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Heparin Immobilized poly(hydroxylethylmethacrylate) Cryogels for Lysozyme Purification

Lizozim Saflaştırılması İçin Heparin İmmobilize Edilmiş poli(hidroksietilmetakrilat) Kriyojeller

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ABSTRACT

Heparin immobilized poly(2-hydroxylethyl methacrylate) PHEMA cryogel was synthesized and applied for lysozyme purification from egg white. Firstly, the PHEMA cryogel was synthesized by cryopolymerization and then heparin was covalently immobilized on to the PHEMA cryogel with cyanogen bromide activation. The modification of PHEMA cryogel structure with heparin was further confirmed by Fourier-transform infrared spectroscopy (FTIR). The surface and inner structure morphologies of PHEMA cryogels were studied and characterized by the scanning electron microscope (SEM). The surface area of PHEMA cryogel was found to be 25.2 m^2/g. Heparin immobilized PHEMA cryogels were used in lysozyme adsorption studies to assess the effects of pH, lysozyme concentration, flow rate, temperature and ionic strength. The maximum lysozyme adsorption on the heparin immobilized PHEMA cryogel was found to be 48.73 mg/g from aqueous solutions under optimized conditions. 1.0 M NaCl solution was used for desorption of lysozyme in a continuous system. The reusability of heparin immobilized PHEMA cryogels was tested for 10 adsorption-desorption cycles. The Langmuir adsorption model was plotted and found fitted for adsorption studies. The purity of lysozyme from egg white studies was analysed by sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% separating gel.

Keywords

Affinity chromatography, lysozyme, heparin, purification.

ÖZ

Yumurta akından lizozim saflaştırılması için heparin immobilize edilmiş poli(hidroksi etilmetakrilat) (PHEMA) kriyojeller sentezlenmiştir. İlk olarak, PHEMA kriyojeller kriyopolimerizasyon yöntemi ile sentezlenmiştir. Heparin, siyanojen bromür aktivasyonu ile PHEMA kriyojeline kovalent olarak immobilize edilmiştir. PHEMA kriyojel yapısının heparin ile modifikasyonu Fourier dönüşümlü kızıl ötesi spektroskopisi (FTIR) ile incelenmiştir. PHEMA kriyojellerin yüzey ve iç yapı morfolojileri taramalı elektron mikroskobu (SEM) ile karakterize edilmiştir. PHEMA kriyojelin yüzey alanı 25.2 m^2/g olarak bulunmuştur. Heparin immobilize edilmiş PHEMA kriyojeller lizozim adsorpsiyon çalışmalarında pH, lizozim derişimi, akış hızı, sıcaklık ve iyonik şiddetin etkilerini incelemek için kullanılmıştır. Optimum koşullar altında, heparin immobilize edilmiş PHEMA kriyojelin sulu çözeltide en yüksek lizozim adsorpsiyonu 48.73 mg/g olarak bulunmuştur. Sürekli sistemde lizozimin desorpsiyonu için 1.0 M NaCl çözeltisi kullanılmıştır. Heparin immobilizasyonuna heparin derişiminin etkisi incelenmiştir. Heparin immobilize edilmiş PHEMA kriyojellerin tekrar kullanılabilirliği adsorpsiyon-desorpsiyon döngüsü 10 kez tekrarlanarak test edilmiştir. Langmuir adsorpsiyon modeli çizilmiş ve adsorpsiyon çalışmaları için uygun bulunmuştur. Yumurta akından lizozim saflığı %12 ayırıcı jel kullanılarak sodyum-dodesil sülfat poliakrilamid jel elektroforezi kullanılarak analiz edilmiştir.

Anahtar Kelimeler

Afinite kromatografisi, lizozim, heparin, saflaştırma.

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INTRODUCTION

Supermacroporous cryogels are a novel generation of chromatographic supports for separation and purification of biomolecules [1-6]. Large interconnected porous structure is a basic characteristic of cryogel. Cryogels have interdepened macropores with pores size of several microns to several hundred of microns [7,8]. Cryogels are cheap materials and have many advantages over traditional matrix such as short diffusion path, large pores structure, low pressure drop and very short retention time for both adsorption and elution [9-12]. Cryogels are used in different biological applications as matrix due to osmotic, chemical and mechanical stability [13-17]. Cryogels have been used in biotechnology such as chromatographic materials, carriers for the immobilization of molecules etc. [18-25].

