Bovine Serum Albumin Adsorption by Dye Derived Poly(hydroxyethyl methacrylate) [PHEMA] Membranes

Boya Takılı Poli(hidroksietil metakrilat) [PHEMA] Membranlarla Sığır Serum Albümin Adsorpsiyonu

Research Article

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ABSTRACT

Procion Brown MX 5BR (PBR) attached poly(hydroxyethyl methacrylate) [PHEMA] membranes were used for adsorption of albumin. The poly(hydroxyethyl methacrylate) was selected as the basic component due to it's inertness, mechanical strength, chemical and biological stability, and biocompatibility. Different amounts of PBR was covalently attached to the poly(hydroxyethyl methacrylate) membrane to prepare poly(hydroxyethyl methacrylate)-Procion Brown [PHEMA-PBR] membrane. PHEMA-PBR membrane was characterized with Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM). Dye attached PHEMA membranes were used in bovine serum albumin (BSA) adsorption studies to assess the effects of pH, protein concentration, flow rate, temperature and ionic strength. The maximum albumin adsorption of PBR attached PHEMA membrane was 14.79 mg/g at pH 7.0 from aqueous solutions. Desorption of albumin was studied with 1.0 M NaCl solution in a continuous system. The reusability of the adsorption medium was tested for 4 adsorption-desorption cycles. Adsorption isotherms fitted Freundlich adsorption model and adsorption kinetics followed pseudo-second order model.

Key Words
Bovine Serum Albumin, Procion Brown MX 5BR, Dye Afinity Adsorption.

ÖZET

Procion Brown MX 5BR (PBR) takılı poli(hidroksietil metakrilat)[PHEMA] membranlar albumin adsorpsiyonu için kullanılmıştır. PHEMA, mekanik direnç, kimyasal ve biyolojik kararlılık ve biyo-uyumluluk gibi özellikleri ile temel bileşen olarak seçilmiştir. Sentezlenen poli(hidroksietil metakrilat)-Procion Brown [PHEMA-PBR] membran için poli (hidroksietil metakrilat) membranlara farklı miktarlarda PBR kovalent olarak bağlanmıştır. PHEMA-PBR membran fourier transform infrared (FTIR) ve taramalı elektron mikroskopisi (SEM) ile karakterize edilmiştir. Boya takılı PHEMA membranlar, sığır serum albümini (BSA) adsorpsiyon çalışmalarında, pH, protein derişimi, akış hızı, sıcaklık ve iyonik şiddetin etkilerini incelemek için kullanılmıştır. Boya takılı PHEMA membrana maksimum albumin adsorpsiyonu pH 7.0 de sulu çözelti ortamında 14.79 mg/g olarak bulunmuştur. Albümin desorpsiyonu sürekli sistemde 1.0 M NaCl çözeltisi ile gerçekleştirilmiştir. Membranların tekrar kullanılabilirliği 4 adsorpsiyon-desorpsiyon döngüsü ile test edilmiştir. Freundlich adsorpsiyon izotermi adsorpsiyon modeline uygundur. Adsorpsiyon kinetiği ikinci dereceden bağlanma kinetiği modelini izlemektedir.

Anahtar Kelimeler
Sığır Serum Albümin, Procion Brown MX 5BR, Boya Afinite Adsorpsiyonu.

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INTRODUCTION

Bovine serum albumin (BSA), the most abundant protein in blood plasma, is one of the most commonly studied protein in the serum albumin family. BSA indicates 52–62% of the total plasma protein fraction [1]. It has many physiological functions in the body such as use in cell culture, protein binding, in drug delivery to transport pharmaceutical drugs like antibiotics and anti-inflammatories [2,3]. BSA has structure similarity to human serum albumin (HSA) and extensively used due to its low cost availability, binding properties [4].

Dye–ligands have been used for the separation and purification of a large number of proteins and enzymes [5]. Due to the good stability and group specificity dye ligands have many advantages in separation and purification of proteins. Dye ligands are cheap and commercially available. It can easily be immobilized on matrices bearing hydroxyl groups. Dyes are all synthetic in nature so they are classified as affinity ligands. They interact with the active sites of many proteins simulating the structure of the substrates and cofactors. Most of the reactive dyes consist of a chromophore, linked to a reactive group (often a mono- or dichlorotriazine ring). The interaction between the dye ligand and proteins are electrostatic, hydrophobic, and hydrogen bonding [6].

In this study, dye attached PHEMA membranes were prepared by ultraviolet (UV)-initiated photopolymerization method. PBR dye ligand was attached onto the PHEMA membranes via nucleophilic substitution reactions under alkaline conditions. The adsorption conditions (i.e., the initial concentration of the albumin, temperature and medium pH) were varied to evaluate their effects on the performances of the affinity membranes. The adsorption kinetics of the albumin on the dye attached membranes were studied to characterize surface complexation reaction. The adsorption isotherm was measured to evaluate the discrepancy between the experimental data and the theoretical equilibrium capacity predicted by the kinetic equations.

