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The Adsorption of Calmoduline via Nicotinamide-Immobilized Poly(HEMA-GMA) Cryogels

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Abstract: The separation and purification of an important biomolecule calmoduline is extremely important. The adsorption technique is quite popular, and the cryogels as adsorbents with the macro porous structure and interconnected flow channels are preferred in this field. In this study, the adsorption of calmoduline via Ca(II) immobilized poly(2-hydroxyethyl methacrylate-glycidyl methacrylate), poly(HEMA-GMA), cryogels was studied through changing interaction time, calmoduline initial concentration, and temperature conditions. For the characterization of cryogels, the swelling test, Fourier Transform Infrared (FT-IR) Spectroscopy, Scanning Electron Microscopy (SEM), surface area (BET), elemental analysis and ICP-OES methods were performed. Nicotinamide molecule was used as Ca(II) being the chelating agent and the adsorption capacity of the cryogels was estimated as 1.812 mg calmoduline/g cryogel. The adsorption models of the process were examined via the Langmuir and Freundlich isotherm models.

Keywords: Adsorption; calmoduline; cryogel; nicotinamide.

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INTRODUCTION

Calmoduline (CaM) is an acidic protein with the molecular weight of 17 kDa. This protein consists of two globular regions or lobe (N-lobe or expressed as C-lobe), each joined by long flexible helix centre. These regions consist of a pair of EF-hand motif (1). It binds to the four calcium ions via the four EF-hand motifs with binding in micromolar range. As a result of binding, there are conformational changes, the hydrophobic fields come into the outer surface (2-4). The calmoduline regulates a variety of cellular functions such as the concentration of abdominal muscle (5), cell cycle (6), and metabolism (7). This protein constitutes at least 0.1% of the total cellular protein concentration in most cells and exists in higher levels in the brain, testicles, and stimulated and rapidly growing cells (8). It also helps the regulation of functions (by contributing the signaling pathway in organisms) such as cell proliferation, learning and memory, growth, exocytosis, endocytosis, and movement (9). The regulation of these functions can be accomplished through the direct interaction of calmoduline protein with a large number of proteins such as kinase, phosphatase, cytoskeleton to increase in calcium concentration within the cell. Moreover, the calmoduline carries the calcium signal in the cell (10-12).

Immobilized metal affinity chromatography (IMAC) has been developed first in 1975 by Porath *et al* (13). This technique is fast, reliable and used generally for the purification of proteins recombined with histidine (14-18). IMAC resins consist of a solid support (*e.g.* agarose), a linker, a chelating compound (iminodiacetic acid, nitrilotriacetic acid or carboxymethyl ethylenediamine) and a divalent transition metal ion (Zn^{2+}, Cu^{2+}, Ni^{2+}, Co^{2+}, *etc.*). The chelating compounds are being immobilized to the solid support via a linker and are coordinated with metal ions (19).

Metal ions interact with nitrogen atoms locating within the structure of amino acids (especially nitrogen atoms in the imidazole ring of histidine amino acids) on the surface of proteins (20).

In this study, the nicotinamide molecules were bonded on the poly(HEMA-GMA) cryogels as a chelating agent. The Ca(II) ion was used as the metal ion. A significant conclusion was achieved as a result of comparison of results with the literature (21). In addition, there was no recent study encountered about the calmoduline adsorption using cryogels. The adsorption of calmoduline from an aqueous solution of this protein was studied and the optimum adsorption conditions (interaction time, initial concentration and temperature) were determined.

MATERIALS AND METHODS

Materials

2-Hydroxyethyl methacrylate (HEMA), glycidyl methacrylate (GMA), ethylene glycol dimethacrylate (EGDMA), calcium chloride dihydrate, ammonium persulfate (APS), sodium dodecyl sulfate (SDS), N,N,N',N'-tetramethylethylenediamine (TEMED), nicotinamide, and calmoduline were supplied from the company Sigma (St. Louis, USA). All other chemicals are of analytical purity and ultra-pure water (18 MΩ.cm) was used in all studies.

Methods

The Synthesis of poly(HEMA-GMA) Cryogels: GMA (500 µL), HEMA (5000 µL) and distilled water (6500 µL) were mixed to obtain the monomeric phase. The disperse phase was prepared using SDS (1 g), distilled water (25.60 mL) and EGDMA (2.4 mL) and then the two phases were mixed with each other. It was cooled in an ice bath for 10-15 minutes. TEMED (100 µL) and APS (20 mg) were added. The mixture remained at -20°C for 24 hours.

