GEDIZ UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

Dye Incorporated Nanopolymers For Albumin Depletion

M.Sc. THESIS

Şule Yeşim YILDIZ

Institute of Science

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Thesis Supervisor: Prof. Dr. Sinan AKGÖL

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GEDİZ ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

Albumin Uzaklaştırması İçin Boya Bağlı Nanopolimerler

YÜKSEK LİSANS TEZİ

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Şule Yeşim YILDIZ, a **M.Sc.** student of Gediz University, **Institute of Science** with student **ID No: 60071004**, successfully defended the **thesis** entitled " **Dye Incorporated Nanopolymers For Albumin Depletion** ", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

We approve the thesis of **Şule Yeşim YILDIZ**

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Ege University

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Date of Submission : **April 2014**
Date of Defense : **April 2014**

To Mr.Bilgi Görkem YILDIZ and my Family

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To my son, husband, my father, my mother, my brother and my aunt for their love & support. I could not have done this without them.

CERTIFICATE OF ORIGINALITY

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

April 2014 Şule Yeşim YILDIZ

ABSTRACT

Proteomics is the understanding of all proteins and their interactions in a cell or an organism. Dynamic nature of the proteome makes the proteome an indicator of physiological processes. The major goal of plasma and serum proteomics is to obtain the most reliable information possible for diagnostic and therapeutic purposes. Since biomarker proteins are usually present at very low concentrations, main goal of protein depletion from blood plasma is to separate the high abundant proteins from the low abundant proteins. Affinity chromatography is a well established method for the purification and seperation of macromolecules and basic on highly specific molecular recognation. A wide variety of functional molecules, including enzymes, coenzymes, antibodies, aminoacids, proteins and nucleic acid may be used as ligands in the design of novel adsorbents. Dye-ligands are important candidate to the natural counterparts for affinity chromatography. Dye-ligands have high binding capacity to the most of proteins. In this thesis, synthetic polymer p(HEMA) that the solid support material used in BSA adsorption, was synthesized by surfactant free emulsion polymerisation method for protein depletion in proteomic studies. Dye Ligand Reactive Red 2, covalently bounded to the p(HEMA) nanopolymers and this material was used for this purpose. p(HEMA)-RR2 nanopolymers were characterized by FTIR, ZETA sizer and SEM. Average size of the nanopoylmer is 100 nm and specific surface area of the nanopolymer is 7122.92 m2/g, BSA adsorption of p(HEMA)-RR2 nanopolymers was found to be 855 mg/g and desorption rate was found as %85.

ÖZET

Proteomik, bir hücre veya organizmadaki bütün proteinleri ve bunların etkileşimlerini tanımlama çalışmasıdır. Proteom, dinamik yapısı ile fizyolojik işlemlerin bir göstergesidir. Plazma ve serum proteomik çalışmalarının ana hedefi, teşhis ve tedavi için en güvenilir bilgileri elde etmektir. Biyomarker proteinler, organizmalarda genellikle çok düşük konsantrasyonlarda bulunmaktadırlar. Kan plazmasında protein uzaklaştırmanın esas amacı, bol miktarda bulunan proteinler ile çok seyrek miktardaki proteinlerin birbirlerinden ayrılmasıdır. Afinite kromatografisi yüksek spesifiklikte moleküler tanımaya dayanan, makromoleküllerin tanınmasında ayrılmasında ve saflaştırılmasında çok kullanılan bir yöntemdir. Enzimler, koenzimler, antikorlar, aminoasitler, proteinler, nükleik asitler gibi çeşitli fonksiyonel moleküller, yeni sorbentlerin geliştirilmesinde affinite ligand olarak kullanılabilirler. Afinite kromatografisinde, boya ligandlar doğal muadillerine karşı önemli bir alternatiftir. Boya ligandlar, birçok proteine yüksek bağlanma kapasitesine sahiptirler. Bu tezde, proteomik çalışmalarda, protein azaltma amacıyla, adsorpsiyonda kullanılan katı destek malzemesi olan sentetik polimer p(HEMA), sürfaktansız emülsiyon polimerizasyonu yöntemiyle sentezlenmiştir. p(HEMA) nanopolimerine, boya ligand Reaktif Red2 kovalent olarak bağlanmış, elde edilen bu malzeme BSA proteinin adsorpsiyonunda kullanılmıştır. p(HEMA)-RR2 nanopolimeri FTIR, ZETA boyut ve SEM ile karakterize edilmiştir. Nanopolimerin ortalama boyutu 100 nm ve spesifik yüzey alanı 7122.92 m2/g olarak hesaplanmıştır. p(HEMA)-RR2 polimerinin BSA adsorpsiyon koşulları optimize edilmiştir. p(HEMA)-RR2 nanopolimerinin maksimum adsorpsiyon kapasitesi 855 mg/g ve desorpsiyon oranı 85% olarak bulunmuştur.

ABBREVIATIONS

PVA: Polyviniyl Alcohol, $[CH_2CH(OH)]_n$ (sometimes polyvinyl acetate)

HEMA: Hydroxyethylmethacrylate , $C_6H_{10}O_3$

EGDMA: Ethylene glycol dimethacrylate, $C_{10}H_{14}O_4$

KPS:Potassium Persulfate

Et-OH:Ethanol,Ethyl Alcohol , C_2H_6O

Reactive Red2: (C19H12Cl2N6O7S2.2 Na)

Na Ac: Sodium acetate, $C_2H_3NaO_2$

FTIR: Fourier Transform Infrared Spectroscopy

UV-VIS: Ultraviolet Visible

SEM : Scanning Electron Microscopy

ZETA Dimensional

BSA : Bovine Serum Albumin

N2 : Nitrogen

NaOH: Sodium Hydroxid

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1. INTRODUCTION

1.1 Blood, Plasma, Serum

Due to its basic role in disease diagnostics blood is the most commonly studied biological fluid. Blood has contact with various organs and tissue which causes some of the proteins to separate and also causes modifications in proteins. These proteins undergo specifically physical and pathological changes. Plasma and serum are used alternately. In human body, blood makes %8 of weight. Mainly blood is composed of plasma, serum, white blood cells and red blood cells. White blood and red blood cells are separated from eachother according to their duties. White blood cells defense the body, so they are very important in immune system, on the other hand red blood cells transport oxygen. Plasma and serum are routinely used for blood testing which are the components of blood. Serology is used in the study of blood serum which is the branch of medical science. In order to understand the difference between plasma and serum, these key points can be helpful. Serum which is a part of blood exclude clotting factors f blood but on the other hand serum and plasma are similar to eachother in composition. There is a protein called fibrinogen which is involved in blood coagulation. Fibrinogen converts its functions into fibrin (active form of fibrinogen), only by this way it can become an active protein, or in actual, it is an inactive protein. Plasma is considered as the medium of blood in which RBCs (red blood cells), WBCs (white blood cells) and other components of blood are suspended. The components of plasma and serum are both contain hormones, glucose, electrolytes, antibodies, antigens, nutrients and certain other particles except clotting factors which are present only in plasma, so they are similar to eachother according to this. If it is composed with plasma and serum the volume of serum is less than plasma in percentage. The % 55 of total volume of blood is blood plasma which is a clear yellow liquid. Water is % 93 of plasma and % 7 of it is composed of blood cells and other parts. It's density is 1025 kg/m^3 (R. Apweiler et al., 2004).

Blood plasma contains a very high concentration of proteins, typically in the range of 60-80 mg of protein per mL. Estimates of the number of proteins in blood plasma start from 10,000, but the actual number of distinct proteins may be several orders of magnitude higher (L.A. Huber, 2003).

1.2 Proteins

Proteins are big structured molecules. Proteins perform various functions such as enzymatic catalysis, transporting ion and molecules between organs, regulating cellular and physiological activities. Protein sequences consist of 20 different amino acids, they are building blocks of proteins. They are biologically important and contain aminoacids. The scarcity and abundance of proteins in the metabolism causes various diseases (M.M. Gromiha, 2010).

1.3 Albumin

In plasma mostly albumin and immunoglobulin are found. Albumin and immunoglobulin plasma make up of 80% of proteins. They mask the rest low concentrated proteins. So, during the analysis the albumin removal is vital in disease-specific protein determination. That is why the removal of these proteins play a crucial role in proteome analysis (A. Denizli, 2007).

The polymer based sudies of albumin and immunoglobulin removal for protein content determination is increasing in the last years. Besides various commercial products which serves in this sector, textile dyes which has a greater affinity against albumin is used for albumin and immunoglubulin removal. For the albumin removal dye-affinity approach is specially used (M. Karataş et al., 2007) (E.B. Altıntaş et al., 2006).

In order to advance in proteome analysis, which brings new innovations, new data input and an increase in sensitivity is required (S. Akgöl

et al., 2007) (E.B. Altıntaş et al., 2007).

Albumin which has many applications in therapeutics and in diagnostics has a role in fluid management of actuely ill patients for more than 50 years (W. Norbert, 1976).

Albumin theraphy is licensed in the United States include hypovolemia or shock burns, hypoalbunemia or hypoproteinemia; surgery; trauma; cardiopulmonary by-pass, the acute respiratory distress syndrome; hemodialysis, acute nephrosis, hyperbilirubinemia, acute liver failure, ascitess and sequestration of proteinrich fluids in acute peritonitis, pancreatitis, mediastinitis and extensive cellulitis (M.M. Wilkes et al., 2001).

In Cohn's classical fractionation procedure, it is seen that albumin is commonly isolated from humanblood (E.J. Cohn et al., 1946).

In Cohn's method, by using ethanol with varying pH, ionic strength and temperature, proteins can be precipitated. This technique which is used for industrial fractionation of blood proteins is known as an oldest method, but it is not highly specific and this technique can give partially denaturated proteins (J.F. Stotz et al., 1990).

There are more reliable and efficient techniques for isolation and purification of proteins because of the rapid developments in biotechnology, biochemistry, pharmaceutical science and medicine (A. Kassab et al., 2000) (E.B. Altıntaş et al., 2006) (N. Özturk et al., 2007).

1.4 Proteomics

With the definition of nucleic acids in Nature Journal in April the 25th 1953 the DNA, code of life, was revealed. So, starting from one cell organisms up to mammals the DNA chain was clarified. After that, the studies over genes brought into light the physical properties, illnesses and various unknown subjects. With the definition of proteins, the molecules that give meaning to the definition of living creatures, have helped the explanation of basic subjects like;

- The survival of cells
- The communication of cells
- The variety and functionality of cells

With the definition of these the target range was narrowed down to proteome analysis for proteomic research (R. Bischoff et al., 2004) (C. Li et al., 2004) (J.L. Hess et al., 2005).