Experimental

Materials

Bovine serum albumin (BSA), was supplied from Sigma Chemical Co. (St Louis, MO). HEMA was obtained from Fluka AG (Switzerland), distilled under reduced pressure in the presence of hydroquirione and stored at 4°C until use. Azobisisobutyronitrile (AIBN) was obtained from Sigma Chemical Co. All other chemicals were of analytical grade and were purchased from Merck AG (Darmstadt, Germany).

Methods

Preparation of poly(2-hydroxyethyl methacrylate) [PHEMA] membranes

The poly(2-hydroxyethyl methacrylate) PHEMA membrane was prepared as previously described [7]. The membrane preparation mixture (5.0 mL) contained 2.0 mL (HEMA), 5 mg AIBN as polymerization initiator and 3.0 mL 0.1 M $SnCl_4$ as pore marker. The mixture was then poured into a round glass mould (diameter 4.5 cm) and exposed to ultraviolet radiation for 20 min, while a nitrogen atmosphere was maintained in the mould. The membrane was washed several times with distilled water and cut into circular pieces (diameter 0.5 cm) with a perforator.

Dye attachment to PHEMA membrane

PBR was covalently immobilized onto the membranes via the nucleophilic reaction between the chloride of its triazine ring and the amide and hydroxyl groups of the membranes under alkaline conditions. Procion Brown MX 5BR (40, 60 and 120 mg) was dissolved in distilled water (10 mL), and transferred to the same medium (90 mL) in which membrane disks (1.5 g) were equilibrated. Sodyum hydroxide (1.0 M, 10 mL) was then added to the medium (about pH 13.0) and heated at 80°C for 4.0 h in a sealed reactor. After the reaction period, the solution cooled down to the room temperature and membrane disks were washed several times with distilled water, 2.0 M NaCl and 10% methanol. At the last stage, membrane were washed again with distilled water. Any remaining chlorine atoms in the dye-derived microspheres due to the dichloro triazinly dye structure, after covalent immobilization, was converted to amino groups by treating with 2.0 M NH_4Cl at pH 8.5 for 24 h at room temperature. PBR immobilized

PHEMA membrane were stored at 4°C until use.

Characterization of PHEMA membrane

PBR attached PHEMA membrane was evaluated by using an elemental analysis instrument (Leco, CHNS-932, USA) by considering the sulphur stochiometry. FTIR spectra of the PHEMA membranes were obtained by using a FTIR spectrophotometer (FTIR 8000 Series, Shimadzu, Japan). The dry membrane (about 0.1 g) was mixed with KBr (0.1 g) and pressed into tablet form. The FTIR spectrum was then recorded. The dried membranes were coated with gold should be incorporated under reduced pressure and their scanning electron micrographs were obtained using a JEOL (J M5 5600) scanning electron microscope. Contact angle measuements were performed with KRÜSS DSA 100 instrument (Hamburg, Germany).

Adsorption-desorption studies with BSA

BSA was selected as a model protein. BSA adsorption of the plain and the PBR attached PHEMA membranes were studied at various pH. The pH of the adsorption medium was changed in the range of 4.0 and 8.0 by using different buffer systems (0.1 M CH_3COONa-CH_3COOH for pH 4.0-6.0, 0.1 M Na_2HPO_4-KH_2PO_4 for pH 7.0-8.0. The initial concentration of BSA was changed between 0.1 and 1.5 mg/ml. In a typical adsorption after dissolving of BSA in 25 mL of buffer solution membranes were added. The adsorption experiments were carried out for 2 h at 20°C at a stirring rate of 100 rpm. The time to reach equilibrium adsorption with continuous stirring was found to be 60 min and in the rest of the study a 120 min adsorption duration was therefore employed. At the end of the equilibrium period (i.e. 2 h), the membranes were separated from the solution. The BSA adsorption capacity of PHEMA membranes was determined by measuring the remaining concentration of BSA in the adsorption medium spectrophotometrically at 280 nm. BSA elution was performed using 1.0 M NaCl solution. The desorption ratio was determined for 1.0 h contact time. The reusability of the adsorption medium was tested for 5 adsorption-desorption cycles.

RESULTS AND DISCUSSIONS

Characterization of PHEMA membrane

PBR attached PHEMA membranes PHEMA-PBR were subjected to elemental analysis. The amount PBR which is bound to PHEMA membrane was estimated by using sulphur stochiometry. Estimated dye amount is 700 μmol per g polymer for 120 mg attached PBR dye. Table 1 shows the amount of PBR attached onto the PHEMA structure. PHEMA-PBR membranes were extensively washed until to ensure that there is no dye leakage from any of the dye-attached PHEMA membrane in any media used at adsorption and/or desorption steps. Specific surface area, macroporosity and water uptake of PHEMA and PHEMA-PBR membranes are presented in Table 1. The specific surface area of PHEMA-PBR was determined by a multipoint BET apparatus to be 8.79 m^2/g polymer. The chemical structure of the Procion Brown MX 5BR is shown in the Figure 1. Surface hydrophilicity of PHEMA-PBR and PHEMA membranes were determined with contact angle instrument via sessile drop method. The contact angle values were calculated using DSA 2 software and summarized in Table 2. When we increased the amount of bound dye, contact angle degree increased. Because there is benzene rings in the PBR dye structure. These rings causes hydrophobicity increment of membranes.