The resulting cryogels were cut in the shape of membrane (disc). The cryogels were washed using a rotator (Multi Bio RS-24 Biosen, Latvia) at 10 rpm with distilled water for the removal of sodium dodecyl sulfate and other unwanted chemicals and then the washing water was changed in every 15 minutes until it becomes clear.

The Immobilization of Nicotinamide and the Binding of Ca(II) to the Poly(HEMA-GMA) Cryogels: The number of 20 cryogel membrane was stirred in NaOH (1 M, 10 mL) for 2 hours. Membranes washed several times were interacted with nicotinamide solution (50 mg/mL, 10 mL) for 24 h. At the end of this process, the color of membranes turned from white to yellow. Then the cryogels was washed again with distilled water and stirred in $CaCl_2.2H_2O$ solution (10 mL, 5 mg/mL). The yellow color of Ca(II) attached membranes was brightened a little bit and washed with distilled water-ethanol mixture several times (Figure 1).

Characterization Studies

Swelling Test: The water retention capacity of poly(HEMA-GMA)@Nic-Ca(II), cryogels was determined using distilled water. The dry cryogel membranes were carefully weighed and then placed into the distilled water in the isothermal water bath and remained at 25°C for 30 minutes. The membranes were placed on a filter paper and the water retained on the surface was removed by clinging. At the end, the water retention capacity was calculated according to the formula given below:

$$\text{Water Retention Capacity \%} = [(W_s - W_o)/ W_o] \times 100 \qquad \text{(Equation1)}$$

Wherein, W_o and W_s are the weights (g) of dry and water retained membranes, respectively.