The proteome analysis lead the transformation stages and tracing theses stages of the correlation of the relation between the structure and functionalities of proteins was establised and thus the determination of healty and infected cells became easier. As a result the determination of these proteins a huge progress was taken in diagnosis and treatement of various diseases like cancer infected cells, cardiovascular diseases, ostreoarthritis, hemofilia etc. (T.C. Petric et al., 2007) (T.D. Veenstra, 2007) (Y. Jmeian et al., 2007).

In the past 50 years the human gene map was determined. Nowadays, the genes coded by those proteins from this gene map are targeted. These protein molecules take a crucial part in various important events like the ordering of chemical reactions up to intercellular communcation in living creatures and thus make up the basis of living creatures. Genomic research is inadequate in defining this flow and its properties. That is why a new field of study was established called proteomic. That is also the reason why proteomic

field is crucial in clinical trials (T. Linke et al., 2007) (C. Fredolini et al., 2008) (A. Luchini et al., 2009).

The proteins expressed of a tissue or organism genome are called proteome. It is formed by the merger of the words Protein and Genome. The aim of the proteome analysis is to define the properties and functionalities of by conducting structural analysis of proteins. The DNA has only 4 building blocks (A,G,S,T) while natural proteins have 20 different aminoacids. Hence, their analysis is more difficult (E.B. Altıntaş et al., 2006).

The plasma proteome consists of composition of proteins in the blood plasma. Blood flows through different organs, so new proteins can be added or some proteins can be removed, or modifications in proteins can occur (E. Petricoin et al., 2003) (L.A. Liotta et al., 2003) (J.T. Lathrop et al., 2003) (J. Lathrop et al., 2005) (M. Cristea et al., 2004) (K.C. Chan et al., 2004) (K. Rose et al., 2004). Categories of proteins in the human body were represented in the blood plasma. So, the plasma proteome is a source of diagnostic markers and therapeutic targets for many human diseases (N. Anderson et al., 2004).

It plays an important role in the definition of disease spesific proteins, the diagnosis, treatment and development of therapuetic drugs from cancer up to cardiovascular diseases. Proteomics is certainly a promising approach to revolutionize clinical diagnostics, improve prognosis, and lead to potentially life-saving medical treatments (L. Urbas et al., 2009).

1.4.1 The kinds of proteome analysis and field of applications

The proteome analysis can be expressed in various chapters within itself;

- Expressed from cell and tissue
- The determination of the 3 dimensional structure

- The determination of the molecule interacting with the cell
- Protein-protein interaction and intercellular location detection

(A. Denizli, Ö.Irfan Küfrevioğlu)

The fields of applications are,

- The profiling of disesases in medicine
- The regulation of gene functioning
- Resistivity mechanism in farming
- Allergy and toxicology
- Finding new drugs
- Studies on diagnosis and treatment

Examples used in proteome analysis,

- Brain
- Heart
- Liver
- Lung
- Muscle
- Pancreas
- Spleen
- Testis
- Human tissue fluids
- Cerebrospinal fluid
- Urine
- Salivary
- Amniotic fluid
- Follicular fluid
- Blood plasma and serum

(Yılmaz, E. et al.)

1.5 Protein Depletion

Plasma contains numerous proteins, hence it plays a crucial role in disease diagnostics. Biomarkers, described by Adkins et al. as "proteins that undergo a change in concentration or state in association with a biological process or disease," can be key factors for early diagnosis, monitoring response to therapy, and detection of relapse of most types of cancers as well as of other diseases. However, due to the wide concentration ranges of proteins in the plasma, the analysis is quite complex. The albumin which is the most abundant protein, is present in blood 30-50 mg/mL contentration. This constitutes over half of the plasma proteins. In contrast, most of the potential biomarkes are present at very low concentration (L. Thadikkaran et al., 2005) (K. Merrell et al., 2004) (G. Tsangaris et al., 2005). For example, cytokines, which are specific proteins for some diseases and prostat specific antigen (PSA) are present in low pg/L levels. The more abundant proteins will certainly mask the detection of the very low abundance proteins. Purpose of the protein depletion is to avoid this masking effect (J. Adkins et al., 2002) (R. Richter et al., 1999).

Several major strategies are available for protein seperation;

- Gel-based seperation techniques, where electrophoretic mobilities of the proteins being used (A.Gorg et al., 2000) (J.Malmström et al., 2002).

- CE (Capillary electrophoresis), faster from traditional electrophoresis techniques (J. Bienvenu et al., 1998).

- RP-HPLC (Reversible Phase High-Performance Liquid Choromatography), the seperation of proteins is based on differences in their hydorphobic properties. (G.Thevenon et al., 1989) (C.Horvath et al., 1976)

In the case of human serum albumin, depletion can be achieved by either dye-ligands such as the widely recognized Cibacron Blue F3GA and derivatives (E.Gianazza et al., 1982) or specific antibodies (Y.Y. Wang et al., 2003).

A dye-affinity resin for removal of human serum albumin has the advantage of high loading capacity as compared to an antibody-based system but has been shown to lack specificity (A.Denizli et al., 2001) (N.Ahmed et al., 2003).

The other challange in serum protein analysis is the depletion of the high concentration of IgGs. The removal of IgG is commonly achieved by immobilizing protein A of protein G onto the affinity resins, which binds to the Fc region of the IgG (H.Yavuz et al., 2005), but specific antibodies can also be used. In addition, the depletion of IgG in human plasma is employed for the treatment of immune disorders (M.Odabaşı et al., 2005) (S.C. Bansal et al., 1978) (M. Haas et al., 2002).

Depletion of plasma proteins can be made using different strategies, but the final goal is to separate the high abundance proteins from the low abundance proteins.

1.6 Affinity Chromatography

Affinity chromatography is based on highly specific molecular recognition and it is a well-established method for identification, purification and separation of biomolecules (M. Wilchek, 2004). The molecule has the capability of specific recognition in this method and this capability is immobilized on a suitable support (C.R. Lowe, 1979).

Affinity chromatography, is a purification technique which is most effective and specific. It provides a rational basis for the refinement of target proteins (Y.D. Clonis, 1987).

Affinity chromatography uses the basic bio-molecular recognition. This means, biologically active macromolecules form specific and reversible complexes with affinity ligands (N.E. Labrou et al., 1994). Affinity chromatography is more expensive and less robust than other types of liquid chromatography and this is an unpleasant situation of it. This technique can be applied to large-scale purification of protein aqeous pharmaceuticals limitedly. The unrealistic expectation of single-stage purifications from crude extracts causes the perception of this limited applicability. A new perception appears because of this situation. For quality therapeutic and other protein products, the number of purification steps should be reduced while there is an increase in product recovery. This makes necessary of the protein purification processes and restore the interest in affinity chromatography (P. Bailon et al.2000) (Y.D. Clonis et al., 2000) (M. Linhult et al., 2005).

This technique is used in the production of various licensed therapeutic plasma products untill now, for example, factors III, XI and von Willebrand, protein C, Antithrombin III, with the most commonly used affinity ligands heparin, gelatin, murine antibodies and Cu2+-chelates (T. Burnouf et al., 2001).

Besides, novel affinity-based purification strategies are considered for genetically engineered antibodies and related molecules. Protein manufactures can successfully cope with the mentioned challenges with the guidance of affinity chromatography. Now this technique can dominate the protein pharmaceutical industry. Procedures based on affinity chromatography and which are more sophisticated and selective take the place of familiar purification protocols for high-value proteins. For this reason, more importance and attention are given to designing and selecting ligands of high affinity and

specifity. 'Synthetic' and 'Biological' are two kinds of ligands for protein chromatography. Synthetic affinity ligands are produced by de novo synthesis or by the modification of existing molecular structures (e.g. purine and pyrimidine derivatives, triazinly nucleotide-mimetics, non-natural peptides, peptoids and peptidomimetics, triazinly dyes and mimidyes, other triazinebased ligands, oligosaccharide and boronic acid analogues). Biological ligands are obtained from natural sources such as RNA and DNA fragments, nucleotides, coenzymes, vitamins, lectins, antibodies, binding or receptor protein and obtained from also biological and genetic packages, imploying display techniques such as oligonucleotides, peptides, protaiin domains and proteins (A.C.A. Roque et al., 2004).

1.6.1 Advantages and Disadvantages of Affinity Ligands

A comparison can be based on some principal factors such as selectivity, binding capacity, chemical and biological stability, and economics, incorporating buying or manufacturing cost and adsorbent at life-time. There is an intense competition between R&D laboratories dedicated to synthetic or biological ligand. They are in a race to prove which is the most promising ligand class, therefore, this competition attracts the attention of protein industry. Main advantage of biological ligands (e.g.(oligo)-nucleotides, peptides, proteins, oligosaccharides) is their being high selectivity and affinity. Such ligands can be selected from large combinatorial ligand libraries which are based on biological/genetic packages and they can be generated from in vitro evolution approaches. Ligands with proteins have an distinguishing advantages over their peptide counterparts, for instance; higher affinities (smaller loss of entropy upon binding); higher proteolytic stability, making them more suitable for recombinant production; and preservation of their biological activity when produced by fusion to a different protein and domain (P.A. Nygren et al., 1997).

1.6.2 Dye-Ligand Systems

Various molecules such as enzymes, coenzymes, cofactors, antibodies, amino acids, proteins may be used as ligands in new design of sorbents. In most cases, these ligands are specific. However, due to the high costs of production and complex purification steps, they are expensive. It is also difficult to immobilize to support material due to their biological activity. Always some precautions had to be taken at usage and storage (E.P. Lillehoj et al., 1982) (H.A. Chase, 1988) (A. Denizli et al., 1995).

Dye-ligands are important candidate to the natural for affinity chromatography. Dye-ligands have binding capacity to the most of proteins. Commercially availability, easy immobilization to support material (especially with having hydroxyl groups), reusability, biological and chemical stability, being inexpensive are some properties provides to be an alternative to other ligands (A. Denizli et al., 2001).

Several techniques (UV visible, FTIR, NMR etc.) have been used to understand dye-protein interactions. According the studies, dye-protein interactions might consist of electrostatic and hyrophobic forces (L. Lascu et al., 1984) (T. Skotland, 1981) (M.M. Federici et al., 1985) (S. Subramanian, 1984).

Dye molecules act as natural ligands and bind at active points to protein. And dye-ligands provide conditions for other interactions also. Most proteins binds by combination of electrostatic, hydrophobic, hydrogen bonding and charge-transfer interactions (A. Denizli et al., 2001).