To examine the nature of the interactions between the PBR and the PHEMA membrane, FTIR spectra for PHEMA and dye immobilized PHEMA were obtained. As shown in Figure 2, FTIR spectra of PHEMA-PBR have an absorption band different from that of PHEMA at 1566 cm^{-1}, this is the characteristic of aromatic rings stretching vibration band (C=C) arising from immobilized dye molecules on the PHEMA membrane. The bands at 3300, 1566, 1090, 1173 and 1247 cm^{-1} representing N-H stretching, N-H bending (scissoring), symmetric stretching of S=O, asymmetric stretching of S=O and aromatic C-N vibration, respectively, are due to the dye Procion Brown MX 5BR bonded to PHEMA membrane.

The SEM micrographs given in Figure 3 show the cross sectional and surface structures of PHEMA membrane, respectively. As clearly seen here, the PHEMA membrane shows a

Table 1. Surface area measurements of PHEMA and PHEMA-PBR membranes.

Polymer	Surface Area (BET)[a] (m^2/g)	Total Pore Volume [b](mL/g)	Average Pore Diameter ($Å^2$/mol)
PHEMA	7.61	0.05	14.7
PHEMA-PBR	8.79	1.51	16.2

a. Determined using multipoint BET method.
b. BJH cumulative desorption pore volume of pores between 20 and 245 Å.
c. BJH desorption average pore diameter of pores between 20 and 245 Å.

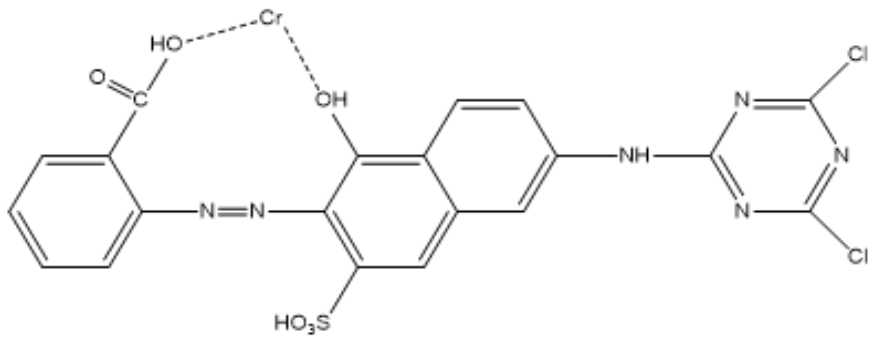

Figure 1. Chemical structure of Procion Brown MX 5BR.

homogeneous molecules highly porous bulk and surface structure, which may provide high mass transfer rate due to high internal surface area (means high amount of dye immobilization and high protein adsorption capacity) with low diffusional mass transfer resistance in the matrix.

Bovine serum albumin adsorption-desorption studies.

Effect of pH

The effects of pH on BSA adsorption are presented in Figure 4. Proteins have no net charge at their isoelectric points, and therefore maximum adsorption from aqueous solutions is usually observed at their isoelectric points [8-10]. The isoelectric pH of BSA used in this study is 4.7. As seen in Figure 4, the maximum adsorption of BSA was observed at pH 7.0. At this pH value, BSA was negatively charged. PBR is dicholorotriazinyl dye and at pH 7.0, sulfonic (-SO_3H), carboxylic (-CO_2H) and secondary amino groups (-NH-) are pronated so we can speculate that dominant force contributing to dye and protein interactions is electrostatic interactions rather than other forces. The conformational changes of protein molecules due to specific interactions at this pH may also contribute the specific interactions. The binding between BSA and PHEMA-PBR may have resulted from charge–charge interactions (ionic) between chelated positively charged Cr(II) ion through the carboxyl and hydroxyl groups of PBR and negatively charged BSA molecules. Hydrogen bonding and hydrophobic interactions may have a cooperative effect on the binding process.

Effect of Initial Concentration of BSA

Figure 5 shows the effect of initial BSA

Table 2. Contact angle values of PHEMA membranes containing different amount of dyes.

