CIRCULATING NUCLEIC ACID (CNA) SEPARATION FROM SERUM BY ELECTROSPUN MEMBRANES

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ABSTRACT

CIRCULATING NUCLEIC ACID (CNA) SEPARATION FROM SERUM BY ELECTROSPUN MEMBRANES

Early detection of diseases is a key factor that can be made provision and successfully treated. There is a wide array of methods to diagnose cancer like biopsy, endoscopy, magnetic resonance imaging (MRI) and blood tests. In blood, while some nucleic acids are found in intracellular fluids, circulating nucleic acids (CNAs) circulate freely in extracellular fluids. They are located at genomic regions and increased levels of CNAs imply a connection with cancer and tumor initiation. For further analysis, separation and concentration of CNAs have high potential in early cancer detection. A model system was constructed with bovine serum albumin (BSA) and single stranded Deoxyribonucleic acid (ss-DNA) for the investigation of membrane separation efficiency. The membranes made of poly styrene (PS) and poly(methylmethacrylate) (PMMA) were fabricated by electrospinning and they were placed in syringe columns. By using the absorption spectroscopy, the sorption efficiency of membranes was determined. The electrospun membranes are promising for BSA uptake over a wide pH range. Under the same circumstances, thanks to PS fibers, ss-DNA uptake is very with respect to BSA uptake. Our results revealed that PS membranes show a better affinity to BSA molecules by hydrophobic interactions. In the mixture of BSA and ss-DNA, ss-DNA cannot be held on the surface of the membrane and pass through with low sorption efficiency. By altering the membrane amount in syringe column and modifiying the surface of the membranes, the separation could be enhanced. The proposed technology promises fast and effective separation of CNAs from whole blood and body fluids.

ÖZET

KANDA SERBEST HALDE DOLAŞAN NÜKLEİK ASİTLERİN ELEKTROEĞİRME LİFLER İLE SERUMDAN AYRILMASI

Hastalıkların teşhisi, bir kişinin sağlık durumu hakkında gerekli bilginin sağlanması için önemli bir faktördür. Kanser teşhisi için biyopsi, endoskopi, manyetik rezonans görüntüleme ve kan testleri gibi bir çok metot bulunmaktadır. Teşhiste en çok kullanılan araçlardan biri ise, insan vücudunda önemli bir yere sahip olan kandır. Kan tüm vücudu dolaşan bir sıvı bağ dokusudur ve içeriğinin %55'inde kan plazmasını bulundurur. Kan proteinlerinin serumdan uzaklaştırılması, kan testlerinde çok önemli bir basamaktır. Kanın içeriğinde bazı nükleik asitler hücre içinde bulunurken, bazıları hücre dışında serbest olarak dolanırlar. Araştırma sonuçları kanser hastalığında kanda serbest halde dolaşan bu nükleik asitlerin onkojenik ve tümör baskılayıcı olarak davrandıklarını göstermektedir. Genomik bölgelerde yer aldıklarından, sayılarındaki artış kanser hastalığının başlangıcıyla ilgili bir bağlantı kurulmasına olanak sağlamaktadır.

Bu tezde kanda birlikte bulunan proteinler ve nükleik asitleri basit, ucuz ve de seçici bir yöntemle ayırmak amacıyla kullanmak için elektroeğirme tekniği ile hazırlanmış çeşitli polimerlerden, hedef biyomakromoleküle karşı seçici membran filtreler elde edilmiştir. Tek zincirli DNA ve Bovine Serum Albümini (BSA) kullanılarak hazırlanmış model bir sistem üzerinde elde edilen filtrelerin ayırma kapasitesi incelenmiştir. Elde edilen filtrelerin ayırma kapasitesi pH, filtre yüzeyindeki modifikasyonlar ve fiber çapı gibi parametreleri değiştirerek artırılmıştır. Hidrofobik filtrelerin proteinleri daha çok tutma özelliğine sahip olduğu ve filtre yüzeyine yapılan modifikasyonların ayırma kapasitesi üzerine büyük etkiye sahip olduğu gösterilmiştir.

Dedicated to my parents…

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LIST OF ABBREVIATIONS

AFD	average fiber diameter
BET	Brunauer-Emmett-Teller
BSA	bovine serum albumin
CB	cibacron blue
CNA	circulating nucleic acid
DLS	dynamic light scattering
DNA	deoxyribonucleic acid
DMF	N,N-dimethylformamide
EDX	energy dispersive X-ray
IARC	Internatonal Agency for Research on Cancer
IEP	isoelectric point
IgG	immunoglobulin G
miRNA	micro ribonucleic acid
MRI	magnetic resonance imaging
NP	nanoparticle
OA	oleic acid
PBS	phosphated buffered saline
PCR	polymerase chain reaction
PMMA	poly methylmethacrylate
PS	polystyrene
RNA	ribonucleic acid
SEM	scanning electron microscope/microscopy
ss-DNA	single stranded deoxyribonucleic acid
TGA	thermogravimetric analysis
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1. Motivation

Today, many people face with the health problems that require a considerable significance and these problems have potential to result in fatality. Recently, fatal diseases such as cancer, liver failure, and immunodeficiency show increment. Cancer disease particularly causes growing number of deaths worldwide. According to International Agency for Research on Cancer (IARC), 14.1 million new cancer cases and 8.2 million cancer deaths occurred in 2012. The explorations of World Health Organization (WHO) show that 28 types of cancer diseases give rise to these deaths and incidences. Among all other types of cancers, 1.6 million new cases are diagnosed in lung cancer each year and this statistics represents 13% of the all cancer diseases. Likewise, with 1.38 million deaths, lung cancer is the most commonly diagnosed cancer type that results in death. (Ferlay et al. 2010)

There is a wide array of methods to diagnose cancer like biopsy, endoscopy, magnetic resonance imaging (MRI) and blood tests. Currently, mammography is a good choice for screening of early breast cancer. However, this technique requires high cost equipment for diagnosis. (William D. Travis 2004) Among the other methods, blood tests have the top priority. Recently, lab-on-a-chip or lab-on-a-paper concepts have great attention for biomedical applications and miniaturization of analytical devices due to low cost, ease of fabrication, and ease of use. (Pham 2012)

Biomarkers play an important role for the development of biosensors or diagnosis of diseases. The important class of biomarkers is circulating nucleic acids (CNAs) which are extracellular nucleic acids found in cell free plasma of humans. They travel several miles every day and diffuse every cell of the body. CNAs are nucleated at the genomic regions, thus they have a unique position to understand "*What is going on in our body?*" They are found in healthy people and originate from lymphocytes. The increased levels of them indicate some diseases and this increase can be used for

diagnosis with a non-invasive, rapid, sensitive and accurate method. (Swarup and Rajeswari 2007)

Considering the critical role of CNAs, separation of these nucleic acids from blood has a great importance. However, using the blood sample as is in a test could be problematic due to the high content of protein that have risk of reducing the testing accuracy. (Schrauder et al. 2012) Protein content in the blood sample may shed the presence of RNA, which play the key role in detection. Separation of proteins from the blood sample can be efficient approach to obtain reliable and reproducible testing. In this study, electrospun fiber membrane systems based on commodity polymers such as polystryrene (PS) and poly(methyl methacrylate) (PMMA) were developed to make an efficient separation of circulating nucleic acid from a model protein.

1.2. Structure and Scope of the Thesis

The first chapter of thesis gives an introduction and purpose of the research. In *chapter 2*, a comprehensive literature review is done. This part includes detailed information about blood, nucleic acids, proteins and macromolecule separation methods. Experimental part is given in *chapter 3*, and in *chapter 4* results will be given with their discussions. In *chapter 5,* concluding remarks will be explained.

1.3. State of the Art: Electrospinning

Electrospinning is as an efficient method that has been recognized for the fabrication of continuous polymer fibers with diameters down to a few nanometers. (Demir et al. 2002) When the diameters of fiber materials decrease from micrometers to nanometers, several characteristics show up like very large surface area to volume ratio, flexibility and mechanical performance. Initially polymers were accepted as fiber forming materials, and then ceramics and glasses were started to be considered as fiber precursors. (Greiner and Wendorff 2007)

By virtue of being a very simple and easily controlled technique, the utilization of electrospinning has spread into all fields of work such as energy storage, biomedicine, tissue engineering, cell adhesion, sensor applications, catalytic applications, and filtration. (Wu et al. 2013)

In a typical electrospinning set up, there are three basic components to consummate the process: a high voltage supplier, a capillary tube with a pipette or needle of small diameter, and a metal collecting screen. During the process, the polymer solution or melt is pumped through the capillary tube and a high voltage is used to create an electrically charged jet of polymer out of the tube. The nozzle of the capillary tube serves as an electrode and 100–500 kV m^{-1} electric field is applied. The polymer solution evaporates and solidifies while it is transported through the electric field. Then, interconnected web of fibers are formed on the collecting screen. (Huang et al. 2003) Figure 1.1 shows the typical electrospinning set up with a horizontal arrangement of electrodes.

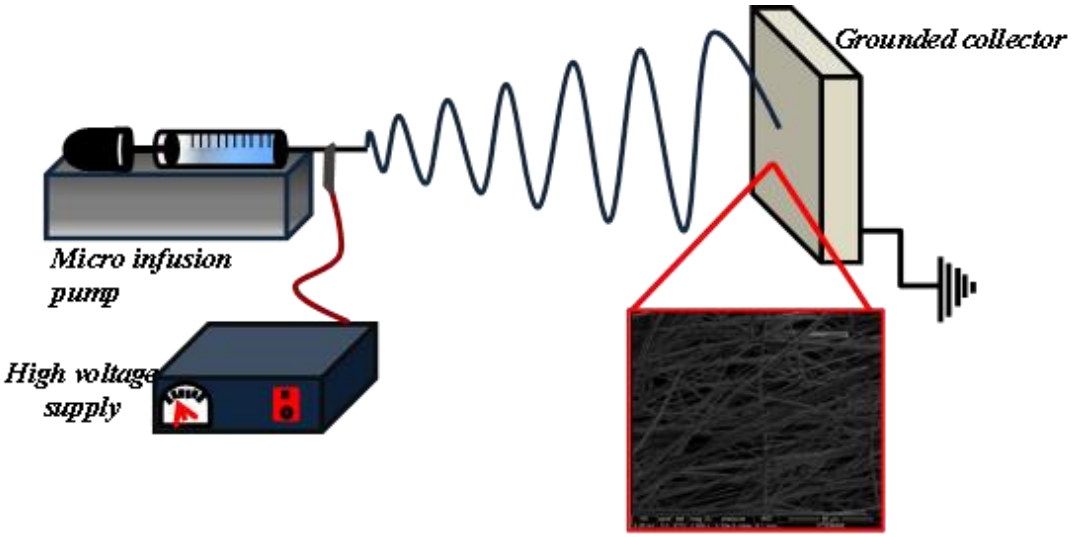

Figure 1.1. Schematic representation of electrospinning set up

CHAPTER 2

LITERATURE REVIEW

2.1. Blood

Blood fluid is a connective red fluid that circulates in our body. It has so many functions like transportation, protection, and regulation. About 45% of blood fluid consists of cells, which are red blood cells, white blood cells, and platelets. The remaining 55% volume consists of plasma, which transports water and other nutrients to our body tissues. (Figure 2.1) (Gopal and Yuejun 2013) While red blood cells carry oxygen by the help of hemoglobin, white blood cells defend the body against infection. Also platelets help in blood clotting. (Sherwood 2004)

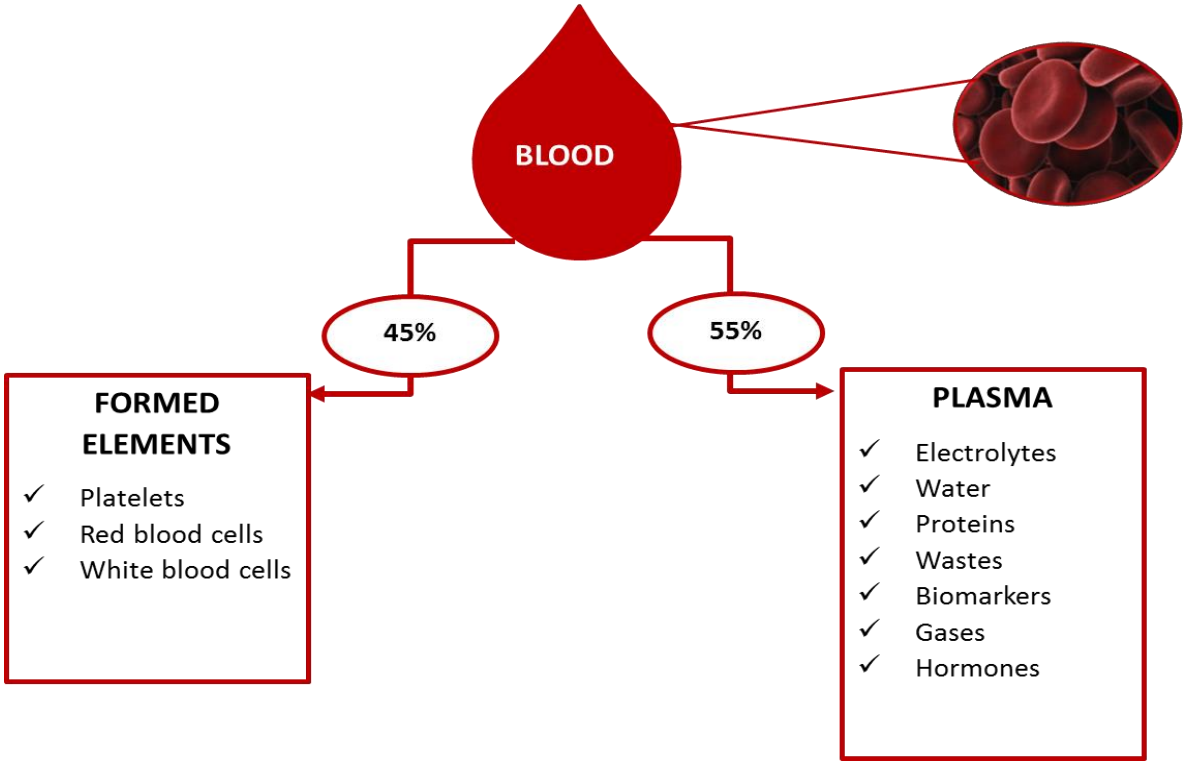

Figure 2.1. The overview of blood components

2.2. Biomarkers

Biomarkers, which are also named as tumor markers, are important measures of a biological state. According to the definition of US National Institutes of Health's (NIH) and Biomarkers Consortium, biomarker is a unique part of biological molecules that can be found in blood, other body fluids (saliva, urine, etc.), or tissues. When a certain type of cancer is present, the amount of biomarkers increases. With the advances in technology, levels of nucleic acid can be measured that are important biomarkers for the disease diagnosis. Table 2.1 shows selected types of biomarkers, their locations in our body and relevant diseases. (William Bigbee 2003)

Table 2.1. Types of biomarkers and their relevant diseases

	Types of Biomarkers	Purpose	Place	Disease
1	Alpha fetoprotein (AFP)	Diagnose and treatment	Blood	-liver cancer -germ cell tumors
2	Beta-2-microglobulin (B2M)	Diagnose	Blood	-myeloma -chronic lymphocytic leukemia
3	Bladder tumor antigen (BTA)	Diagnose	Urine	-bladder cancer
4	CA 15 - 3	Diagnose	Blood	-breast cancer
5	BRAF	Diagnose	In BRAF gene	-melanoma -thyroid cancer
6	Carcinoembriyonic antigen (CEA)	Diagnose	Blood	-colorectal cancer
7	Human chorionic gonadotropin (HCG)	Diagnose	Blood	-germ cell tumors
8	Immunoglobuling	Diagnose	Blood	-bone marrow cancer (myeloma)
9	Lactate dehydrogenase (LDH)	Diagnose and detect tissue damage	Blood	-testicullar cancer -germ cell tumors
10	Neuron specific enolase (NSE)	Diagnose	Blood	-small cell lung cancer -carcinoid tumor
11	Prostate specific antigen (PSA)	Diagnose	blood	-prostate cancer
12	Epidermal growth factor receptor (EGFR)	Diagnose	In EGFR protein	-lung cancer -colon cancer -pancreas cancer -breast cancer
13	Thyroglobulin	Diagnose	Blood	-thyroid cancer
14	Anaplastic lymphoma kinase (ALK)	Diagnose	In ALK gene	-lung cancer

Biomarkers are helpful in a number of ways like early detection of cancer or monitoring its evolution.

- *Early detection of cancer:*

  Early detection indicates the finding the disease at early stages, when it's less likely to spread and relatively easier to treat. The most widely used tumor marker is the prostate specific antigen (PSA) blood test to screen men for prostate cancer.

- *Diagnosing cancer:*

  Biomarkers cannot be used alone for diagnosis; they can help to figure out where it started. For an example, the level of alpha fetoprotein (AFP) rises up with some liver diseases.

- *Prognosis for certain diseases:*

  Some cancer types grow and spread faster than others and the level of biomarker can help the prediction of behavior of cancer. In testicular cancer, very high levels of human chorionic gonadotropin (HCG) sign a more aggressive cancer treatment.

- *Seeing how treatment works:*

  If the level of biomarker in the blood decreases, this indicates that treatment helps recovery. However, increasing levels of biomarkers indicate that treatment must be changed.

- *Monitoring the recurrent cancer:*

  Biomarker levels must be periodically checked after the treatment to prevent the recurrent cancer.

