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Benchmarking Polymeric Cryogels for Immobilized Metal Affinity Chromatography

Immobilize Metal Afinite Kromatografisi için Polimerik Kriyojellerin Karşılaştırması

Yeşeren Saylan

Hacettepe University, Department of Chemistry, Ankara, Turkey.

ABSTRACT

Cryogels are polymers prepared in frozen milieu, and garnered significant attention in the field as new separation matrices. They have denoted significant benefits including supermacroporosity, short diffusion path, low pressure, and resistance for both adsorption and elution. Macro- and connected pores give polymeric cryogels a unique spongy structure. Immobilized metal affinity chromatography (IMAC) is a standard analytical separation method for the purification of biomolecules. Several transition ions generate stable complexes with electron-rich compounds. IMAC sorbent is obtained by complexing first-order transition metal ions over chelating agents. On the other hand, lysozyme is an enzyme found in various vertebrate cells and secretions. Common applications include its use as a cell disrupting agent, as an anti-bacterial agent, as a meal additive, and as a medicine against infections and ulcers. In this study, cryogel-based polymeric material was prepared by free-radical polymerization method with hydroxyethyl methacrylate/glycidyl methacrylate monomer pair that were covalently interacted with iminodiacetic acid metal chelating agent. The regions showing affinity for lysozyme enzyme were formed by binding with Ni(II) metal ions. The polymeric cryogel was first characterized using Fourier transform infrared spectrophotometer, scanning electron microscopy, thermal gravimetric analysis, X-ray photoelectron spectroscopy and swelling degree test. Then, the effects of pH, concentration, temperature, salt concentration and flow rate on enzyme adsorption capacity were evaluated, and optimum conditions were found. According to the optimization experiments, the maximum adsorption capacity of polymeric cryogel was reported as 11.82 mg/g at pH 7.4 and 25°C with a 0.5 mL/min flow rate and without ionic strength.

Key Words

Enzyme adsorption, immobilized metal affinity chromatograpy, cryogel.

ÖZ

Kriyojeller, donmuş ortamda hazırlanan polimerlerdir ve yeni ayırma matrisleri olarak bu alanda büyük ilgi görmüştür. Süpermakrogözenekliliği, kısa difüzyon yolu, düşük basıncı ve hem adsorpsiyona hem de elüsyona karşı gösterdiği az direnç gibi önemli faydalar sağlamışlardır. Büyük ve bağlı gözenekler polimerik kriyojellere özgün süngersi yapı özelliği kazandırır. İmmobilize metal afinite kromotografisi (IMAC), biyomoleküllerin saflaştırılması için kullanılan standart analitik bir ayırma yöntemidir. Birçok geçiş iyonu elektronca zengin bileşiklerle kararlı kompleksler oluştururlar. Birinci sıra geçiş metal iyonları şelatlayıcı ajanlar üzerinden kompleksleştirilerek IMAC sorbenti elde edilir. Lizozim çeşitli omurgalı hücreleri ve salgılarında bulunan bir enzimdir. Yaygın uygulamaları arasında bakteri hücre içi ürünlerin çıkarılması için bir hücre parçalayıcı madde, oftalmolojide anti-bakteriyel bir madde, süt ürünlerinde bir gıda katkı maddesi, enfeksiyon ve ülser tedavisi için bir ilaç olarak kullanılmaları sayılabilir. Bu çalışmada, hidroksietil metakrilat/glisidil metakrilat monomer çifti ile serbest radikal polimerizasyonu yöntemi ile hazırlanan kriyojel-temelli polimerik malzeme iminodiasetik asit metal şelatlayıcı ajanı ile kovalent olarak etkileştirilmiş ve Ni(II) metal iyonları ile bağlanarak lizozim enzimine afinite gösteren bölgeler oluşturulmuştur. Hazırlanan polimerik kriyojel önce Fourier transform spektrofotometresi, taramalı elektron mikroskopisi, termal gravimetrik analiz, X-ışını fotoelektron spektroskopisi ve şişme derecesi testi ile karakterize edilmiştir. Daha sonra, lizozim adsorplama kapasitesine pH, derişim, sıcaklık, tuz derişimi ve akış hızı etkileri araştırılarak optimum koşullar bulunmuştur. Optimizasyon deneylerine göre, polimerik kriyojelin maksimum adsorpsiyon kapasitesi, pH 7.4'de, 25°C'de, 0.5 mL/dk akış hızında ve iyonik kuvvet olmadan 11.82 mg/g olarak rapor edilmiştir.

