILs in the presence of additives (water and/or protein case)[21]

1.6. Albumin – Imidazolium Cation based IL Interactions

Various studies were conducted to understand interactions between IM based ILs and BSA. The results of these studies are very important for placing IM based ILs in new bionanomaterials production methods and biological applications.

Yan et al. showed that hydrogen bond and van der Walls interactions have major roles between BSA and [C_nMIM] (n=4,6,8) cation.[22] Zhu et al. investigated this interplay more deeply by microcalorimetry and circular dichroism techniques and proposed following two interactions: (1) electrostatic interaction of BMIM cation and negatively charged sites of BSA and (2) hydrophobic interactions between imidazole ring and BSA such as pi-pi interactions.[23]

Akdogan et al. studied that effects of ILs on the tertiary structure of HSA by continuous wave electron paramagnetic resonance (EPR) spectroscopy and nanoscale distance measurement for detecting the change in the protein structure by double electron-electron resonance (DEER) spectroscopy.[24-25] The group observed that $BMIMBF_4$ and ethanol have the similar effects on protein unfolding. Ethanol is one of the desolvating agent for albumin and it is used in synthesis of albumin NPs. Also, they reported that ILs with more hydrophobic alkyl chains unfold the HSA more than the ILs with less hydrophobic alkyl chains. [24]

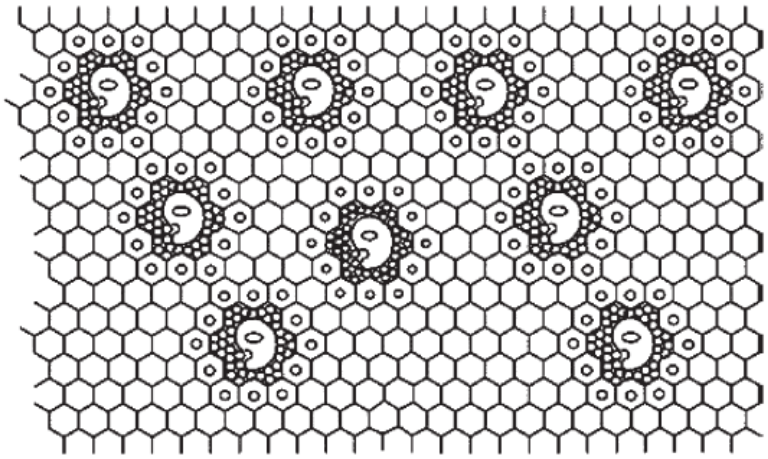


Figure 1.10. Proteins with a small amount of water are firmly trapped in the networks of ILs.[26]

Zhao et al. resulted that the addition of other molecules (such as probes for UV-VIS or EPR analyses) or biomacromolecules into the $BMIMBF_4$/water binary mixtures ($0.25<Xw<0.75$) results polar and non-polar regions formation.[27] Also, the Dupont group reported that when proteins and/or enzymes are suspended in the IL, these macromolecules are surrounded by the IL network (Fig. 1.10) which protects the native structures of macromolecules by preserving the ample water of proteins and the desired solvophobic interactions; particularly hydrophobic and Coulombic interactions.[21] When the protein-in-water droplets are dispersed into the IL network, highly ordered supramolecular structure of IM-ILs conserves the stability of proteins by keeping them in their active conformations.[28]

1.7. The Scope of Thesis

Albumin nanoparticles are frequently used in biological applications like drug delivery due to its various advantages. Production methods of these nanoparticles involve volatile, flammable and toxic organic solvents which are harmful for the environment and human health. Also, the waste of them causes storage problems. For solving this problem, imidazolium based ionic liquids selected for development of a greener method. This study represents the first example of using ionic liquids as alternative to the traditional organic solvents for the albumin nanoparticle preparation.

Interactions between ILs and albumin are affected by the kinds of anions and cations in ILs. For example, imidazolium based ILs have denaturation effects on the albumin, but choline based ionic liquid such as choline dihydrogen phosphate (dhp) has a stabilizing effect on the albumin structure.[24] The denaturing effect of the ILs could be used to initiate albumin nanoparticle formation. Therefore, the chosen anions and cations regulate the albumin nanoparticle formation, and also affect the physical properties of albumin nanoparticles such as size, size distribution, morphology and stability. Also, for drug transportation, especially for the poor water soluble drugs, ILs could be very effective to obtain drug loaded albumin nanoparticles in the future. Different types of drugs are soluble in ILs depending on the anions and cations.[29] This could help in encapsulating drugs in the albumin nanoparticle during the nanoparticle preparation. For these reasons, we aim to prepare albumin nanoparticles in an IL based system.

In the literature, ILs have been used in microemulsion systems for the replacements of aqueous phase, oil phase or surfactant molecules. For example, starch nanoparticles were prepared separately in IL/oil, water/IL and water/oil (IL is surfactant) microemulsion systems.[30-32] In this thesis, a microemulsion-like technique was developed by using IM based ILs. Mixture of selected ILs and albumin aqueous solution in the presence of surfactants and glutaraldehyde is exposed to a high speed homogenizer to yield albumin nanoparticles. Different parameters mentioned in Chapter 3 were studied to understand formation of nanoparticles.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

BSA (Mw = 66 000 Da, lyophilized powder), [EMIM][BF_4], [BMIM][BF_4], [HMIM][BF_4], Triton™ X – 100, 1 – butanol, Glutaraldehyde solution (Grade II, 25% in H_2O), and absolute ethanol were purchased from Sigma – Aldrich. All chemicals and solvents were analytical grade and used without any further purification processes. All aqueous solutions were prepared in Milli-Q water. The pH of aqueous solutions was adjusted with 0.1 M HCl or 0.1 M NaOH using an OHAUS STARTER3100 pH meter.