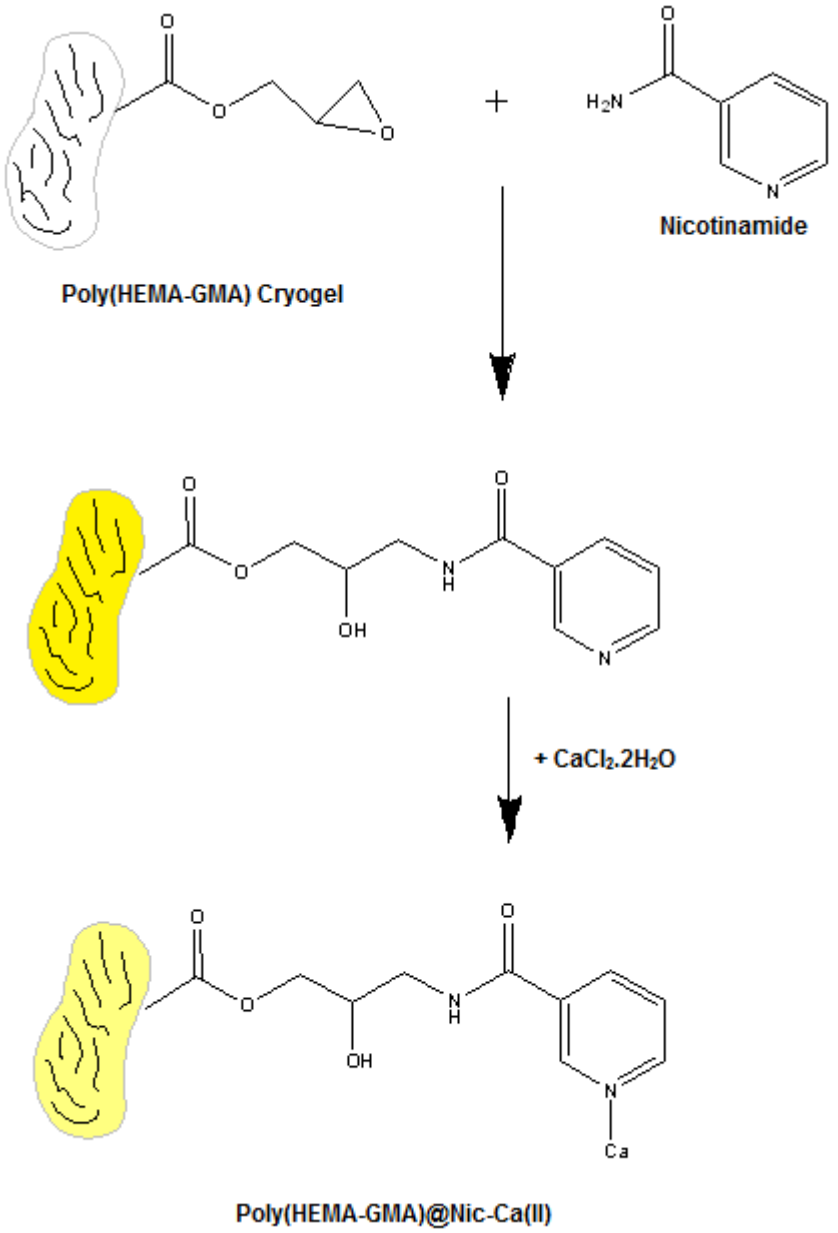


Figure 1. The schematic representation of Poly(HEMA-GMA)@Nic-Ca(II) cryogel synthesis.

SEM Analysis: The surface morphology of cryogel membranes was examined using Scanning Electron Microscopy (SEM, FEI / Quanta 450 FEG, USA). The membrane dried via lyophilization was tailored for SEM analysis and attached on double-sided carbon tape on the SEM holder. The sample was then coated under vacuum with a thin gold layer. The sample obtained was then placed in the SEM device and imaged.

FT-IR Analysis: The characteristic functional groups of poly(HEMA-GMA) cryogels were determined using Fourier Transform Infrared (FT-IR, Thermo Scientific Nicolet 6700 FT-IR spectrometer, USA). The cryogels were dried and pulverized primarily (about 2 mg) and made into pellets by mixing homogeneously by anhydrous potassium bromide powder (KBr) (98 mg, IR grade, Merck, Germany) and the FT-IR spectrum was obtained in the wavenumber range of 400-4000 cm^{-1}.

Elemental Analysis: The dry sample was weighed and placed into the elemental analysis (Elementar Vario PYRO cube, Germany) container. The analysis was performed under the condition of the combustion tube at 1120°C and the reduction tube at 850°C and then the N% value obtained.

The Determination of Ca(II) Amount Bound to the Cryogels : To determine the amount of Ca(II) attached to the poly(HEMA-GMA)@Nic cryogels, the ICP-OES (Spectro Arcos, Germany) were used. In this technique, the sample was excited by electromagnetic induction to the temperature of 10,000 K by argon plasma and the quantity of elements excited was determined by the specific wavelength emitted by these elements. Plasma is obtained by the stimulation of argon gas electromagnetically using a radio frequency (RF) generator on the induction coils. This process was performed by the ionization of incoming gas via hot plasma successively.

Surface Area Analysis: The specific surface area of the membranes was determined via Brunauer-Emmett-Teller (BET; Quantachrome Autosorb® iQ-Chemi, USA) device. The cryogels samples dried with lyophilization remained at 35°C under 100 mbar vacuum for 6 hours to eliminate oxygen and moisture in the pores. Then, the cryogel samples were treated with nitrogen gas at room temperature.

The Adsorption-Desorption Studies: The calmoduline adsorption onto the poly(HEMA-GMA)@Nic-Ca(II) was studied in a batch system. For the adsorption experiment, the buffer solution (4 mL, pH 7.5, 0.1 M Tris-HCl) and the calmoduline solution (1 mL) were mixed and stirred in a rotator for 15 minutes. To determine the calmoduline concentration prior to adsorption, the sample (200 µL) was taken. A cryogel was placed into the adsorption medium and stirred in a rotator at 20 rpm. The membrane was then removed from the adsorption medium and sample (2 mL) was taken to be analyzed in UV-Vis to determine the protein concentration after adsorption. The adsorbed amount of calmoduline was determined using a UV-Vis spectrophotometer (Double Beam PC 8 Auto Cell Scanning UVD-3200 Labomed, INC., USA) at 280 nm wavelength. The calmoduline adsorption capacity of cryogel was determined according to the formula given below.

$$q = [(C_i - C_f) \times V] / m \quad \text{(Eq. 2)}$$

Where q is the amount of adsorption (mg / g), C_i is the concentration of calmoduline solution before adsorption (mg / L), C_f is the concentration of calmoduline solution after adsorption (mg / L), V is the volume of the adsorption medium (L) and m is the amount of dry adsorbent (g).