Cancer biomarkers can be divided into 3 categories. (Figure 2.2.) Nucleic acids have been identified as important biomarkers for biosensor development and disease diagnosis. Ideally, the biomarkers should be easily accessible for diagnosis. Thus, taking them from body fluids, like serum or urine, is more desirable. Recent researches indicate that in serum both cell free deoxyribonucleic acid (DNA) and micro ribonucleic acid (miRNA) are present and they called as circulating nucleic acids (CNAs) which are potential biomarkers.(Gilad et al. 2008)

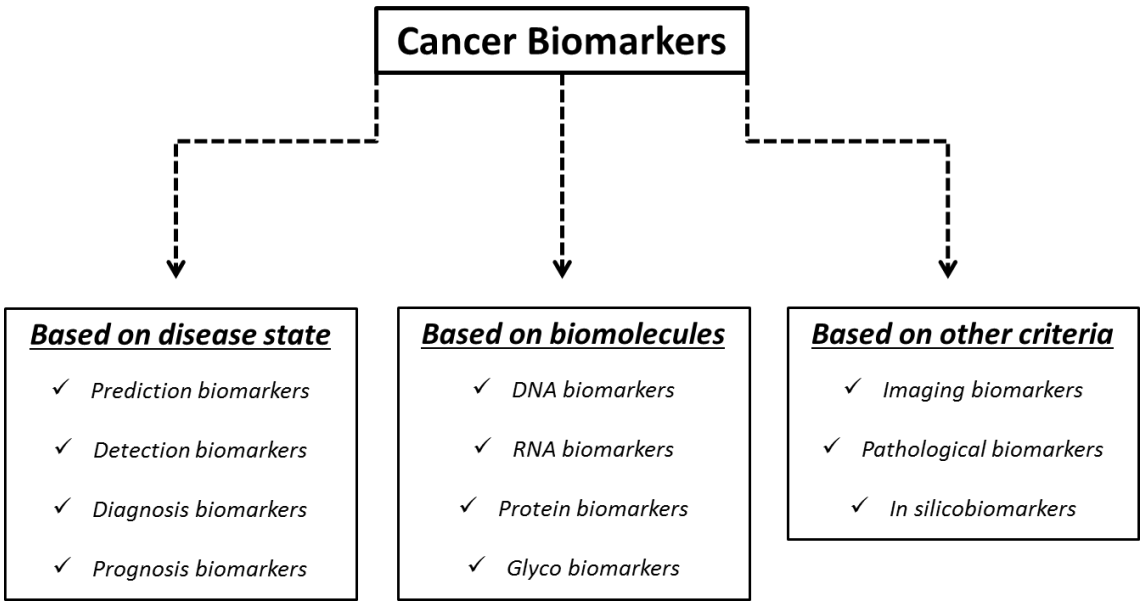

Figure 2.2. Classification of cancer biomarkers
(Verma 2010)

2.3. Nucleic Acids

Nucleic acids are large biopolymers and their repeating unit is nucleotide that has three components as 5-carbon sugar, a phosphate group and a nitrogenous base. Based on the sugar type, nucleic acids are classified into two groups as deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). They are found in all living organisms and have important functions such as encoding, transmitting, and genetic information expression. (Dahm 2008)

The structure of nucleic acids shows different characteristics under different pH media. While nucleic acids are denatured at high pH, they have depurination at low pH and double helix melts which results in the loss of information. The phosphodiester backbone of nucleic acid hydrolyzes by breaking the glycosidic bond between base and deoxyribose at acidic environment. (Brennan 2015) Hydroxyl ion concentration has a very important contribution for changes in nucleic acid structure. At high pH, hydroxyl ion concentration increases and hydroxyl groups interact with the hydrogen bonds leading to the separation of the strands. The stability of DNA is better than the RNA, because DNA loses the hydroxyl group on the 2′ position during the separation. In contrast to DNA, hydroxyl group of RNA can give its hydrogen to the solution and a highly unstable alkoxide is formed. (Brennan 2015)

RNA differs from DNA due to its 2 important chemical properties;

i. It has a ribose sugar due to having a hydroxyl group attached to 2′ position
ii. It has a uracil base rather than thymine. (Neidle 2007)

Most of the nucleic acids are located within cells; however the segments of DNA or RNA are also found circulating freely in the blood called Circulating Nucleic Acids (CNAs)**.** (Figure 2.3)

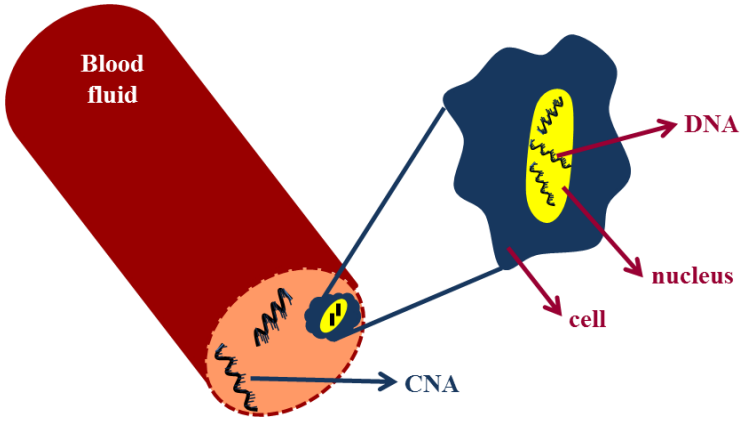

Figure 2.3. Schematic representation of circulating nucleic acids (CNAs)

The origin of CNAs was a concern and it was found that they originate from apoptotic and necrotic cells according to a range of studies. (Gahan and Swaminathan 2008) Leon *et al*. reported that the amount of CNA during the breast cancer ranged from 0 to 2 μg mL^{-1} in patients serum. (S. A. Leon 1977) Pathak *et al.* demonstrated that cell death and necrosis were induced after radiation therapy. CNA levels decreased for 40% of the patients. (Pathak et al. 2006)

MicroRNA's are a kind of single stranded RNA molecules with 20-23 nucleotides that they are a novel class of endogenous, non-coding RNAs. They play key roles in the regulation, transformation, and degradation of messenger RNA. (Yanaihara et al. 2006) Primary miRNA transcripts are generated by RNA polymerase II. (Kimura 2013, Jansson and Lund 2012) In 1993, the first miRNA was investigated and there are several types of miRNAs that each of them indicates different diseases as biomarkers. (Esquela-Kerscher and Slack 2006)

In various human cancers, the circulating miRNAs have been investigated. (Croce 2012, Lu et al. 2005) Fayyad-Kazan *et al*. studied plasma miR150 and miR342 biomarkers for the diagnosis of acute myeloid leukima (AML). (Fayyad-Kazan et al. 2013) The decrease in miRNA levels indicates that miRNAs act as suppressor of oncogenes in human cancers and they prevent cell growth. (Malumbres 2012, Yanaihara et al. 2006) For example; miR125 and miR34 are related with breast cancer, miR10 relates with colorectal and liver cancers. The first identified oncogenic miRNA is miR21 and it takes place on the basis of many cancer types including lung cancer. (Díez 2010, Morat; 2014, Le Quesne and Caldas 2010) The detection of miRNAs was monitored by a paper based biosensing platform. (Yildiz, Alagappan, and Liedberg 2013) They used luminescent reporters to detect mir21 and different optical signals were obtained to enable rapid, sensitive and selective determination of miRNA.

2.4. Proteins

The blood plasma contains 90% of water and other blood proteins comprise an important part of the plasma. Protein molecules contain α-helical and β-sheet structures, while α-helices are rigid peptide bonds and β-sheets are extended forms. The protein level in blood plasma is approximately 70 g L^{-1} and there are 3 types of proteins.

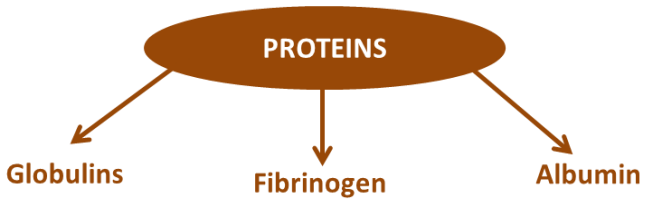

Figure 2.4. Types of proteins

2.4.1.Globulins

They are small portion of the blood proteins; however they perform very significant functions such as providing antibodies, transporting vitamins and hormones. They are divided into categories according to their electrophoretic mobility as α-globulins, γ-globulins and β-globulins. (Hamlett 2015)

2.4.2.Fibrinogen

This class of proteins has a mission for blood clot formation and produced in the liver. Also, they are important biomarkers for the determination cardiovascular disease risk factors. (Hamlett 2015)

2.4.3.Albumin (Serum Albumin)

Serum albumin molecules compose the largest amount among the other blood proteins and have important properties such as acidity, high solubility and stability. Serum albumin protein is synthesized in the liver. (Curry et al. 1998)

Serum albumin molecule has 4.2 × 14.1 nm dimensions when it is in native structure at pH 7, and it is a multidomain protein that contains 9 loops which are subdivided into 3 domains. It is a polar molecule with 100 negative and 82 positive charges. The highest charge is found in the N- terminal and the lowest is in the C-terminal. (Peters 1985)

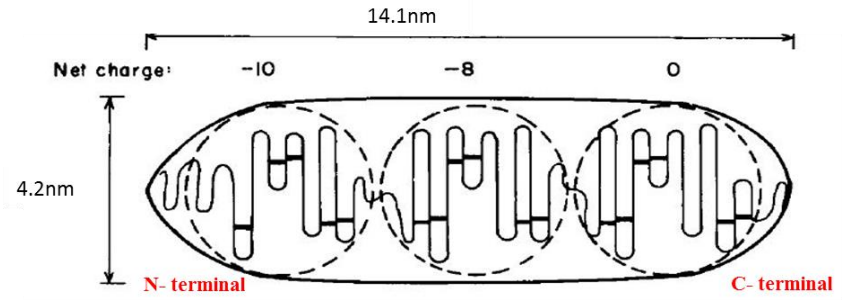

Figure 2.5. Serum albumin molecule domains
(Peters 1985)

The domains of serum albumin molecule are dominantly α-helical and they include 17 disulphide bonds. While α-helical bonds are rigid peptide bonds but they are free to rotate, β-sheets are extended conformations. (Lehninger 1993, El Kadi et al. 2007) Disulphide bonds are derived from 2 thiol groups of cysteine and they have covalent characteristics. Cleavage of these bonds results in unfolding while the formation makes molecule folded. (Nakamura et al. 1997)

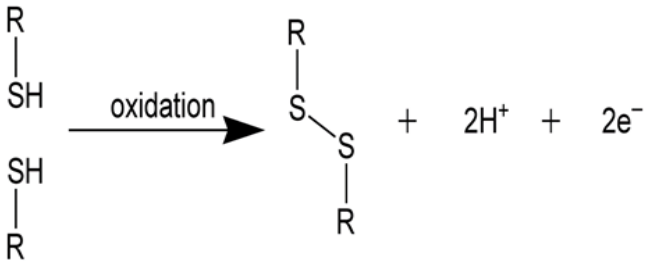

Figure 2.6. Disulphide bond cleavage
(Nakamura et al. 1997)

The structure of BSA contains approximately 600 amino acid units. (Eckenhoff and Johansson 1997)Non-polar amino acids comprise a large amount of molecule while aromatic amino acid residues have low content. These low contents of aromatic acids are responsible for the UV-absorbance of serum albumin molecule at 280 nm. (Boehm et al. 2007)

MKWVTFISLL LLFSSAYSRG VFRRDTHKSE IAHRFKDLGE EQFKGLVLIA FSQYLQQCPF DEHVKLVNEL TEFAKTCVAD ESHAGCEKSL
HTLFGDELCK VASLRETYGD MADCCEKQEP ERNECFLSHK DDSPDLPKLK PDPNTLCDEF KADEKKFWGK YLYEIARRHP YFYAPELLYY
ANKYNGVFQD CCQAEDKGAC LLPKIETMRE KVLASSARQR LRCASIQKFG ERALKAWSVA RLSQKFPKAE FVEVTKLVTD LTKVHKECCH
GDLLECADDR ADLAKYICDN QDTISSKLKE CCDKPLLEKS HCIAEVEKDA IPENLPPLTA DFAEDKDVCK NYQEAKDAFL GSFLYEYSRR
HPEYAVSVLL RLAKEYEATL EECCAKDDPH ACYSTVFDKL KHLVDEPQNL IKQNCDQFEK LGEYGFQNAL IVRYTRKVPQ VSTPTLVEVS
RSLGKVGTRC CTKPESERMP CTEDYLSLIL NRLCVLHEKT PVSEKVTKCC TESLVNRRPC FSALTPDETY VPKAFDEKLF TFHADICTLP
DTEKQIKKQT ALVELLKHKP KATEEQLKTV MENFVAFVDK CCAADDKEAC FAVEGPKLVV STQTALA

Figure 2.7. Bovine serum albumin sequence
(Boehm et al. 2007)

The conformation of proteins depends on the nature of the dispersing medium. Shear and thermal stress, pH and temperature affect the stability and conformation of proteins. As an example, Figure 2.8 shows the relationship between the hydrodynamic radius and the scattered light intensity for a protein mixture. (Arzenšek 2010)

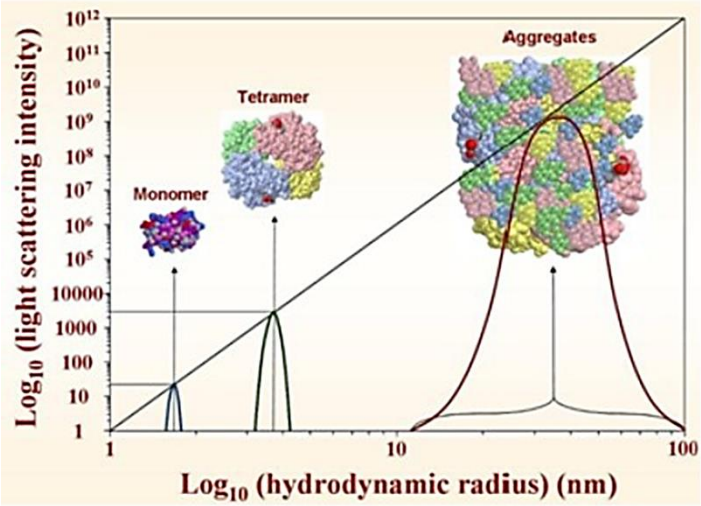

Figure 2.8. Dynamic light scattering spectra of a BSA-Lysozyme mixture (Arzenšek 2010)

Serum albumin molecule shows different characteristic properties at different pH media. Isoelectric point (IEP) of molecule is about 4.8 and conformation of protein changes around the IEP. (Fu et al. 2014a) When the pH is around the IEP, the backbone of polypeptide chain is extended to a zigzag character and α-helix content decreases and this conformation results in unfolded structure. When the pH of the medium is away from the IEP, α-helical content increases and folded structure is formed. (Lehninger 1993) When the pH is above the 8, serum albumin molecule undergoes structural isomerization and structure becomes linear. After pH 10, second structural isomerization and deamidation occurs by forming an aged structure which is more linear. During the deamidation, an amide functional group is removed and a carboxylate group is formed. (Robinson and Robinson 2001, Burt et al. 2004, Clarke 1987)

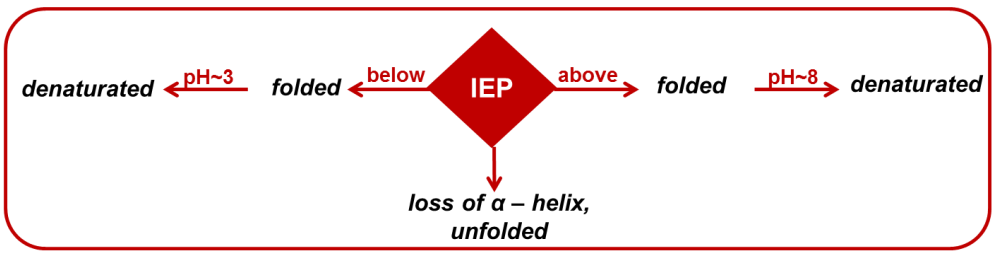

Figure 2.9. Behaviour of proteins at different pH media

Serum albumin molecules prefer hydrophobic surfaces rather than hydrophilic ones and adsorption of molecules occurs randomly. Serum albumin binding occurs in the following order;

Hydrophobic $> COO^- > NH_3^+ > OH^-$ > Ethylene glycol (Brewer et al. 2005)

Protein adsorption is driven by different sorbent-surface interactions including electrostatic and hydrophobic forces which make this process complex. The decrease in the Gibbs free energy governs the adsorption process under constant temperature and pressure. (S. Ghosh A. 1963)

$$\Delta G_{ads} = \Delta H_{ads} - T\Delta S_{ads} < 0 \quad (2.1)$$

The abbreviations G, H, and S indicate Gibbs free energy, enthalpy and entropy, respectively. Also, T is the absolute temperature in Kelvin. The decrease in the enthalpy (ΔH_{ads}) or the increase in the entropy (ΔS_{ads}) results in the negative change in the Gibbs free energy.

The conformational changes are the primary driven forces for the adsorption by decreasing or increasing the conformational entropy. These changes arise from the side chain orientations and alteration of main torsional angles in polypeptides. (Gerstein, Lesk, and Chothia 1994) Serum albumin is a globular protein having a highly ordered and low entropy structure. During the adsorption, protein molecules form various contacts with the surface and the structure may break down. The breakage of the structure leads to a change in the secondary structure and conformational entropy increases. (Norde 1998) The large entropy gain characterizes the hydrophobic interactions and the release of the water molecules from the hydrophobic components has a small enthalpy effect. The increase in the entropy and a small change in the enthalpy lead to a decrease in the Gibbs free energy in equation 2.1.