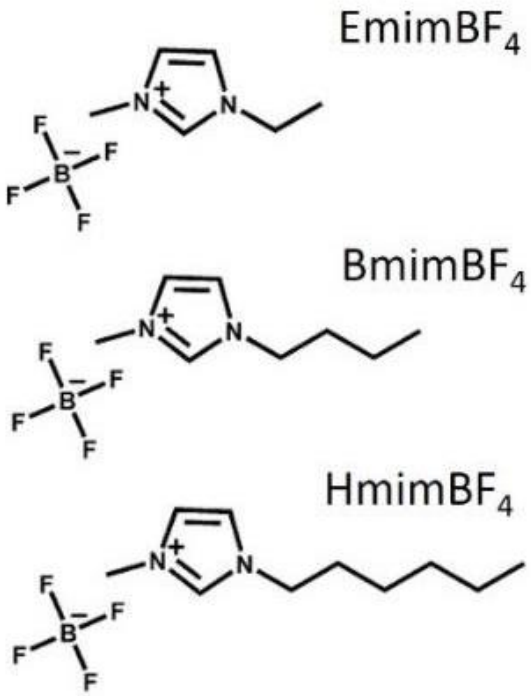


Figure 2.1. Structures of selected ILs.

2.2. Preparation of BSA NPs

3 g of $BMIMBF_4$, 90 mg of TX-100 and 30 mg of 1-butanol were stirred in a glass vial for 15 min. at 1200 rpm. Then, 240 μL of 5% (w/v) BSA aqueous solutions at different pH=9.0 and 60 μL of glutaraldehyde solution (Grade II, 25% in H_2O) were added dropwise to the stirred IL mixture at 1200 rpm. After addition, the mixture was stirred at 22 000 rpm for 3 min. by a homogenizer (IKA, T 18 TURRAX). Next, mixture was stirred for 15 min. at 1200 rpm to bring the reaction medium to thermal equilibrium. BSA NPs solution was transferred to the eppendorf tubes for centrifugation. After centrifugation at 10 000 rpm for 15 min, pellets were washed 2 times with ethanol and once with Milli-Q water for eliminating $BMIMBF_4$, unreacted glutaraldehyde, TX-100, and 1-butanol. Finally, pellets were prepared as described in the characterization part of the article for further analyses.

The following parameters were studied by applying the same procedure: (1) pH of the BSA solution (pH = 4, 7, 9), (2) aqueous phase amount (120, 240 and 480 mg of dH_2O), (3) BSA concentration (0.5, 1.5, 4.0 and 12 mg of BSA), (4) glutaraldehyde effect, (5) amount of surfactant(s), (6) co-surfactant effect, and (7) number of carbon in alkyl chain of $[C_nMIM]^+$ (n = 2, 4, 6).

2.3. Characterization of BSA NPs

After centrifugation step, obtained pellets were dissolved in the same amount of Milli – Q water. For SEM (FEI QUANTA 250 FEG) analyses, nanoparticle solutions were diluted 1000 times from their original volumes with Milli-Q water. After that, 10 μL of nanoparticle solutions were dropped on a clean aluminium foil and SEM samples were left in the fume hood for drying. Dried SEM samples were coated with gold using EMITECH K550X in a vacuum before SEM analyses. The accelerating voltage was 10 kV. Particle size analyses of SEM images were done by using ImageJ program. A Malvern dynamic light scattering (DLS) Nano-ZS instrument (Worcestershire, UK) was used for size and zeta potential measurements.

CHAPTER 3

RESULTS AND DISCUSSION

In C_nMIMBF_4 ionic liquids with n≥4, aggregation of the hydrophobic alkyl side chains of imidazolium ring leads to a nano-phase separation from ionic domains consisting of the imidazolium ring and the anion.[33] Therefore, a micelle-like structure is observed. For the water-IL binary system, polar water molecules can interact with ionic domains of C_nMIMBF_4. For example, by addition of water into the $BMIMBF_4$, water molecules are first dissolved and then form water aggregates around the ionic domains of $BMIMBF_4$ especially for the intermediate water contents (50 mol%).[20,34,35] Although, these water rich domains can be investigated by ^1H NMR spectroscopy, their sizes could not be detected by DLS in here due to their dynamic and swinging structures. Since the mixture of C_nMIMBF_4, n≥4, and water form unstable mesostructures composed of water-rich and IL-rich domains, we developed a microemulsion-like method to synthesize albumin nanoparticles in the water-in-ionic liquid (w/IL) system. For albumin nanoparticle preparation, various experimental parameters and the effect of alkyl side chains of ILs were studied in detail.

3.1. Effect of Surface Charge of BSA (pH of BSA Solution)

For [CnMIM]+ (n=4,6,8) based ILs, H-bond and van der Walls forces play major role in interaction of [CnMIM]+ with BSA.23 Hence, the hydrophobic/hydrophilic properties and therefore charge type of BSA at different pHs become important for understanding the nanoparticle formation mechanism. It is known from the literature studies that zeta potential of BSA varies with changing pH and these change surface properties, conformational changes and aggregation characteristics of BSA.36 The influence of surface charge of the BSA on the nanoparticle formation was studied with three different pH values including pH 4.0, 7.0 and 9.0. From SEM images (Figure 3.1), it is obviously seen that the best pH for the BSA nanoparticle formation is pH 9.