	Free Dye	Dye Bound PBR-1	Dye Bound PBR-2	Dye Bound PBR-3
Amount of dye (mg)	-	40	60	120
Contact Angle (°)	76,2	103	112	139

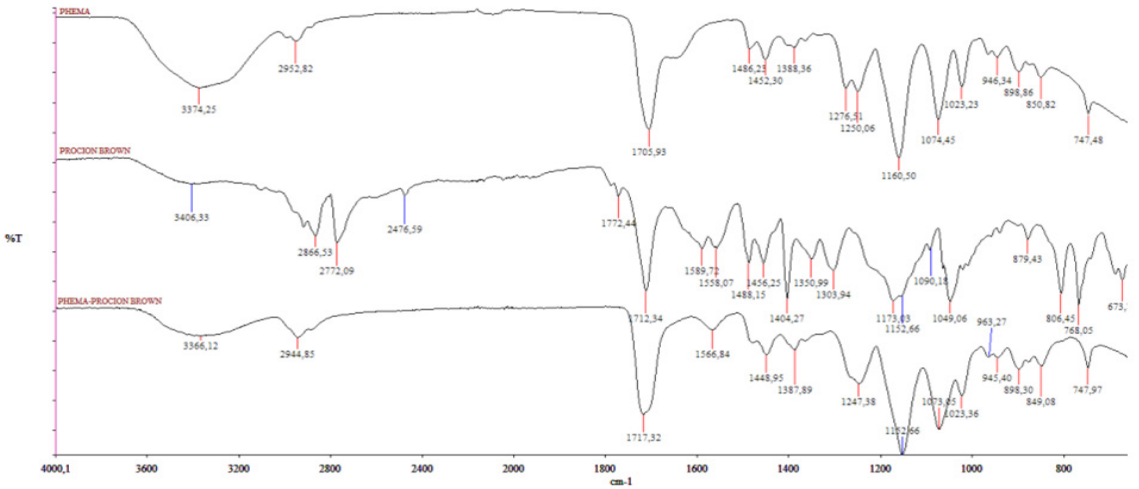

Figure 2. FTIR spectrum of PHEMA, PBR and PBR immobilized PHEMA membrane.

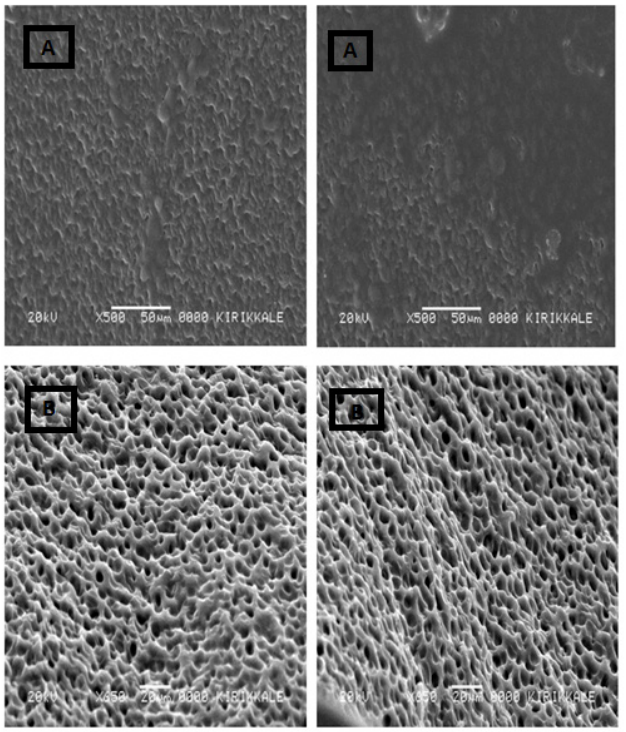

Figure 3. SEM micrographs of PHEMA (A) and PBR immobilized PHEMA (B) membranes.

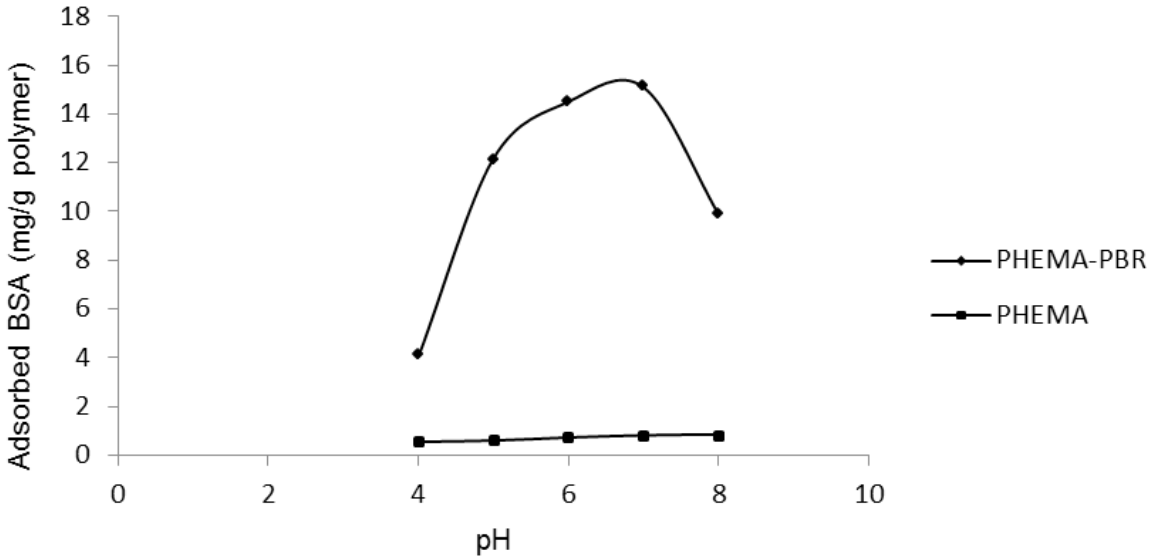

Figure 4. Effect of pH on BSA adsorption on the PHEMA and PHEMA-PBR membranes. PBR loading: 120 mg/g polymer, Initial concentration of BSA: 1.0 mg/mL, T: 25°C.