1.7 Nanotechnology

Nanotechnology originates from the Greek word meaning “dwarf”. A nanometre is one billionth (10^{-9}) of a metre, which is only the length of ten hydrogen atoms. Although scientists have manipulated matter at the nanoscale for centuries, calling it physics or chemistry, it was not until a new generation of microscopes were invented in the nineteen eighties.

Nanotechnology is the construction and use of functional structures designed from atomic or molecular scale with at least one characteristic dimension measured in nanometers. Their size allows them significantly improved physical, chemical, and biological properties. When characteristic structural features are intermediate between isolated atoms and bulk materials in the range of about one to 100 nanometers, the objects often display physical attributes substantially different from those displayed by either atoms or bulk materials. Nanotechnology can provide understanding about materials and devices and is likely to impact many fields. By using structure at nanoscale, we can greatly expand the range of performance of existing chemicals and materials. Alignment of linear molecules in an ordered array on a substrate surface (self-assembled monolayers) can function as a new generation of chemical and biological sensors. Entirely new biological sensors facilitate early diagnostics and disease prevention of cancers (L.Z. Wang, 1999).

1.7.1 Nanopolymers and Affinity Interactions

In ligand systems, selection of the support material is the most important parameter. Support material must have functioanal surface groups for the immobilization of ligands. Also, it has to be resistant to extreme conditions (low and high temperature, pH etc.) and it has to stable chemically and physically (A. Denizli et al., 2001).

Porous materials like nanoparticules have quite high surface area, allows high amount of ligand immobilization and so high adsorption capacity could be provided. Porous structure is suitable for adsorption but pore sizes must carefuuly selected. Pores should be large enough to adsorb and desorb large size ligands and proteins (A. Denizli et al., 2001).

Non-porous nanoparticles as support for adsorption of proteins is progressively increasing nowadays (D.A. Uygun et al., 2009) (S. Akgöl et al., 2008). These supports have very small size and despite being nonporous, they permit to immobilize big amount of protein. Additionally, an advantage of nonporous nanoparticles is that they dont have external diffusion problems (L. Betancor et al., 2005).

In recent years, nanoparticles have received great attention for biotechnological applications such as separating cells, proteins, and DNA, medical diagnosis and controlled drug delivery (V. Rousseau et al., 1998) (B.Yoza et al., 2003) (D.A. Uygun et al., 2012). The use of nanoparticles for purification and immobilization of biomolecules, have the following advantages: (1) high specific surface area can be obtained to bind large amount of protein samples; (2) selective targeting of biomolecules with desired surface characteristics and easy separation from the reaction mixture and (3) elimination of several steps in the purification process such as centrifugation, filtration (C. Okoli et al.,2011).

2.EXPERIMENTAL AND METHODS

2.1 Chemicals and Materials

2-Hydroxyethylmethacrilate (HEMA), Polyvinylalcohol (PVA), Potassium persulfate (KPS), Ethyleneglycol dimethylacrilate (EGDMA), Reactive Red2 (RR2), SodiumHydroxide (NaOH), Sodium Acetate Buffer (0.1M), Sodium Chloride (NaCl),Hydrochloric Acid (Hcl), Phosphate Buffer (0,1M), Ethanol (EtOH), Methanol (MeOH), BSA used in this work were purchased from Sigma Aldrich.

2.2 Devices and Laboratory Tools

The laboratory apparatus and devices used in this study are given below. Precision Scales (Kern ABS), Shaking Water Bath (Wise-bath), Spectrophotometer, Magnetic Stirrer (Wisd Laboratory Instrument), Thermal Precipitator, pH meter (Istek), N_2 , Vacuum Furnace (Memmert), Centrifuge (Eppendorph Mini Spin Plus), UV-Visible Spectrophotometer (Thermoscientific Evolution 60), Sonicator (Bandelin Sonorex), ZETA Size Measurement Device (Malvern Instruments Nanoseries Zeta Sizer, Hacettepe University Chemistry Department), Scanning Electron Microscopy (SEM-EDX)(Quanta 250s FEG, IYTE MAM), Fourier Transform Infrared Spectroscopy (FTIR) (Perkin Elmer 100 FTIR).

2.3 Synthesis of p(HEMA) nanopolymer

Surfactant free emulsion polymerization method was used at the preparation of the p(HEMA) nanopolymer (Öztürk et al., 2007). 0.275 mg PVA (stabilizator) is dissolved in distilled water with heating. Solution was transferred to the polymerization reactor then 0.6 mL HEMA (monomer) and 0.3 mL EGDMA (crosslinker) was added. Finally, 0.198 g of KPS (initiator)

solved in 45 mL distilled water was added. Nitrogen gas was passed 1-2 minutes to remove dissolved oxygen from the mixture. Reactor was placed in shaking water bath and stirred at $70^{o}C$. After 2 hours, polymer formation was observed (Figure 2.1).

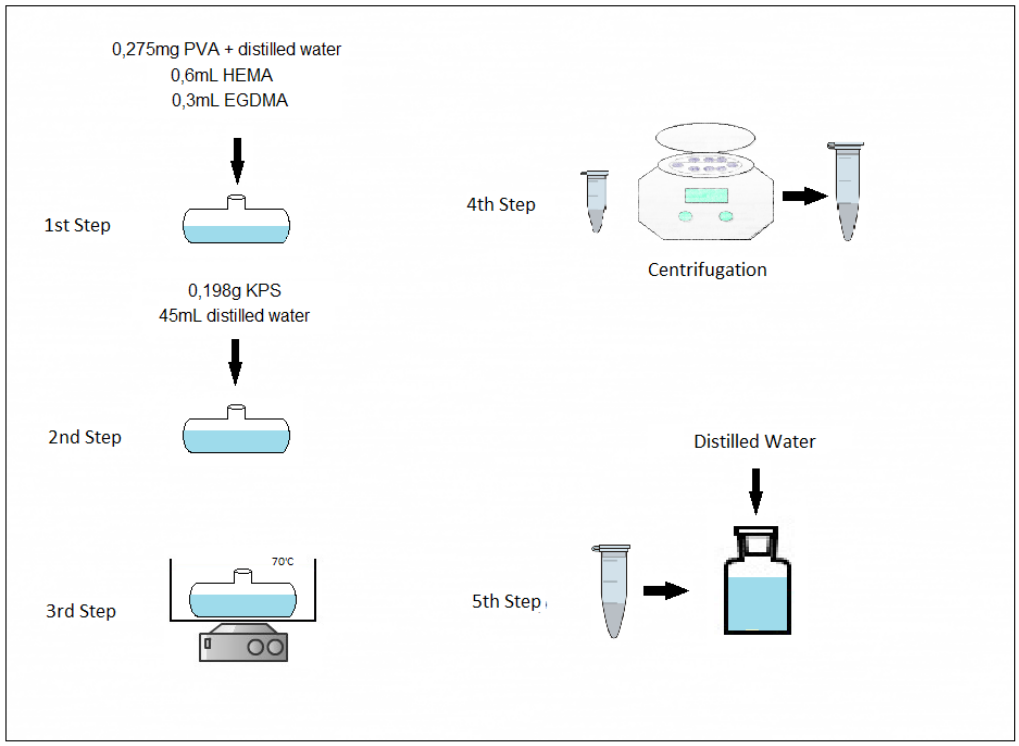

Figure 2.1. Synthesis of p(HEMA) nanopolymers

Obtained p(HEMA) nanopolymer washed 9 times with distilled water and ethanol to remove unreacted initiator and monomer. During the washing steps, nanopolymer centrifuged at 9000rpm for 20 min to obtain precipitated nanoparticles. After washing, the particles was dispersed with distilled water in sonic bath. The particles were stored at $+4^{o}C$ after washing process.

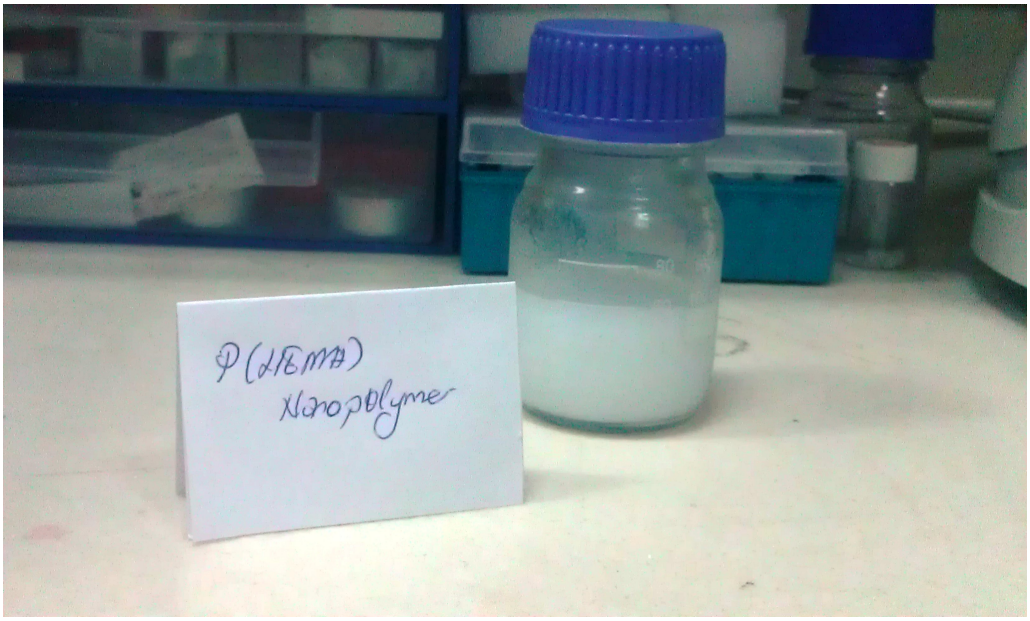

Figure 2.2. p(HEMA) (poly-2-hydroxyethyl methacrylate) nanopolymer

2.4 Modification of p(HEMA) nanopolymer with Dye-Ligand via Covalent Bond

As the ligand, Reactive Red2 which is textile dyes was used in the dyeing of the nanopolymer p(HEMA). The dye-binding procedure given in the literature was performed with minor modifications.