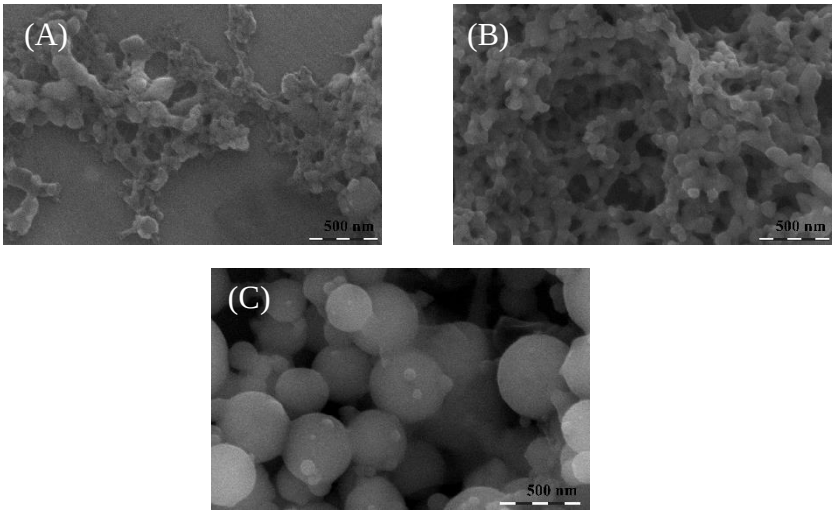


Figure 3.1. SEM images of pH 4 (A), 7 (B), 9 (C) experiments (12 mg of BSA in 240 mg of water)

These observations are in good agreement with the earlier works in which smaller albumin nanoparticles were obtained at pH 8.0 - 9.0 with the desolvation technique.[37,38] The influence of pH on the isolated albumin nanoparticle formation can be explained by the charge effects. The structure of albumin remains stable between pH values of 4.0 and 9.0 but the charge of albumin changes from positive to negative with reported isoelectric points in the range from 4.8 to 5.6.[36] At pHs 4.0, 7.0 and 9.0, the reported charges of BSA are +10, -13, and -26, respectively.[36] The absolute value of charge of BSA at pH 9.0 is about twice the absolute value of charges of BSA at pH 4.0 and 7.0. Therefore, higher electrostatic repulsive forces at pH 9.0 keep the formed albumin nanoparticles away from each other which avoids the agglomeration. In addition, the albumin nanoparticle formation can be affected by the types of intermolecular interactions between $BMIMBF_4$ and BSA which are also pH dependent. In addition to hydrogen bonding and hydrophobic interactions which exist between BMIM cation and BSA, at pH 9.0, a higher electrostatic attraction between BMIM cation and BSA takes place due to a net higher negative charge on albumin. The combination of these forces at pH 9.0 possibly supports albumin nanoparticle formation and stabilization.

3.2. Effect of the Aqueous Phase Amount

After pH optimization experiments, the effect of aqueous phase amount was investigated. Change in aqueous phase to IL phase ratio results change in system viscosity and distribution of applied energy via shear stress (which varies with volume and viscosity), which affects nanoparticles characteristics like size, morphology, etc.[39]

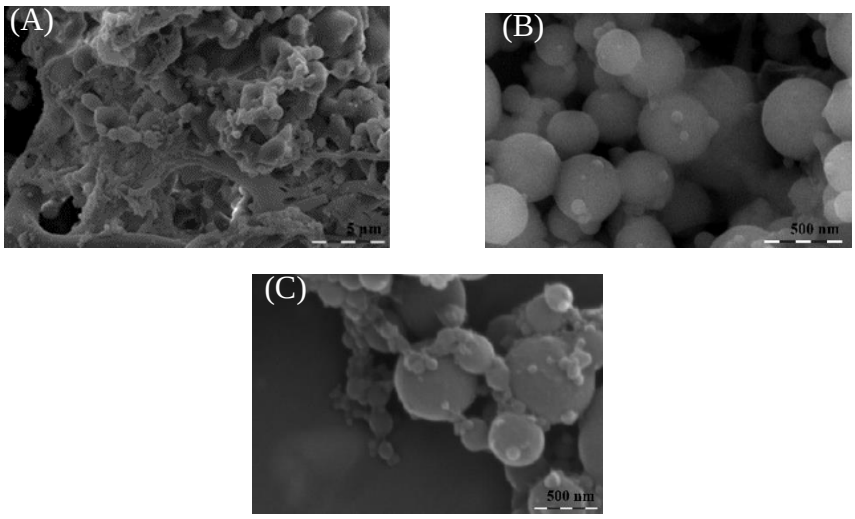


Figure 3.2. SEM images of experiments prepared with the use of 12 mg of BSA in 120 (A), 240 (B) and 480 (C) mg of water at pH=9.

Table 3.1. Amounts of the used water and BSA in the experiments. (n_{IL} = 0,013 mol and max. solubility of BSA in water is ~2-3 mM.)

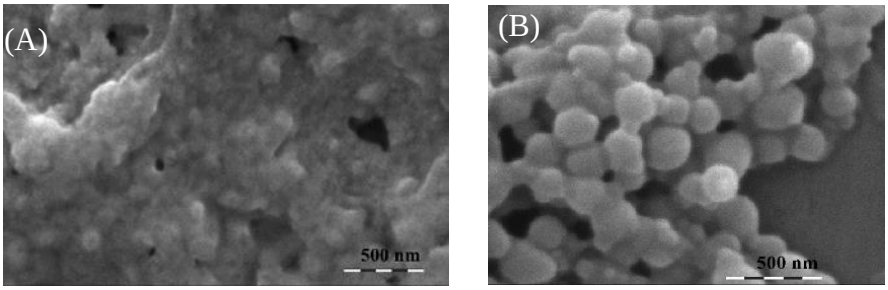
Amount of Water (mg)	**n_w (moles)**	**X_w**	**BSA Concentration (mM)**
120	0.0065	0.33	1.5
240	0.013	0.5	0.75
480	0.026	0.67	0.375

Insoluble aggregates were obtained when 12 mg of BSA was dissolved in 120 mg of H_2O. Fail of nanoparticle formation may be sourced from microviscosity concept

which means the viscosity in the core of the nano-capsules. By decreasing the water content from 240 mg (Fig. 3.2 (B)) to 120 mg (Fig. 3.2 (A)), BSA concentration increases from 0.75 mM to 1.5 mM. (Table 3.1.) It seems that during the albumin nanoparticle formation, close interactions between BSA proteins turned into larger aggregates with the help of glutaraldehyde cross linking process. On the other hand, by keeping the BSA amount constant, increasing the volume of water phase from 240 mg to 480 mg produced larger BSA nanoparticles with non-uniform size distributions (Fig. 3.2 (C)). This can be explained by the formation of larger water aggregates with increasing the water content. Also, applying the homogenizer with the same speed and time to a higher volume of water contained system might not be energetically successful to lead small nanoparticles with uniform size distributions.