Even if the globular proteins have conformational changes, they are always subjected to a compact structure and do not adsorb in a train-loop-tail like conformation. (Figure 2.10)

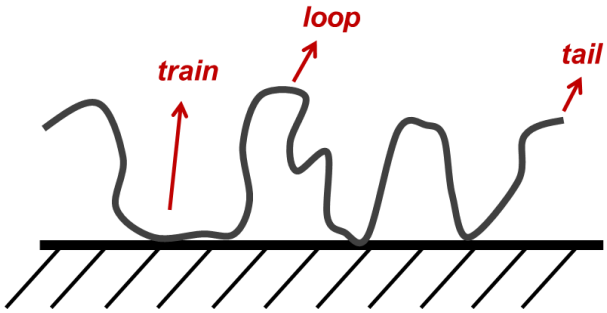

Figure 2.10. Partially adsorbed polymer chain on a surface

The equilibrium relationship between the surface and adsorbate can be described by adsorption isotherms. There are three adsorption isotherm models for bovine serum albumin (BSA) adsorption on the surfaces. Langmuir adsorption isotherm is one of the most commonly used adsorption model to quantify the amount of adsorbate on the surface as a function of solution concentration at a given temperature. (Latour 2015) This isotherm model assumes that the formation of monolayer adsorbate on the surface continues until no adsorption takes place and it is based on a reversible process. Another adsorption isotherm model is Freundlich isotherms which are empirical relations between the concentration of adsorbent on the surface and the solute in solution. Freundlich isotherms do not have a restriction as Langmuir isotherms and describes the adsorption characteristics for heterogeneous surface. (Asnin, Fedorov, and Chekryshkin 2000) Temkin isotherm models consider the interactions between the adsorbent and adsorbate. Also, this model assumes the decrease in heat of adsorption as linear. (Dada 2012)

Langmuir model: $$\frac{C_{eq}}{Q_{eq}} = \frac{1}{K_L Q_m} + \frac{1}{Q_m} C_{eq} \quad (2.2)$$

Freundlich model: $$\ln Q_{eq} = \frac{1}{n} \ln C_{eq} + \ln K_F \quad (2.3)$$

Temkin model: $$Q_{eq} = K_T \ln C_{eq} + K_T \ln f \quad (2.4)$$

In these equations Q_{eq} and Q_m represent the equilibrium and maximum adsorbed mass per unit mass substrate, respectively. C_{eq} represents the equilibrium concentration of adsorbent, and other parameters indicate the constants. (Han et al. 2009)

Hydrophobic surfaces can thermally stabilize the folded state of adsorbed proteins, because unfolded state experiences the loss of energy during the adsorption. (Sharma, Berne, and Kumar 2010) The reason of having more extended conformations with surface is that loops and tails of serum albumin molecules have more entropic freedom. When the net charge of molecule is high, the molecule will be in its extended conformation. Having small energy forms more globular conformations. (Lee and Ruckenstein 1988)

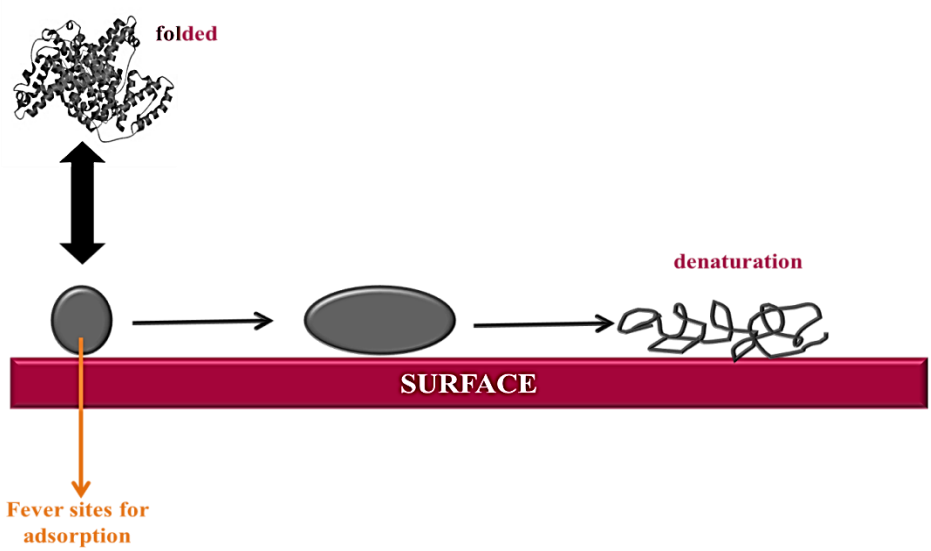

Figure 2.11. Schematic representation of protein adsorption on a surface

The existence of globular protein molecules requires low entropy when comparing with unfolded and extended structures. Conformational entropy, hydrophobic-electrostatic and dispersion interactions are important factors that determine the protein adsorption on the surfaces. (Norde 1996)

2.5. Methods for Macromolecule Separation

Purification and isolation of nucleic acids are significant steps in most molecular biology studies. There are number of available methods for purification and isolation like extraction/ precipitation, chromatography, electrophoresis, and affinity separation. (Roche 2011)

- *Extraction/precipitation:*

For the removal of proteins, a combination of phenol and chloroform is used as a solvent extraction technique. Compaction precipitation is another method for the recovery of small DNA or RNA fragments from the blood. Compaction agents are cationic molecules that interact with the phosphate backbone of nucleic acids. The conformation of nucleic acid changes to a compact form and can be precipitated out of the solution. However, using higher concentration of these agents reduces the sensitivity. (Vu et al. 2012)

- *Chromatography:*

The molecular sieving properties make privilege the porous gel particles in gel filtration technique. While ion exchange chromatography uses the electrostatic interactions between the target molecule and functional groups on column, adsorption chromatography takes the advantage of selective adsorption of nucleic acids on silica or glass in the presence of a chaotropic salt. Also, affinity chromatography is an exclusive technique that uses an immobilized ligand to bind a part on biomolecule and removes unbound components. Then, a competitor molecule releases the bound molecules from ligand. (Roche 2011) The affinity membrane chromatography is performed by immobilization of specific ligands on to the membrane surface and adsorption of target biomolecules on these ligands. (Saxena et al. 2009) (Figure 2.12)

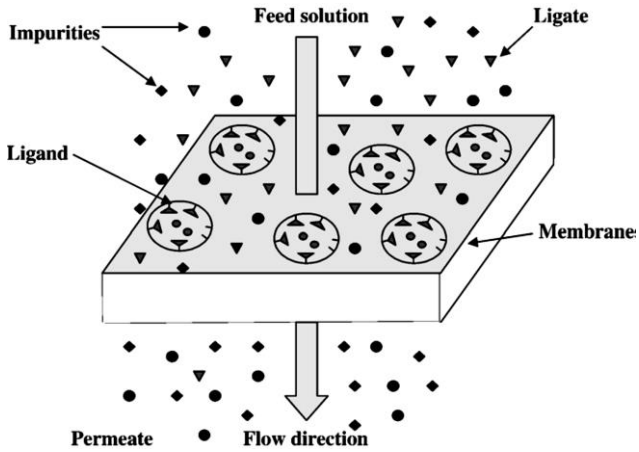

Figure 2.12. Schematic representation of membrane chromatography (Saxena et al. 2009)

- *Electrophoresis:*

  By using the electrophoretic properties of nucleic acids, they can be purified in terms of their size. Another method is dielectrophoretic isolation which has a considerable potential for rapid, simple, and cost effective diagnostics. It requires small amount of samples. (Sonnenberg et al. 2014)

- *Affinity purification:*

  Several centrifugation, extraction, and phase separation steps are replaced with a single, rapid magnetic separation step. (Roche 2011)

Polymerase chain reaction (PCR) and microfluidic systems are other important techniques for the separation and isolation of nucleic acids. (Boom et al. 1990)

Before the modern membrane industry come to the fore, membranes have been used for bioseparation processes. In 1936, a review article was published that described the utilization of membrane technology for enzymes, cell and protein free ultrafiltrates, and sterile filtration. (Ferry 1936)

Membranes act as an interphase and a barrier to the flow of molecular and ionic species in liquids or vapors. In the design of a membrane, selectivity, volumetric flux, and the system capacity are the key characteristics. (van Reis and Zydney 2007) Due to their selective transportation and efficient separation properties, there is a no requirement for additives during the separation processes. (Saxena et al. 2009)

Even though all membranes can be used for bioseparation, the pressure driven processes like ultrafiltration, microfiltration, and virus filtration have major concern among the other techniques. (Figure 2.13) While ultrafiltration membranes have pore sizes between 1 and 20 nm, microfiltration ones have 0.05 and 10 μm, and virus filtration membranes have 20 and 70 nm. (van Reis and Zydney 2007)

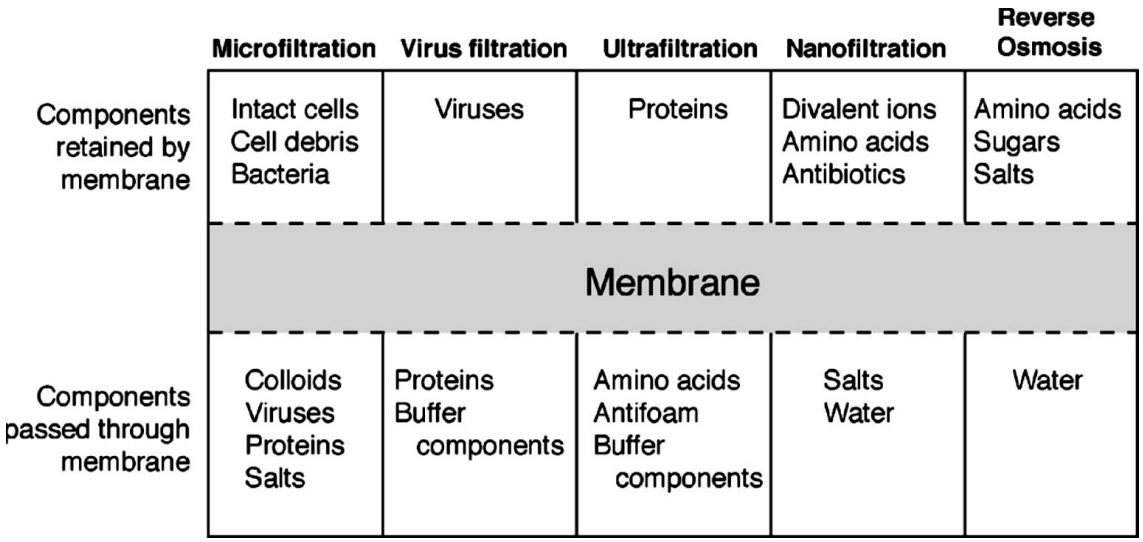

Figure 2.13. Applications of different membrane processes
(van Reis and Zydney 2007)

Mathew *et al.* submitted a study for the fabrication for protein resistant and adhesive surfaces with chitosan/ polystyrene sulfonate (CHI/ PSS) multilayer membranes. They demonstrated that the variation of pH, number of bilayers, time and mode of adsorption can change the adsorption behavior of bovine serum albumin (BSA) and lysozyme. (Mathew et al. 2012)

Protein adsorption is a complicated issue, because there are several factors affecting the process involving van der Waals forces, hydrophobic and hydrophilic interactions, and hydrogen bonding. Roach *et al.* indicated that adsorption on to the hydrophobic surfaces induces higher deformation rather than hydrophilic surfaces.(Roach, Farrar, and Perry 2005) By changing the surface properties like size, chemistry and curvature, the adsorption characteristics of proteins can be explored. In another work, Roach *et al.* demonstrated the surface curvature effect on hydrophobic and hydrophilic surfaces for globular and rod like proteins. Serum albumin was adsorbed in higher amounts with the decreasing substrate size. However, fibrinogen has two possible adsorption orientations: side on and end on. Thus, when the substrate size is small, fibrinogen loses its secondary structure. (Roach, Farrar, and Perry 2006)

Electrospinning is a versatile technique for the fabrication of nanofibers with a controllable composition, diameter, and porosity for a variety of applications. Electrospun nanofibrous membranes are privileged among the other membranes due to having some interesting properties;

i. High porosity that comes from the interconnected structure

ii. Enhanced specific surface area and hydrophobicity

iii. Unique topography

iv. Differing surface chemical properties and composition

By virtue of its high porosity and great surface area, electrospun fiber mats are studied in several applications such as drug delivery, fuel and solar cells, textile industry, sensor and filtration applications. (Figure 2.14)(Kaur et al. 2014)

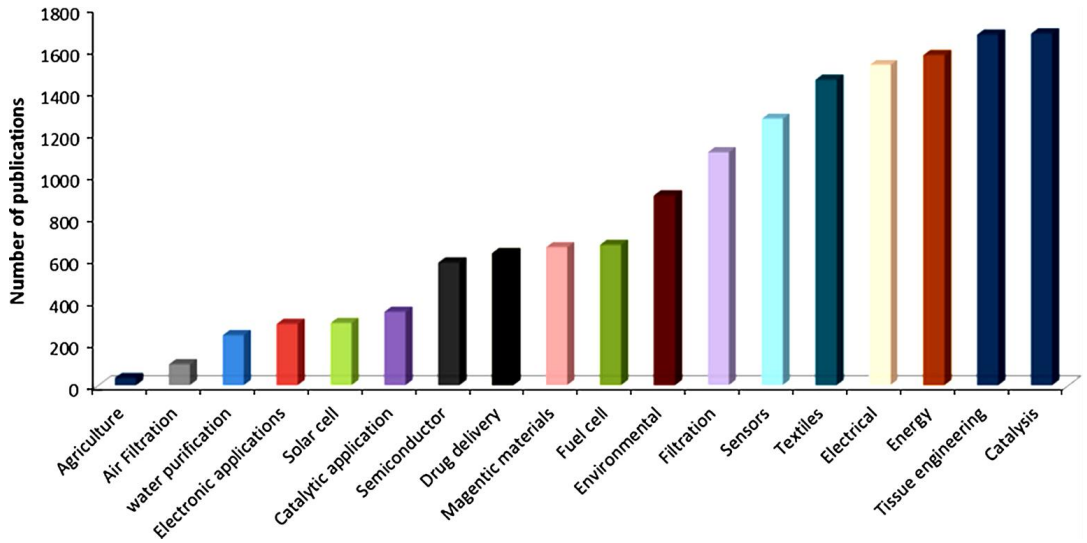

Figure 2.14. Applications of electrospun membranes
(Kaur et al. 2014)

In recent years, liquid filtration applications have gained interest and affinity membrane preparation was developed. In this technique, purification of molecules is based on the physical, chemical or biological functions rather than the molecular weight or size. The affinity membranes provide selectivity to capture the molecules by the immobilization of specific ligands on the membrane surface. (Ma, Masaya, and Ramakrishna 2006)

An approach developed for the adsorption of bovine serum albumin (BSA) and bilirubin by using the regenerated cellulose acetate (CA) membranes. The cellulose acetate membranes have poor mechanical strength and they treated by heat to improve the tensile modulus and strength. (Figure 2.15)

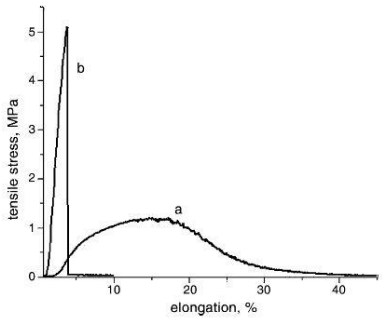

Figure 2.15. Tensile stress curve of (a) the untreated CA nanofiber membrane and (b) the heat treated CA nanofiber membrane
(Ma, Kotaki, and Ramakrishna 2005)

Before the immobilization of cibacron blue (CB) dye, which captures protein, the surface of the membrane became hydrophilic by alkaline treatment. Finally, CB immobilized regenerated cellulose nanofiber affinity membranes were used for the BSA and bilirubin adsorption tests. (Figure 2.16) (Ma, Kotaki, and Ramakrishna 2005)

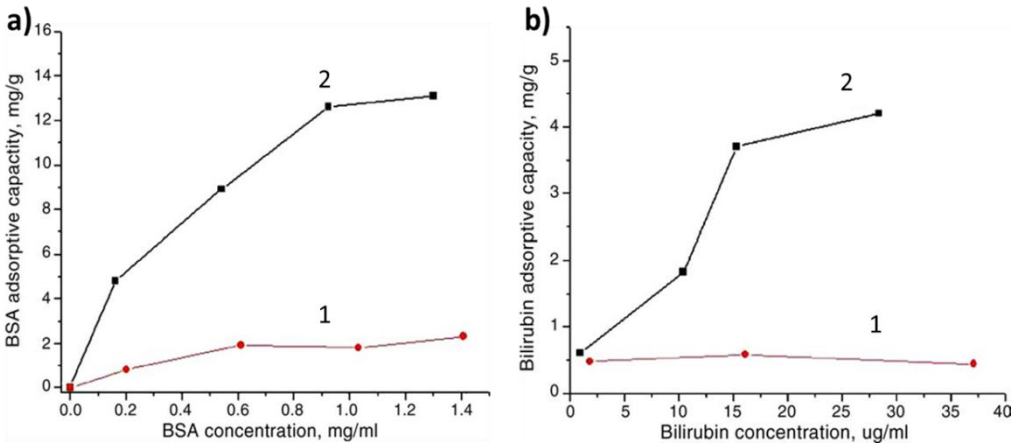

Figure 2.16. BSA (a) and bilirubin (b) adsorption capacity on the (1) regenerated cellulose membrane and (2) CB immobilized regenerated cellulose membrane
(Ma, Kotaki, and Ramakrishna 2005)

Ma *et al.* proposed a research which aims the purification of Immunoglobulin G (IgG) from a model impurity protein by using an affinity membrane. Figure 2.17 shows the chemical reaction scheme for ligand immobilization on the membrane. They prepared a spin column with round shaped membranes and performed IgG adsorption though. For the investigation of the dynamic binding efficiency of membranes, bovine serum albumin (BSA) was used as a model protein.