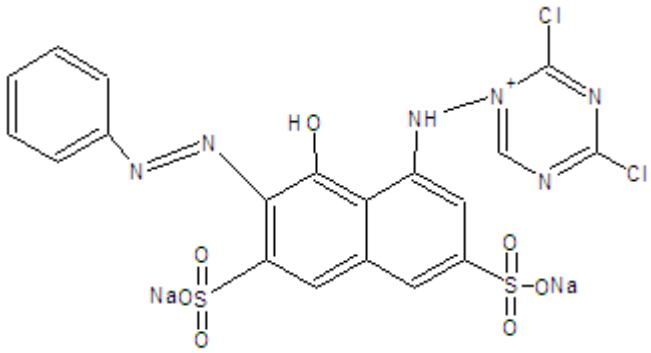

Figure 2.3. The chemical structure of Reactive Red2

To avoid excess of dye waste, 3 mg/mL dye concentration was selected as initial concentration. 218.6 mg Reactive Red2 (final dye concentration to be 3 mg/mL) was added to 70 mL nanoparticle solution. Then a mixture of 4 g

NaOH and 30 mL distilled water added and stirred at sonicator. Solution was stirred in a water bath at $80^{o}C$ for 4 hours. Dyed particules were washed with distilled water, methanol and 1M NaCl solution. Dye leakage was controlled at 538 nm and washing process ended at no leakage observed. To determine the amount of ligand bounded dye;

$$Q = \frac{(C_{initial} - C_{final})(mg/mL) * V_{medium}(mL)}{Dry\ Polymer\ Mass(g)} \quad (2.1)$$

equation was used

(Q :adsorbed amount of dye per g of polymer(mg), $C_{initial}$:initial dye concentration, C_{final} :dye concentration as determined by spectrophotometry.)

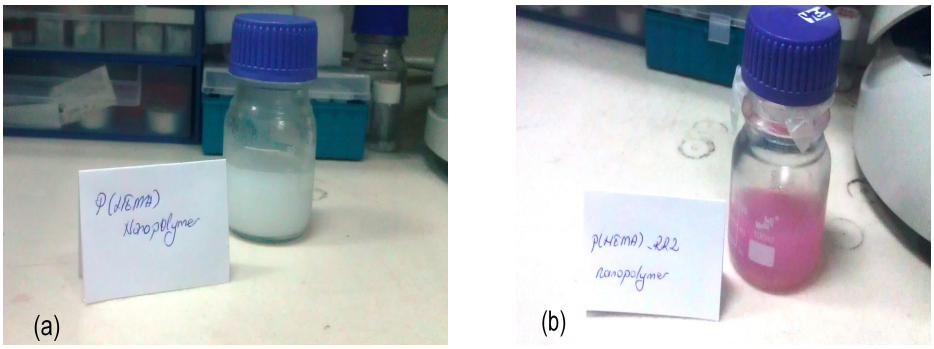

Figure 2.4. p(HEMA) nanopolymer; (a) Prior to dye (b) After dye image

2.5 Characterization of p(HEMA)-RR2 nanopolymer

p(HEMA)-RR2 nanopolymer characterization studies includes; FTIR was used to determine whether dye ligand participated in nanopolymer structure or not, elemental analysis was performed to determine the dye ligand content of the nanopolimer, SEM was used to determine the spherical structure, ZETA size analysis was performed to determine the size. Also to determine the dry weight of the nanopolimer per mL , the dry polymer mass in 1 mL was calculated in mg (Akgöl et al., 2010).

2.5.1 FTIR (Fourier Transform Infrared Spectroscopy) Measurements of the p(HEMA)-RR2 nanopolymer

FTIR spectrums of p(HEMA) and p(HEMA)-RR2 nanopolimers were obtained using FTIR spectrometer (FTIR Perkin Elmer 100). Dried nanoparticles(2 mg) were brought into tablets and FTIR spectrum was taken in the wave number range 4000 to 650 cm^{-1}.

2.5.2 ZETA Dimensional Analysis of the p(HEMA)-RR2 nanopolymer

Zeta dimensional analysis of the synthesized p(HEMA)-RR2 nanopolimer was made with Nano Zetasizer (NanoS, Malvern Instruments, London, England). Measurement was performed by injecting 1 mL dilute polymer into chamber of the device.

Zetasizer working principle is based on Brownian motion of the particles in the liquid. Particle size measurements is made by the property of the particles which is moving the fluid. The larger particles are moving more slowly than the small particules. Based on this principle, dynamic light is sent by the device to sample. Looking at the size of the movement of the particles, information about size of the particles obtained.(Malvern Instruments Ltd., 2013)

2.5.3 SEM images (Scanning Electron Microscope) of the p(HEMA)-RR2 nanopolymer

SEM device (Quanta 250S FEG) at Izmir Institude of Technology was used for the SEM images of the p(HEMA) nanopolymers. Polymeric nanostructures were dried by lyophilisation then to increase the conductivity of the polymer, surface was gold coated. Morphological characteristics and size of the particles were examined.

2.5.4 Determination of the Dry-Mass of p(HEMA)-RR2 nanopolymer

For the determination of the dry mass of polymer, 0.5-2 mL nanopolymer solution were added to the tared eppendorf tubes. After centrifuging, the upper phase of the samples were taken then samples were oven-dried at $35^{o}C - 40^{o}C$. Eppendorf weight was removed from the final mass and mass-volume graph was drawn. The amount of grams of polymer per unit volume was calculated.

2.5.5 Calculation of the Specific Surface Area of p(HEMA)-RR2 nanopolymer

Determination of the surface area of the polymer was made out with the following equation which gives the number of particles in 1 ml of suspension. (Bangs 1987).

$$N = \frac{6 * 10^{10} * S * \rho_L}{\pi * \rho_S * d^3} \qquad (2.2)$$

Where N represents the number of particles in 1 mL of suspension; S shows the % solid form; d indicates the diameter (μm); ρ_s the density of polymer (g/mL).

The amount of nanoparticles (mg) in suspension (mL) was determined theoretically via standart graph of mass-volume belonging to the nanoparticles. The specific surface area of p(HEMA)-RR2 nanoparticles were calculated in terms m^2/g, using below equation of surface area of the sphere.

$$surface\ area\ of\ the\ sphere = 4 * \pi * r^2 \qquad (2.3)$$

(r: radius of nanoparticles(m), π=3.14)

2.6 Determination the Amount of BSA Adsorption to p(HEMA)-RR2 nanopolymer

Spectrophotometric measurements were performed at 595 nm wavelength in UV region to determine the amount of BSA. By taking the measurements with different concentrations of BSA, calibration graph was created. All measurements were performed at room temperature. Amount of BSA adsorption was calculated with below equation;

$$Q = \frac{(C_{initial} - C_{final})(mg/mL) * V_{medium}(mL)}{Dry\ Polymer\ Mass(g)} \quad (2.4)$$

(Q : adsorbed amount of BSA per g of polymer,(mg), $C_{initial}$: initial BSA concentration (mg/mL), C_{final} : BSA concentration (mg/mL) as determined by spectrophotometry.)

2.7 Optimization of the BSA Adsorption Conditions to the p(HEMA)-RR2 Affinity Nanopolymer

At the optimization experiments, the effect of time, pH, temperature, ionic strength and the initial concentration to the BSA Adsorption on p(HEMA)-RR2 nanopolymer were examined. In the adsorption experiments 2mg/2 mL BSA solution was prepared. At pH trials, 0.1M acetate buffer was used for pH 4 and 5, for pH 6,7 and 8 phosphate buffer was used. After trials, protein determination was done with Bradford Method and adsorption capacity value Q (mg), amount of BSA (mg) adsorbed per gram of polymer were calculated.

2.7.1 Bradford Method

The Bradford method is very effective at protein determination. The absorbance of acidic solution of Coomassie Blue shifts from 465 nm to 595 nm, due to binding to protein.

The preparation of the Bradford reagent: 40 mg of Coomassie Blue were dissolved in 50 mL of ethanol (%95). The solution was stirred for 45 min in a sealed manner then 45mL of phosphoric acid(%88) was added. The solution was stirred until all dye were completely dissolved then diltued to 1 L.

2 mL of Bradford reagent was added to 100 µL of sample of protein solution, measurement of absorbance was done after about 10 min at 595 nm.

2.7.2 The effect of Time on the BSA Adsorption

For the purpose of examinig effect of time, 2mL BSA solution was prepared in acetate buffer at pH 5 then 100 µL of dyed polymer solution was added and mixture was put at angle stirrer. Samples were taken from adsorption medium at 5,10,15,30,45,60,120,150 minutes and were centrifuged for 20 min at 14500 rpm. Protein determination was made by the Bradford Method at the supernatant. So that time-dependent variation of adsorption was investigated.

2.7.3 The effect of pH on the BSA Adsorption

In order to examine the effect of pH on the BSA adsorption, 2mg BSA was dissolved in 2 mL buffer solution at pH range 4-8. 100 µL p(HEMA)-RR2 and p(HEMA) polymer solution was added onto BSA solution and mixture was put at angle stirrer for 2 hours. Samples were centrifuged for 20 min at 14500 rpm. Protein determination was made by the Bradford Method at the supernatant.

2.7.4 The effect of the Initial Concentration to the BSA adsorption

In order to examine the effect of initial concentration on the BSA adsorption, 1 mg/mL of BSA solution was prepared at range 2-10 mg/mL initial concentration in 2 mL 0.1M acetate buffer at pH 5. 100 μL p(HEMA)-RR2 and p(HEMA) polymer solution was added onto BSA solution and mixture was put at shaker for 2 hours. Samples were centrifuged for 20 min at 14500 rpm. Protein determination was made by the Bradford Method at the supernatant.

2.7.5 The effect of Ionic Strength on the BSA Adsorption

In order to examine the effect of ionic strength on the BSA adsorption, 1 mg/mL BSA solution was prepared in 2 mL 0.1M acetate buffer at pH 5 containing 0.01 to 1.5M of NaCl. 100 μL p(HEMA)-RR2 and p(HEMA) polymer solution was added onto BSA solution and mixture was put at shaker for 2 hours. Samples were centrifuged for 20 min at 14500 rpm. Protein determination was made by the Bradford Method at the supernatant.

2.7.6 The effect of Temperature on the BSA Adsorption

In order to examine the effect of temperature on the BSA adsorption, 1 mg/mL BSA solution was prepared in 2 mL 0.1M acetate buffer at pH 5. 100 μL p(HEMA)-RR2 and p(HEMA) polymer solution was added onto BSA solution and samples were put at water-baths with temperatures regulated to $4,25,37,45^{o}C$ for 2 hours. Samples were centrifuged for 20 min at 14500 rpm. Protein determination was made by the Bradford Method at the supernatant.