Anahtar Kelimeler

Enzim adsorpsiyonu, immobilize metal afinite kromatografisi, kriyojel.

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Correspondence to: Y. Saylan, Hacettepe University, Department of Chemistry, Ankara, Turkey.

E-Mail: yeseren@hacettepe.edu.tr

INTRODUCTION

Conventional chromatographic techniques have some restrictions including high pressure drop, reduced flow rate, low prolificacy, and complexities [1]. For overcoming these restrictions, new techniques have been advanced and utilized successively in polymeric forms [2]. Cryogels are supermacroporous polymeric materials prepared at sub-zero Celsius degrees employing a suitable activator/initiator pair [3]. The free-radical polymerization happens in the solvent and frozen monomer solution in the forming sides between the ice crystals. These ice crystals behave as porogen suppliers and a supermacroporous polymer is obtained following the thawing step [4]. They show low pressure drop and high flow rate compared with conventional methods. Moreover, they are low-cost and easy-going materials and have sponge-like forms with high mechanical stability and durability [5,6]. These properties offer distinctive applications to cryogels for several adsorption studies [7-9]. Immobilized metal ion affinity chromatography called as IMAC is a significant analytical technique for various biomolecules [10]. This method contains covalently bonded chelating sides to interact with metals. These metals are utilized for various biomolecules adsorption by coordinative binding features of amino acid sides of proteins [11]. IMAC also has more benefits including high healing yield with usage of non-denaturing desorption condition [12]. Different transition metals can obtain consistent complex with electron-rich compounds and arrange O-, N-, and S-containing molecules via ion-dipole bindings [13]. Metal-ion coordination with biomolecules is well suited for molecular recognition due to its specificity and stability, directing the biomolecules as a mediator to form a stable complex with high selectivity [14]. The tight binding and bond strength can be adjusted by selecting the appropriate metal ion for the biomolecule. The cost-friendly metal ions and ease-regeneration of adsorbents are charming properties of metal-chelate separation [15]. As a biomolecule, lysozyme is a hydrolase enzyme found in body secretions such as saliva, tears, sweat, mucus, and also milk, and egg whites. It cleaves β-1-4 glycosidic bonds, contains 129 amino acids, and weighs 14.4 kDa [16]. Common usage fields include anti-bacterial, anti-viral, anti-tumor, anti-inflammatory, anti-histaminic agent, and antimicrobial agent [17, 18]. High purity needs for therapeutic and natural biomolecules along with pressures to decrease costs have warned more simple, efficient, and less expensive adsorption methods in the last years [19,20].

Here, poly(2-hydroxyethyl methacrylate (HEMA)/glycidyl methacrylate (GMA)) cryogel was prepared with a free-radical polymerization method. The iminodiacetic acid (IDA) was modified to this cryogel as a chelating agent and then Ni(II) ions were incorporated into this cryogel for affinity binding of lysozyme. The cryogel was characterized employing scanning electron microscope (SEM), Fourier transform infrared spectroscopy (FTIR), thermal gravimetric analysis (TGA), X-ray photoelectron spectroscopy (XPS), and swelling degree studies. Following the characterization experiments, optimum lysozyme adsorption conditions (pH, concentration, temperature, salt concentration, and flow rate) were investigated in an aqueous solution. The mathematical calculations were utilized to compare experimental data with theoretical data for clarifying adsorption properties.

MATERIALS and METHODS

Chemicals

2-Hydroxyethyl methacrylate (HEMA), glycidyl methacrylate (GMA), ammonium persulfate (APS), N,N'-methylene bisacrylamide (MBA), N,N,N',N'-tetramethylethylenediamine (TEMED), iminodiacetic acid (IDA), nickel nitrate hexahydrate and lysozyme were acquired from Sigma. Other chemicals were reagent grades and the water utilized in the experiments was purified by Barnstead ROpure LP®.