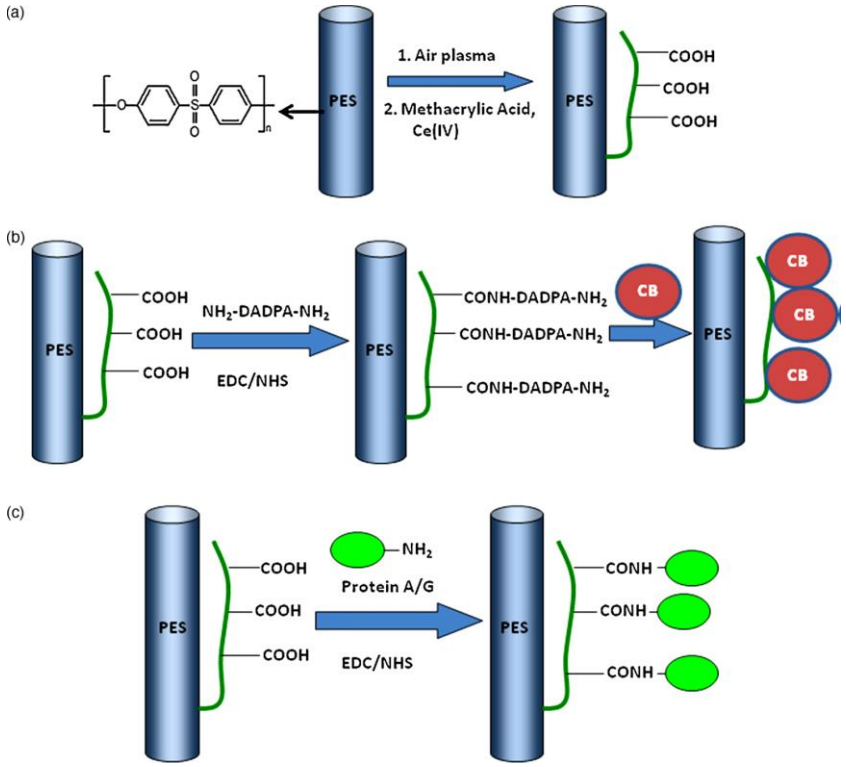

Figure 2.17. Immobilization of polyethersulfone membrane with cibacron blue (CB) and protein A/G (Ma et al. 2009)

Figure 2.18 shows that protein A/G immobilized affinity membranes are good candidates for the small-scale and fast protein purification. (Ma et al. 2009)

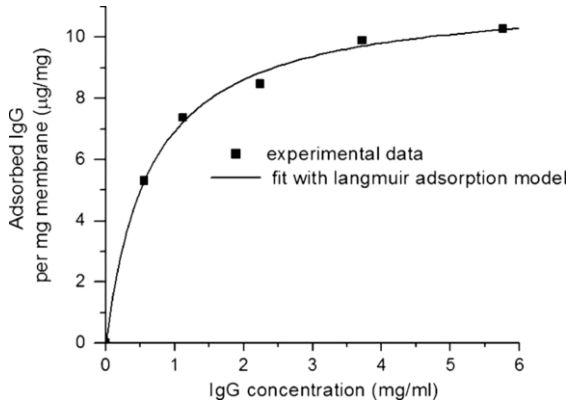

Figure 2.18. Adsorption curve of IgG on protein A/G immobilized PES affinity membranes (Ma et al. 2009)

Leong *et al.* published a paper which investigates the protein adsorption by various types of scaffolds. Electrospun fiber nano porosity has an outstanding effect on cell attachment and protein adsorption. Figure 2.19 shows the amount of proteins adsorbed by different scaffolds. (Leong et al. 2009) Porous electrospun scaffolds shows the highest adsorption capacity among the others.

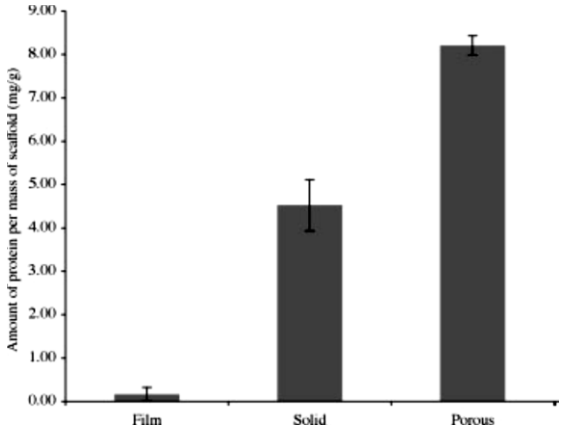

Figure 2.19. Adsorption profile of BSA on different scaffolds (Leong et al. 2009)

Regarding the purification of enzymes, Landry and Levin proposed an affinity membrane system with DNA - coated polyvinylidene fluoride (PVDF). By virtue of the affinity between DNase and DNA, DNase could be purified from the proteins with this membrane system. Figure 2.20 shows the preparation of affinity membrane system. (Landry and Levin 2014)

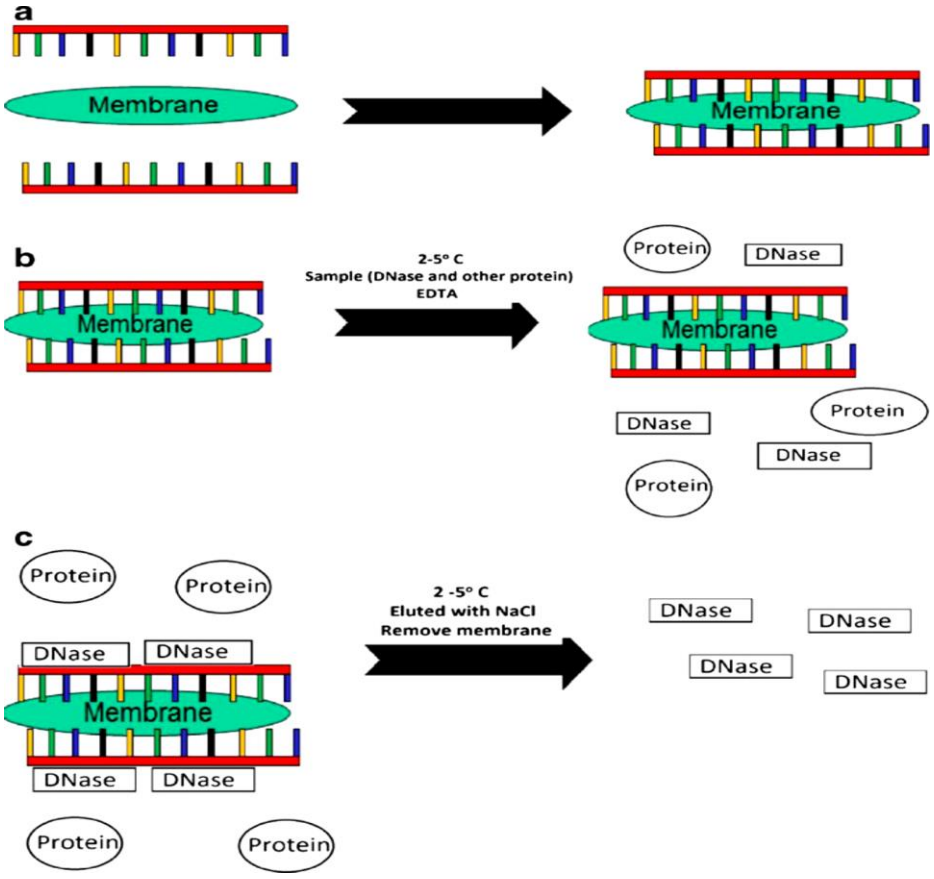

Figure 2.20. Schematic representation of the affinity membrane purification system (Landry and Levin 2014)

The affinity membranes are not only used for the filtration of biomacromolecules but also for the filtration of polyatomic heavy metal ions from the aqueous solutions. Horzum *et al.* demonstrated that polyacrylonitrile (PAN) fibers could be used for the removal of aqueous uranyl ions. Figure 2.21 shows the modification of PAN fibers by amidoximation reaction to transform the surface of the membrane from hydrophobic to hydrophilic. Thereby, the nitrile groups on the acrylonitrile side chain are converted to amidoxime groups and the membrane network provided a high metal binding affinity.

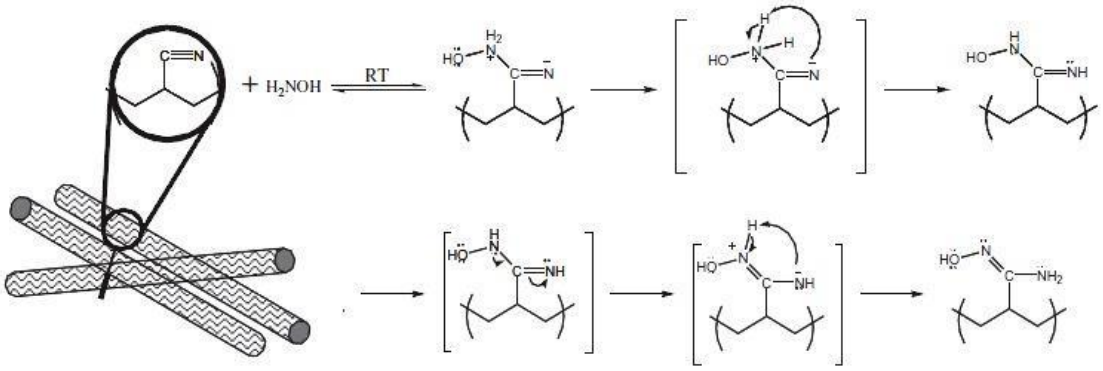

Figure 2.21. The conversion of nitrile groups to amidoxime groups in PAN fiber (Horzum, Shahwan, et al. 2012)

They studied the repetitive usability of modified membranes at different concentrations. At high ion concentrations, the mobility of ions increases due to the ionic repulsions and they can compete for sorption sites readily. (Figure 2.22) (Horzum, Shahwan, et al. 2012)

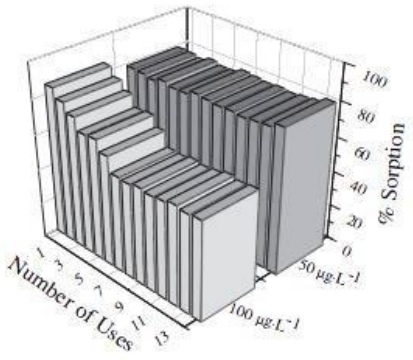

Figure 2.22. Variation of percent sorption of uranyl ion with the number of repetitive usages of the same fiber sorbent at the initial uranyl ion concentrations (Horzum, Shahwan, et al. 2012)

CHAPTER 3

EXPERIMENTAL SECTION

3.1. Materials

Poly(methyl methacrylate) (PMMA, *Mw* ≈ 350,000 g mol^{-1}, Aldrich), polystyrene (PS, *Mw* ≈ 350,000 g mol^{-1}, Aldrich), Bovine Serum Albumin (BSA, ≥98%, Sigma-Aldrich), and single strand-DNA (ss-DNA) oligonucleotide 5'-TAG-CTT-ATC-AGA-CTG-ATG-TTGA-3' (Sigma-Aldrich) were used without any further purification. Phosphate Buffer Saline (PBS, Sigma), sodium phosphate (20 mM, pH 9.0, Fluka), tris-EDTA (100x for molecular biology, Sigma), and acetate (pH 5.12, Fluka) buffer solution, hydrochloric acid (HCl, 37%, Sigma-Aldrich) and sodium hydroxide (NaOH, ≥98%, Sigma-Aldrich) were used for pH adjustment. Gold (III) chloride hydrate ($HAuCl_4$, 99.99%, Aldrich), trisodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$,>99%, Sigma-Aldrich) and oleic acid (OA, 90%, Alfa Aesar) were used for surface modification experiments. *N*,*N*-Dimethylformamide (DMF, 99%, Carlo Erba), ethanol (≥99.5%, Sigma-Aldrich) and water (molecular biology reagent, Sigma) were used as solvents. For the fluorescence spectroscopy measurements, fluorescent tag DNA 5'- / 56-FAM/GGT-TGG-TGT-GGT-TGG/ 3Dab/ -3' was purchased from Integrated DNA Technologies (IDT) and used without further purification. Coomasie blue stain was supplied from Sigma. Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$, Merck), calcium chloride ($CaCl_2$, Aldrich), D (+) Glucose (Sigma), potassium chloride (KCl, Merck), urea (ACS reagent, 99.0 – 100.5 %, Sigma Aldrich), and sodium chloride (NaCl, Merck) were used without further purification.

3.2. Fabrication of nanofiber membranes

PMMA (10, 15, 20 wt%) and PS (15, 25, 30 wt%) were separately dissolved in DMF at different concentrations, and stirred for 24 hours until homogeneous solutions were achieved. For the fabrication of PS/OA fibers, oleic acid were added into PS (15wt %) solution. The volume ratio of PS:OA was 100:1 and 10:1.

The polymer solutions were transferred to 20 mL plastic syringe with stainless steel needle (18 gauges) and the needle was connected horizontally to a high voltage supply under 15 kV potential (Gamma High Voltage Research Ormond Beach, FL, US). By using a microsyringe pump (LION WZ-50C6), the flow rate was kept at 5 mL h^{-1} and tip to collector distance was 15 cm. Fluffy PS and mat like PMMA fibers were obtained on the grounded aluminum plate. Figure 3.1 shows typical electrospinning setup for fabrication of fibrous membranes.

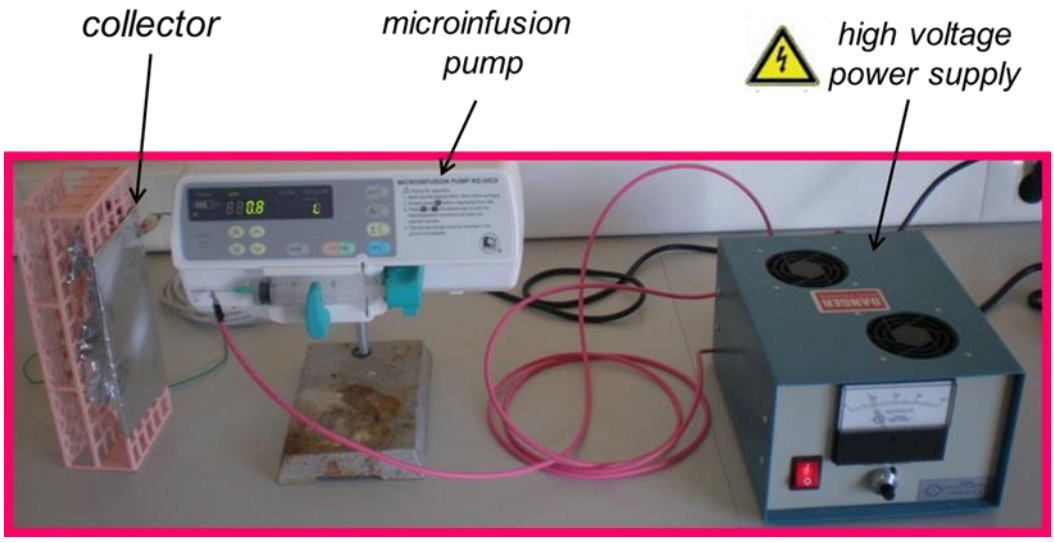

Figure 3.1. Schematic representation of electrospinning setup

The PS and OA modified PS electrospun membranes were immersed in gold salt solution (3.25 mM, 40 mL/g fiber) at room temperature and immobilization process took 68 hours on an orbital shaker at 240 rpm (IKA-WERKE Yellow Line, Germany). Subsequently, the reduction of gold ions on membranes was induced by adding trisodium citrate solution (8.2 mM, 40 mL/g fiber) and the solution were continued to shake for further 4 hours. Then, the membranes were removed from the solution and dried under room temperature.

The morphology of electrospun membranes was observed using Scanning Electron Microscope (FEI Quanta250 FEG, Oregon, USA). EDX results were obtained for elemental analysis and fiber diameter distributions were estimated by statistically from SEM micrographs using ImageJ software. The surface area of electrospun membranes was measured using Brunauer-Emmett-Teller (BET) gas adsorption method (Micromeritics Gemini V, Georgia, USA).

3.3. Syringe columns packing and filtration

The electrospun membranes were used as sorbent for the removal of BSA molecules from model solution. As-prepared membranes were placed into the homemade syringe column having 15 mm inner diameter (Figure 3.2).

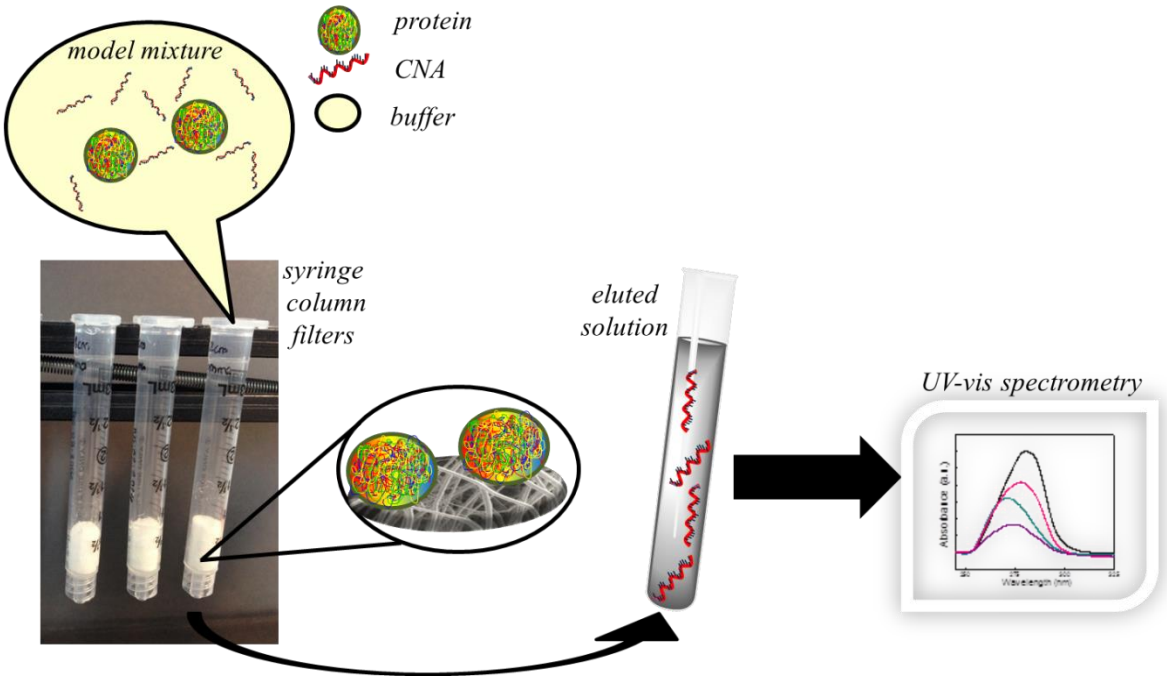

Figure 3.2. Scheme of filtration process

PBS was dissolved in 200 mL deionized water and yielded 0.01 M phosphate buffer. Tris-EDTA buffer was received in 100x concentration and diluted to 1x in water to have pH 8 buffer solution, and HPCE was diluted to 1 mM with water to get a pH 9 buffer. Sodium acetate buffer solution was used as received for pH 5.12, and for other pH mediums HCl and NaOH were used.