2.8 Adsorption Isotherm of p(HEMA)-RR2 Affinity nanopolymer

Adsorption isotherm is a process which is used to characterize the interaction of each protein molecule with adsorbent. It describes the relationship between the amount of protein adsorbed by solid phase and the concentration of protein in solution while two phases are in equilibrium. Langmuir adsorption model accepts that adsorption is equal and adsorbent is standard in all regions. These regions are equal in terms energetics, adsorbed molecules will not interact with each other. Langmuir adsorption model is defined as:

$$Q=(Q_{max}*b*C_d)/(1+b*C_d) \tag{2.5}$$

whereas Q: the amount of the removed molecules (mg/g), C_d: equilibrium molecule concentration (mg/L), b: Langmuir constant (mL/mg), Q_{max}: maximum molecular adsorption capacity (mg/g). When this equation is linearized;

$$1/Q=[1/Q_{max}]+[1/(Q_{max}*b)]*(1/C_d) \tag{2.6}$$

equation is obtained. Intersection of the line with y-axis at $(1/C_d)$ versus $(1/Q)$ graph, gives the value of $(1/Q_{max})$, the slope of the line gives the value of $[1/(Q_{max}*b)]$ (S. Akgöl et al., 2010).

Freundlich adsorption model, unlike the Langmuir adsorption model, accepts the exponential adsorption system. After surface adsorption, it shows strong solute-solute interaction with condensation effects. Freundlich adsorption model is defined as:

$$Q=K_F*C_{eq}*(1/n) \tag{2.7}$$

Where K_F and n are Freundlich constants, $1/n$ indicates the surface heterogeneity and takes values between 0 and 1. The surface heterogeneity

increases while value approaches to 0. Equation is linearized with taking the logarithm,

$$lnQ = \ln K_F + (1/n) * lnC_{eq} \quad (2.8)$$

Intersection of the line with y-axis at lnQ versus lnC_{eq} graph, gives the value of lnK_F, the slope of the line gives the value of $1/n$ (Bayramoğlu and Arıca, 2009).

The adsorption isotherm of p(HEMA)-RR2 nanopolymer was determined with data of the effect of initial BSA concentration.

2.9 Reusability assays of p(HEMA)-RR2 Affinity nanopolymers

For the reusability assays of p(HEMA)-RR2 nanopolimer, 0.1M NaOH desorption solution was prepared. 1 mg/mL BSA solution with 5 mg/mL initial concentration was prepared in acetate buffer at pH 5. 100 µL p(HEMA)-RR2 and p(HEMA) polymer solution was added onto BSA solution and mixture was held at room temperature for 2 hours. Samples were precipitated via centrifuging for 20 min at 14500 rpm. Protein determination was made by the Bradford Method at the supernatant. 1 mL of desorption solution was added onto the precipitated nanopolymer and putted at angle stirrer for 2 hours. Precipitated nanopolymer via centrifuging at 14500 rpm, was washed with 1M NaOH for regeneration and sterilization and washed with acetate buffer at pH 5 for balancing after each adsorption-desorption process. Second cycle was performed in the same way by adding BSA solution after regeneration. To determine the reusability of nanopolymer synthesized in this way, adsorption - desorption cycle was repeated 5 times with same polymer. Desorption rate was calculated using the following equation;

$$Desorption\ Rate\ (‰) = \frac{BSA\ release\ desorption\ environment}{Absorbed\ BSA} * 100 \quad (2.9)$$

3. RESULTS AND DISCUSSION

3.1 Covalent Modification of p(HEMA) nanopolymer with Dye-Ligand

Hypothetic structure of synthesised p(HEMA) nanopolymer is shown in the below figure (Figure 3.1).

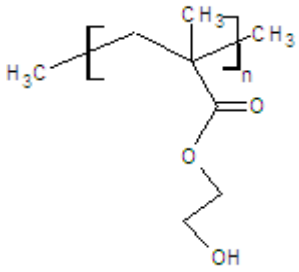

Figure 3.1 p(HEMA) structure

Accordingly, covalent binding of nanopolymer and dye shown in the below reaction scheme (Figure 3.2).

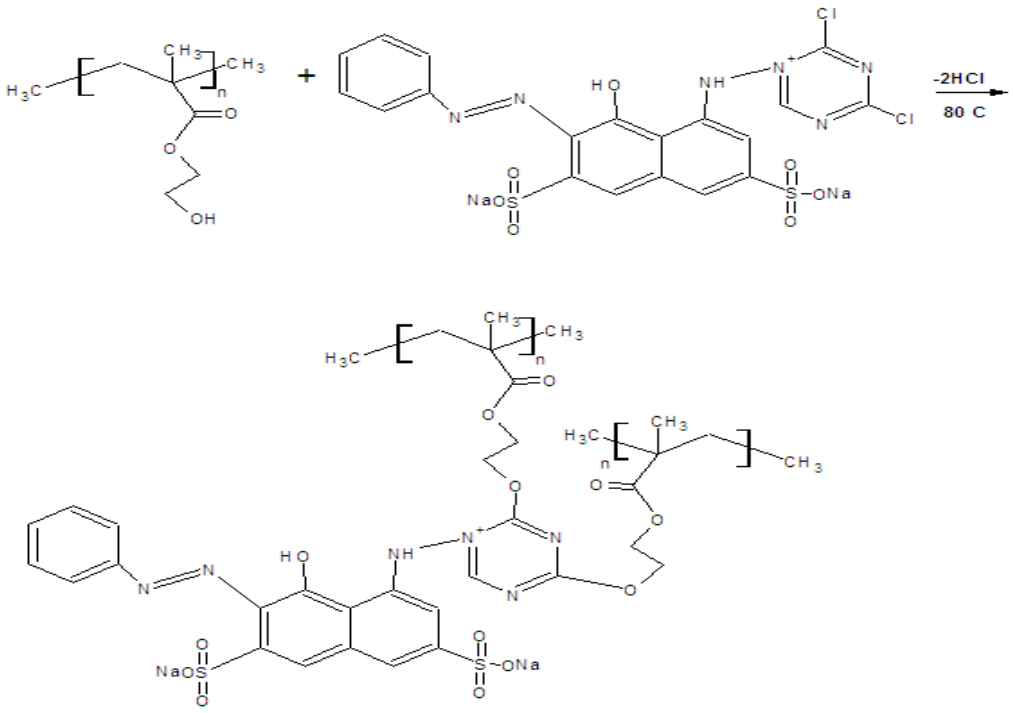

Figure3.2 Binding Reaction of the Reactive Red2 to the p(HEMA) nanopolymer

Under alkaline conditions (pH 10-11 0.1M NaOH), binding has been realized between the reactive chloride of the RR2 molecules and hydroxyl groups by nucleophilic substition reaction. HCl molecule is exposed as a result of the reaction.

The dye-ligand adsorption capacity of p(HEMA) nanopolymer increase with increasing the Reactive Red2 concentration. Nanopolymer reaches to the maximum adsorption capacity whereas free -OH groups on the surface of nanopolymer reaches to saturation. To avoid excess of dye waste, 3 mg/mL dye concentration was selected and nanopolymers were dyed. The amount of adsorbed dye was measured sphectrophotometrically at 538 nm and 0.82 mmole/(g nanopolymer) (110.5g/g nanopolymer) was calculated.

3.2 Characterization of p(HEMA)-RR2 nanopolymer

Some special methods were used for characterization of the polymers. These methods listed as; FTIR, ZETA, SEM and The Determination of Dry Mass.

3.2.1 FTIR (Fourier Transform Infrared Spectroscopy) Measurements of the p(HEMA)-RR2 and p(HEMA) nanopolymers

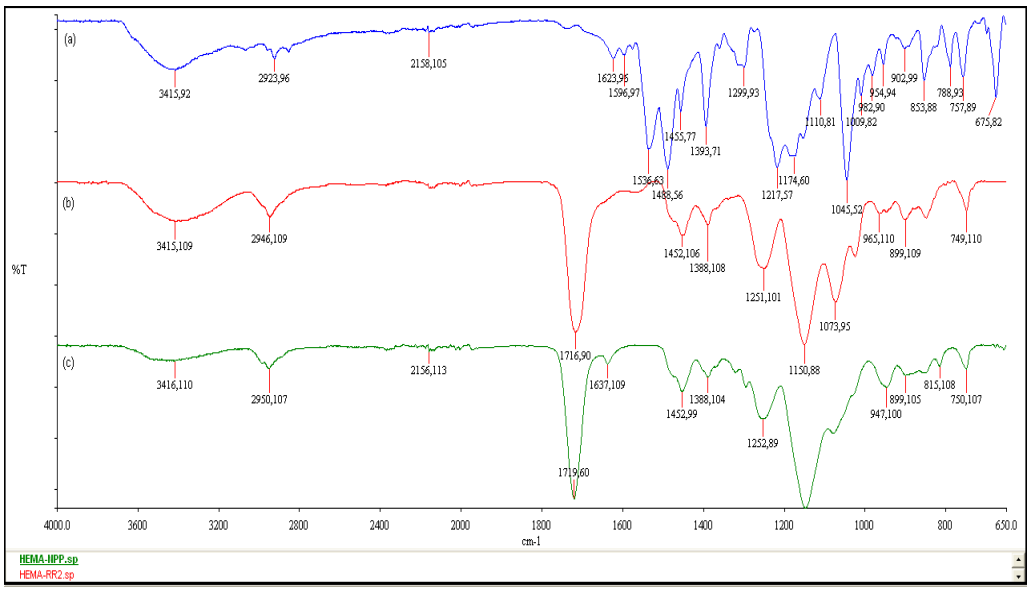

Figure 3.3 (a) RR2, (b) p(HEMA)-RR2, (c) p(HEMA) spectrum of FTIR

FTIR spectrums of RR2, p(HEMA) and p(HEMA)-RR2 nanopolymers are shown in the Figure 3.3. Resulting from O-H groups, near by $3500\,cm^{-1}$, the characteristics O-H streching vibration band was observed. With additon of RR2 to p(HEMA), absorption is significantly increased, because of the existance of N-H bending in RR2 structure. All spectrums contains aliphatic CH_2/CH_3 streching vibration band at $2950\,cm^{-1}$. At p(HEMA) polymer spectrum, streching vibrations band of carbonyl (C=O) groups is observed around $1720\,cm^{-1}$. Characteristics streching vibrations bands of ester (C-O) are around $1250\,cm^{-1}$ and $1150\,cm^{-1}$.

In addition to the bands of p(HEMA), p(HEMA)-RR2 spectrum contains symetric S=O stretching at $1073\,cm^{-1}$, asymetric S=O stretching at $1150\,cm^{-1}$, aromatic C-N stretching at $1251\,cm^{-1}$. However, since p(HEMA) have absorption bands at same regions, these bands are not seen as distinctly.

The physical appearance of the nanopolymer being pink is also an indication of binding of the dye ligands to the polymer. Also, p(HEMA)-RR2 nanopolymer samples were hold in buffers with different pH for 1 hour, then washed. No ligand leakage observed at washing process.