The most common applications of cryogel are bioseparation, biocatalysis and affinity chromatography. Affinity chromatography is a more promising method for purification and separation of biomolecules, the isolation and identification of specific components based on most specific molecular recognition [26,27]. In affinity chromatography, the molecule having a specific recognition capability is immobilized on an available support. The molecule to be isolate is selectively enslaved by the component ligand immobilized on the matrix. Ligand stability is an important consideration. Thus, there is need to replace high molecular mass biological ligands with small molecular mass pseudospecific ligands [28,29]. PHEMA based supermacroporous cryogel was chosen as the basic component due to its mechanical strength, inertness, biocompatibility, biological and chemical stability. Heparin used as a bio-ligand for biomedical applications. Heparin known as glycoaminoglycans with an average molecular weight of 15 kDa [30]. Generally, heparin used as an anticoagulant as long as surgical operations and extracorporeal therapies [31]. Heparin is an anionic linear polysaccharides having β-D-glucuronic acid, 2-O-sulfo-α-L-iduronic acid, 2-acetamido-2-deoxy-α-D-glucose, 2-deoxy-2-sulfamino-6-O-sulfo-α-D-glucose, α-L-iduronic acid as important saccharide units. Heparin has the ability to unite proteins [32]. Heparin immobilization on the surface of PHEMA cryogels improves their surface properties, biocompatibility and blood compatibility [30,33]. It is widely used in tissue, engineering and protein adsorption. One lysozyme molecule has binding sites per 3 disaccharide units of heparin. The heparin functional polymers have strong adsorption ability with lysozyme by strong electrostatic interactions [34]. Lysozyme (EC 3.2.1.17) is a hydrolytic enzyme in a major bacterial cell wall polymer. It cleaves the β-(1,4)-glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in peptidoglycan. Lysozyme (Lyz) is commercially important enzyme and is extracted from egg white. The egg white proteins are ovalbumin (54%), conalbumin (13%), ovomucin (3.5%) and lysozyme (3.5%) [35, 36]. Amoung egg white proteins, Lyz is the most widely isolated protein due to its important properties and functions [37, 38]. Lyz is used as an antimicrobial, anti-inflammatory agent in the pharmaceutical and food industries. [39]. In this work, PHEMA cryogel is one of the new generation polymeric systems is prepared as the polymer support and low molecular weight heparin, which has a high affinity for lysozyme was chosen as the ligand. PHEMA cryogels were synthesized by cry-polymerization of HEMA monomer. Heparin was covalently attached onto the PHEMA cryogel via cyanogen bromide (CNBr) activation. Heparin immobilized PHEMA cryogels were characterized using scanning electron microscope (SEM), fourier transform infrared spectrophotometer (FTIR) and sweeling tests. Several parameters effecting adsorption capacity were examined and optimized for the adsorption of lysozyme from egg white. Then, purity of lysozyme purified from egg white was determined by sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-page).

MATERIAL and METHODS

Materials

Model protein (Lysozyme (Lyz)) and crosslinker (N,N'-methylene-bis(acrylamide) (MBAAm)) were supplied by Sigma (St Louis, MO, USA). Monomer (Hydroxyethyl methacrylate (HEMA)) and the initiator-activator pair (N,N,N',N'-Tetramethylene diamine (TEMED) and ammonium persulfate (APS)) were obtained from Fluka A.G. (Buchs, Switzerland). All other chemicals were of reagent grade and were purchased from Merck AG (Darmstadt, Germany).

Preparation of Poly(2-hydroxyethylmethacrylate) (PHEMA) Cryogel

The supermacroporous cryogel based on HEMA was prepared by cryopolymerization. HEMA (1.6 mL) and MBAAm (0.3 g) were used as monomer and cross-linker in cryogel preparation. After, mixing monomer and cross-linker, APS (25 µL) and TEMED (20 mg) were added into this solution. The polymerization solution was stirred for 1 min. This solution was poured into a plastic syringe with closed outlet at the bottom and