DNA was dissolved in PBS buffer in 100 mM concentration. To avoid freeze and thaw cycle, 100 µM DNA was diluted to 1 µM fractions in 1.5 mL volumes, and molten samples were not frozen again. All of the BSA solutions were prepared in PBS buffer except that in different pH mediums. The behavior of BSA in different pH medium was investigated by Dynamic Light Scattering measurements (Malvern Nano ZS, Worcestershire, UK).

The mixture of BSA and DNA solutions was prepared in 1:1 volume with 1 μM and 4 μM concentrations, respectively.

As-prepared syringe columns were wetted with 1 mL of PBS buffer before filtration study. Then, the analyte solutions were passed through the electrospun membranes and eluted solutions were analysed with UV – visible spectrometer (UV SHIMADZU 2450, Japan). For ethanol treated PS membranes, the columns were wetted with ethanol instead of PBS.

For the investigation of membrane amount effect, columns were prepared with 5, 10, 20, 40 and 80 mg membranes and the same process was applied for filtration study.

Coomasie Blue solution (prepared in 50%methanol-10%acetic acid-40%water mixture) was immobilized on electrospun membranes after BSA filtration for 30 min at 150 rpm. Then, the dye was removed and de-staining solution was treated for 10 min.

Artificial serum solution was prepared by using 4.5 mM KCl, 5 mM CaCl2, 1.6 mM MgCl2, 4.7 mM d(+)-glucose, 2.5 mM urea, 0.1% human serum albumin, and 145 mM NaCl. (Uygun and Sezginturk 2011) The separation efficiency of membranes was investigated with the same procedure before mentioned.

Bradford Assay is used as a second spectroscopic analytical technique to measure the protein amount in the solutions. The Bradford Reagent was prepared in a known protocol by dissolving 0.1g Coomasie in 50 mL absolute ethanol and 100 mL phosphoric acid, then the solution was diluted to 250 mL to get 5x reagent. Before the analysis, the reagent was diluted to 1x with water. The reagent and unknown were mixed in 9:1 (v : v) ratio, then the absorbance of the samples were measured at 595nm wavelength by UV spectrophometry.

After filtration study, the electrospun membranes were analyzed by SEM to investigate the changes on fibers. Also, FTIR-ATR measurements were done for the membranes before and after filtration.

Figure 3.3 demonstrates the approach of this study in a schematic representation. The enrichment of the DNA in eluted solution will be provided by the increasing the BSA amount on the fiber.

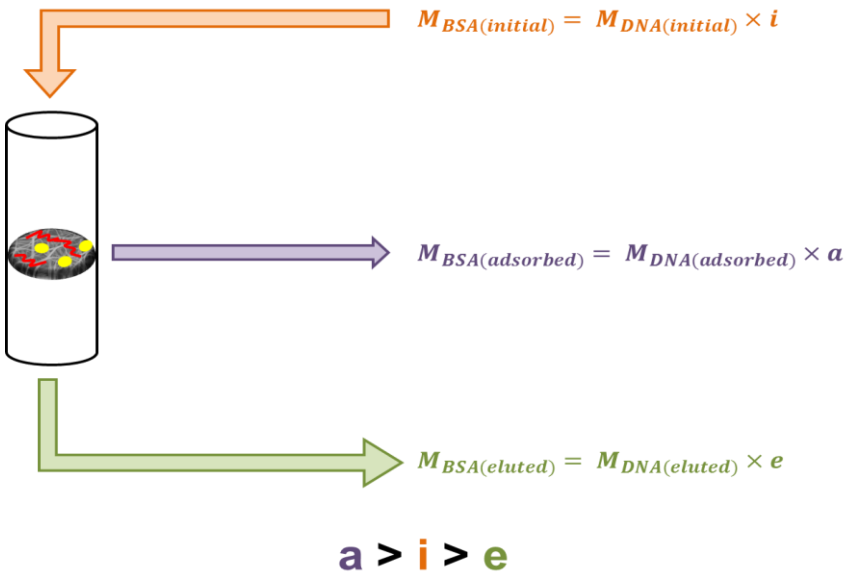

Figure 3.3. Schematic representation of the approach for the enrichment of DNA in eluted solution

CHAPTER 4

RESULTS AND DISCUSSION

In body fluids, DNA and RNA are located within the cells, but a small amount of nucleic acid is found circulating freely in the blood. Circulating nucleic acids (CNAs) are extracellular nucleic acids found in cell-free plasma/serum of humans. The increased levels of CNA in blood of patients indicated that CNA can be used as a non-invasive, rapid, sensitive and accurate method of diagnosis of several diseases such as cancer and tumor. This study asserts a convenient approach for the development of affinity membrane for the separation of CNAs. The separation efficiency of electrospun polymeric membranes was examined with bovine serum albumin (BSA) as a model protein and single stranded DNA (ss-DNA) as CNA. BSA is a commonly used serum albumin protein in immunodiagnostic procedures, protein chemistry researches, and molecular biology laboratories. The conformation of BSA is thought to be similar to human serum albumin (HSA), because 76% of the amino acid sequence is identical. (Huang, Kim, and Dass 2004) The membranes based on isotactic polystyrene (PS) and poly(methyl methacrylate) (PMMA) were fabricated by electrospinning. Our approach is based on the decreasing the BSA amount in eluted solution by increasing the adsorbed BSA amount on membranes. Hereby, the amount of DNA in the eluted solution will be higher than that of BSA. Figure 4.1 demonstrates the cross-sectional view of the polystyrene membrane. The thickness of the membranes and its equivalent weight are approximately 150 μm and 5 mg, respectively.

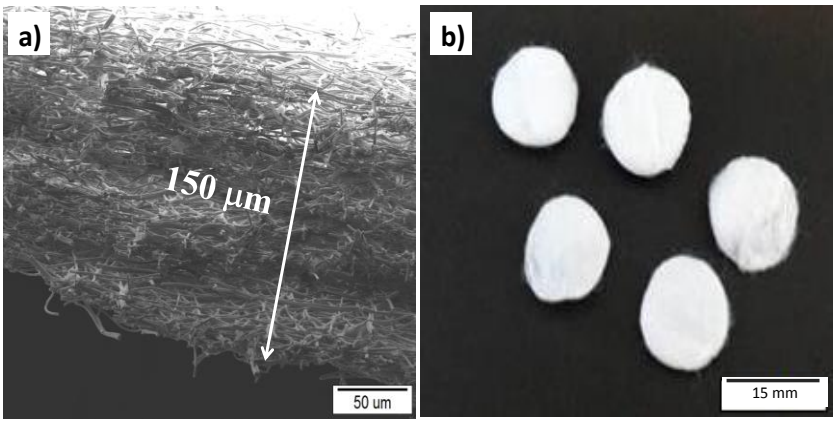

Figure 4.1. (a) SEM micrograph of the prepared electrospun membrane showing the thickness at the cross section, and (b) Photographic image of the membranes placed into the syringe columns

To determine the working concentration range of BSA solution, a set of absorbance measurements were performed. Figure 4.2 presents calibration curve of aqueous BSA solutions at different concentrations ranging from 5×10^{-7} to 1×10^{-5} mol L^{-1}. A linear function was fit to the experimental data points. The regression value of the linear function exceeds 0.99 indicating that there is a perfect agreement between the concentrations of BSA and its absorbance value. The working concentration range was selected as 10^{-5} and 10^{-6} mol/L for this study in line with the physiological conditions of human body. (Liu et al. 2004) The equation given in Figure 4.2 was used for the quantitative determination of BSA in supernatant solution after filtration process.

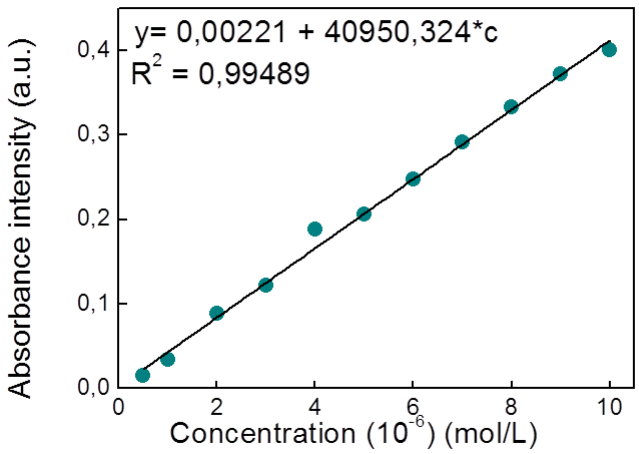

Figure 4.2.Calibration curve of BSA

Both poly (methyl methacrylate) (PMMA) and polystyrene (PS) nanofiber membranes were prepared by employing similar pathways. PMMA and PS were dissolved separately in *N*,*N*-Dimethylformamide (DMF) at different concentrations. The resulting homogeneous solutions were electrospun using a horizontal set up at 1.0 kV cm^{-1}. Figure 4.3(a-c) shows the SEM micrographs of PMMA fibers with the diameter distribution histogram. The increase in PMMA solution concentration (from 10wt % to 20wt %) results in the increment in average fiber diameter (AFD) from 1.1 to 9.5 µm. Higher solution concentration gives rise to an increase in viscosity and thicker fiber. Due to the increasing viscoelastic forces, stretching of the jet may be difficult. Therefore, the electrical charges cannot provide adequate strength to stretch the solution to form fibers. These results are compatible with the literature that indicates effect of concentration on electrospun fiber diameter. (Gu and Ren 2005, Mit-uppatham, Nithitanakul, and Supaphol 2004) Demir *et. al.* reported the dependency of AFD on solution concentration as a power law relationship. (Demir et al. 2002)

$$AFD = (Concentration)^3 \tag{4.1}$$

The concentration of polymer solution plays a critical role in determining the fiber diameter during the electrospinning. We also found that fiber diameter of PMMA increases as the third power of solution concentration. (see Appendix A1 for the plot showing AFD as a function of concentration)

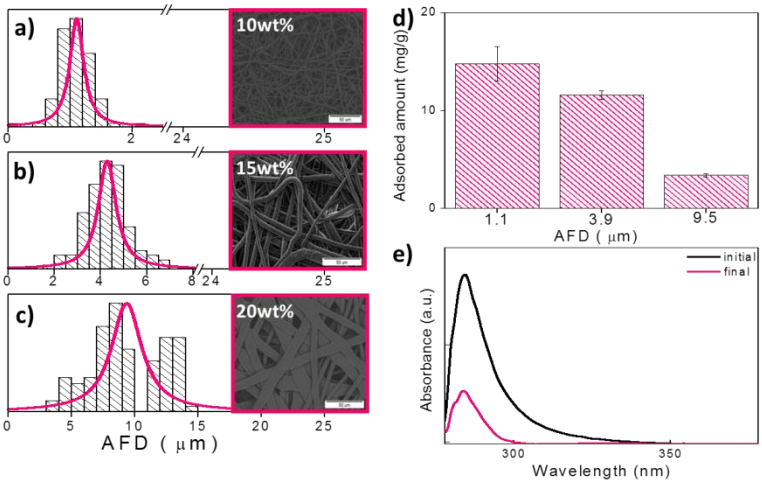

Figure 4.3.(a-c) SEM micrograph and fiber diameter distribution of the PMMA membranes, (d) The effect of AFD on BSA adsorption, and (e) UV-vis spectra for initial and final BSA solutions

The adsorbed amount of BSA solution on fibers with different AFD is illustrated in Figure 4.3d. The extent of adsorbed amount of BSA solution decreased with the increase in AFD of the fibers. As AFD increases, the sieve size of the membranes formed by the space between electrospun fibers increases, and surface area decreases. (Horzum, Munoz-Espi, et al. 2012) Therefore, BSA molecules can pass through these gaps and cannot be adsorbed on the fiber surface. On the other hand, the thinner fibers with larger surface area have more adsorption capacity. The representative UV spectra of BSA solution before and after filtration process are shown in Figure 4.3e. The apparent decline in the absorbance intensity suggests the adsorbed BSA molecules on the membrane.

The electrospun micrographs and diameter distributions of PS fibers are presented in Figure 4.4(a-c). Not surprisingly, the mean of fiber distribution shifts to larger diameters with the increase in polymer solution concentration. The fiber diameters lie between 0.8 and 9.5 µm.

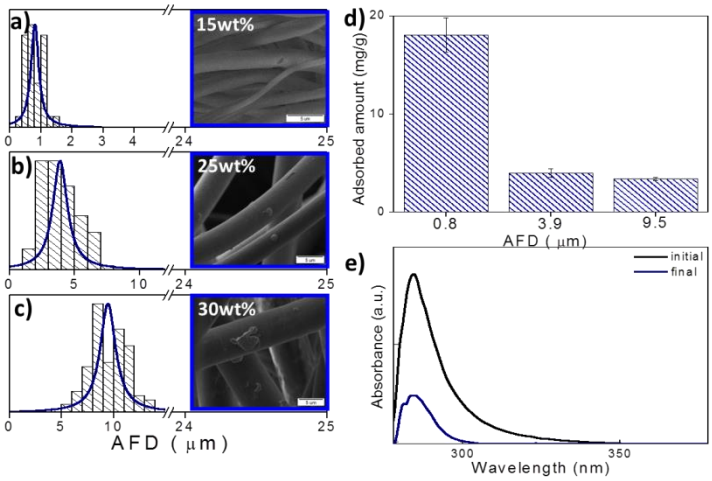

Figure 4.4. (a-c) SEM micrograph and fiber diameter distribution of PS membranes, (d) The effect of AFD on BSA adsorption, and (e) UV-vis spectra for initial and final BSA solutions

Figure 4.4(d-e) shows the BSA adsorption efficiency of the PS membranes as a function of AFD. Similar to PMMA membranes, with the decreasing fiber diameter and increasing surface area of the fibers, the extent of adsorbed amount of BSA solution on PS membranes increased. On the other side, PS membranes show higher sorption capacity than that of PMMA. The reason might be explained by four main cases of

forces and interactions; *i)* ionic or electrostatic interaction, *ii)* hydrogen bonding, *iii)* hydrophobic interaction, and *iv)* charge-transfer interactions.

BSA is composed of ~600 amino acid residues (Eckenhoff and Johansson 1997) and each of them has neutral or charged pendant groups. The charged amino acid subunits contribute protein interaction via ionic or hydrogen bonds. On the other hand, the neutral-polar side chains mainly make hydrogen bonds, and the neutral-nonpolar side chains commonly take part in hydrophobic interactions with other molecules. Both protein molecules and PS surface have aromatic residues in their structures. Polystyrene (PS) surfaces are preferred by serum albumin molecules due to π-π interactions. Interaction between the serum albumin and PS arene rings increases the binding property.

Conformational rearrangements of BSA also affect adsorption on the membranes because of varying polarity. The illustration of folding/unfolding of a protein chain is depicted in Figure 4.5. The folding allows hydrophilic amino acids (blue spheres) to interact with water, and the hydrophobic amino acids (pink spheres) gather together within the protein core.

Unfolded structures of proteins show high hydrophobicity, flexibility and reactivity. (Lai 2006, Lomakina and Mikova 2006) While the sorption of serum albumin occurs on hydrophobic surfaces, secondary structure shows irreversible changes. The α-helix content is reduced and β-turns increases. However β-sheet percentage remains same before and after adsorption of serum albumin molecule. (Norde and Giacomelli 2000, Lynch and Dawson 2008) Thus, at IEP, electronic repulsions are minimal and tendency to hydrophobic surfaces increases. Also increasing the surface hydrophilicity results in the resistance to protein binding. (Liang and Kristinsson 2005, Cho et al. 2012)

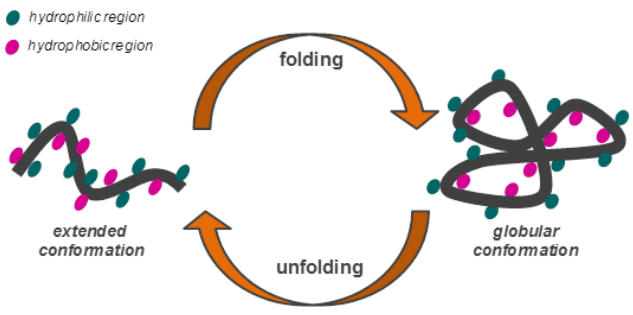

Figure 4.5. Folding and unfolding mechanism of protein structure in an aqueous medium

From a thermodynamical point of view, Gibbs free energy (ΔG_{ads}) of the system must be negative for the spontaneous adsorption of proteins to the surfaces. When non-polar groups present in both protein and PS structure are exposed to the aqueous solutions, the hydrophobic regions get dehydrated and the Gibbs free energy decrease. According to the Gibbs free energy expression (Equation 4.2), an increase in the entropy (ΔS_{ads}) makes Gibbs energy negative. (Dietschweiler 2008, Lee and Ruckenstein 1988)

$$\Delta G_{ads} = \Delta H_{ads} - T\Delta S_{ads} < 0 \qquad (4.2)$$

Since adsorption is a surface-based phenomenon, surface morphology affects the BSA adsorption properties. Figure 4.6(a-b) shows the SEM micrographs of PS and PMMA fiber membranes indicating the surface dissimilarities of these membranes. While PS fibers have porous and rough surfaces, PMMA ones have smooth and non-porous surface.