Preparation of cryogel

The monomer mixture was made ready by mixing 0.283 g of MBA added in 10 mL of H_2O, 1.3 mL of HEMA, and 100 µL of GMA prepared in 3.7 mL of H_2O for preparation of cryogel. After that, 20 mg of APS and 25 µL of TEMED were stirred to this mixture. The final mixture was added into a syringe with a covered outlet. The syringe was kept at -12°C for a one day for free-radical polymerization and thawed at 25°C. Following the washing step for removing the impurities and unreacted monomers, the cryogel was used for the next steps. For the next step, 10 mg/mL of IDA solution (pH 11.0) was covalently modified to the PHEMA-GMA cryogel utilizing a peristaltic pump at 75°C for 6 h. Finally, PHEMA-GMA-IDA cryogel was cleared with CH_3COOH and H_2O to eliminate excess IDA and the pH 4.5 of Ni(II) solution interacted with PHEMA-GMA-IDA cryogel at 25°C for 2 h to chelate Ni(II) ions. The PHEMA cryogel was also prepared as a control polymer by employing same method with no GMA, IDA and Ni(II) ions.

Characterization of cryogel

The characterization experiments of cryogel were performed using SEM (QUANTA 400F Field Emission), XPS (Thermo Fisher Scientific, K-Alpha), TGA (Q500 V20.13), FT-IR (Thermo Fisher Scientific, Nicolet iS10), and swelling degree analysis for the examination of topology, elemental, physical and chemical properties, respectively. The FT-IR spectrum was obtained between 400 cm^{-1} and 4000 cm^{-1} wavenumbers. The surface topology and pore structure of cryogel were examined by SEM analysis. The cryogel was covered with a gold layer before being placed in the sample holder. Thermal stability of cryogel was performed at a heating rate of 10°C/min under N_2 by means of a thermogravimetric analyzer. The elemental property of cryogel was analyzed with XPS. Dried cryogel was attached to the sample holder with copper tapes and vacuumed for 30 min. Following surveying the general elemental map of the composition, the analysis was finalized. Dried cryogel was weighed (W_0) before swelling to determine equilibrium swelling degree of cryogel and then dropped into 10 mL of water and swollen cryogel was weighed (W_{sw}) at regular intervals (0-30 min) and calculated the following equation:

$$Swelling\ degree = \frac{W_0 - W_{sw}}{W_0} x100\%$$

Lysozyme adsorption of cryogel

The benchmarking adsorption of cryogel was performed in accordance with the effects of pH (4.0-8.0), lysozyme concentration (0.05-2.0 mg/mL), temperature (4-45°C), salt concentration (0-1.0 M NaCl) and flow rate (0.25-2.0 mL/min). For adsorption studies, the lysozyme was prepared in 10 mL of pH solutions, and reacted with cryogel using peristaltic pump for 2 h. Following this experiment, the adsorbed lysozyme was desorbed with desorption solution (0.1 M NaCl) for 1 h and cleaned with water for 1 h. The ultraviolet-visible absorbance values of samples were evaluated at 280 nm. The adsorbed lysozyme was calculated following equation:

$$Q = \left(Ci - Cf\right) \times \frac{V}{m}$$

Q is adsorbed lysozyme amount (mg/g), C_i and C_f are first and last lysozyme concentration (mg/mL), V is volume (mL) and m is cryogel mass (g). All adsorption experiments were done three times.

Mathematical calculations

The interaction between lysozyme and cryogel was determined with two adsorption models including Langmuir and Freundlich using experimental data. Langmuir adsorption model interests homogeneous interaction between adsorbed biomolecules and polymers and is defined by equation:

$$\frac{1}{Qeq} = \left[\frac{1}{QmaxbCeq}\right] + \left[\frac{1}{Qmax}\right]$$

Q_{max} and Q_{eq} are maximum and equilibrium adsorbed lysozyme amount (mg/g), C_{eq} is an equilibrium concentration (mg/L), and b is Langmuir constant belongs to adsorption energy. Q_{max} and b can be computed from the slope and intercept of plot of C_{eq} against C_{eq}/Q_{eq}.

The Freundlich adsorption model interests to heterogeneous binding between adsorbed biomolecules and polymers and is described by equation:

$$lnQeq = lnKf + \left(\frac{1}{n}\right) \times lnCeq$$

1/n and K_f are Freundlich mathematical constants belongs to heterogeneity and adsorption capacity and they can be computed from the slope and intercept of plot of $\ln Q_{eq}$ against $\ln C_{eq}$.