3.2.2 ZETA Dimensional Analysis of the p(HEMA)-RR2 nanopolymer

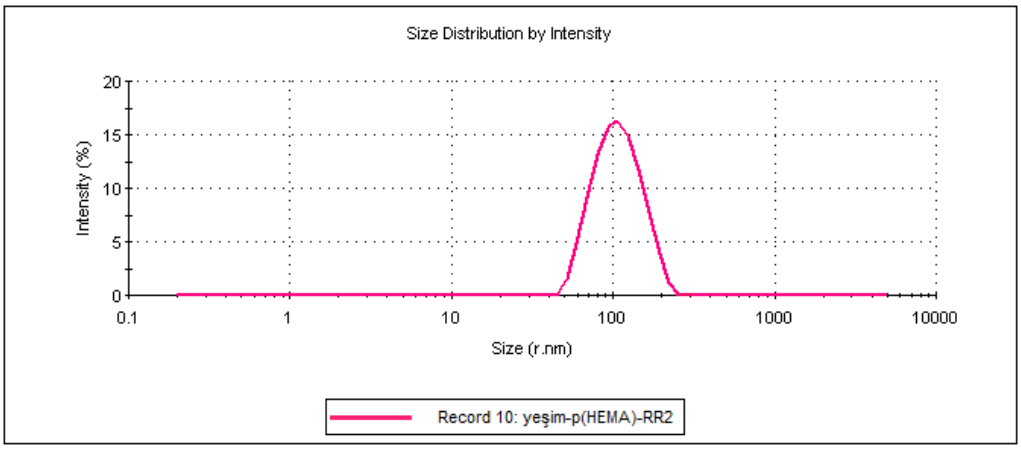

Figure 3.4 ZETA Dimensional analysis of the p(HEMA)-RR2 nanopolymers

ZETA analysis of p(HEMA)-RR2 nanopolymers was performed with Nano Zetasizer (NanoS, Malvern Instruments, London, England). Measurement was performed by injecting 1 mL dilute polymer into chamber of the device. As a result of analysis, mean particle size is 104 nm and polydispersity index value (PDI) is 0.179 and PDI width is 43.30 nm.

3.2.3 SEM images (Scanning Electron Microscope) of The p(HEMA)-RR2 nanopolymer

The morphology of the p(HEMA)-RR2 nanopolymer was observed in Scanning Electron Microscopy. Spherical shapes of the particles was observed. Also observed that their size was compatible with the dimensional size of ZETA (Figure 3.5).

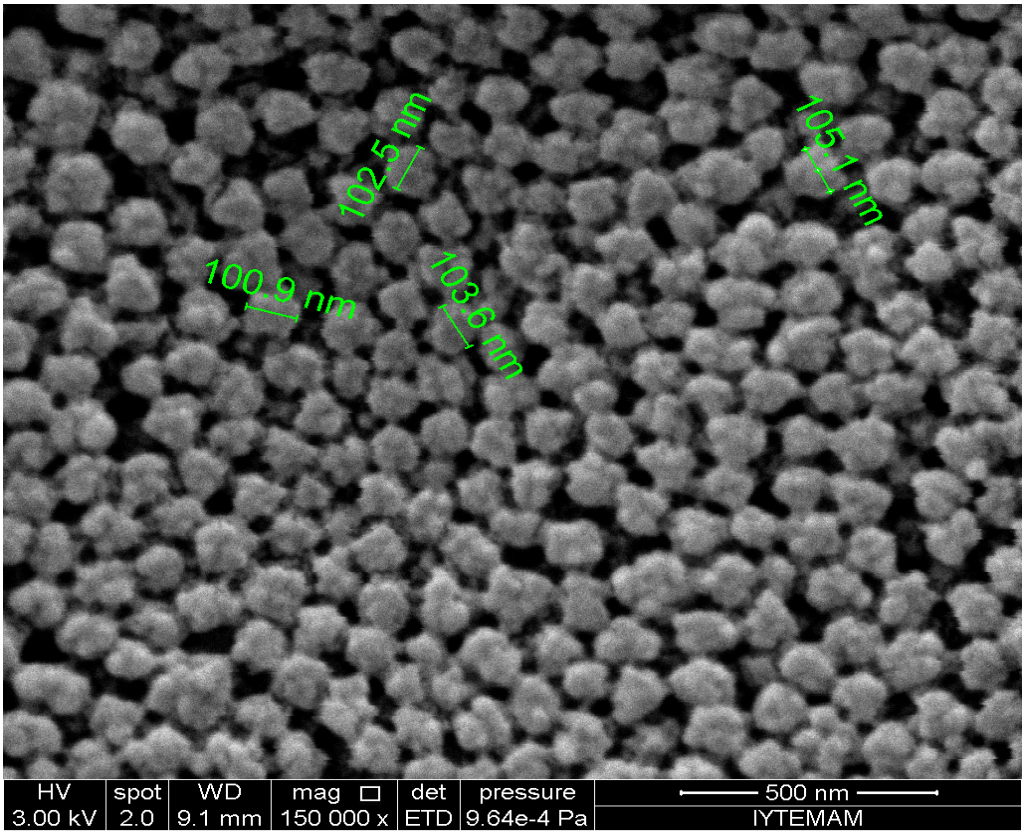

Figure 3.5 SEM images of p(HEMA)-RR2 nanopolymer

3.2.4 Determination of the Dry-Mass of p(HEMA)-RR2 nanopolymer

Aid of the obtained volume-mass graph, the amount of polymer(g) per unit volume was calculated as $0.85*10^{-4}\ polymer/mL$.

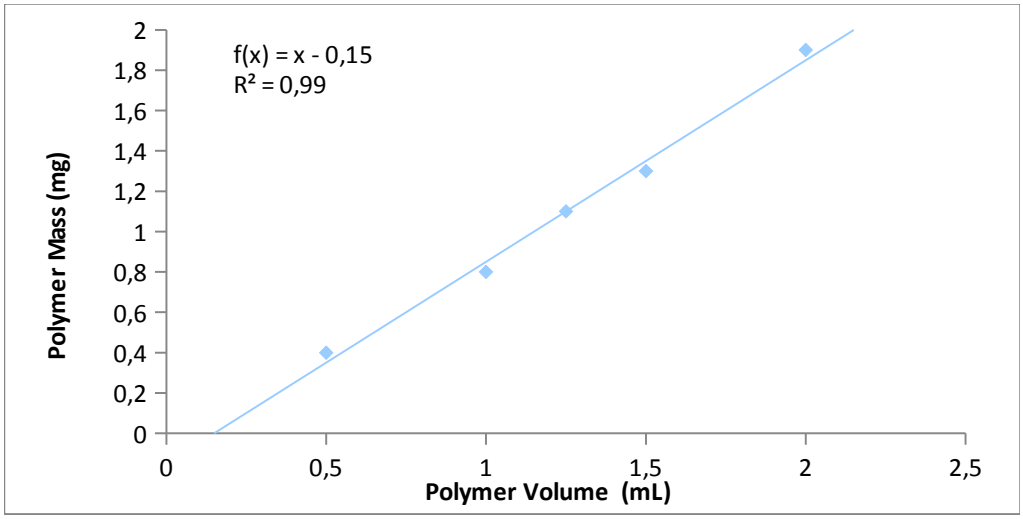

Figure 3.6 Volume-Mass Graph of p(HEMA)-RR2 nanopolymer

3.2.5 Calculation of the Specific Surface Area of p(HEMA)-RR2 nanopolymer

Diameter of the p(HEMA)-RR2 nanospheres was determined as 100nm and specific surface area calculated as $7122.92\,m^2/g$ using the equations 2.2 and 2.3;

$$N=\frac{6*10^{10}*S*\rho_L}{\pi*\rho_S*d^3} \qquad ()$$

$S=10\,;\ \rho_L=0.99899\mathrm{g}/mL\,;\ \rho_S=0.99\mathrm{g}/cm^3\,;\ d=0.100\,\mu m\,;\ \pi=3.14$

$N=1928.18*10^{11}$ Number of particles in 1 mL.

Surface area of the spheres $A=4*\pi*r^2$

$A=4*3.14*(50*10^{-9})^2$

$A=3.14*10^{-14}m^2$

Dry-Mass of p(HEMA)-RR2 nanopolymer per mL = 0.85 mg = $8.5*10^{-4} g$

Specific Surface Area = $(N*A)/dry-mass\ of\ nanopolymer\ per\ mL$

Specific Surface Area = $7122.92\, m^2/g$

3.3 Optimization of the BSA Adsorption Conditions of p(HEMA)-RR2 nanopolymer

3.3.1 The effect of Time on the BSA Adsorption

Effect of time on BSA adsorption were studied with samples which were taken at 5,10,15,30,45,60,120 and 150 minutes. BSA solution in (pH5 2mL) Na Acetate buffer prepared and 100 µL p(HEMA)-RR2 nanopolymer added onto BSA solution. Mixture was put at angle stirrer. The experiment was performed at room temperature. Samples were taken from adsorption medium at 5,10,15,30,45,60,120,150 minutes and were centrifuged for 20 min at 14500 rpm. Protein determination was made at the supernatant with Bradford Method.

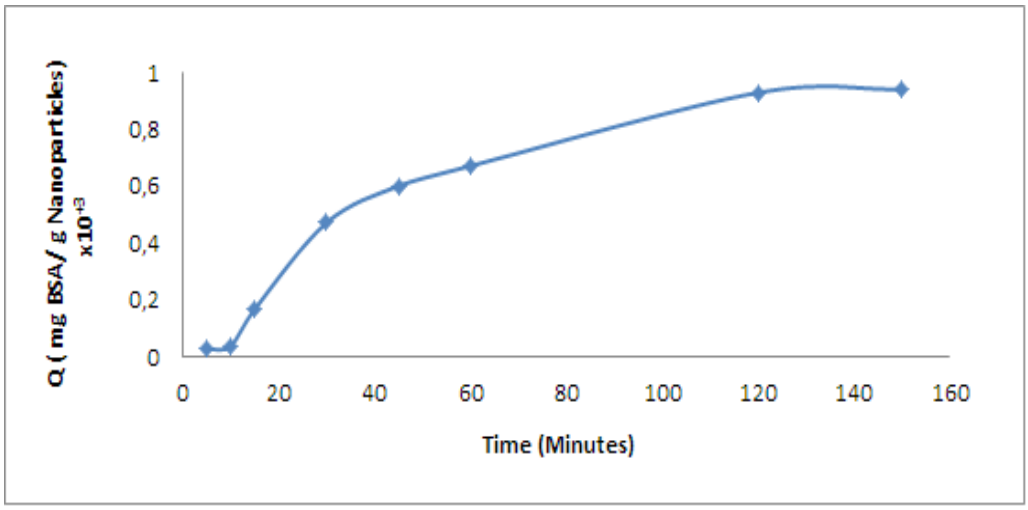

Figure 3.7 The effect of Time on the BSA Adsorption *(C BSA-initial: 2 mg/mL, pH5, $T: 25^{o} C$)*

Adsorption capacity values Q increases with time to a maximum value then started to decrease. After 2 hours, as shown in Figure 3.9, the value of maximum adsorption has been reached.