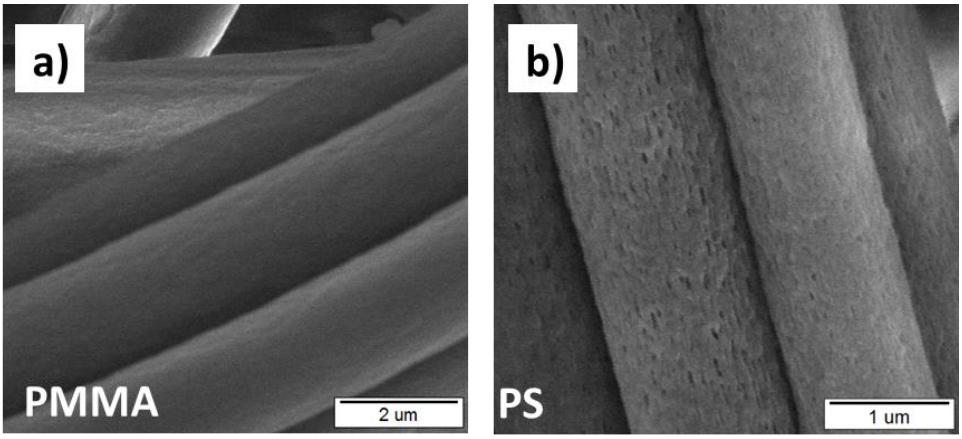

Figure 4.6. SEM micrographs showing (a) The smooth surface of PMMA membrane, (b) The porous surface of PS membrane

BET analysis of the measured nitrogen adsorption on PS and PMMA membranes given in Table 4.1. The surface area of PS and PMMA fibers are 27.1 and 7.3 $m^2\ g^{-1}$, respectively. PS fibers, due to its porous nature, have 4-fold larger surface area than the PMMA fibers. The increment in surface area leads to higher BSA adsorption capacity on PS fibers.

Table 4.1. BET results for liquid nitrogen adsorption on electrospun PMMA and PS membranes

Membrane type	AFD (µm)	BET surface area(m^2/g)	Langmuir surface area(m^2/g)	Pore size (A)	Single point area (m^2/g)
PS	3.9	27.1	37.9	582	22.7
PMMA	3.9	7.3	9.7	208	6.6

BSA molecules have their maximum size as about 12 nm at the pH of 5.1, which is around the IEP of BSA, and the pore size of PS membranes is 2-fold larger than the BSA molecule. This result can explain the reason of enhanced adsorption capacity of PS membranes.

The Langmuir, Temkin and Freundlich adsorption isotherms are the most commonly used models for the protein adsorption experiments. These isotherms could be obtained only by the batch adsorption experiment results, because the required time for the interaction of sorbent and the adsorbent could not be supplied during column adsorption. Also, most of the adsorption experiments in the literature preferred batch sorption to make assumption about the adsorption isotherm models. (Ma et al. 2009, Deng and Yu 2015, Horzum et al. 2010) Figure 4.7 demonstrates the breakthrough curves for PMMA and PS membranes during the 40 cycles of BSA passage through the syringe columns.

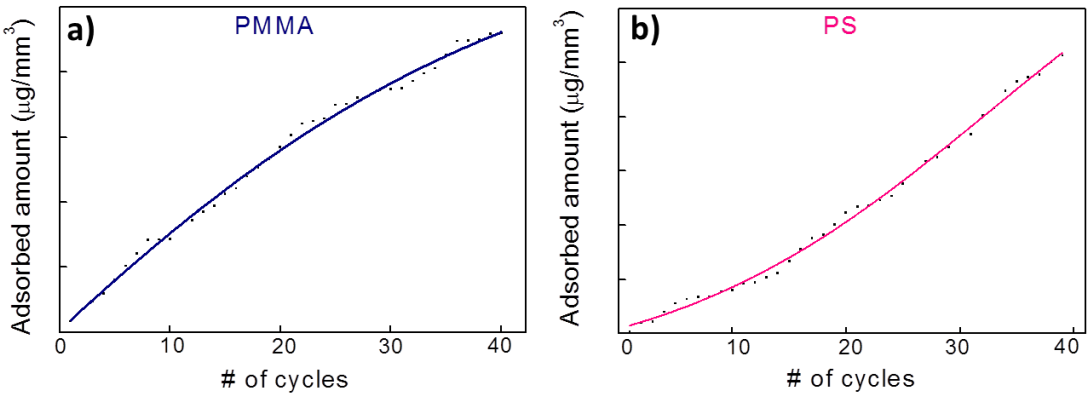

Figure 4.7. Breakthrough curves for (a) PMMA and (b) PS membranes after 40 cycles of BSA adsorption

Both PMMA and PS membranes never reach a plateau level after so many adsorption experiments, thus any of the isotherm models could not be applied for this results. (see Appendix A2 for the plots and equations showing adsorption isotherms)

Figure 4.8 shows the SEM micrographs of PMMA and PS membranes after BSA adsorption. In general, both membranes preserved fiber morphology after sorption process. PS membranes showed more fracture resistance compared to PMMA ones. PS fibers were selected for further experiments because of the exclusive properties, such as high adsorption capacity and resistance to fracture.

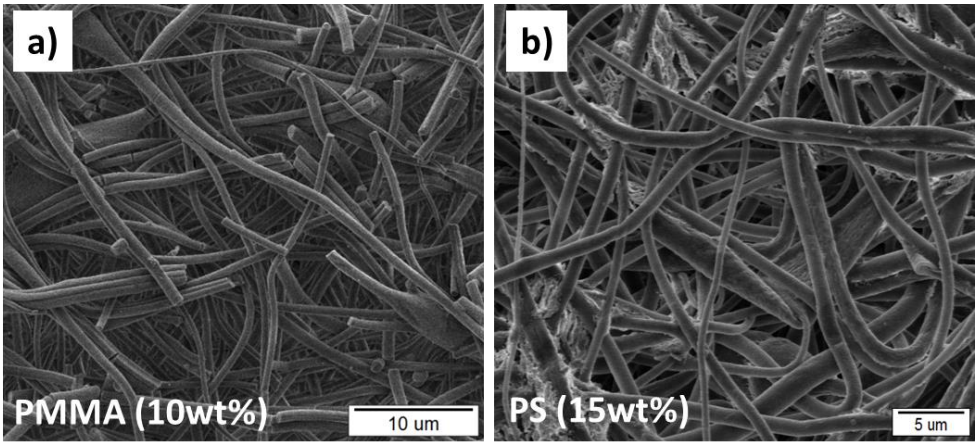

Figure 4.8. SEM micrographs of (a) PMMA and (b) PS membranes after BSA adsorption

The amount of membranes that were used during the experiments has a major effect on the BSA adsorption. To test the sorption efficiency of syringe columns, the amount of the membranes were varied from 5 mg to 80 mg. Figure 4.9 showed the change in adsorbed BSA amount as a function of the membrane amount and average fiber diameter. A significant increase was observed for both PS and PMMA membranes with the increase in the membrane amount. PS membranes showed a sharp increase with a higher adsorption capacity compared to the PMMA membranes. Moreover, higher adsorbed amount is often achieved by using thinner fibers that have higher surface area. (see also in Figures 4.3d and 4.4d) This result confirms the significance of the surface area and sieve size of the fibers.

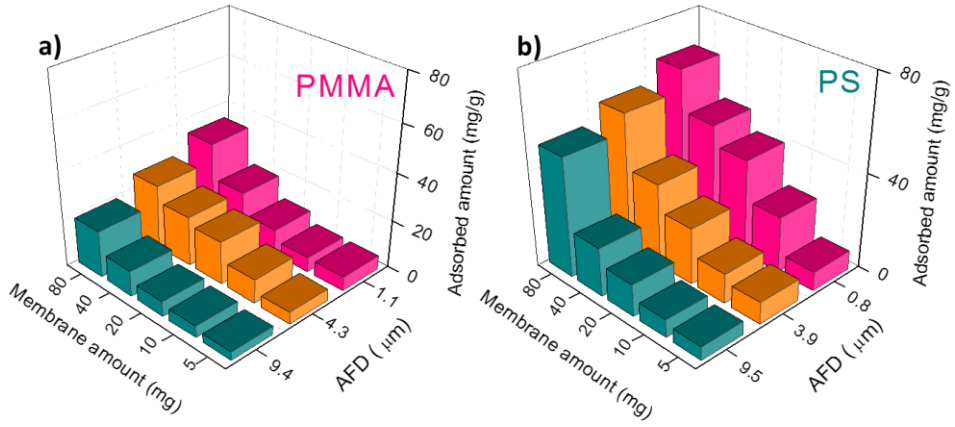

Figure 4.9. Adsorbed amount profiles of (a) PMMA and (b) PS membranes with respect to the membrane amount and average fiber diameter

The conformation of proteins is dependent on the pH of the dispersing medium. Figure 4.10 shows the adsorbed amount profile of BSA on PS membranes and the hydrodynamic radius of BSA molecule at different pH values.

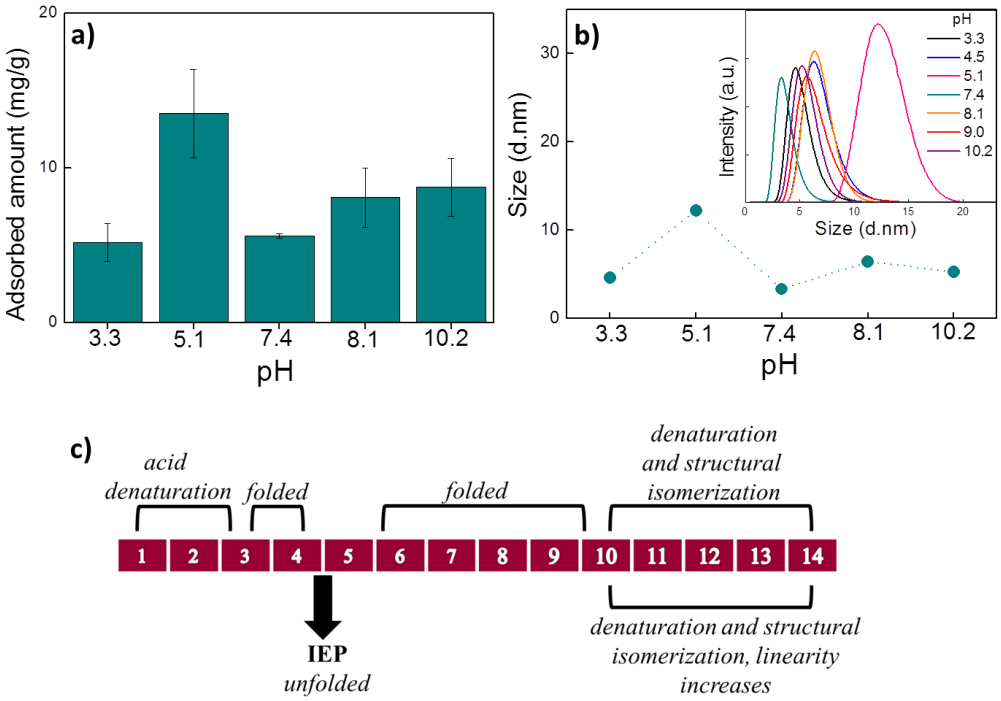

Figure 4.10.(a) Effect of pH on the adsorption of BSA on PS electrospun membranes, (b) The DLS results for BSA solutions at different pH media, (c) Structural changes of BSA around IEP

Figure 4.10a demonstrates the adsorption of BSA as a function of pH. BSA has IEP about 5.1 in which there is no net charge on the protein and electrostatic interactions are negligible. Therefore; BSA molecule becomes unfolded and decreases its α-helical content. The DLS profile and the inset showing the intensity versus size of BSA solution at different pH range are represented in Figure 4.10b. The BSA molecule takes more extended conformation and the size of molecule reaches its maximum value at its IEP. When the size of molecules increases, they could be held on the surface of the membrane and the highest adsorbed amount was achieved at IEP. Below and above the IEP, BSA molecules carry a net charge. As the pH becomes away from the IEP, the intensity of hydration forces increases and the surface becomes more hydrophilic. The folded structure was achieved due to the gain of α-helix structure and rigid peptide bonds. (Figure 4.9c) The size of BSA molecules decreases and the adsorbed amount on the fiber membranes decreases below and above the IEP. At pH 3.3, the hydrodynamic volume of BSA molecule was 28% higher than the volume at pH 7.4. In the study of Valle-Delgado et al. (Valle-Delgado et al. 2004) reported that in the acid denaturated state (at pH 3.6) serum albumin molecule have about 25% higher hydrodynamic volume than the volume at the native state (about pH 7.4). At higher pH, proteins introduce negative charges and this fact leads to structural isomerisation and deamidation. Above the pH 9, disulphide cleavage occurs and proteins become unfolded. Thus, the adsorbed amount of BSA on fiber increases.

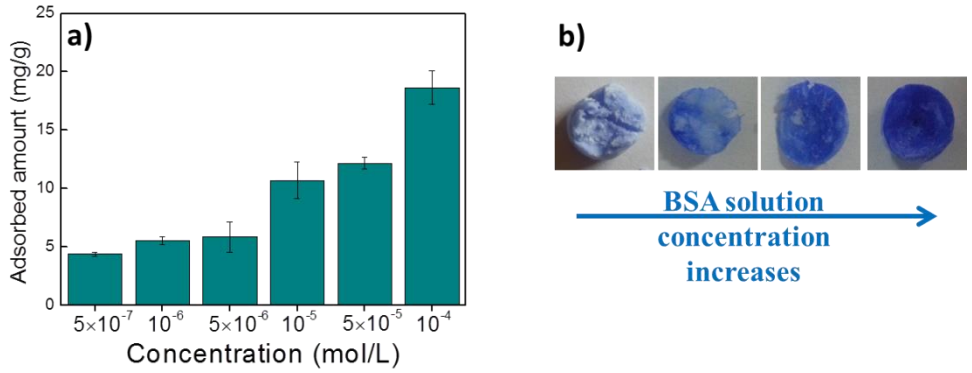

Figure 4.11. (a) Effect of initial concentration on the adsorbed BSA amount, and (b) Naked eye detection of filtrated BSA through a Coommasie blue staining of the PS membranes

Initial concentration is another important effect on adsorption of BSA onto the fiber membranes. At higher concentrations, surface would be occupied easily and the

highest adsorbed amount of BSA was achieved. (Figure 4.11a) However, at high concentrations the number of BSA molecules is higher and they can cover the surface of the membranes rapidly. This case would affect the equilibrium time of PS fiber membranes. Figure 4.11b shows the Coomassie blue stain on the membranes that adsorbed solutions. The color intensity of the stain increases with the BSA concentration.

Absorbance measurements of DNA allowed us to determine the working concentration range with a linear correlation coefficient > 0.992. Figure 4.12 represents the calibration curve of aqueous DNA solutions in the 7×10^{-7} to 5×10^{-6} mol L^{-1} concentration interval. The equation was linear fit with the experimental data and used for the quantitative analysis of DNA. The working concentration was selected as 10^{-6} mol L^{-1} during this study. For the quantitative determination of DNA in supernatant solutions, the fitting equation given in Figure 4.12 was used.

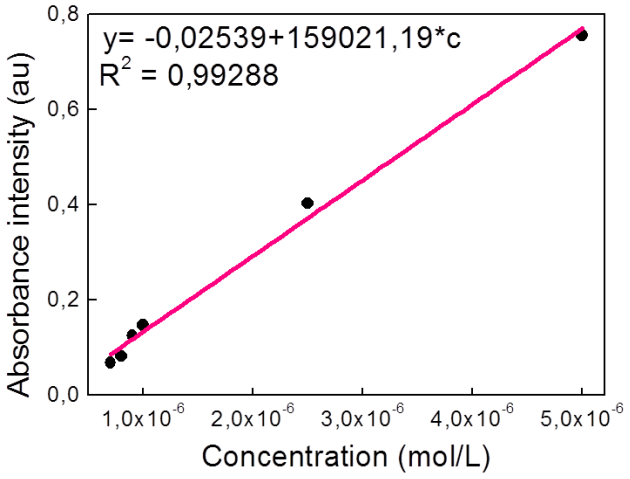

Figure 4.12.Calibration curve of DNA

The average fiber diameter is an important parameter for the adsorption process due to the sieve size and surface area of the electrospun membranes. The curvature effect of the nanofibers has also significance, because it is associated with the diameter of nanofibers. While this effect is straightforward for proteins and peptides, ss-DNA shows different behaviour during the adsorption. (Cederquist and Keating 2009) Cederquist *el al.* indicated that the DNA strands adsorbed on the surfaces as curve away from each other and curvature of the surface could reduce the adsorbed amount of DNA. In another study, Mirkin and co-workers demonstrated the curvature effect on

gold nanoparticle sizes. While the size of nanoparticles increases, the curvature decreases and DNA strands get close to eachother. The interstrand phosphate repulsions come in sight, and the adsorption process is blocked by these interactions. (Hurst, Lytton-Jean, and Mirkin 2006) Figure 4.13 shows the profiles of adsorbed amount of PS and PMMA membranes for aqueous ss-DNA solutions. The increase in the AFD of the membranes reduces the surface curvature and the adsorbed amount of ss-DNA increases for both PMMA and PS.

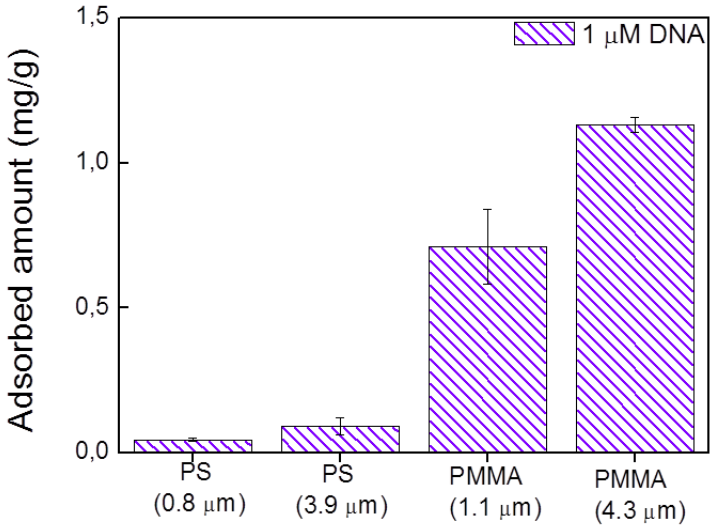

Figure 4.13. Sorption profile of DNA on PS and PMMA membranes and inset figure shows the size of DNA measured by DLS

Single stranded DNA is an amphiphilic polyelectrolyte. Thus, it can be adsorbed both on hydrophobic and hydrophilic surfaces. While hydrophilic surfaces form hydrogen bonds with ss-DNA, hydrophobic surfaces experience a weak attraction. (Elder and Jayaraman 2014) PMMA membranes can make hydrogen bonds with the side chains of BSA and has a significant sorption when compared with the PS membranes. Also, DLS measurement shows the size of ss-DNA in aqueous medium as ≈ 100 nm. When compared with BSA, ss-DNA has larger size due to its extended conformation.