3.3. Effect of BSA Concentration

After pH and amount of aqueous phase optimization experiments, the effect of BSA concentration was investigated in constant amount of aqueous phase (240 mg of water) at pH 9. BSA concentration affects directly the microviscosity. Increasing of the microviscosity in the nano-capsules (with increasing BSA concentration) may cause additional and high viscous resistance against applied shear stress during homogenization part of the method. When BSA concentration was increased at constant amount of aqueous phase, increased viscous forces resisted breakdown of droplets and hence, size of the obtained nanoparticles was increased. [39]



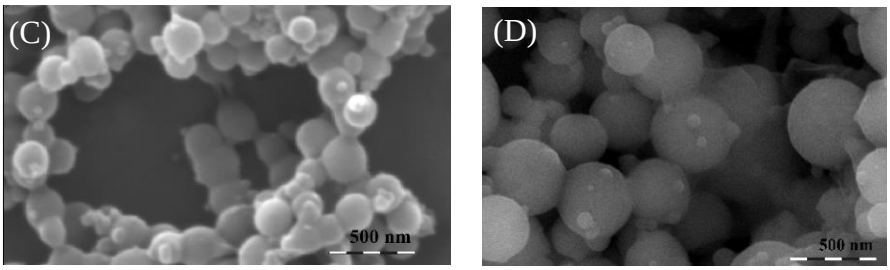
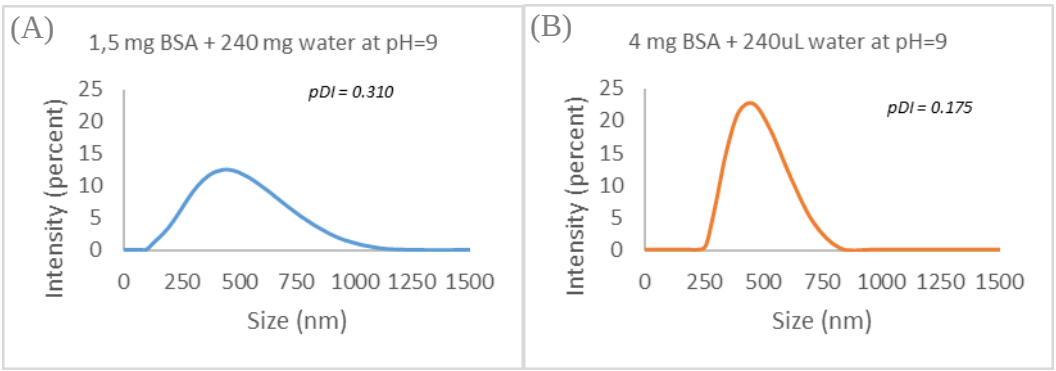


Figure 3.3. SEM images of experiments prepared with 0.5 mg (A), 1.5 mg (B), 4. 0 mg (C) and 12 mg (D) of BSA in 240 mg of water at pH=9.

After pH and amount of aqueous phase optimization experiments, the effect of BSA concentration was investigated in constant amount of aqueous phase (240 mg of water) at pH 9. BSA concentration affects directly the microviscosity. Increasing of the microviscosity in the nano-capsules (with increasing BSA concentration) may cause additional and high viscous resistance against applied shear stress during homogenization part of the method. When BSA concentration was increased at constant amount of aqueous phase, increased viscous forces resisted breakdown of droplets and hence, size of the obtained nanoparticles was increased. [39]

From SEM images, one can clearly see that when 0.5 mg BSA was used for nanoparticle synthesis, film-like formation was obtained. This may be sourced from insufficient amount of BSA to interact and support each other. Interactions between IL-BSA, especially Coulombic interactions were stronger than the BSA-BSA interactions and so, nanoparticles were not formed.



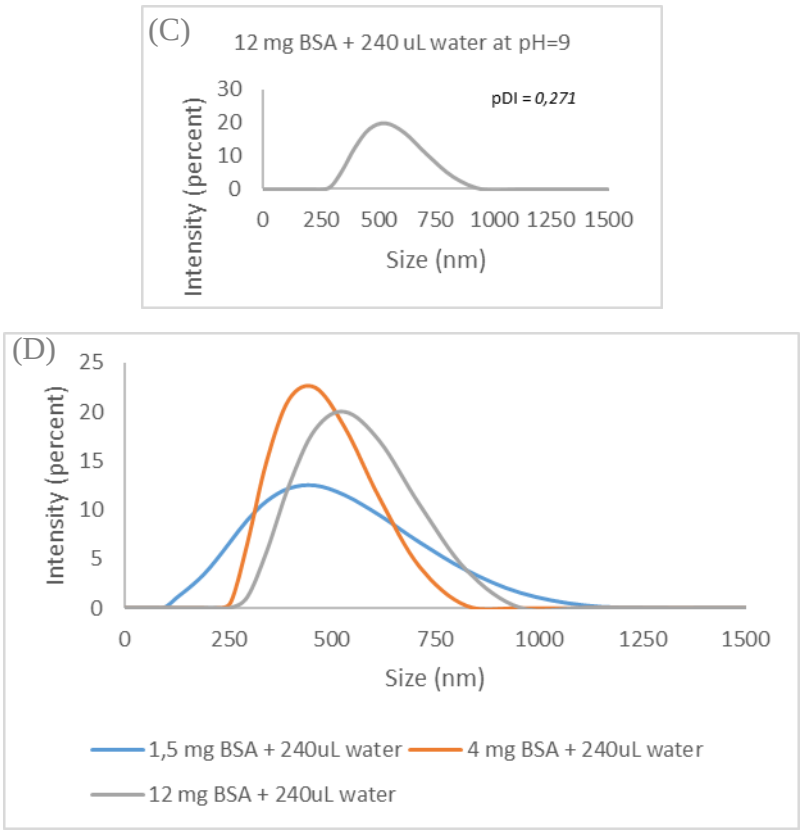


Figure 3.4. DLS results of samples prepared with 1.5 mg (A), 4.0 mg (B), 12 mg (C) of BSA in 240 mg of water at pH=9 and their display in the same graph (D).