3.3.2 The effect of pH on the BSA Adsorption

In order to examine the effect of pH on the BSA adsorption, 2 mg BSA was dissolved in 2 mL buffer solution. Buffer solutions are respectively used,

0.1M pH 4 NaAc buffer
0.1M pH 5 NaAc buffer
0.1M pH 6 Phosphate buffer
0.1M pH 7 Phosphate buffer
0.1M pH 8 Phosphate buffer

100 µL p(HEMA)-RR2 and p(HEMA) polymer solution was added onto BSA solution and mixture was put at angle stirrer for 2 hours at room temperature. Samples were centrifuged for 20 min at 14500 rpm. The samples were diluted 10 times with distilled water. Protein determination was made at the supernatant. UV analysis was completed by addition of Bradford reagent.

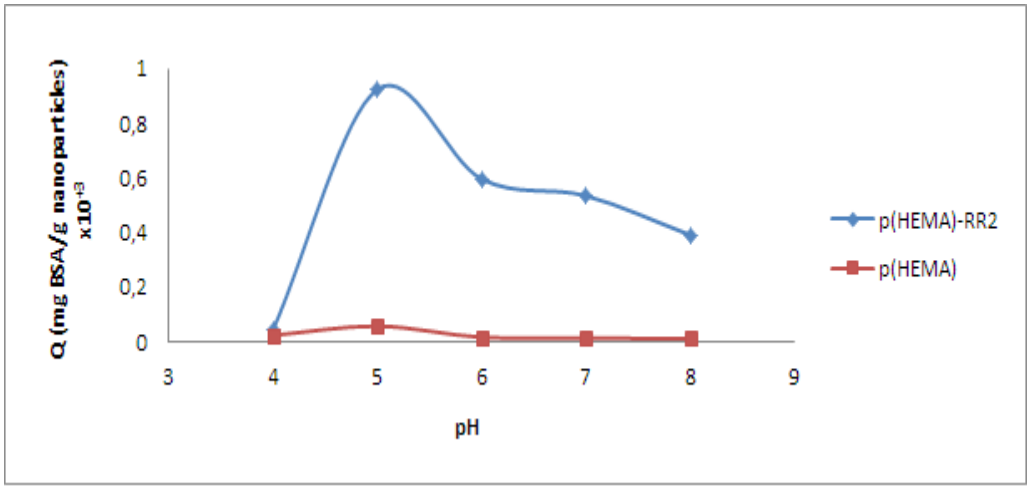

Figure.3.8 The effect of pH on the BSA adsorption *(C BSA -initial:2 mg/mL, T:25^{o}C, t adsorption:2 Hours)*

As shown in the graph, effect of pH on BSA adsorption to p(HEMA) nanopolimer is negligible. From the neutral to acidic region, the adsorption capacity of p(HEMA)-RR2 nanopolymer increases. Maximum BSA adsorption

was found at pH5 as 929.5 mg/g. From pH5 to alkaline region, BSA adsorption capacity to p(HEMA) nanopolimer decreases. The lowest value of adsorption capacity was observed at pH8.

The isoelectric point of the BSA is known as (pI) ≈4.7, 5 (Ge S, Kojio K, Takahara A, Kajiyama T (1998)). The specific interactions (hydrophobic, electrostatic, or hydrogen bonds) between BSA and the dye molecules at pH:5 were due to ionization states of the groups (sulphonic acid and amino groups) in RR2 and the groups in the amino acid sidechain of BSA.

3.3.3 The effect of the Initial Concentration to the BSA Adsorption

To investigate initial concentration effect on BSA adsorption, in 2mL buffer solution, BSA solutions were prepared at 0.2, 0.5, 1, 2, 4, 6, 8, 10 mg/mL concentrations. Then 100 µL of p(HEMA)-RR2 and p(HEMA) solutions were added on each. After 2 hours with angle stirrer, samples were centrifuged 20 minute at 14500 rpm and protein identification was made at supernatant.

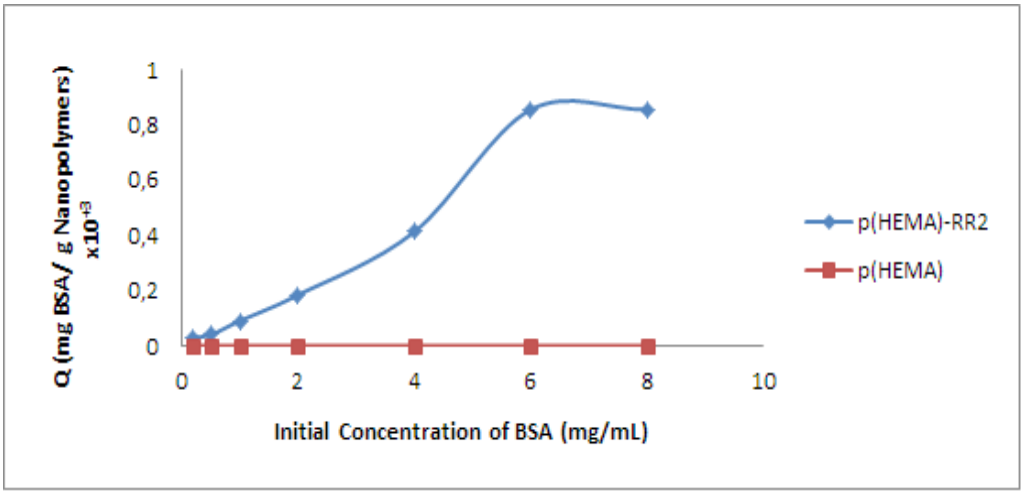

Figure 3.9 The effect of the initial concentration on the BSA adsorption *(pH5, T:25^{o}C, t adsorption:2 hours)*

The effect of the initial concentration on BSA adsorption is given on in Figure 3.9. Referring to the graph, the amount of absorbed BSA by per unit mass of nanopolymer increases with increasing the initial concentration of BSA and reached a plateau at a value 6 mg/mL. The active adsorption sites of the p(HEMA) nanopolymer reaches to saturation at 6 mg/mL initial concentration. Under optimization conditions, BSA adsorption capacity of the p(HEMA)-RR2 nanopolymer was found as 855 mg/g. This high value of the BSA absorption of the nanopolymer per unit mass is related with the large surface area of the nanostructure and adsorption capacity is increased with the dye immobilization to the nanopolymer.

3.3.4 The effect of Ionic Strength on the BSA Adsorption

In order to examine the effect of ionic strength on the BSA adsorption, 1 mg/mL BSA solution was prepared in 2 mL 0.1M acetate buffer at pH5 containing 0.01,0.1,0.5,1.0,1.5M of NaCl. 100 µL p(HEMA)-RR2 and p(HEMA) polymer solution was added onto BSA solution and mixture was put at shaker for 2 hours. Samples were centrifuged for 20 min at 14500 rpm. Protein determination was made with Bradford Method at the supernatant.

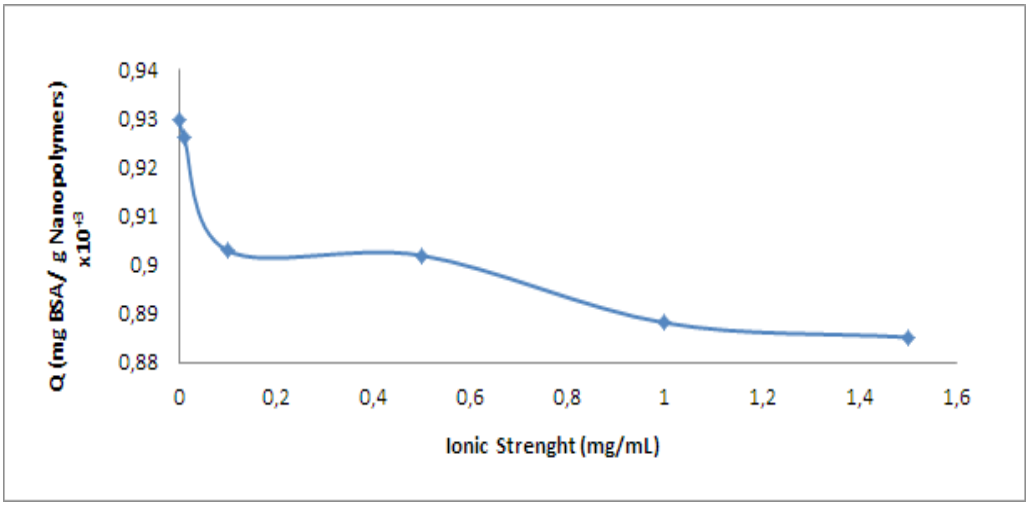

Figure 3.10 The effect of Ionic Strength on the BSA adsorption *(C BSA initial:1 mg/mL, pH5, T:25^{o}C, t adsorption:2 hours)*

Effect of NaCl concentration on the adsorption of BSA is shown in Figure 3.10. According to graph, with increasing NaCl concentration 0M to 0.01M NaCl, the amount of absorbed BSA by p(HEMA)-RR2 nanopolymer reduced sharply. The increase of the NaCl concentration may increase the absorption of the dye to the polymer surface with hydrophobic interactions. It also may increase the hydrophobic interactions between the dye molecules. The free dye molecules in dye solution observed to be sacked with the addition of salt. So that reachable number of dye molecules by BSA decreased and with increasing ionic strength, BSA adsorption to dye molecules becomes difficult. Also electrostatic interactions decreases as the NaCl concentration increases (Öncel et al.,2005).

3.3.5 The effect of Temperature on the BSA Adsorption

In order to examine the effect of temperature on the BSA adsorption, 1mg/mL BSA solution was prepared in 2 mL 0.1M acetate buffer at pH 5. 100µL p(HEMA)-RR2 and p(HEMA) polymer solution was added onto BSA solution and samples were put at water-baths with temperatures regulated to $4,25,37,45^{o}C$ for 2 hours. Samples were centrifuged for 20 min at 14500 rpm. Protein determination was made with Bradford Method at the supernatant.