The next step for the sorption experiments was to investigate the separation efficiency of pre-defined membranes. Thus, a model mixture was prepared with BSA and DNA. The important point for the membranes was to get a high protein adsorption while DNA is held on the surface in low amounts. Figure 4.14a shows absorption

spectra of BSA, DNA and BSA/DNA mixtures for before and after the filtration. The decrease in the absorbance spectrum of the eluted BSA/DNA mixture suggests that electrospun membranes provide a filtration medium for BSA molecules. The shift in the spectrum to the smaller wavelengths, which close to the wavelength of DNA, indicates that BSA amount in the eluted solution is getting smaller.

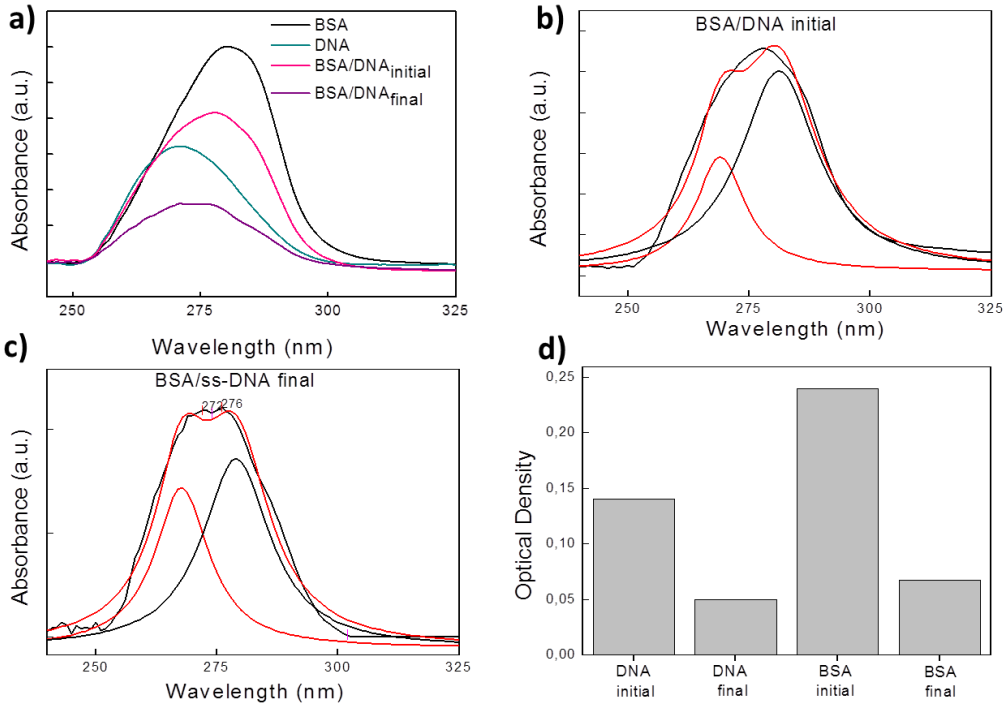

Figure 4.14. (a) The difference between the initial and final absorbance spectra of BSA and DNA, (b-c) spectra of BSA and DNA mixture after deconvolution, and (d) optical density profiles of BSA and DNA

Figure 14b and c show the spectra of mixtures after deconvolution. While the difference between the absorbance of BSA and DNA is significant in the initial solution, this difference becomes small in the eluted solution. The optical density profiles for BSA and DNA in mixture also indicates that BSA is more efficiently adsorbed compared to DNA.

Figure 4.15 demonstrates the comparison between the proposed membranes and commercial membranes. The effect of AFD is obvious for the separation experiments. The difference between adsorbed BSA and DNA is significant, because PS and PMMA membranes with a smaller AFD have a better separation due to the sieve size and surface area as mentioned earlier.

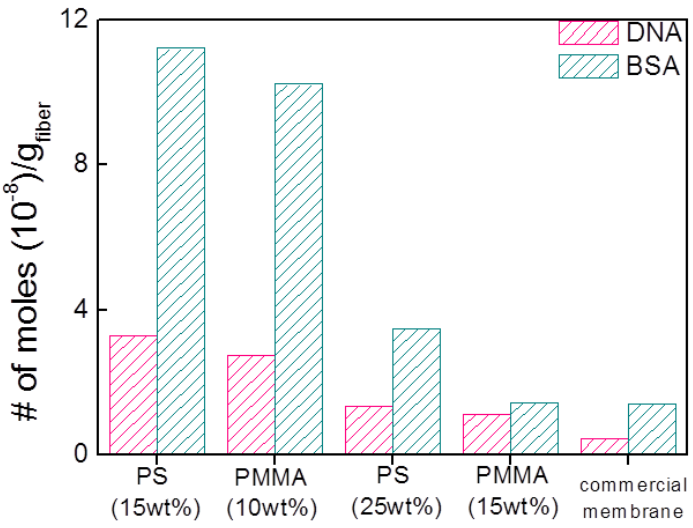

Figure 4.15. The sorption efficiency comparison of PS and PMMA membranes according to their AFD with BSA and DNA mixture

Also, PS membranes have better sorption and separation among the other membranes due to the π-π interactions and their porous surfaces to capture BSA molecules. As in the other results, PS membranes with the lowest AFD have the highest BSA sorption and best separation efficiency among the other membranes.

To improve the separation and sorption efficiencies, some surface modifications were performed to the membranes. PS membranes were chosen for modification, because its neat form has already better properties. Figure 4.16 shows the comparison of adsorption profiles for the membranes after **oleic acid** and **gold nanoparticle** modifications.

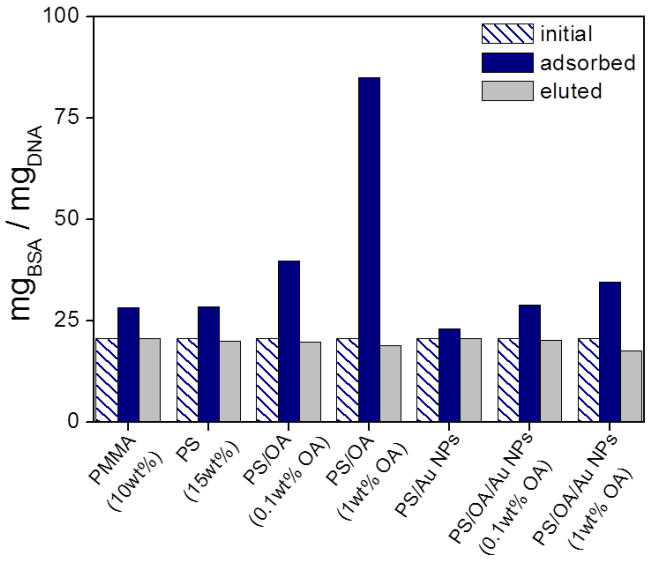

Figure 4.16. BSA/DNA ratios in eluted solutions and on modified membranes with BSA and DNA mixture in 2:1 ratio

Serum albumin molecules are major transporters of fatty acids in blood plasma and they are bounded to fatty acids. We prepared PS/OA solution mixtures and these mixtures were subjected to electrospinning. (Sarver, Gao, and Tian 2005) Figure 4.16 shows that the presence of oleic acid increases the BSA adsorption and improves the separation efficiency. When neat PS membrane is compared with PS/OA membrane, it is obvious that there is a decrease in DNA sorption and an increase in the BSA sorption. Also, the oleic acid concentration is effective for the adsorption experiments. The reason for the enrichment of DNA in decantate could be the result of the attractive hydrophobic interactions with oleic acid and PS and repulsion between negatively charged OA and DNA molecules.

According to some researches, BSA can be adsorbed on gold nanoparticles (NPs) through hydrophobic effect and its thiol group can bind with gold by enhancing the adsorption. (Fu et al. 2014b) Serum albumin molecule has positively charged residues like lysine, arginine and histidine, therefore negatively charged gold surfaces have high binding capacity. Also, thiol groups on serum albumin sequence increase the binding efficiency to the gold surfaces. (Zhong et al. 2014)

Another surface modification is performed with gold NPs for PS and PS/OA membranes. However, they did not have a better separation efficiency than oleic acid modified membranes. (see Appendix A3 for the FTIR-ATR spectrum of membranes)

Syringe columns were prepared with 5 mg membranes for previous experiments. For the investigation of the separation efficiency, the membrane amount in syringe columns were increased from 5 to 80 mg. Figure 4.17a shows the effect of membrane amount on BSA/DNA ratio in adsorbed and eluted solutions. When the amount of membrane was 80 mg, there is a remarkable difference between BSA/DNA ratios for adsorbed and eluted solutions. The increase in the adsorbed BSA/DNA ratio indicates that BSA amount increases on the membranes and decreases in eluted solution. Thus, the enrichment of DNA in eluted solution can show a remarkable increase.

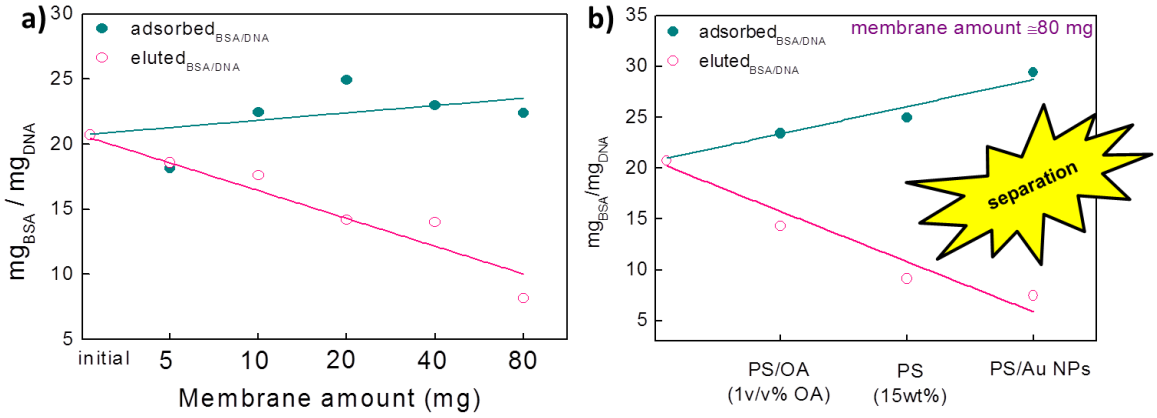

Figure 4.17. BSA/DNA ratios in eluted solutions with respect to membrane amount in syringe column

Figure 4.17b shows the effect of oleic acid and Au nanoparticle modifications for the 80 mg membranes. Au nanoparticle modified membranes enhanced the BSA adsorption on the membranes. The decrease in the BSA/DNA ratio in eluted solution indicates that DNA adsorption is low when compared with BSA. However, oleic acid modified PS membranes did not show a better separation than neat PS membranes. Although their BSA adsorption is very high in Figure 4.16, separation efficiency of these membranes are weak due to the DNA adsorption. These results showed that membrane amount in syringe columns are another important factor affecting the separation efficiency.

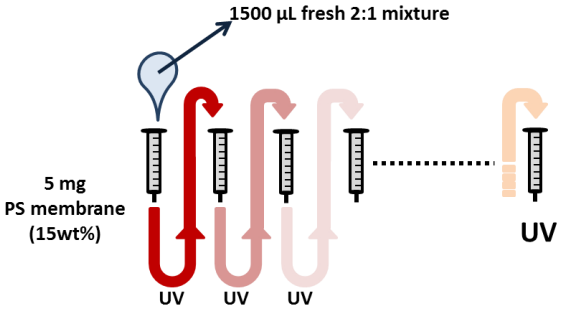

Figure 4.18. Schematic representation of an alternative mixture separation system. The solutions were passed through the membrane many times consecutively

To understand the difference between using a syringe column with big amounts of membrane and different number of syringe columns with 5 mg membrane, an alternative syringe column system was prepared as shown in Figure 4.18. The BSA/DNA mixture was passed through a syringe column containing 5 mg membrane and UV spectrum of eluted solution was taken. Then, the same solution was passed through a new syringe column and this process continued to twelfth cycle. Then the UV spectra of solutions changed and solution was deteriorated.

Figure 4.19 indicates the separation results of the consecutive separation system. As well as the previous results, BSA/DNA ratio increases for the adsorbed amounts and decreases for eluted solutions. After the twelfth cycle the solution has been interacted with the different membranes excessively, thus the UV absorbance spectrum indicated that there was denaturation.

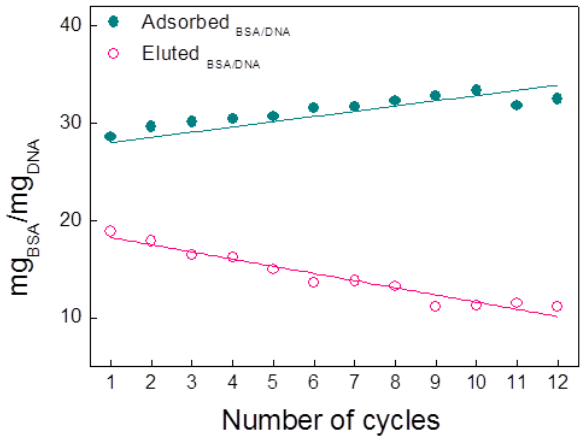

Figure 4.19. BSA/DNA ratios in eluted solutions with a different syringe column system

Also, at the twelfth cycle the solution was used for only 60 mg membranes and the separation efficiency is not good as the 80 mg membranes. However, the cycle cannot continue to sixteenth cycle, which equals to 80 mg membrane, due to deterioration. As a result, using syringe columns with high amounts of membranes have a better efficiency.

Figure 4.20 shows the effect of pH on the separation efficiency for two different membrane systems. For the neat PS membranes, BSA/DNA adsorbed and eluted amounts showed the same attitude with the only BSA adsorption experiments. (Figure 4.10) Around the IEP of BSA, there is a highest separation between BSA and DNA. However, at neutral pH there is nearly no separation due to the too low BSA adsorption. Above the pH 8, the solutions were denaturated. (Figure 4.20a)

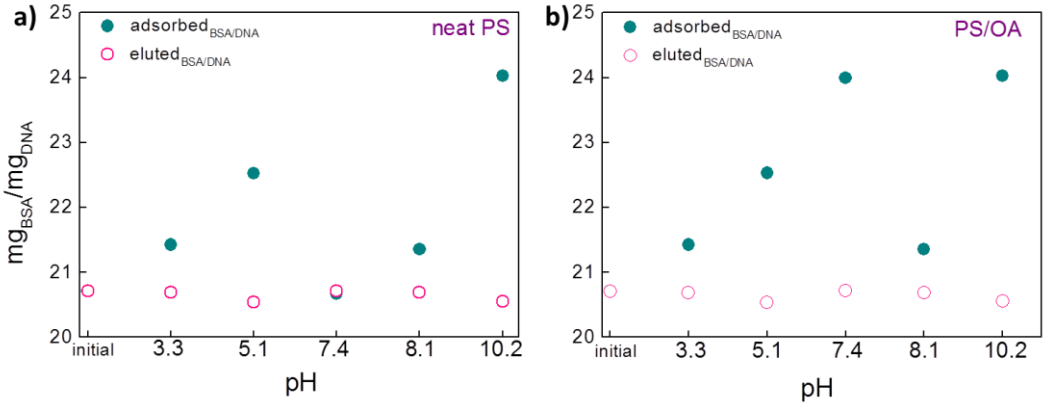

Figure 4.20. pH effect on BSA/DNA separation for (a) PS and (b) PS/OA membranes

Oleic acid modified membranes showed a different attitude over the same pH range due to the carboxylic acid groups on the oleic acid. On the acidic pH scale, carboxylic acid is protonated and the surface became positive. Thus, the BSA adsorption was restricted due to the electrical forces. However, on the basic pH ranges the carboxylic acid group turned to the carboxylate group and the surface became negative. At pH 7.4 the maximum separation was obvious; because positively charged BSA molecules could be adsorbed on the negative surface while negatively charged could not be adsorbed. This attitude of the PS/OA surface enhanced the separation efficiency of the membrane. (Figure 4.20b)

Fluorescence spectroscopy is a more sensitive technique for the quantitative analysis when compared with the UV-vis spectroscopy, because UV-vis has an absolute unit of measurement called absorbance. However, in fluorescence spectroscopy there are a wide variety of ways with which sensitivity has been described. A molecule stays

in excited state for a long time before the emission and the fluorescence intensity is the consequence of this time. (Ashutosh Sharma 1999, Douglas A. Skoog 2006).