For desorption studies, NaCl solution (1 M, 10 mL) was used. To determine the reusability of cryogels, the adsorption-desorption cycle was performed using NaOH solution (50 mM, 10 mL) for the regeneration of cryogels exposed to the desorption process.

RESULTS and DISCUSSIONS

Characterization Studies

The swelling ratio of cryogels was calculated as 764% which is quite a good result as compared to the literature (22, 23). Accordingly, the dry cryogel (1 g) has retained about 7.64 g of water. The SEM image of cryogels shows the macro-pores and the interconnecting flow channels (Figure 2). When the FT-IR spectra are examined, the peaks at 3423 cm^{-1} (alcohol, -OH), 2947 (alkane C-H), 1731 (carboxylic acid, C = O), 1619 and 1558 cm^{-1} (amine, NH) are obvious for the poly(HEMA-GMA)@Nic-Ca(II) cryogels (Figure 3). In the case of elemental analysis results, the poly(HEMA-GMA)@Nic-Ca(II) cryogels have the N% value corresponding the ligand amount of 32.9 mg/g cryogel (nicotinamide 1.175 mmol/g cryogel). The surface area of cryogels was estimated as 8.736 m^2/g which quite comparable with the literature (24, 25). The Ca(II) amount immobilized on the cryogels was found as 455 mmoles/g.