RESULTS and DISCUSSION

Characterization of cryogel

The cryogels were characterized using SEM, XPS, TGA, FT-IR, and swelling degree analysis for examination of topology, elemental, physical, and chemical properties, respectively. The surface morphologies of cryogels were investigated at several magnifications via SEM analysis (Figure 1). It could be noticed that the cryogels had supermacroporous structures and the pore size was highly bigger than size of lysozyme, letting it to penetrate simply. The existence of channels with a diameter (around 50-100 µm) was obtained in the voids of dense thick polymeric walls. Moreover, the roughness difference between the PHEMA and PHEMA-GMA-IDA cryogels showed that the modification was successfully accomplished in the polymer structure [21].

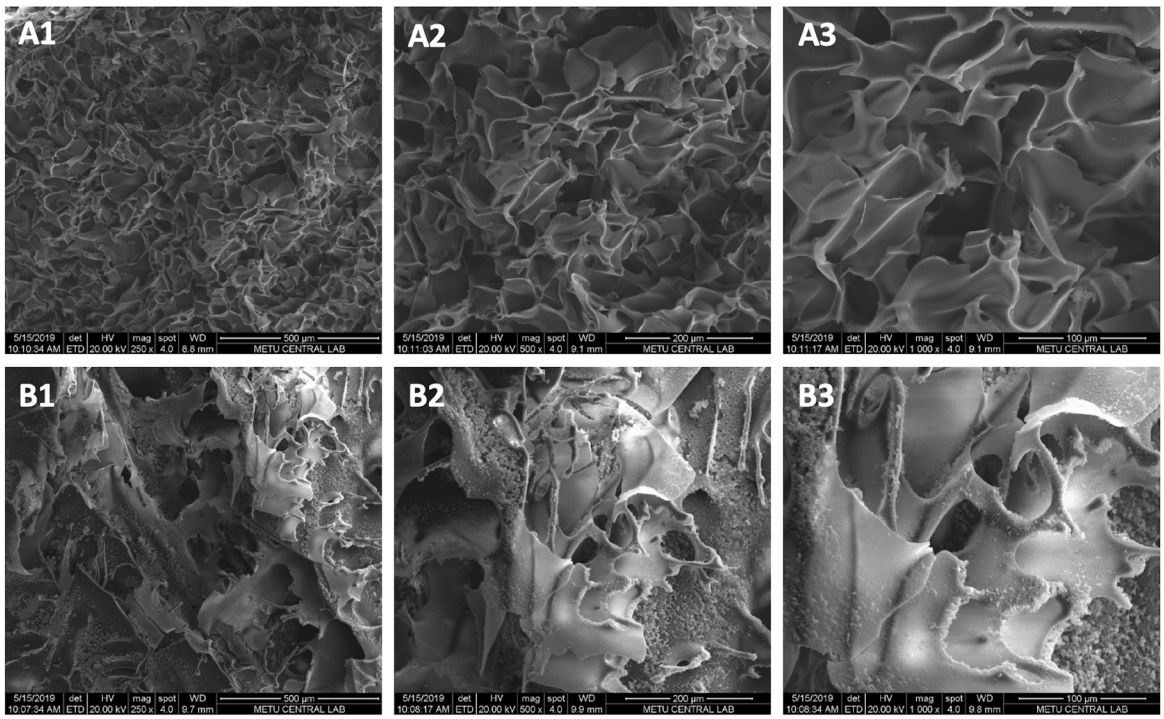

Figure 1. SEM images of PHEMA (A1-A3) and PHEMA-GMA-IDA (B1-B3) cryogels.

As depicted in Figure 2, the peaks belonging to functional groups were observed in the XPS spectra of the C, O, and N elements of the PHEMA-GMA-IDA cryogel. The corresponding chemical groups along with their binding energies (eV) were stated on the plots. Briefly, the presence of C=O was observed at 288.29 eV and 533.46 eV from carbon and oxygen data. The shoulder on the main peak at 286.25 eV and 532.18 eV correlated with the C-O groups in the cryogel [22].