When 1.5 and 4.0 mg BSA was used, spherical BSA nanoparticles with particle size ~200 nm (based on SEM images) were obtained. Smaller albumin nanoparticles with a narrow size distribution was obtained with 4 mg of BSA compared to the results of albumin nanoparticles synthesized with 12 mg of BSA. Lomis et al. showed that decreasing the BSA concentration in chloroform/water solution yields smaller BSA nanoparticles in the emulsion-solvent evaporation method.[40] Decreasing the BSA concentration leads to a decrease in the BSA-BSA interactions. This avoids the agglomeration of BSA clusters. Also, BSA concentration directly affects the microviscosity of the system as mentioned previously. Interestingly, use of 1.5 and 4.0 mg of BSA provided the same size of nanoparticles and this situation may be sourced from the thermodynamic stabilization of a preferential size of the nanoparticles.[41] DLS results yielded the similar average size of BSA NPs (~450 nm) for 1.5 mg and 4.0 mg of BSA, which had 0.310 and 0.175 pDI values, respectively. The reason of obtaining greater nanoparticle size in DLS (Fig. 3.4) is a common issue for organic nanoparticles

and related with the hydration shell of nanoparticles and the presence of surfactant(s) on the surface of nanoparticles.

3.4. Effect of Glutaraldehyde

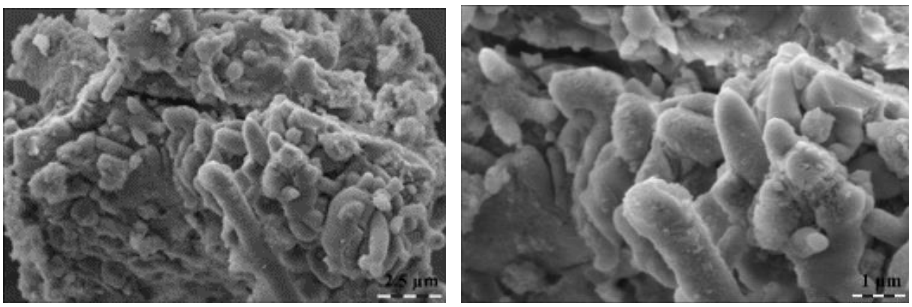


Figure 3.5. SEM images of experiment prepared with 12 mg of BSA in 240 mg of water at pH=9 without GLA addition.

Glutaraldehyde (GLA) is a common cross-linker to stabilize the formed BSA nanoparticles and mainly reacts with primary amine groups of the protein (e.g. lysine).[37] According to the SEM analyses, function of GLA in our method was the stabilization of the formed nanoparticles. Fiber-like structures were obtained in the absence of GLA which were another indirect proof of the stabilization role of GLA.

3.5. Effect of Surfactant(s) Concentration

Stabilization of the BSA contained nano-vesicles in the IL matrix is one of the crucial factors in our method. The amount of surfactant(s) plays a significant role since surfactant(s) molecules can avoid the coalescence of the nano-vesicles. When the amount of them was exceeded their CAC values, the mechanism of our method obviously changed and it can be seen from the SEM images. (Fig. 3.6.)

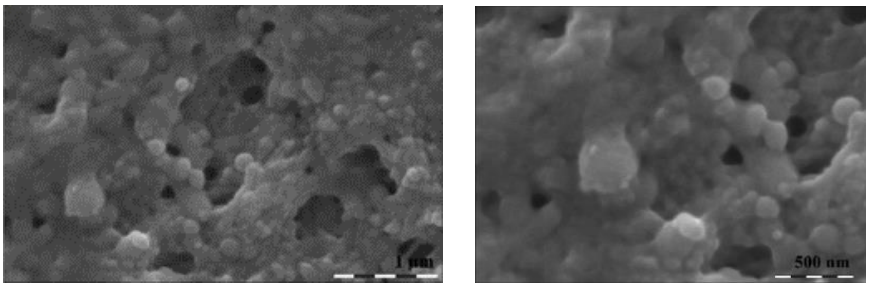


Figure 3.6. SEM images of experiment prepared with 4 mg of BSA in 240 mg of water at pH=9 with 2.5 times more surfactant(s) condition.

Surfactant molecules can decrease the interfacial tension between immiscible liquids in a system. This increases miscibility, colloidal stability and dispersion of nanoparticles in liquid.[20] Using a 50 mM of non-ionic surfactant TX-100 (with 140 mM of 1-butanol) below its critical micelle concentration (CMC) in $BMIMBF_4$ yielded BSA nanoparticles. Besides, using 125 mM of TX-100 (with 420 mM of 1-butanol) caused BSA aggregation. Behera et al. showed that the CMC value of TX-100 increases with $BMIMBF_4$ concentration in the aqueous solution. At 30 wt% of $BMIMBF_4$ solution, TX-100 does not form aggregation up to 50 mM of TX-100.[42] Therefore, in here, it is not expected to observe TX-100 (50 mM) aggregation in the 85 wt% of $BMIMBF_4$ aqueous solution. But, 125 mM of TX-100 may form aggregation in the system and may cause the coalescence of BSA nanoparticles. This shows that the ratio of surfactants in the system plays a significant role for the BSA nanoparticle formation.