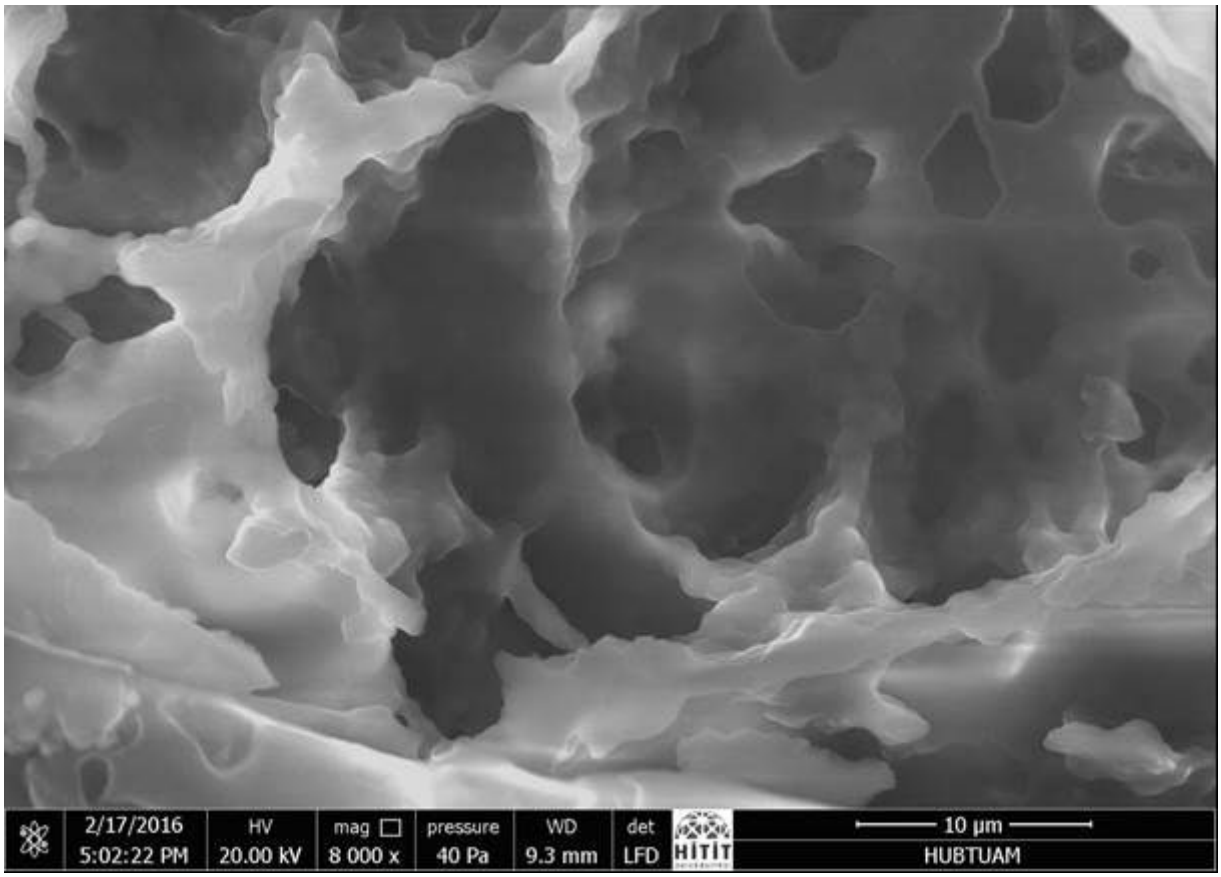


Figure 2. The SEM images of poly(HEMA-GMA)@Nic-Ca(II) cryogels.

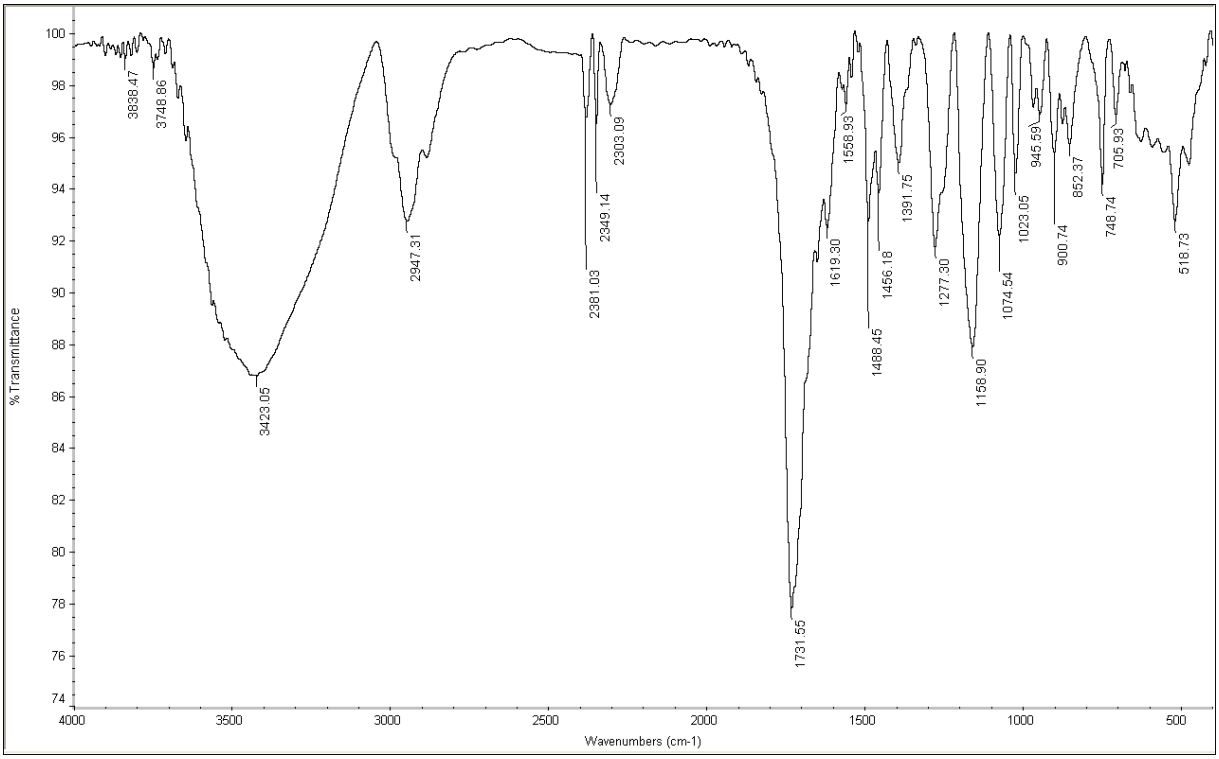
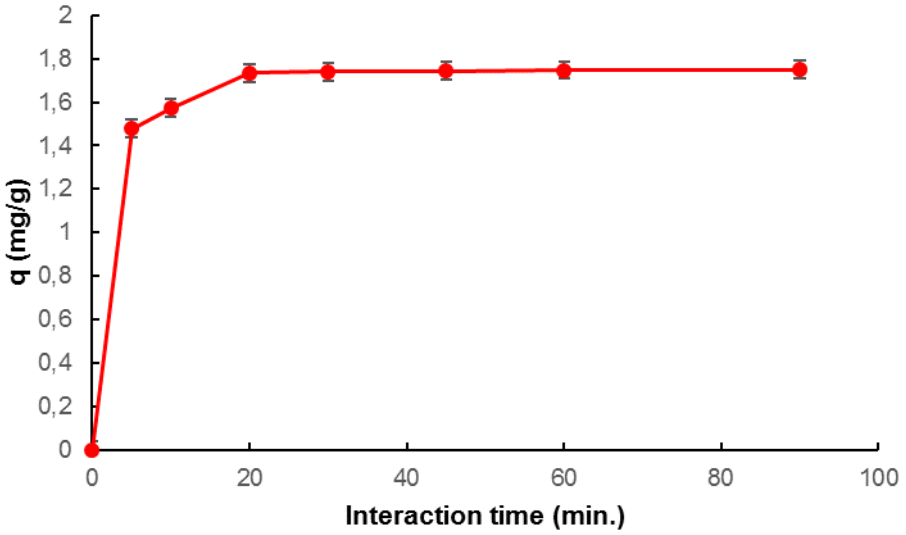