concentration on adsorption. The adsorption studies were carried out at pH 7.0 and 25°C. The concentration of BSA in the adsorption medium was varied between 0.1 and 1.5 mg/mL. The results of the adsorption studies for PHEMA-PBR membranes were presented in Figure 5. We observed that the amount of adsorbed BSA increased with increasing BSA concentration. Maximum adsorption capacity was found to be 14.79 mg/g for PHEMA-PBR membrane and the adsorbed amounts per unit mass of PHEMA-PBR membrane reached a plateau at about 0.75 mg/mL BSA concentration. This increase is likely due to the formation of a ternary complex between the dye and BSA molecules. PBR immobilization significantly increased the BSA binding specificity and capacity of the PHEMA membranes (up to 14.79 mg/g). This can explain that the strong binding of the dye ligands to BSA may have resulted from a cooperative effects of different mechanisms such as hydrophobic, hydrogen bonding, van der Waals and electrostatic interactions caused by the aromatic structures and functional groups on the dye molecules and on the BSA molecules.

Effect of Ionic Strength

To determine the effect of ionic strength on BSA adsorption studies were carried out in the range of 0.1-1.0 M NaCl concentration. As shown in Figure 7, the amount of BSA adsorbed onto the PHEMA/PBR membranes decreased significantly as the NaCl concentration was increased from 0.1 to 1.0 M. The increase in NaCl concentration could promote the adsorption of the dye molecules onto the polymer surface through hydrophobic interactions. Moreover, the hydrophobic interactions between the immobilized dye molecules themselves would also become strong, because it has been observed that the addition of salt to a dye solution caused the stacking of the free dye molecules. Thus, the number of the immobilized dye molecules accessible to BSA would decrease as the ionic strength increased, and the adsorption of the BSA to the immobilized dye would become difficult. It is also suggested that an increase in NaCl concentration result in the reduction of electrostatic interactions [11].

Effect of Temperature

It is well known that hydrophobic interaction is an entropy driven process, and hydrophobic interactions increase with increasing temperature. The lowest adsorption capacity was determined at 4°C as 9.03 mg/g whereas the highest capacity value at 37°C was 17.05 mg/g membrane (Figure 8). The increase in adsorption capacity with increasing

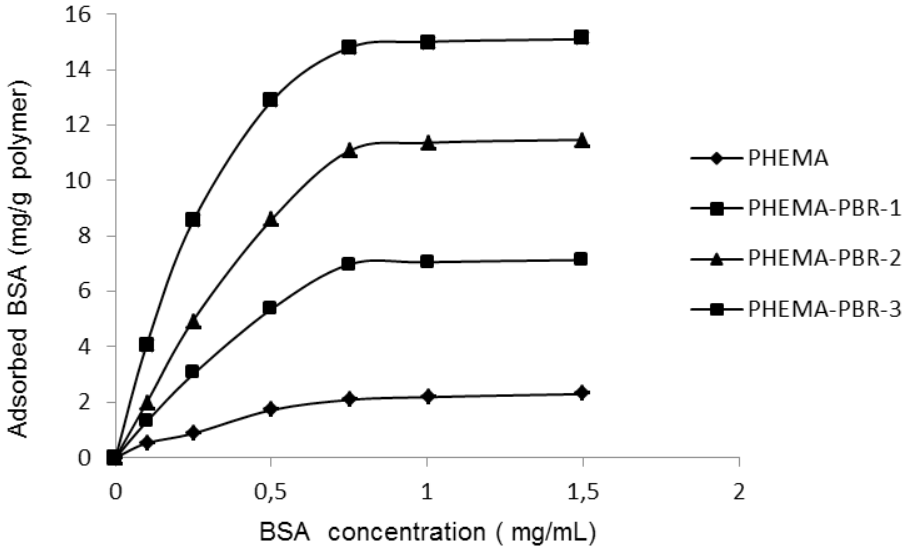

Figure 5. Effect of initial BSA concentration on the PHEMA and PHEMA-PBR membranes. PBR loading: 120 mg/g polymer, pH: 7.0, T: 25°C.