When our method was tested for the absence of surfactant(s), solution stability of obtained particles was very low and fast coagulations were observed within a few seconds. So, this was related with the interface of the nanoparticles. This is an indirect interpretation of the role of surfactant(s) in this system because in the presence of surfactant(s), stability and solubility of nanoparticles showed that surfactant molecules are used to cover interface of nanoparticles. The high surface energy of the nanoparticles led to aggregation and presence of surfactant(s) provided steric barrier on the surface of the nanoparticles.[39]

3.6. Effect of Cosurfactant

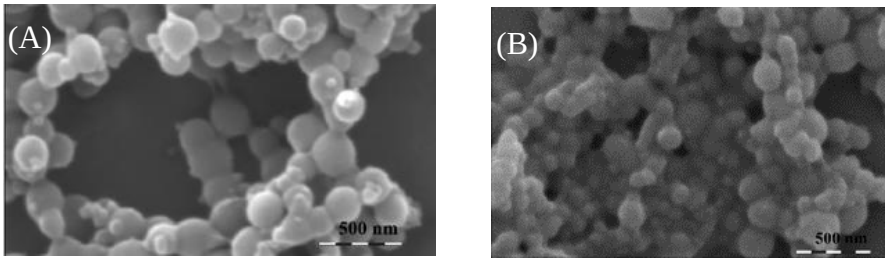


Figure 3.7. SEM images of experiment prepared with 4 mg of BSA in 240 mg of water at pH=9 with (A) and without (B) 1-butanol addition

According to experimental observations and also SEM images (Fig. 3.7.), the role of the cosurfactant (1-butanol) is the enhancement of water solubility of obtained nanoparticles. 1-butanol molecules may have aligned on the interface of the nano-vesicles along with the TX-100 molecules and reduce the interfacial tension and hence increase in water solubility was observed.

3.7. Effect of the Number of Carbon in [C_nMIM] Cation (n=2 and 6)

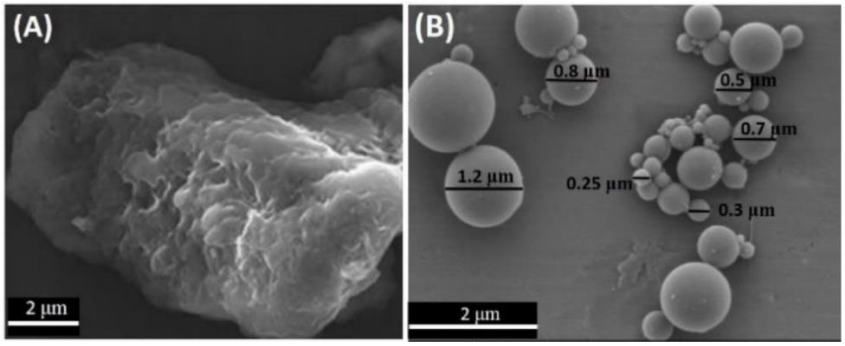


Figure 3.8. SEM image of experiment prepared with the use of $EMIMBF_4$ (A) and $HMIMBF_4$ (B).

According to the experimental observations and SEM images (Fig. 3.8 (A)), when $EMIMBF_4$ was used in our method, BSA aggregates were obtained. Fail of $EMIMBF_4$ used in our method may be explained by the following reason: increasing the alkyl side chain of imidazolium cation favors the polar/nonpolar nanophase separation in ILs. Therefore, water molecules can be aggregated in the polar regions of BMIMBF4 which

stimulate the nanoparticle formation. Since the hydrophobicity of ethyl groups is not strong enough to create robust nonpolar domains in IL, water molecules can be homogeneously distributed in $EMIMBF_4$ more than in $BMIMBF_4$. Thus, they do not form water pools extensively in $EMIMBF_4$ which are necessary for the albumin nanoparticle formation.

Further increasing the length of alkyl side chain of imidazolium cation from butyl to hexyl, water solubility decreases. Thus, this leads to larger water pools formation around the polar regions of $HMIMBF_4$. Addition of 4 mg BSA solution (240 mg of water) into 3 g of $HMIMBF_4$ in the presences of glutaraldehyde and surfactants produce BSA nano- and micro- particles after applying the high-speed homogenizer (Fig. 3.8 (B)). As expected, larger water pools formed in the water-$HMIMBF_4$ system leaded to the larger BSA nanoparticles compared to the those formed in the water-$BMIMBF_4$ system. Spherical BSA nano- and micro- particles with a broad size distribution between 0.2 μm and 1.2 μm were obtained in the water-$HMIMBF_4$ microemulsion like system.

Change in the alkyl chain length of the imidazolium cation caused pronounced effect on the mechanism. Balance of the all forces are very important for the formation of albumin nanoparticles. All of the results showed that successful creation of nm-sized mesostructures (water pools in IL network) is the touchstone of the nanoparticle formation mechanism.

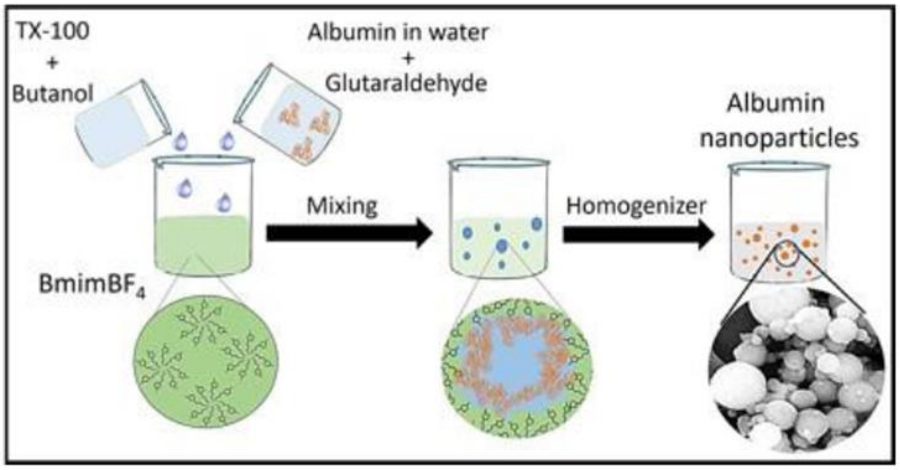


Figure 3.9. Proposed mechanism for BSA NP formation in our developed method.[43]

CHAPTER 4

CONCLUSION

Organic solvents have been commonly used to prepare albumin nanoparticles. Alternatively, here imidazolium based ionic liquids having BF_4 anion were used to prepare albumin nanoparticles. Ionic liquids have been considered as "green" replacements for organic solvents due to their unique properties in addition to their designability features.