was frozen at -12°C for 24 h. The cryogel was activated with cyanogen bromide (CNBr) in order to create active binding sites for heparin immobilization. 2 mL of 0.5 M sodium carbonate buffer (pH 10.5) was prepared in a fume hood. CNBr was added into solution and was adjusted to 11.5 with 4.0 M NaOH. The CNBr solution was recirculated through the column at 1.0 mL/min at room temperature for 60 min. After, PHEMA cryogel was washed with 0.1 M $NaHCO_3$. Any remaining active groups on the PHEMA cryogel were removed with ethanol amine (pH 9.1) and $FeCl_3$ solution for 1.0 h. The CNBr-activated PHEMA cryogel was washed 4 times with 0.5 M NaCl solution. In the last stage, the PHEMA cryogel was washed with cold sodium citrate buffer (0.1 M; pH 6.5). CNBr-activated cryogels were continuous system at a constant temperature of 25°C for about 2.0 h with 100 mL of a heparin solution. First, it was also examined the effect of heparin immobilized on CNBr concentration. The CNBr initial concentration was changed between 10-75 mg/mL in 0.1 M phosphate buffer pH 7.4. Heparin was covalently bound to the free hydroxyl and amine groups of CNBr-activated PHEMA cryogels via imidocarbonate groups of heparin. The initial concentration of heparin was varied between 0.5-8.0 mg/mL. In different heparin (HP) ratio immobilized cryogels were named as HP-1 (0.5 mg/mL), HP-2 (1.0 mg/mL), HP-3 (2.0 mg/mL), HP-4 (4.0 mg/mL) and HP-5 (8.0 mg/mL), respectively. The heparin-immobilized PHEMA cryogels were washed with 0.1 M $NaHCO_3$ and distilled water. The amount of heparin immobilization on the cryogel was determined at 280 nm by a UV-visible spectrophotometer.

Characterization of PHEMA Cryogels

Swelling ratio (S) of PHEMA and heparin immobilized PHEMA cryogels were determined as following procedure. First, the water uptake capacity of cryogels was determined using deionized water. Cryogels surface was dried and swollen gels were weighed ($m_{wet\ gel}$). After that cryogels were dried completely and the mass of dried cryogel was determined ($m_{dry\ gel}$). The swelling ratio was calculated using the equation (1):

$$S = m_{wetgel} - m_{drygel} / m_{drygel} \quad (1)$$

Scanning electron microscope (SEM) analysis was used to analyze morphology of a cross section of the dried cryogel. Dried cryogel samples were coated with gold–palladium (40:60) and examined using a JEOL JSM 5600 (Tokyo, Japan) scanning electron microscope. The chemical composition of PHEMA cryogels were characterized using a Fourier transform infrared spectrophotometer (FTIR, 8000 Series, Shimadzu, Tokyo, Japan). The 98 mg of dried cryogel were prepared with 2 mg of anhydrous potassium bromide powder (KBr, IR Grade, Merck, Germany) and pressed into a pellet form and spectra were taken in frequency range 400-4000 cm^{-1}.

Adsorption–Desorption Studies with Lysozyme

Lysozyme adsorption of heparin immobilized PHEMA cryogels was studied flow rate, pH, ionic strength, lysozyme concentration and temperature were optimized for maximum adsorption of lysozyme. Lysozyme concentration was determined at 280 nm by a UV-visible spectrophotometer (UV mini-1240, Shimadzu, Japan). The pH of the adsorption medium was varried between 4.0 and 8.0 by using different acetate-phosphate buffer systems. The initial concentration of lysozyme was changed between 0.1 and 2.0 mg/mL. Lysozyme elution was studied using 1.0 M NaCl solution for 1.0 h contact time. The reusability of cryogel was tested in 10 adsorption-desorption cycles. The lysozyme purification was performed from egg white. In the first step, the egg white was separated from a fresh egg. The egg white was diluted with phosphate buffer (0.1 M pH 6.5). The diluted egg white was homogenized and centrifuged at 14.500 rpm at 4°C for 30 min. The larger molecular weight proteins were removed from supernatant after centrifugation with saturated $(NH_4)_2SO_4$ solution. The cryogels were performed with the prepared egg white solution for 2.0 h. Adsorbed lysozyme was eluted using 1.0 M NaCl solution. The purity of lysozyme in the purified samples was assayed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% separating gel (9×7.5 cm) and 6% stacking gels were stained with 0.25% (w/v) Coomassie Brillant R 250 in acetic acid–methanol–water (1:5:5, v/v/v) and destained in ethanol–acetic acid–water (1:4:6, v/v/v). Electrophoresis was run for 2h with a voltage of 110 V.