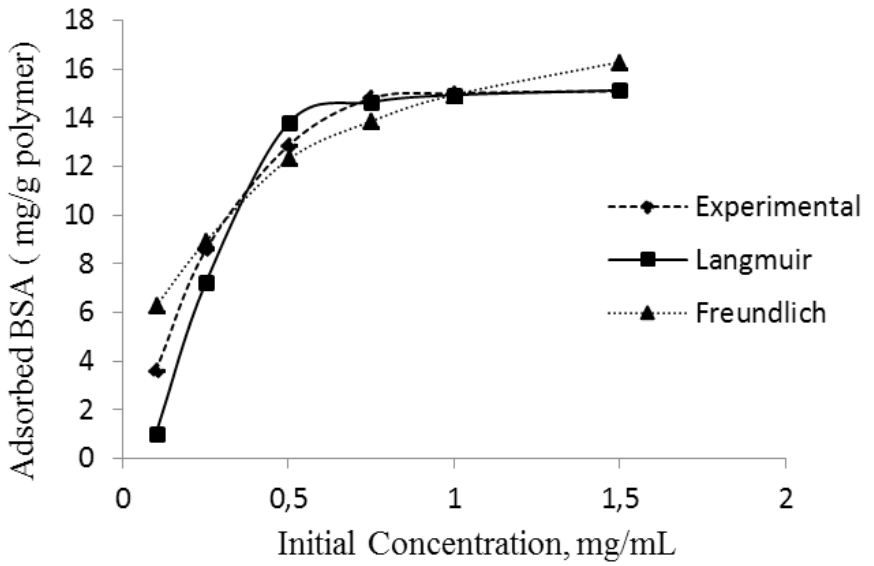

Figure 6. Experimental, Langmuir and Freundlich.

temperature can also be caused by conformational changes of biomolecules due to temperature change. During these changes, accessibility of hydrophobic groups and hydrophobic residues promote hydrophobic interactions and cause an increase in adsorption capacity.

The Adsorption Equilibrium Time

The adsorption equilibrium time of BSA on the PHEMA-PBR membrane was studied with 1.0 mg ml^{-1} BSA concentration at 25°C. The adsorption rate curve that was obtained by following the decrease of the concentration of BSA within the adsorption medium with time was shown in Figure 9. As seen in the figure, there was relatively faster adsorption rate was observed during first 60 min adsorption process, and then adsorption equilibrium was achieved in about 120 min. The time to reach equilibrium of adsorption which was found to be 120 min and was selected as a duration for the rest of the study.

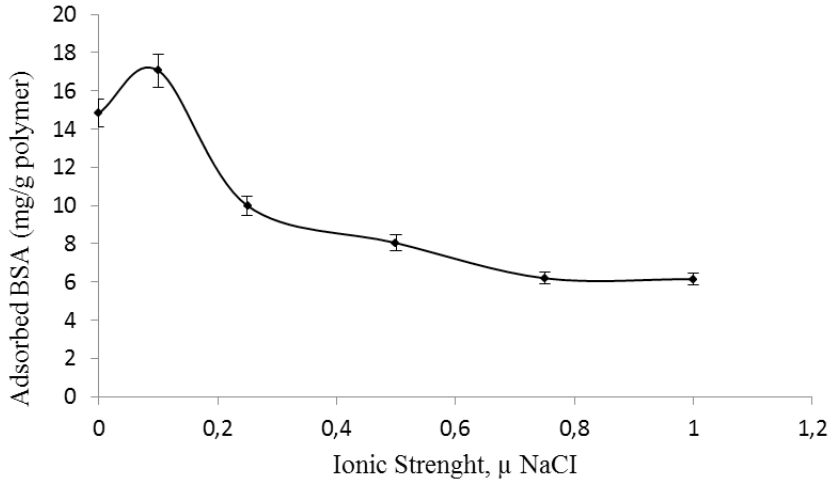

Figure 7. Effect of the ionic strength on the amount of adsorbed BSA on the PHEMA-PBR membrane. PBR loading: 120 mg/ g polymer, initial concentration of BSA: 1.0 mg/mL; pH: 7.0; T: 25ºC.

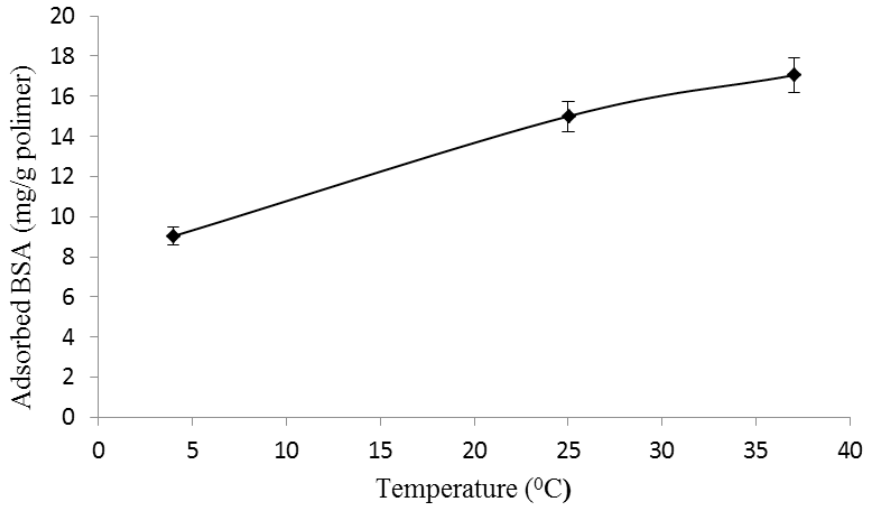

Figure 8. Effect of temperature on the PHEMA-PBR membrane. PBR loading: 120 mg, Initial concentration of BSA: 1.0 mg/mL, pH: 7.0.