The thermal stability of PHEMA-GMA-IDA cryogel was tested with TGA analysis (Figure 3A). TGA spectra result showed that cryogel lost only 3.6% of water and impurities up to 200°C and the sharp mass loss between 300-500°C was due to the breakdown of bonds in cryogel. Furthermore, it was observed that 4.0-4.7% mass of PHEMA-GMA-IDA cryogel was preserved at 800°C [23]. FT-IR spectrum of

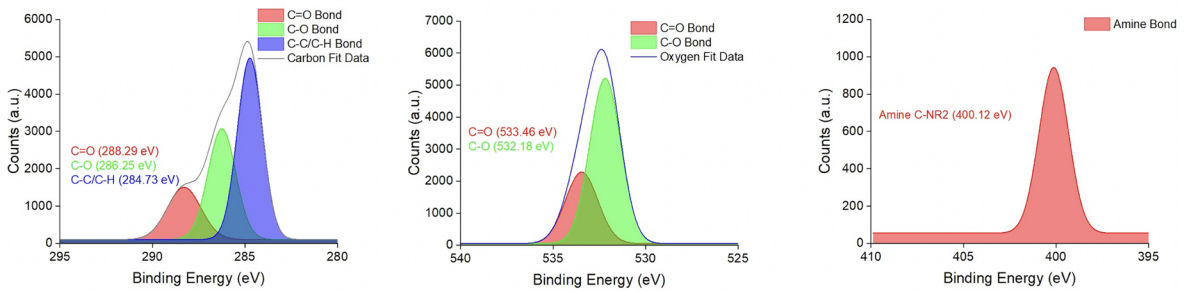

Figure 2. XPS spectra of PHEMA-GMA-IDA cryogel.

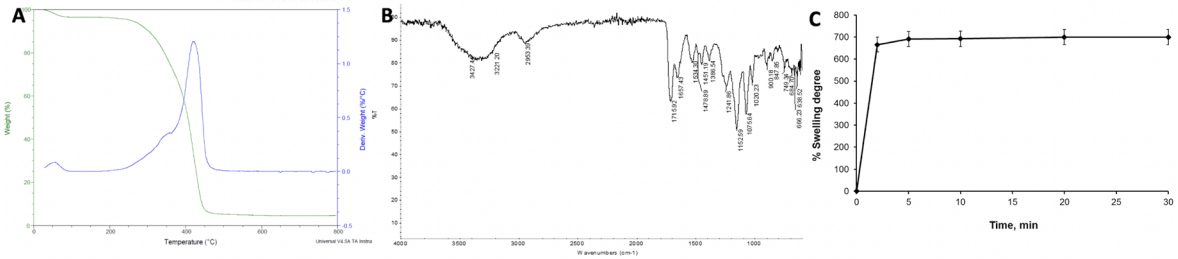

Figure 3. TGA (A) FT-IR (B) spectrum and swelling degree (C) of PHEMA-GMA-IDA cryogel.

PHEMA-GMA-IDA cryogel was demonstrated in Figure 3B. According to the spectra, the characteristic peaks of cryogel were broad peaks from 3200 to 3500 cm^{-1} designing to OH stretching, the peaks at 2953 cm^{-1} and 3221 cm^{-1} designing to CH and CH_3 stretchings. A peak at 1716 cm^{-1} indicated C=O configuration of HEMA and GMA. The C-O stretching of the carbonyl showed up at 1240 cm^{-1}. In addition, 1076 cm^{-1} was belonged to C-N functional group and the peak of the epoxy, C-O-C weakened at 900 cm^{-1} and 750 cm^{-1} that indicating the presence of IDA in the cryogel [24]. Finally, %swelling degree of PHEMA-GMA-IDA was calculated as 714.1g H_2O/g cryogel, respectively. It was observed that PHEMA-GMA-IDA cryogel began to swell in a short time (2 min) and after 10 min, its water holding capacity was stabilized (Figure 3C) [25].