RESULTS and DISCUSSION

Characterization Studies

The scanning electron microscope (SEM) micrographs of the PHEMA cryogels are shown in Figure 1. The SEM images shown that PHEMA cryogels have wide flow channels having thin polymeric walls and large supermacropores. Interconnected flow channels and

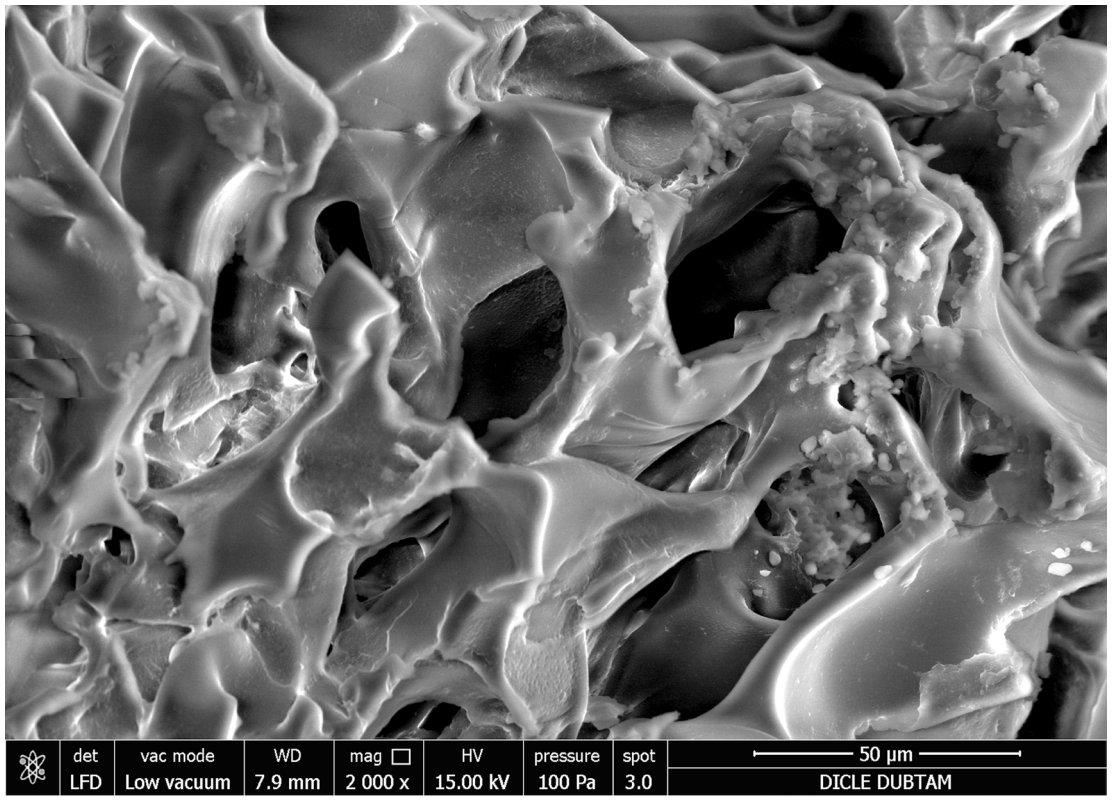

Figure 1. SEM images of the PHEMA cryogels.

supermacropores provide easy diffusion of molecules into the structure and effective interaction with protein molecules. Due to the convective flow of the mobile phase through the pores, the mass transfer resistance was practically negligible. Cryogels have the property of very low back pressure at high flow rates in chromatographic applications. The pore size of the cryogel was much larger than the size of the protein. This property allows them to pass and diffuse easily through the pores.