It has been known that water in $BMIMBF_4$ at intermediate levels (e.g. 50 mol%) forms water aggregates/pools.[20,34,35] Here, addition of BSA aqueous solution with glutaraldehyde into $BMIMBF_4$ (with TX-100 and 1-butanol) forms an microemulsion-like system upon applying high speed homogenizer. Interactions between $BMIMBF_4$ and BSA at the water/$BMIMBF_4$ interface denature the BSA. After that, high speed homogenization converted denatured BSA into BSA nanoparticles with the help of glutaraldehyde. Equilibrium IL network was disrupted by shear application and IL network solubilization of BSA and other molecules in this swelling nanoheterogenous media, leads to nanoparticle formation after quenching. Shear stress was applied to induce deformation of nanostructures in the IL network leading to their breaking into smaller ones of approximately uniform sizes. This proposal was schematized in Figure 3.9.

By choosing the right experimental parameters, which were examined in the results and discussion part, like the pH value, concentration of BSA, volume of aqueous solution, right IL type, surfactant/co-surfactant ratios, etc. smaller BSA nanoparticles (~200 nm) with uniform size distribution can be obtained.

This thesis study showed that IM based ionic liquids can be good candidates for use in the preparation of albumin nanoparticles. Their environmentally friendly properties and also low toxicity values renders them as powerful future solvents.

REFERENCES

[1] Loureiro, A.; Azoia, N.; Gomes, A.; Cavaco-Paulo, A., Albumin – based Nanodevices as Drug Carriers. *Current Pharmaceutical Design* **2016**, *22*, 1-20.

[2] Lohcharoenkal, W.; Wang, L.; Chen, Y.; Rojanasakul, Y., Protein Nanoparticles as Drug Delivery Carriers for Cancer Therapy. *BioMed Research International* **2014**, *2014*, 1-12.

[3] Elzoghby, A.; Samy, W. and Elgindy. N., Albumin – based nanoparticles as potential controlled release drug delivery systems. *Journal of Controlled Release* **2012**, *157*, 168 – 182.

[4] Bamrungsap, S.; Zhao, Z.; Chen, T.; Wang, L.; Li, C.; Fu, T.; Tan, W., Nanotechnology in Therapeutics. *Nanomedicine* **2012**, *7*, 1253 – 1271.

[5] Tatlidil, D.; Ucuncu, M.; Akdogan, Y., Physiological Concentrations of Albumin Favor Drug Binding. *Phys. Chem. Chem. Phys.* **2015**, *17*, 22678 – 22685.

[6] Akdogan, Y.; Emrullahoglu, M.; Tatlidil, D.; Ucuncu, M.; Cakan – Akdogan, G., EPR Studies of Intermolecular Interactions and Competitive Binding of Drugs in a Drug – BSA Binding Model. *Phys. Chem. Chem. Phys.* **2016**, *18*, 22531 – 22539.

[7] Hauenschild, T.; Reichenwallner, J.; Enkelmann, V.; Hinderberger, D., Characterizing Active Pharmaceutical Ingredient Binding to Human Serum Albumin by Spin-Labeling and EPR Spectroscopy. *Chem. Eur. J.* **2016**, *22*, 12825 – 12838.

[8] Akdogan, Y.; Reichenwallner, J.; Hinderberger, D., Evidence for Water-tuned Structural Differences in Proteins: An Approach Emphasizing Variations in Local Hydrophilicity. *PLOS ONE* **2012**, *7*, e45681.

[9] Ren, K.; Dusad, A.; Dong, R.; Quan, L., Albumin as a Delivery Carrier for Rheumatoid Arthritis. *J Nanomed Nanotechol* **2013**, *4*, 176 – 178.

[10] Karimi, M.; Bahrami, S.; Ravari, S.; Zangabad, P.; Mirshekari, H.; Bozorgomid, M.; Shahreza, S.; Sori, M.; Hamblin, M., Albumin nanostructures as advanced drug delivery systems. *Expert Opinion on Drug Delivery* **2016**, *13*, 1609-1623.

[11] Chemfiles Volume 5 Article 6 - Ionic Liquids https://www.sigmaaldrich.com/technical-documents/articles/chemfiles/ionic-liquids1.html

[12] Gilmore, B. F. (**2011**). Antimicrobial Ionic Liquids. In Ionic Liquids: Applications and Perspectives (pp. 587-604). Rijeka, Croatia: InTech.

[13] Wang, J. (**2014**). Aggregation in Systems of Ionic Liquids. In Structures and Interactions of Ionic Liquids (pp. 39-77). Berlin, Germany: Springer.

[14] Varela, L. (**2011**). Physical Properties of Binary Mixtures of ILs with Water and Ethanol. A Review. In Ionic Liquids: Theory, Properties, New Approoaches (pp. 111 – 136). Rijeka, Croatia: InTech.

[15] Triolo, A. (**2014**). Structural Organization in Neat Ionic Liquids and in Their Mixtures. In The Structure of Ionic Liquids (pp. 39 – 61). Berlin, Germany: Springer.

[16] Cascão, J.; Silva, W.; Ferreira, A.S.D.; Cabrita, E.J., Ion pair and solvation dynamics of [Bmim][BF4] + water system. *Magn Reson Chem.*, **2018**, *56*, 127–139.

[17] Zanatta, M.; Girard, A.; Marin, G.; Ebeling, G.; Dupont, J., Confined water in imidazolium based ionic liquids. *Phys Chem Chem Phy*, **2016**, *18*, 18297 – 18304.

[18] Wu, B.; Zhang, L.; Zhang, Y.; Wang, H., Unstable Supramolecular Structure of [Bmim][BF_4] in Aqueous Solution. *Chem. Eur. J.*, **2009**, 1, 1-6. DOI:10.1002/chem.200901098

[19] Murgia, S.; Monduzzi, M.; Lopez, F.; Palazzo, G., Mesoscopic structure in mixtures of water and 1-butyl-3-methyl imidazolium tetrafluoborate: a multinuclear NMR study, *J. Solution Chem.*, **2013,** *42*, 1111–1122.