Lysozyme adsorption of cryogel

The optimum parameters effects were carried out for evaluating the performance of PHEMA-GMA-IDA cryogel for the calculation of lysozyme adsorption capacity values. The pH effect on the adsorbed lysozyme was examined using several buffers (pH 4.0, 5.0, 6.0, 7.0, 7.4, and 8.0) including 0.5 mg/mL of lysozyme at 25°C without ionic strength (I.S.) at a 0.5 mL/min flow rate (F.R.). As Figure 4A demonstrated the maximum adsorbed lysozyme capacity was obtained at pH 7.4. The isoelectric point of lysozyme molecule is around pH 11.0, it carries a net negative charge at a pH above its pI owing to the surface-accessible amino acid residues. Thus, the PHEMA-GMA-IDA cryogel was simply electrostatically bonded lysozyme with at this pH [26].

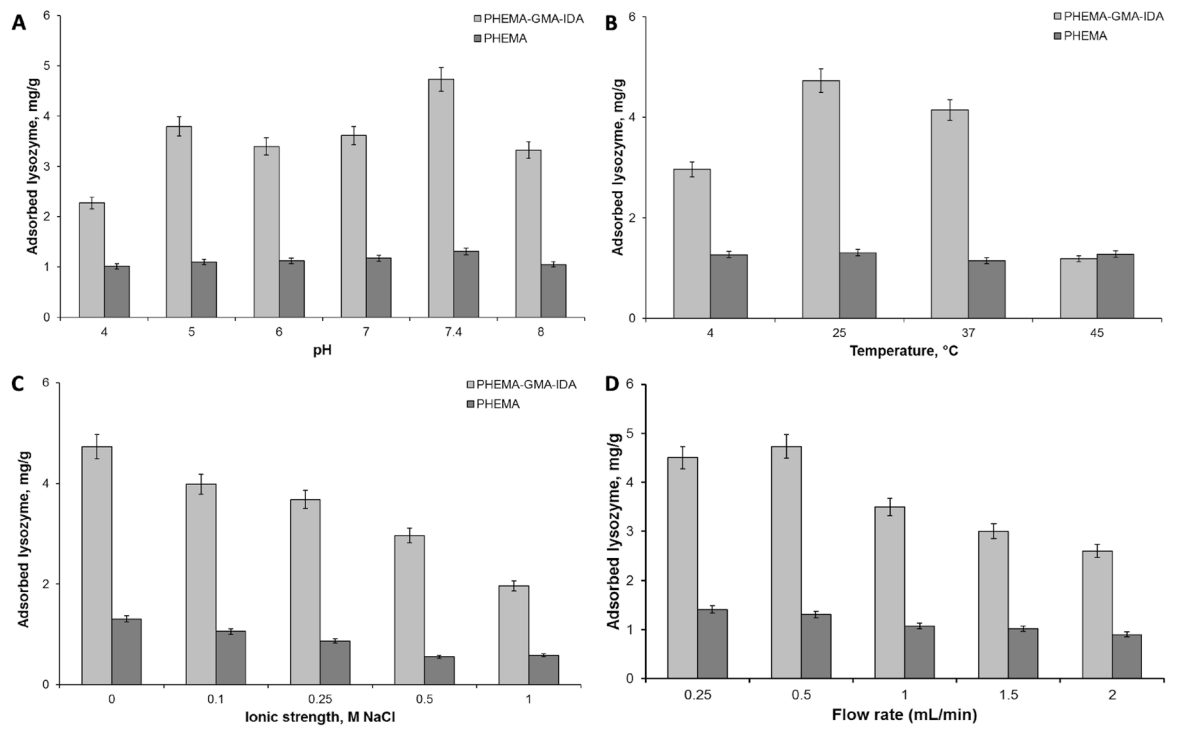

Figure 4. pH (A), temperature (B), ionic strength (C) and flow rate (D) effects on the adsorbed lysozyme. The adsorption experiments conditions: (A): $C_{lysozyme}$=0.5 mg/mL, T=25°C, I.S.=0 M, F.R.= 0.5 mL/min (B): $C_{lysozyme}$=0.5 mg/mL, pH 7.4, I.S.=0 M, F.R.= 0.5 mL/min, (C): $C_{lysozyme}$=0.5 mg/mL, pH 7.4, T=25°C, F.R.= 0.5 mL/min, (D): $C_{lysozyme}$=0.5 mg/mL, pH 7.4, T=25°C, I.S.=0 M.