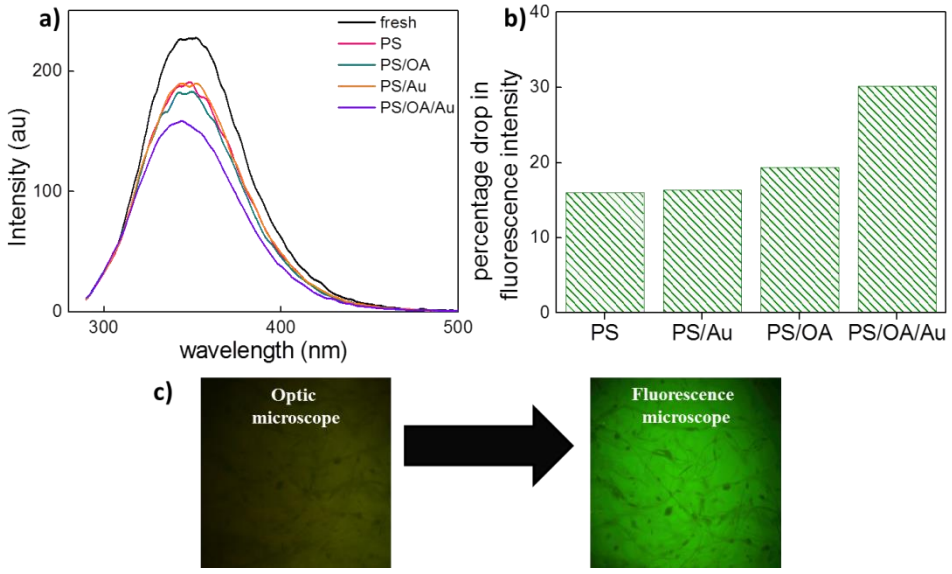

Figure 4.21. (a-b) The fluorescence intensity drop of fluorescent tag DNA after the filtration and (c) The fluorescent microscopy results of the membranes after filtration

Figure 4.23a shows the decrease in the fluorescence intensity after the filtration of BSA/DNA mixture with different membranes. It is obvious in the Figure 4.23b that the membranes showed nearly the same percentage drop with the absorbance measurements after the filtration. (see Appendix A3 for the comparison of fluorescent intensity and absorbance profiles for DNA) Only PS/Au membranes showed a significant difference from the absorbance measurements, but this result could be explained by the thiol groups on the fluorescent tag DNA. (Xue et al. 2014) Au nanoparticles could be interacted with the thiol groups on the DNA sequence and the adsorption of the DNA increased. Figure 4.23c demonstrates the presence of fluorescent tag DNA on the membranes by giving a comparison of optical microscope and fluorescent microscope.

Bradford Assay is a spectroscopic technique for the quantification of proteins in unknown samples. The principle is based on the binding of proteins to Coomasie blue dye and detected at 595 nm by using a spectrophotometer or microplate reader. The calibration graph of standard solutions was plotted and the concentration of proteins in unknown samples was predicted from the equation of this calibration graph. (See

Appendix A5 for calibration graph of Bradford Assay standard solutions) Figure 4.22 shows the comparison of Bradford Assay and Beer's Law results for modified membranes.

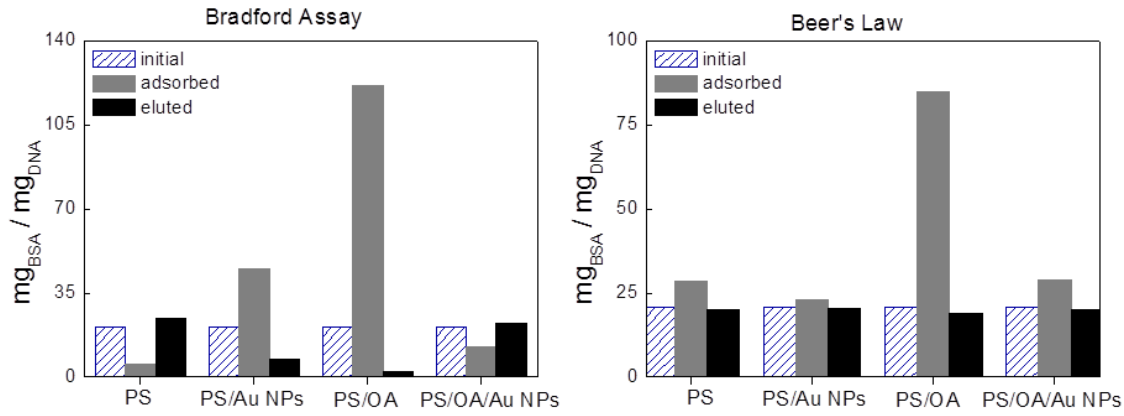

Figure 4.22. The comparison of BSA/DNA ratios for Bradford Assay and Beer's Law measurements

Oleic acid modified poly styrene membranes show the highest separation efficiency among the other membranes, and this result is compatible with the previous results calculated by Beer's Law. The small differences between two histograms may come from the sensitivity difference of these methods.

Until now, the separation efficiency of membranes was obtained in a model system without using any biological medium. Artificial serum contains different components to mimic the real serum in human body. To see the effect of these components and the effectiveness of membranes in near-real conditions, artificial serum sample was prepared in a given protocol.

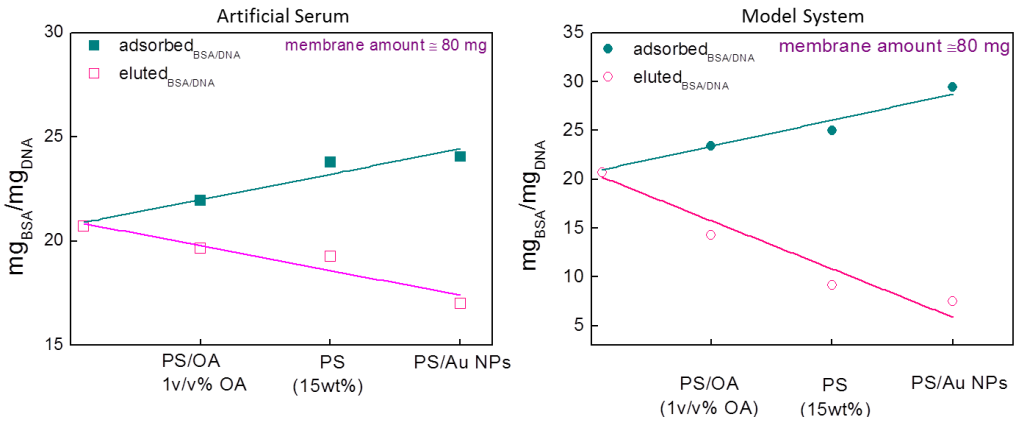

Figure 4.23. The comparison of BSA/DNA ratios in artificial serum and model system

While the model system shows a half-and-half drop for BSA/DNA ratio in the eluted solution, artificial serum has lower separation efficiency with 25% drop in BSA/DNA ratio. The other components in artificial serum affect the separation efficiency of membranes. Neat, oleic acid modified and gold nanoparticle modified PS membranes again show the same attitude on separation efficiency. The syringe column with 80 mg PS/Au NPs modified membrane has the best separation efficient with respect to other columns.

Among the all results, PS/Au NPs modified membranes show the best separation efficiency when the membrane amount is kept constant at 80 mg. To give a concluding remark, efficiency of this membrane was given with a comparison of artificial serum and model system quantitatively.

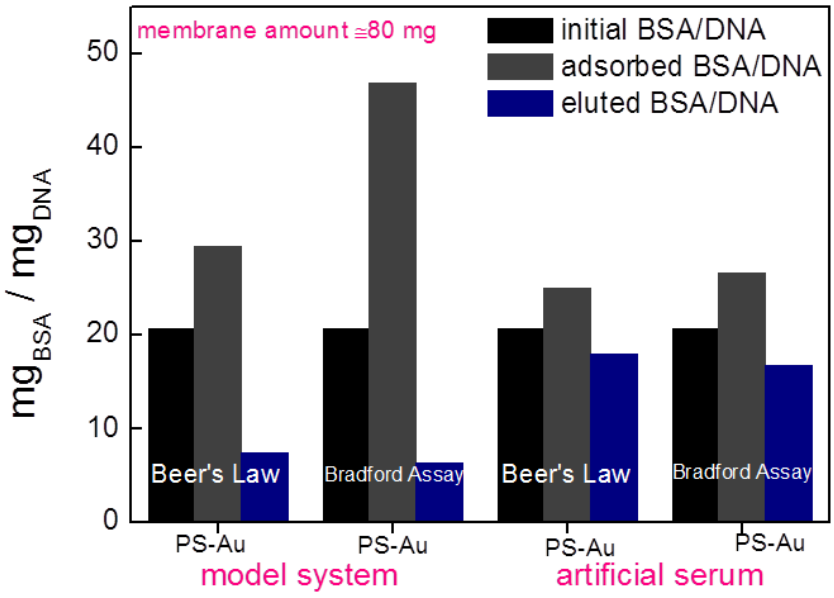

Figure 4.24. The comparison of BSA/DNA ratios of Bradford Assay and Beer's Law for artificial serum and model system

PS/Au NPs modified membranes has high separation efficiency in the model system, while this efficiency decreases in artificial serum medium but it is still high when compared with the other membranes. (Figure 4.24) Also, Bradford Assay results show the same attitude with the Beer's Law results. While the BSA amount in the eluted solution decreases from 0.9470 to 0.5001 mg · mL^{-1}, DNA amount decreases from 0.0236 to 0.0144 mg · mL^{-1}. Two different analytical spectroscopic methods prove the separation efficiency of membrane.

CHAPTER 5

CONCLUSION

Electrospinning provides a simple and versatile method for generating ultrathin fibers from a rich variety of materials that include polymers. Since electrospun fibers have high specific surface area, high porosity, and small pore size, the unique fibers have been proposed for wide range of applications. The aim of this thesis is to develop an electrospun membrane system for the separation of protein and nucleic acid from the synthetic solutions.

Two basic polymers, PMMA and PS were employed in this study. The homogeneous solutions of the polymers were subjected to electrospinning at 1 kV cm^{-1} with various concentrations. The electrospun mats were modified with gold nanoparticles and oleic acid to improve the separation efficiency. By using a model solution prepared with ss-DNA/ BSA or artificial serum, the separation efficiency of the membranes investigated. Fiber diameters, fiber amount, surface modification and pH were the experimental parameters through this study.

We concluded that electrospun nanofibrous mats are promising candidates for the filtration of biomacromolecules from the solutions. The controllable surface morphology and high surface area of the nanofibrous membranes enhance the sorption capacity of these membranes. Overall results indicate that oleic acid modified PS membranes have the best adsorption capacity to BSA when the membrane amount is kept as 5 mg. However, increasing the membrane amount in syringe columns enhanced the separation efficiency of gold modified PS membranes. It was found that gold nanoparticles have strong influence on the BSA sorption and DNA enrichment in eluted solution. Also, altering the pH of the medium has a significant effect on the separation efficiency. At the isoelectric point of the BSA, the maximum separation efficiency was achieved due to the extended conformation of globular protein.

Bradford Assay results have compatibility with the Beer's Law results with respect to the effect of fiber modification and BSA/ DNA separation efficiency. Artificial serum contains different components to mimic the real serum in human body and changes the separation efficiency of membranes. The Bradford Assay and Beer's Law results indicate that BSA amount in the eluted solution decreases from 0.9470 to 0.5001

mg · mL^{-1}, while DNA amount in the solution decreases from 0.0236 to 0.0144 mg · mL^{-1} in the artificial serum when PS/Au membranes were used as membrane. This result indicates the enrichment of DNA in the eluted solution. However, the isolation of DNA from the synthetic solutions could not be achieved completely. The morphological changes on the nanofiber membranes can be studied for future study to improve the separation efficiency of the system.

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APPENDIX A

SUPPORTING INFORMATION

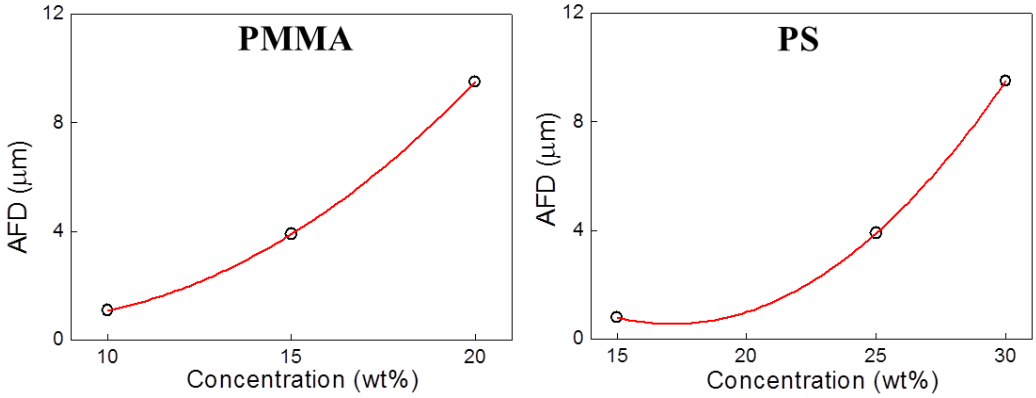

Figure A.1. Effect of solution concentration on average fiber diameter (AFD)

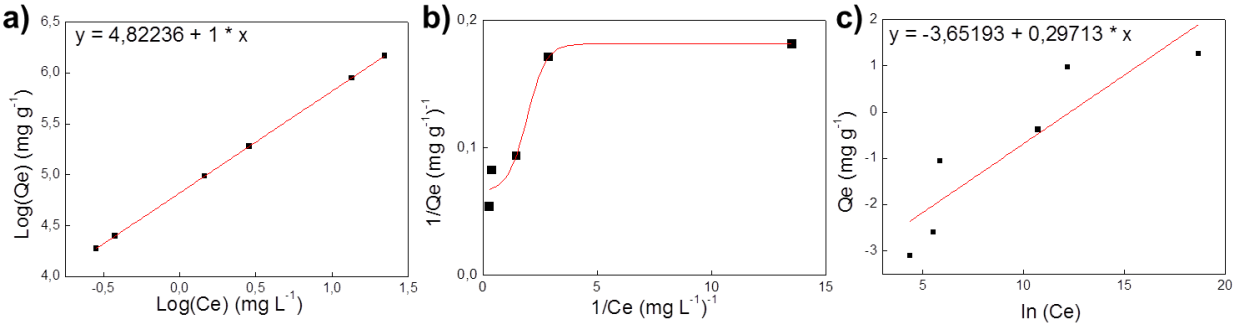

Figure A.2. Adsorption isotherms of BSA (a) Freundlich, (b) Langmuir, and (c) Temkin

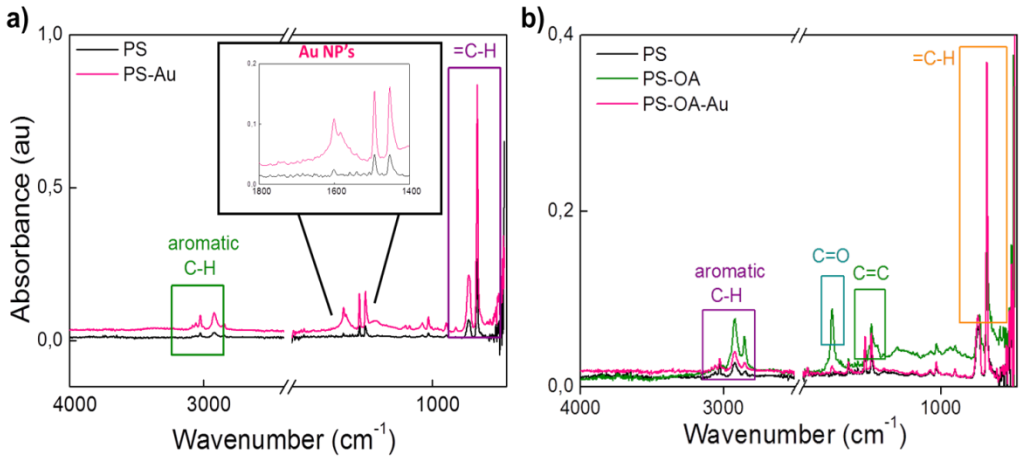

Figure A.3. Comparison of FTIR-ATR spectra of (a) PS and gold modified PS membranes, and (b) PS, oleic acid modified PS, gold and oleic acid modified membranes

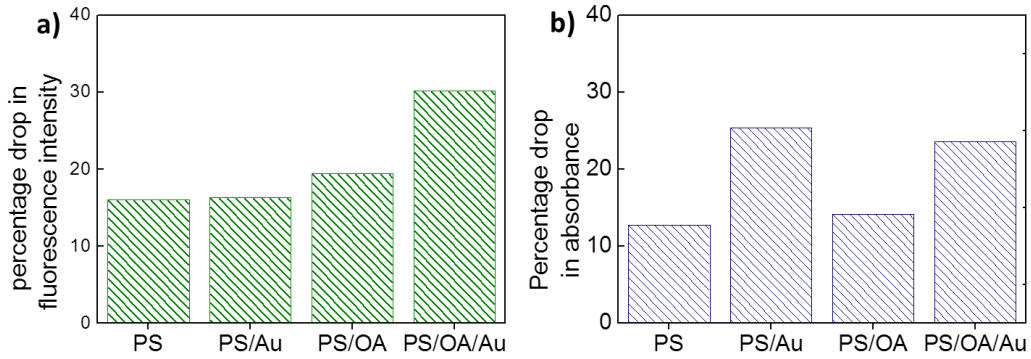

Figure A.4. The comparison of (a) Fluorescent intensity drop and (b)The percentage adsorption profiles of fluorescent tag DNA and DNA, respectively

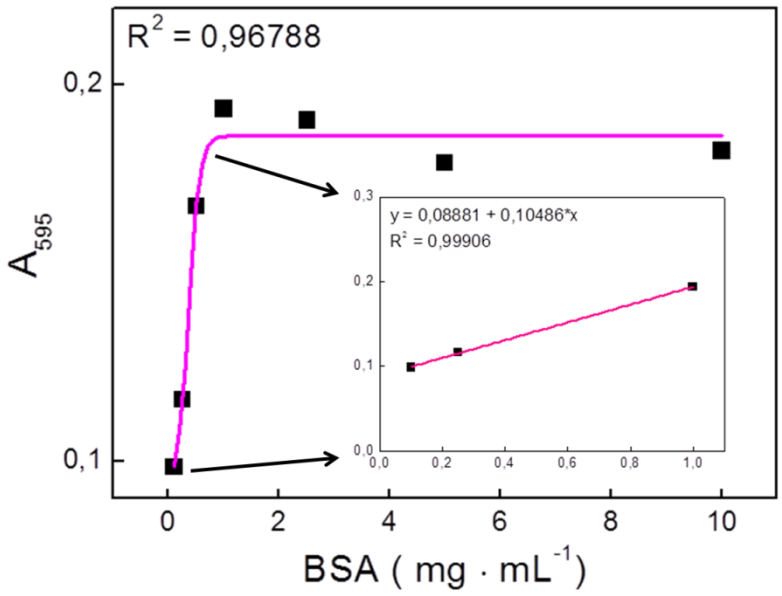

Figure A.5. Calibration graph and equation for Bradford Assay standard solutions

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