The chemical composition and characteristic structure of the FTIR spectrum of PHEMA cryogel presented that the C-O band was observed at 1729 cm^{-1} as a sharp peak. The peaks at 1623 cm^{-1} and 1159 cm^{-1} characterized the C-O (ester) stretching vibrations. In addition, the band originated from hydroxyl groups stretching vibrations was determined at 3522 cm^{-1} as a broad band. The physicochemical properties of the PHEMA and HP-3/PHEMA cryogel were given in table 1. The equilibrium swelling degree of the HP-3/PHEMA cryogels was 11.08 g H_2O/g cryogel. PHEMA and HP-3/PHEMA cryogels are opaque, sponge-like and elastic. These cryogels can be easily compressed by hand to remove water accumulated inside the pores. When the compressed piece of cryogels was submerged in water, it soaked in water and within 1–2 s restored its original size and shape. The equilibrium swelling degree of the PHEMA cryogel was 6.31 g H_2O/g cryogel. Compared with HEMA cryogel, the equilibrium swelling degree of HP-3/PHEMA cryogel increased. Several explanations can be offered. First, formation of molecular cavities in the polymer structure introduces more hydrodynamic volume into the polymer chain.

Second, reacting of lysozyme-heparin complex with HEMA effectively increased the length of polymer chains.

Table 1. Physicochemical properties of the PHEMA and HP-3/PHEMA cryogel.

	PHEMA	HP-3/PHEMA
Macroporosity	73.96 %	83.83 %
Swelling degree	6.31 g H_2O/ g cryogel	11.08 g H_2O/ g cryogel
Flow resistance	430 cm/h	485 cm/h

Therefore, water molecules penetrate into the polymer chains more easily, resulting in an improvement of polymer swelling degree in aqueous solutions.

The amount of CNBr for activation of cryogel was also optimized; thus activation at performed different concentrations of CNBr (10-75 mg/mL). In order to optimize heparin immobilization onto the PHEMA cryogels, the effects of initial concentrations of CNBr and heparin on the immobilization were investigated in adsorption-equilibrium studies (Figure 2). The initial concentration of CNBr was changed between 10 and 75 mg/mL. Heparin immobilization capacities were increased with increasing the initial concentration of heparin, up to 8 mg/mL and above this value, the effect was less pronounced or even plateau values were achieved.

Adsorption-Desorption Studies

Effect of pH, flow rate, concentration, heparin loading, ionic strength and temperature on lysozyme adsorption for heparin immobilized PHEMA cryogels are shown in Figure 3. These parameters were optimized for maximum adsorption of lysozyme using HP-3 cryogels. The adsorption experiments to optimize maximum adsorption capacity were performed on all cryogels. The pH was varied in the range of 4.0–8.0 (acetate-phosphate buffer) in 0.5 mg/mL lysozyme concentration. Lysozyme has isoelectric point at pH 11.0. The strong electrostatic interactions are expected between the negatively charged surface cryogel and positively charged lysozyme. Lysozyme adsorption capacity was low above pH 5.5 in phosphate buffer and the maximum lysozyme adsorption capacity were found to be 12.78 mg/g. Lysozyme molecules were adsorbed nonspecificially at lower pH, while were adsorbed weakly at higher pH. The adsorption capacity decreased from 15.59 to 1.47 mg/g of polymer with the increase of the flow-rate from 0.5 to 2.5 mL/min. An increase in the flow-rate reduces the protein solution volume treated efficiently until breakthrough point and therefore decreases the retention time of the cryogel. The initial lysozyme concentration in the adsorption studies was varied between 0.1 and 2.0 mg/mL. Maximum adsorption capacities were determined as 48.73 mg/g for heparin immobilized PHEMA cryogels and the adsorbed amounts of cryogel reached a plateau at 1.0 mg/mL. Lysozyme adsorption capacity for PHEMA cryogels was found to be 0.68 mg/g of polymer. The effect of ionic strength on lysozyme adsorption was investigated in the range of 0.01-1.0 µM NaCl. The adsorption capacity of lysozyme decreased from 12.77 mg/g to 4.12 mg/g which shows that anions and cations affect the interaction between lysozyme and cryogel. The result revealed that the adsorption of lysozyme decreased with increasing temperature. The adsorption capacity of heparin immobilized PHEMA cryogel for adsorption of lysozyme was decreased upto 64.4% when the temperature was raised from 4 to 45^{0}C. The decrease of adsorption capacity with the increase in temperature indicated that the dominant interactions between lysozyme and cryogel were hydrogen bonds and electrostatic interactions. Desorption of lysozyme