The relation between adsorbed lysozyme and temperature provided significant information to appoint the interaction. As depicted in Figure 4B, several temperatures (4°C, 25°C, 37°C, and 45°C) were investigated with 0.5 mg/mL of lysozyme sample solutions at pH 7.4 without I.S. at a 0.5 mL/min F.R. The maximum adsorbed lysozyme amount was noticed at 25°C. As the bindings between lysozyme and PHEMA-GMA-IDA cryogel started to decline as temperature rise continued, the adsorbed lysozyme amount also reduced. The adsorption on IMAC supported an effect of electrostatic, hydrophobic, and/or donor-acceptor interactions [27]. Moreover, the I.S. effect (0.1 M NaCl, 0.25 M NaCl, 0.5 M NaCl and 1.0 M NaCl) on lysozyme adsorption was assessed with 0.5 mg/mL of lysozyme sample solutions at pH 7.4 at 25°C with a 0.5 mL/min F.R. Increment of the amount of NaCl followed in the adsorbed lysozyme reduction due to the bonds weakening between lysozyme and PHEMA-GMA-IDA cryogel. The NaCl molecules may react with lysozyme using electrostatic interactions and prevent the binding sites [28] (Figure 4C). Finally, the interaction between lysozyme and PHEMA-GMA-IDA cryogel varied at several F.R. As observed in Figure 4D, the maximum lysozyme adsorption was found at a 0.5 mL/min F.R. At higher rates, the lysozyme and PHEMA-GMA-IDA cryogel could not interact each other [29].

Mathematical calculations

The PHEMA-GMA-IDA cryogel interacted with lysozyme samples with different concentrations (0.05 mg/mL, 0.1 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 0.75 mg/mL, 1.0 mg/mL, 1.5 mg/mL and 2.0 mg/mL) at pH 7.4 and 25°C with a 0.5 mL/min F.R. and no I.S. The adsorbed lysozyme amount increased with concentration increment (Figure 5A). It was observed that the PHEMA-GMA-IDA cryogel showed a high lysozyme capacity owing to binding sites being well-fitted with lysozyme [30]. The maximum adsorbed lysozyme amount was obtained as 11.82 mg/g for PHEMA-GMA-IDA cryogel. But, the PHEMA cryogel had only 2.19 mg/g adsorption capacity because of the non-specific interactions. It was also concluded from these results that affinity of lysozyme to the cryogel was increased specifically with attachment of IDA and Ni(II). The adsorption type of PHEMA-GMA-IDA cryogel was estimated by Langmuir and Freundlich models (Figure 5B, Figure 5C, Figure 5D, and Table 1). In accordance with the mathematical calculations, the regression coefficients (R^2 = 0.9911 for Langmuir, R^2 = 0.9867 for Freundlich) and the maximum adsorbed lysozyme amounts (Q_{max} = 12.86 mg/g for Langmuir, Q_{max} = 30.14 mg/g for Freundlich) were compared and noticed that the interaction between lysozyme and PHEMA-GMA-IDA cryogel was coherent with Langmuir adsorption mo-