Adsorption Models

An adsorption isotherm was used to characterize the interactions of each BSA molecule with the PHEMA-PBR adsorbent. This provides a relationship between the concentration of the BSA in the solution and the amount of BSA adsorbed on the solid phase when the two phases are at equilibrium. Adsorption isotherms are used to describe adsorption type of adsorbents. Amount of adsorbed BSA (Q_e) against equilibrium concentration of BSA (C_e) is plotted. Langmuir and Freundlich isotherm models are used to figure out the eqilibrium data for BSA adsorption. According to Langmuir adsorption model, molecules are adsorbed with equivalent well-defined binding sites which far from each other and their is no interaction-adsorbing between molecules. Therefore, energies and entalpies are equal. Langmuir model expression can be written by Eq. 1.

This equation can be converted to linear form as:

$$C_e/Q_e = 1/ (Q_{max}.b) + (C_e/Q_{max}) \quad (1)$$

where b is the adsorption equilibrium contant, C_e and Q_e are unadsorbed BSA concentrations in solution and adsorbed BSA on the adsorbent of equilibrium, respectively. Q_{max} is the maximum amount of BSA per unit weight. Freundlich isotherm is another form of Langmuir isotherm

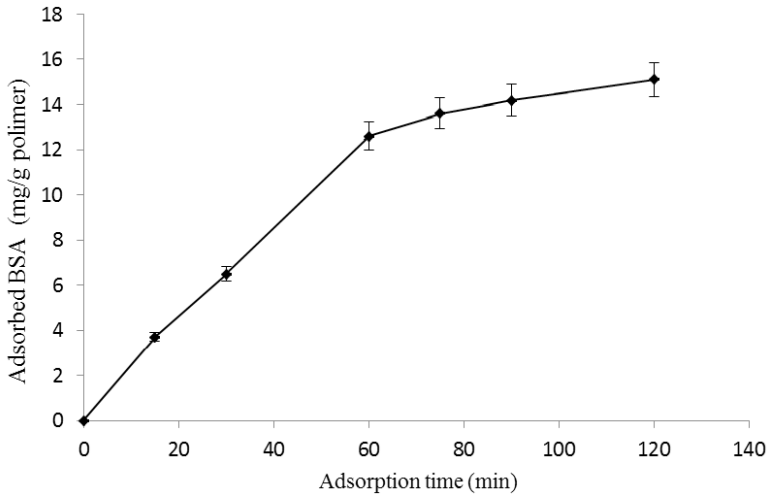

Figure 9. Adsorption equilibrium time on the PHEMA-PBR membrane. PBR loading: 120 mg, Initial concentration of BSA: 1.0 mg/mL, pH: 7.0, T: 25ºC.

for adsorption on heterogeneous surface with a non-uniform distribution of heat of adsorption over surface. Summation of BSA concentration adsorbed by all binding sites is equivalent BSA concentration adsorbed totally. Freundlich adsorption model denotes reversible adsorption and is not limited with monolayer adsorption whereas Langmuir model is. The equation given below was used for Freundlich model:

$$\ln Q_e = \ln K_F + 1/n \ln C_e \quad (2)$$

where Q_e is the equilibrium BSA adsorbed amount; C_e is the residual BSA concentration at equilibrium; and K_F and n are the Freundlich constant related to the adsorption capacity and adsorption intensity of the adsorbent, respectively. The Langmuir adsorption capacity, Q_{max}, obtained in this study was found to be 15.36 mg/g. The adsorption capacity value (K_F) for BSA obtained from Freundlich model was 16.05 mg/g and this value indicated that the PHEMA/PBR have a good affinity for BSA. The order of fitness of these isotherm models to the experimental data was found to be Langmuir (R^2: 0.999) > Freundlich (R^2: 0.942).

In order to calculate thermodynamic parameters, the Langmuir isotherm at different temperatures was examined and used to relate Langmuir constant to thermodynamic parameters according to following equation (Table 3).

$$\ln b = \Delta H^o/RT - \Delta S^o/R \quad (3)$$

Where, b, ΔS^o,R, ΔH^o, ΔG^o and T are Langmuir constant (mL/mg), standart formation entropy (J/molK), standart formation enthalpy (J/mol), temperature (K) and standart formation Gibbs free energy (J/mol), respectively.

$$\Delta G^o = RT \ln b \quad (4)$$

The enthalpy and entropy changes of the calculations were calculated from the gradient and intercept of lnb vs. 1/T curve, respectively. The results calculated were tabulated in Table 4. It is obvious that an increase in temperature caused an increase in Gibbs free energy of the process proving the hydrophobic character of interactions (Table 4).