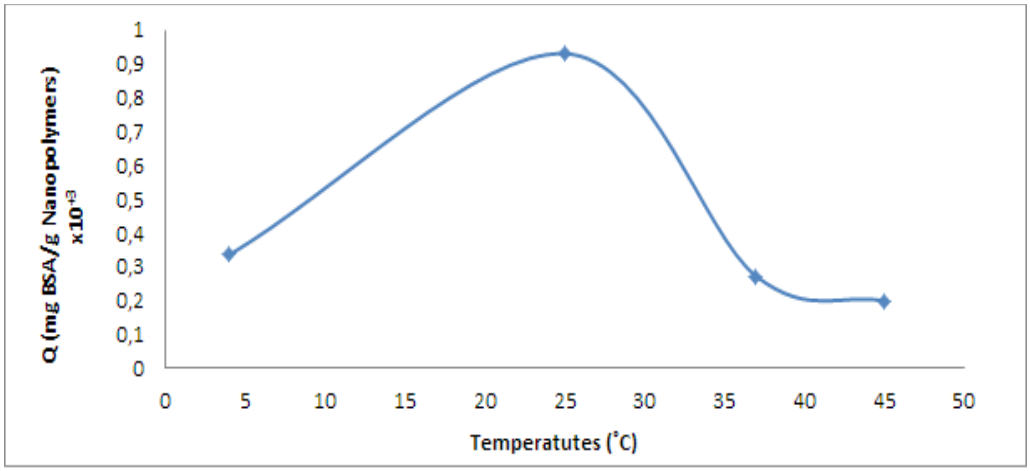

Figure 3.11 The effect of Temperature on the BSA Adsorption *(C BSA-initial: 1 mg/mL, pH5, t adsorpsiyon: 2 hours)*

The effect of temperature on the BSA adsorption is given in Figure 3.11. According to graph, BSA adsorption capacity of the p(HEMA) nanopolymers increases with increasing temperature to the $25^{o}C$ then it starts decreasing. In addition, in physical observations made during the experiment, as the temperature increases nanopolymers come together and create agglomeration. Temperature rise can increase the hydrophobic interactions between the dye molecules. Therefore, nanopolymers could be come together and tend to cluster. This reduces the BSA absorption. The effect of temperature on BSA adsorption on p(HEMA) nanopolymer is negligible.

3.4 Adsorption Isotherm of p(HEMA)-RR2 Affinity nanopolymer

Adsorption isotherm of p(HEMA)-RR2 nanopolymer was determined using the data of effect of inital concentraiton of BSA.

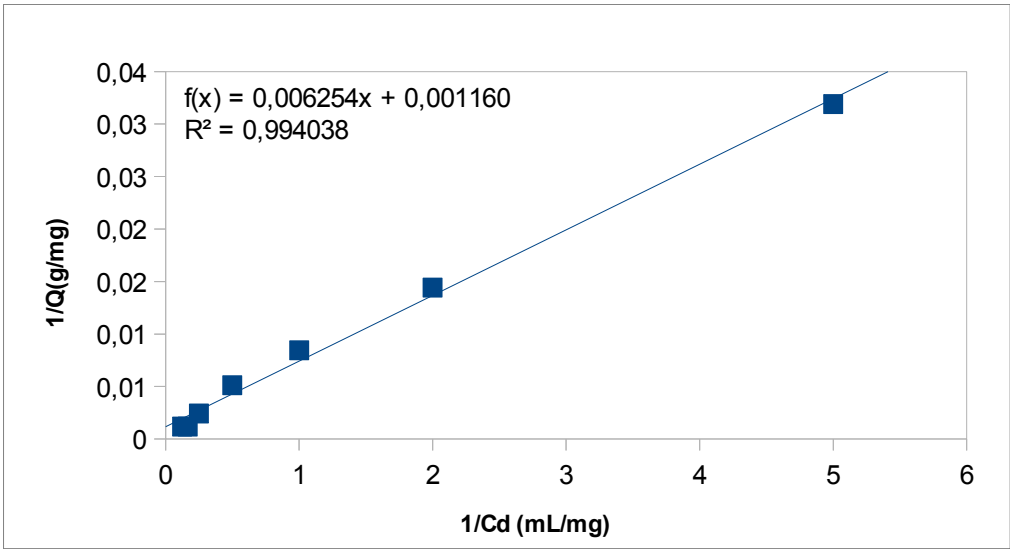

Figure 3.12 Langmuir Isotherm of p(HEMA)-RR2 nanopolymer *(pH5, T:25^{o}C, t adsorption:2 hours)*

According the graph, with the correlation value 0,994, BSA adsorbtion to nanoploymer had proved to be suitable to Langmuir adsorbtion model. Langmuir adsorption model behavior can be seen also at BSA adsorption versus inital concentration graph, at beginning capacity rapidly increase due to

high affinity, then with filling the surface of the adsorbent, capacity increase is slowing down and reach to an equilibrium point. İnteractions between adsorbed BSA molecules and free BSA molecules in solution are too weak, so capacity reach a maximum value after the surface filled with first layer of BSA molecules.

At initial concentration experiments, maximum BSA adsorption capacity was found as $Q_{max} = 855\, mg/g$. According Langmuir isotherm graph, BSA adsorption capacity is $Q_{max} = 862{,}1\, mg/g$. Langmuir constant calculated as $0{,}18\, mL/mg$.

3.5 Reusability of p(HEMA)-RR2 nanopolymers

For the reusability assays of p(HEMA)-RR2 nanopolimer, 0.1M NaOH desorption solution was prepared. BSA solution with 1 mg/mL initial concentration was prepared in 2 mL acetate buffer at pH 5. 100 µL p(HEMA)-RR2 and p(HEMA) polymer solution was added onto BSA solution and mixture was held at room temperature for 2 hours. Samples were precipitated via centrifuging for 20 min at 14500 rpm. Protein determination was made by the Bradford Method at the supernatant. 1 ml of desorption solution was added onto the precipitated nanopolymer and putted at angle stirrer for 2 hours. Precipitated nanopolymer via centrifuging at 14500 rpm, was washed with 1M NaOH for regeneration and sterilization and washed with acetate buffer at pH 5 for balancing after each adsorption-desorption process. Second cycle was performed in the same way by adding BSA solution after regeneration. To determine the reusability of nanopolymer synthesized in this way, adsorption - desorption cycle was repeated 5 times with same polymer.
Desorption rate was calculated using the equation 2.9;

$$Desorption\ Rate(\%) = \frac{BSA\ release\ desorption\ environment}{Absorbed\ BSA} * 100$$

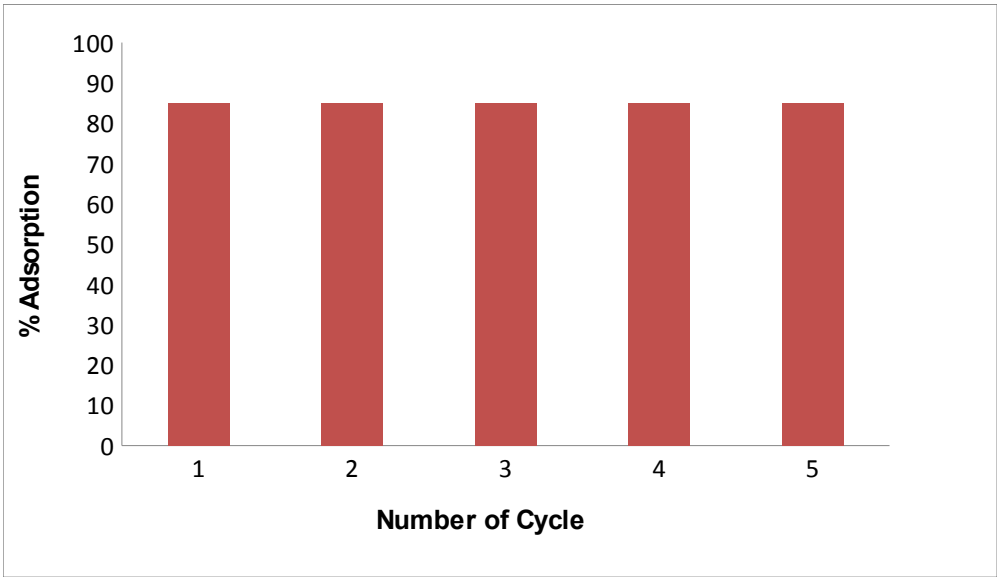

Figure 3.13 Reusability of p(HEMA)-RR2 nanopolymer

Desorption rate was found as %85.

Repeatedly use of a material is most wanted property for a support material. This property, defined as regeneration or reusability, reduces significantly the cost. No leakage of dye-ligand observed at the desorption and sterilization processes. Adsorption - desorption cycle was repeated 5 times and observed that no significant reducing at adsorbtion capacity.

4. CONCLUSION

Synthesized p(HEMA)-RR2 nanopolymer have high specific surface. This property allow both binding high amount of dye RR2 (0.82 mmole/g) and adsorbing huge amount of BSA per unit mass. With 6 mg/mL inital concentration, adsorbtion capacity of nanopolymer reached to 0.855 g/g .

Reusabilitiy of the nanopolymer makes it suitable cost. After 5 cycles of adsorbtion-desorbtion, there is no significant reduction in adsorbtion capacity.

The amount of bounded dye to p(HEMA) nanoploymer, in this work, is very high compared with similar works in literature. In a work at 2008, Başar et al. determined the bounded amount of Cibacron Blue F3GA dye ligand to p(HEMA) as 28.5 μmole/g. Again in a similar work made by Tüzmen et al., they determined the bounded amount of Cibacron Blue F3GA dye ligand to p(HEMA) as 68.3 μmole/g.

Synthesized p(HEMA)-RR2 nanopolymer have very high protein adsorbtion capacity compared other dye ligands. Denizli et al. at 2004 determined HSA adsorbtion capacity of p(HEMA)-CB nanopolymer as 138.3 mg/g. Denizli et al. at 2006 determined HSA adsorbtion capacity of p(GMA)-CB nanopolymer as 189.8 mg/g.

In this thesis, a new dye bounded synthetic polymer was synthesized by surfactant free emulsion polymerisation method as solid support material to use in adsorption works. p(HEMA) nanopolymer, having functional groups at surface, was selected as support material which is physically and chemically stable at extreme conditions such as low and high temperatures, pH etc. Reactive Red2 was used as dye ligand. Obtained p(HEMA)-RR2 nanopolymer is stable, easy to produce and it has low cost. Having high adsorbtion capacity,

p(HEMA)-RR2 nanopolymer is candidate support material in proteomic studies.

5. FUTURE WORK

In this thesis, a new dye bounded synthetic nanopolymer was synthesized as solid support material to use in adsorption works. Proteomic researches planned in future will be very effective and have low cost, and these researches will be take a short time. These considerations are predictable through the works done during this thesis period.

The depletion of the most abundant proteins of the plasma could be realized at one step with polymeric nanomaterials developed within this thesis.

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