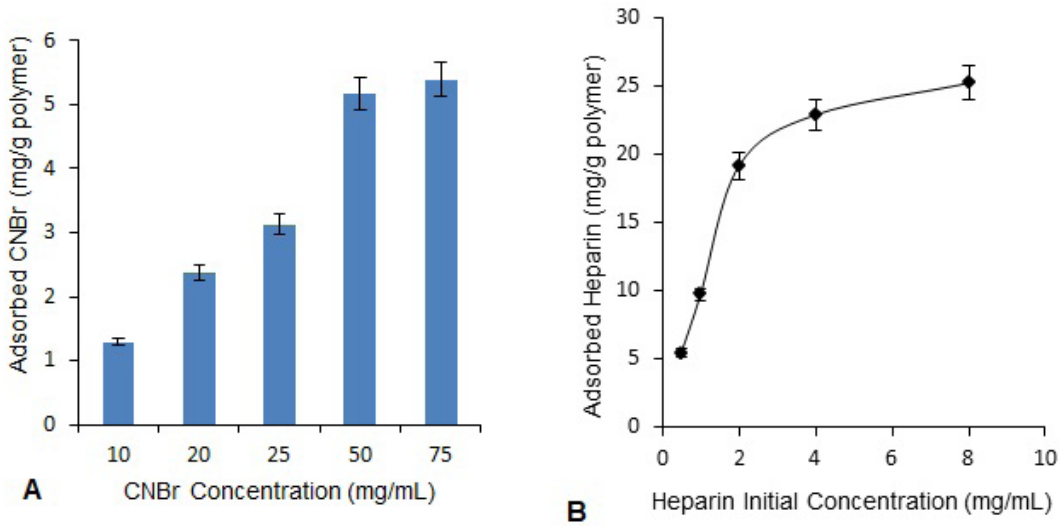

Figure 2. Effect of concentration of CNBr (A) and heparin initial concentration on heparin immobilization (B) flow rate: 0.5 mL/min; T: 25°C.