Kinetic Studies

In order to examine the controlling mechanism of adsorption process such as mass transfer and chemical reaction, kinetic models were used to test experimental data. The kinetic models (Pseudo-first and second-order equations) can be used in this case assuming that the measured concentrations are equal to adsorbent surface concentrations. The first-order rate equation of Lagergren is one of the most widely used for the adsorption of solute from a liquid solution.

Table 3. Langmuir and Freundlich isotherm parameters for BSA on the PHEMA-PBR and PHEMA membranes.

Polymer	Experimental Q_{max}(mg/g)	Langmuir constants			Freundlich constants			
		Q_{max}	b	R^2	K_f	n	1/n	R^2
PHEMA-PBR	14.79	15.36	59.18	0.999	16.05	7.10	0.141	0.942
PHEMA	2.299	3.10	0.045	0.969	2.25	1.75	0.571	0.947

Table 4. Thermodynamic variables for adsorption process.

Temperature (°C)	Q_{max} (mg/g)	ΔG^0 (kJ/mol)	ΔH^0 (kJ/mol)
4 (°C)	11.69	-14.25	91.26
25 (°C)	15.36	-23.65	ΔS^o (J/mol.K)
37 (°C)	17.33	-26.47	382.13

$$\log[Q_{eq}/(Q_{eq}-Q_t)] = (k_1 t)/2.303 \quad (5)$$

Equation (6) can be rearranged to obtain a linear form;

$$\log(Q_{eq}-Q_t) = \log(Q_{eq}) - (k_1 t)/2.303 \quad (6)$$

In addition, a pseudo-second order equation based on equilibrium adsorption capacity may be expressed in the form;

$$dQ_t/dt = k_2 (Q_{eq}-Q_t)^2 \quad (7)$$

A plot of t/Q_t versus t should give a linear relationship for the applicability of the second-order kinetics. The rate constant (k_2) and adsorption at equilibrium (Q_{eq}) can be obtained from the intercept and slope, respectively. The theoretical Q_e value estimated from pseudo-first and second order kinetic models were close to the experimental values and the correlation coefficients were high. Results indicate that R^2 values for different concentration is general close to 1.0. This PHEMA/PBR membrane was obeyed the second order kinetic model.

Desorption and Reusability

The desorption experiments were performed by using 1.0 M NaCl solution. Figure 10 shows the reusability of the PBR dye attached PHEMA membrane. The results showed that about 89% of the adsorbed BSA was successfully desorbed. Adsorption-desorption cycles were repeated 5 times by using same PHEMA-PBR membrane. No significant decrease in adsorption capacity was observed. This is an important feature indicating the possibility of reversible immobilization onto PHEMA membrane.

CONCLUSIONS

The present paper demonstrates the preparation and application of PHEMA membranes for the dye-ligand affnity chromatography of proteins. The PHEMA membranes were prepared by UV-initiated photo-polymerization. Repeated adsorption–desorption processes showed that these novel dye-attached membranes are suitable for albumin adsorption. These features make the dye-ligand affinity membranes very good candidates for use in purification of proteins and would be effective in processing large volumes of biological fluid containing target protein.

Table 5. Rate constants, calculated and experimental adsorbed amounts for BSA on the PHEMA-PBR.

Initial Concentaration (mg/ml)	Experimental	Pseudo-first order			Pseudo-second order		
	q_{eq}(mg/g)	k_1(1/dk)	q_{eq}(mg/g)	R^2	k_2(1/dk)	q_{eq}(mg/g)	R^2
1	15.00	0.037	21.8	0.993	0.00039	28.24	0.908

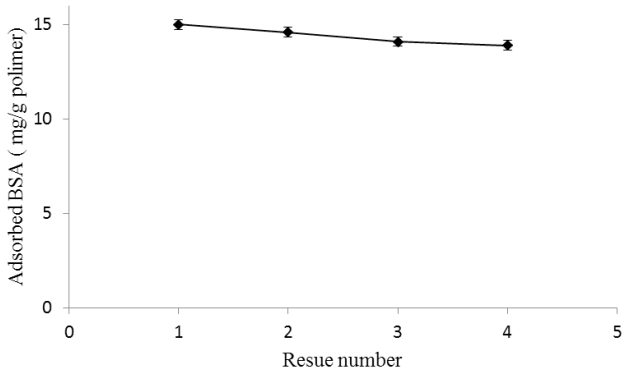

Figure 10. Repeated use of the PHEMA-PBR membrane. PBR loading: 120 mg, Initial concentration of BSA: 1.0 mg/mL, T: 25°C, pH: 7.0.

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