[20] Saihara, K. et al. Properties of Water Confined in Ionic Liquids. *Sci. Rep.* **2015**, *5*, 10619 – 10629.

[21] Dupont, J., On the Solid, Liquid and Solution Structural Organization of Imidazolium Ionic Liquids, *J. Braz. Chem. Soc.* **2004**, *15*, 341 – 350.

[22] Yan, H.; Wu, J.; Dai, G.; Han, D., Interaction mechanisms of ionic liquids [Cnmim]Br (n=4, 6, 8, 10) with bovine serum albumin, *Journal of Luminescence* **2012**, *132*, 622 – 628.

[23] Zhu, L.; Li, G.; Zheng, Y., Interaction of bovine serum albumin with two alkylimidazolium-based ionic liquids investigated by microcalorimetry and circular dichroism, *Journal of Biophysical Chemistry* **2011**, *2*, 146 – 151.

[24] Akdogan, Y.; Junk, M.; Hinderberger, D., Effect of Ionic Liquids on the Solution Structure of Human Serum Albumin, *Biomacromolecules* **2011**, *12*, 1072 – 1079.

[25] Akdogan, Y.; Hinderberger, D., Solvent-Induced Protein Refolding at Low Temperatures, *J. Phys. Chem. B.* **2011**, *115*, 15422 – 15429.

[26] Paterová, J.; Rembert, K.B.; Heyda, J.; Kurra, Y.; Okur, H.I.; Liu, W.R. et al., Reversal of the Hofmeister series: specific ion effects on peptides. *J Phys Chem B* **2013**, *117*, 8150–8158.

[27] Zhao, H., Protein stabilization and enzyme activation in ionic liquids: specific ion effects, *J Chem Technol Biotechnol* **2016**, *91*, 25–50.

[28] Lozano, P.; De Diego, T.; Gmouh, S.; Vaultier, M.; Iborra J.L.; Dynamic structure–function relationships in enzyme stabilization by ionic liquids., *Biocatal Biotransform* **2005,** *23***,**169–176.

[29] Mizuuchi, H.; Jaitely, V.; Murdan, S.; Florence, A.T., Room Temperature Ionic Liquids and Their Mixtures: Potential Pharmaceutical Solvents., *European Journal of Pharmaceutical Sciences* **2008**, *33*, 326 – 331.

[30] Zhou, G.; Luo, Z.; and Fu, X.; Preparation of starch nanoparticles in a water-in-ionic liquid microemulsion system and their drug loading and releasing properties., *J. Agric. Food Chem.* **2014**, *62*, 8214 – 8220.

[31] Zhou, G.; Luo, Z.; and Fu, X., Preparation and characterization of starch nanoparticles in ionic liquid-in-oil microemulsions system., *Industrial Crops and Products* **2014**, *52*, 105-110.

[32] Wang, X.; Chen, H.; Luo, Z.; Fu, X., Preparation of starch nanoparticles in water in oil microemulsion system and their drug delivery properties., *Carbohydr Polym* **2016**, *138*, 192 – 200.

[33] Akdogan, Y.; Heller, J.; Zimmermann, H.; Hinderberger, D., The solvation of nitroxide radicals in ionic liquids studied by high-field EPR spectroscopy, *Phys Chem Chem Phys* **2010**, *12*, 7874 – 7882.

[34] Kattnig, D.R.; Akdogan, Y.; Lieberwirth, I.; Hinderberger, D.; Spin probing of supramolecular structures in 1-butyl-3-methyl-imidazolium tetrafluoroborate/water mixture, *Mol. Phys.* **2013**, *111*, 2723-2737.

[35] Kattnig, D.R.; Akdogan, Y.; Bauer, C.; Hinderberger, D., High-Field EPR Spectroscopic Characterization of Spin Probes in Aqueous Ionic Liquid Mixtures, *Z. Phys. Chem.* **2012**, *226*, 1363 - 1378.

[36] Barbosa, L.R.S.; Ortore, M.G.; Spinozzi, F.; Mariani, P.; Bernstorff; S.; Itri, R., The importance of protein-protein interactions on the pH-induced conformational changes of bovine serum albumin: a small-angle X-ray scattering study, *Biophys. J.* **2010**, *98*, 147 – 157.

[37] Langer, K. et al. Optimization of the preparation process for Human Serum Albumin (HSA) nanoparticles, *Int J Pharm* **2003**, *12*, 169 – 180.

[38] Sebak, S. et al. Human serum albumin nanoparticles as an efficient noscapine drug delivery system for potential use in breast cancer: preparation and in vitro analysis, *Int J Nanomedicine* **2010**, *20*, 525 – 532.

[39] Sharma, N.; Madan, P.; Lin, S.; Effect of process and formulation variables on the preparation of parenteral paclitaxel-loaded biodegradable polymeric nanoparticles: A co-surfactant study, *Asian Journal of Pharmaceutical Sciences* **2016**, *11*, 404 – 416.

[40] Lomis, N.; Westfall, S.; Farahdel, L.; Malhotra, M.; Shum-Tim, D.; Prakash, S., Human Serum Albumin Nanoparticles for Use in Cancer Drug Delivery: Process Optimization and *In Vitro* Characterization, *Nanomaterials*, **2016**, *6*, 1 – 17.

[41] Destree, C. et al. Preparation of organic nanoparticles using microemulsions: their potential use in transdermal delivery, *Langmuir* **2007**, *13*, 1965 – 1973.

[42] Behera, K. et al. Unique role of hydrophilic ionic liquid in modifying properties of aqueous Triton X-100, *J Chem Phys* **2007**, *14*, 184501 – 184510.

[43] Demirkurt, B. and Akdogan, Y., Development of an Ionic Liquid Based Microemulsion – like Method for the Preparation of Albumin Nanoparticles, *ChemistrySelect*, **2018**. Submitted.