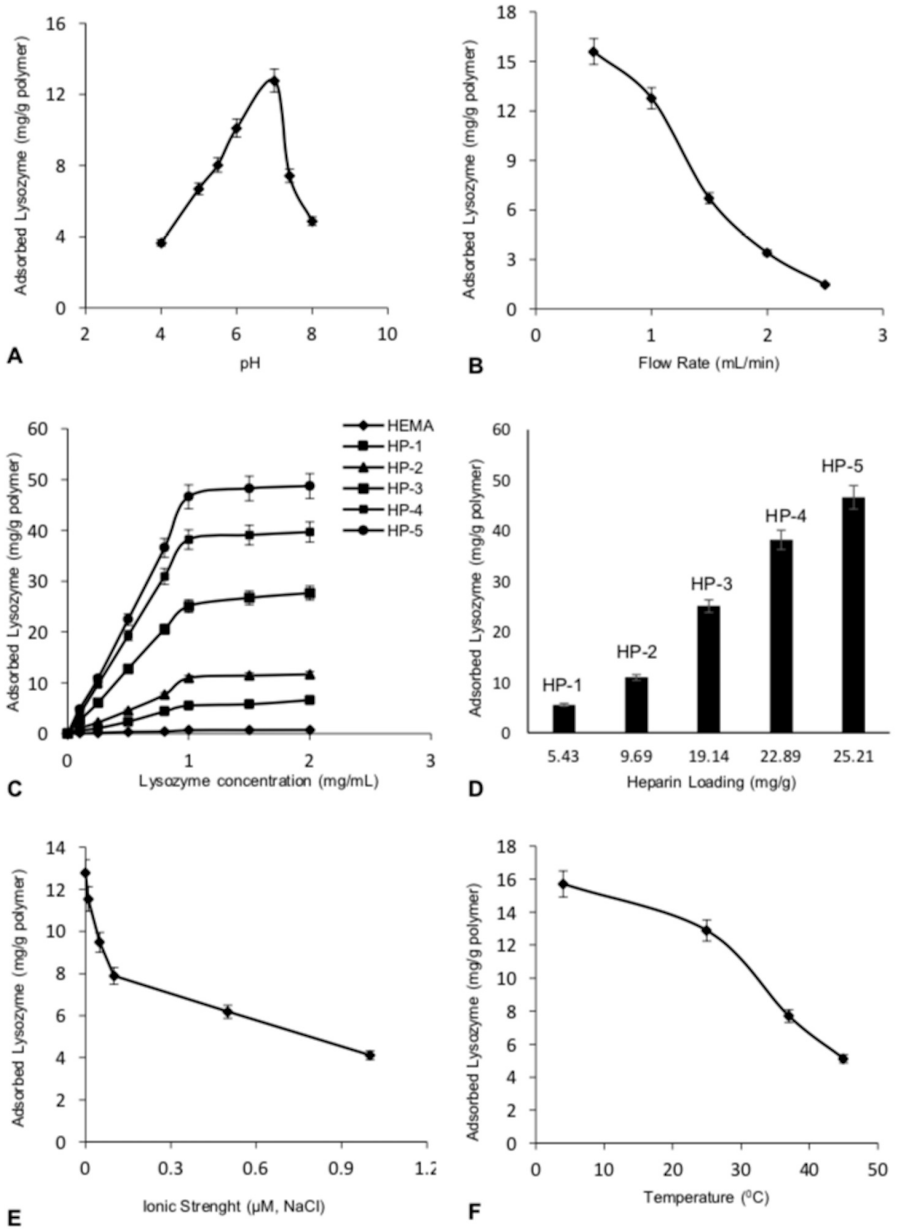

Figure 3. Effect of pH ($C_{lysozyme}$: 0.5 mg/mL, T:25°C) (A), flow rate ($C_{lysozyme}$: 0.5 mg/mL, T:25 °C) (B), lysozyme concentration (flow rate: 0.5 mL/min, T:25°C) (C), heparin loading (D), ionic strength ($C_{lysozyme}$: 0.5 mg/mL, flow rate: 0.5 mL/min, T:25°C) (E) and temperature ($C_{lysozyme}$: 0.5 mg/mL, flow rate: 0.5 mL/min, T:25°C) (F) on lysozyme adsorption.

was studied with 1.0 M NaCl solution for 1.0h. The cryogels were used for 10 repeated cycles and it was observed that there is negligible effect on their adsorption capacity. The adsorption capacity, 12.77 mg/g reported for the first adsorption/desorption cycle, changed to 12.88 mg/g following 10 cycles proving the reusability of the cryogels. Even after 10 cycles of resue the cryogels retained 97.74% of the first adsorption capacity.

The egg white's protein components were lysozyme and other proteins. SDS-PAGE experimentations were performed by spiking 0.5 mg/mL solutions in egg white. The purification of the lysozyme was performed in the SDS-PAGE analysis (Figure 4). The SDS-PAGE shows purity of lysozyme before and after treatment with cryogel. Lane 1 was egg white before adsorption. Lane 2 corresponds to the molecular weight marker (Da). The separation process ratio and the selectivity of cryogel was relatively high. The

eluate after lysozyme adsorption was presented in Lane 3. It can be concluded that the cryogel selectively removed the target protein (Lyz) in egg white, leaving the other proteins almost unchanged. The purity of the desorbed lysozyme was about 87.4% with recovery about 79.6%.

In order to evaluate adsorption phenomenon: the Langmuir and Freundlich isotherms were plotted using equilibrium adsorption data. The Langmuir and Freundlich equations are given below [31].

$$C_e/Q_e = 1/(Q_{max}.b) + (C_e/Q_{max}) \quad (2)$$

$$\ln Q_e = \ln K_f + 1/n \ln C_e \quad (3)$$

where C_e is equilibrium lysozyme concentration in solution (mg/mL), Q_e represents adsorbed lysozyme (mg/g), Q_{max} the maximum theoretical adsorption capacity (mg/g) and b, Langmuir constant (mL/mg).

The higher correlation coefficient value (R^2:0.997) indicated that Langmuir adsorption isotherm is more suitable for experimental data than the Freundlich isotherm (Table 2). The maximum adsorption capacity (27.66 mg/g) obtained from experimental results is also very close to the theoretical Langmuir adsorption capacity (28.41 mg/g). Comparison of all theoretical approaches used in this study shows that the Langmuir equation