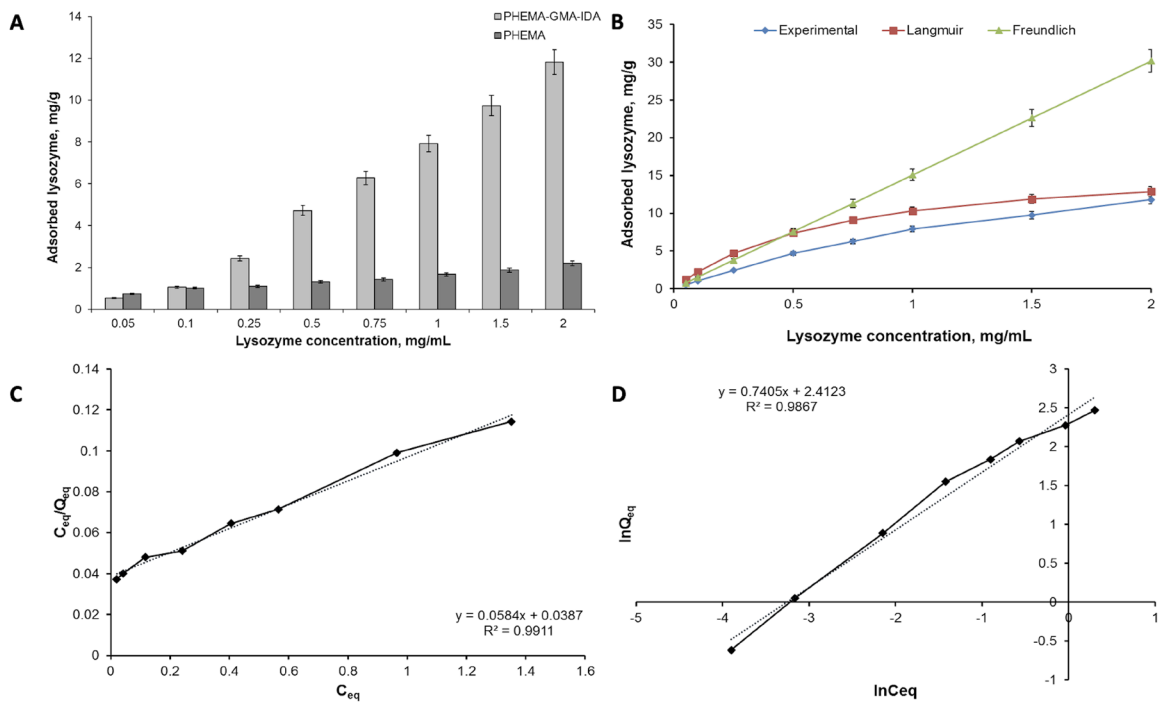

Figure 5. The effect of concentration on the adsorbed lysozyme (A), comparison experimental results with adsorption models (B), Langmuir (C) and Freundlich (D) models. The adsorption experiments conditions: pH 7.4, T=25°C, I.S.=0 M, F.R.= 0.5 mL/min.

Table 1. Adsorption model coefficients.

Langmuir				Freundlich		
Q_{eq} (mg/g)	Q_{max} (mg/g)	b (mL/mg)	R^2	Kf	1/n	R^2
11.82	12.84	1.5	0.9911	11.16	1.35	0.9867

Table 2. Comparing polymers in terms of the metal type, interaction, polymer, target, and adsorption capacity.

References	Metal	Interaction	Polymer	Target	Capacity
[32]	Cu^{2+}	PHEMA-IDA	Magnetic beads	Human serum albumin	28.4 mg/g
[33]	Cu^{2+}	PHEMA-MAH	Beads	Cytochrome c	31.7 mg/g
[34]	Cu^{2+}	PGMA-IDA	Beads	Immunoglobulin G	171.2 mg/g
[35]	Cu^{2+}	PHEMA-GMA-IDA	Bead-embedded cryogel	Immunoglobulin G	257 mg/g
[36]	Cu^{2+}	PGMA-IDA	Beads	Hemoglobin	130.3 mg/g
[37]	Ni^{2+}	PHEMA-GMA-IDA	Cryogel	Urease	11.30 mg/g
[38]	Zn^{2+}	PHEMA-GMA-IDA	Cryogel	Yeast alcohol dehydrogenase	9.94 mg/g
[39]	Cu^{2+}	PGMA-IDA	Magnetic beads	Human serum albumin	37.7 mg/g
[40]	Fe^{3+}	PAAm-GMA-IDA	Cryogel	Catalase	12.99 mg/g
This study	Ni^{2+}	PHEMA-GMA-IDA	Cryogel	Lysozyme	11.82 mg/g

del which referred the interaction type was monolayer, homogeneous, and binding sites had similar affinity for lysozyme. So, lysozyme reached to the interaction sites with no diffusion problems [31].

CONCLUSION

In this study, the preparation of PHEMA-GMA-IDA cryogel for the efficient adsorption of lysozyme was reported. The PHEMA-GMA-IDA cryogel was synthesized using free-radical polymerization and then modified for IMAC technology to create binding sites for lysozyme. This simple, stable, and low-priced preparation and adsorption process can be more distant used for various polymers-based systems for purification, separation, adsorption and sensor applications. Other polymers-based systems were summarized in Table 2 to compare the polymers in terms of the metal type, interaction, target, and adsorption capacity. As compared with existing polymer-based systems, this study was described for the first time and carries great potency for enzyme adsorption. Furthermore, this work demonstrates an adaptable method which can be distributed to the other biomolecules separation and adsorption and supplies a novel platform to synthesize new-generation biomaterial.

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