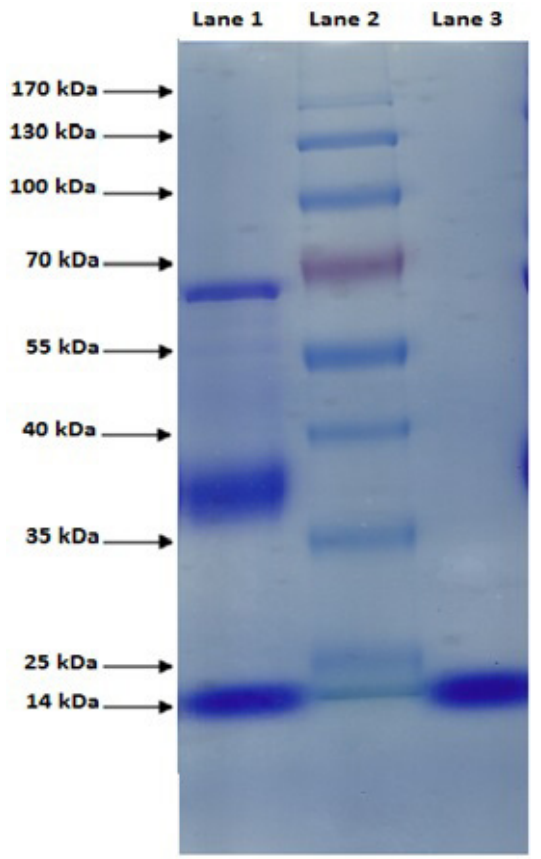

Figure 4. SDS-PAGE of egg-white samples. The samples were assayed by SDS-PAGE using 12% separating gel. Separating gels were stained with 0.25% (w/v) Coomasie Brillant R250 in acetic acid.

Table 2. Constants and coefficients of isotherms for the PHEMA and heparin immobilized PHEMA cryogels.

	Experimental	Langmuir Constant			Freundlich Constant			
Polymer Code	Q_{max} (mg/g)	Q_{max} (mg/g)	b (mL/mg)	R^2	K_f	n	1/n	R^2
PHEMA	0.73	1.34	0.909	0.967	4.30	1.458	0.686	0.928
HP-3/PHEMA	27.66	28.41	0.000035	0.997	22.78	3.126	0.319	0.881

Table 3. The kinetic constants for PHEMA and HP-3/PHEMA cryogels.

	Experimental	Pseudo first-order			Pseudo second order		
Polymer Code	Q_{eq} (mg/g)	k_1 (1/min)	Q_{eq} (mg/g)	R^2	k_2 (min.g/mg)	Q_{eq} (mg/g)	R^2
PHEMA	0.73	0.0363	2.22	0.793	0.0672	0.79	0.963
HP-3/PHEMA	27.66	0.0207	22..4	0.746	0.00092	33.56	0.979

confirmed the best experimental data. It can be concluded that lysozyme adsorbed onto the heparin immobilized PHEMA cryogels in a monolayer fashion.

To evaluate the binding kinetics of PHEMA cryogels pseudo first-order and pseudo second-order kinetic models were applied to data. Lagergren's modified equation for pseudo first-order change is given by the equation below.

$$\log[q_{eq}/(q_{eq}-q_t)] = (k_1 t)/2.303$$

where k_1 is rate constant (1/min) and q_{eq} and q_t are the amounts of adsorbed lysozyme (mg/g) at equilibrium and at any time t, respectively. The equation was applied to test the usability of pseudo second-order kinetic model, where k_2 is the rate constant (min.g/mg).

$$(t/q_t) = (1/k_2 q_{eq}^2) + (1/q_{eq})t \quad (4)$$

The higher correlation coefficient showed that the pseudo second-order kinetic model was best fit to the experimental data (Table 3). The adsorption process did not occur with any diffusion limitation and accomplished by chemical interaction at adsorption sites.

CONCLUSION

In this study, PHEMA cryogel that is one of the new generation polymeric systems was prepared as the polymer support and low molecular weight heparin, which has a high affinity for lysozyme was chosen as the ligand. The hydroxyl groups of PHEMA cryogel were activated using CNBr, then different amounts of low molecular weight heparin was immobilized. Lysozyme adsorption from egg white and regeneration was investigated using heparin immobilized PHEMA cryogel. The maximum lysozyme adsorption on the heparin immobilized PHEMA cryogel was found to be 48.73 mg/g from aqueous solutions under optimized conditions. The reusability of heparin immobilized PHEMA cryogels was tested for 10 adsorption-desorption cycles. The purity of lysozyme from egg white studies was analysed by sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% separating gel.

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