Figure 3. The FT-IR spectrum of poly(HEMA-GMA)@Nic-Ca(II) cryogels.

Adsorption-Desorption Experiments

To determine the environmental condition within the scope of adsorption studies, the adsorption time, calmoduline initial concentration, and temperature parameters were investigated. At the end of the studies performed, the 20 minute-interaction time was sufficient to achieve the maximum adsorption capacity (Figure 4-a). During this time, the calmoduline adsorption capacity of poly(HEMA-GMA)@Nic-Ca(II) cryogels was reached the maximum value and not changed significantly at subsequent time intervals because of the saturation of binding zone on the surface of cryogels. The adsorption capacity of cryogels was increased with the concentration of calmoduline solution used in adsorption, but there was no significant change observed in the adsorption capacity after the concentration value of 5 mg/L (Figure 4-b). This situation can also be explained by the saturation of cryogels in terms of target molecules. The decrease observed on the adsorption capacity with increasing temperature has shown that the interaction between poly(HEMA-GMA)@Nic-Ca(II) cryogels and calmoduline molecules were electrostatic interaction (Figure 4-c). The electrostatic interaction was weakened with increasing temperature and decreased the adsorption capacity by causing the partial breaking of the bonds.



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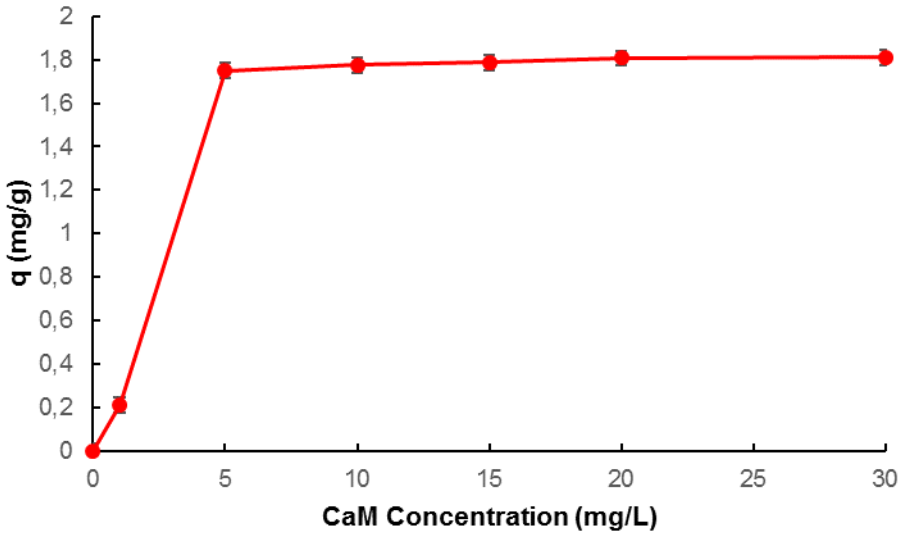

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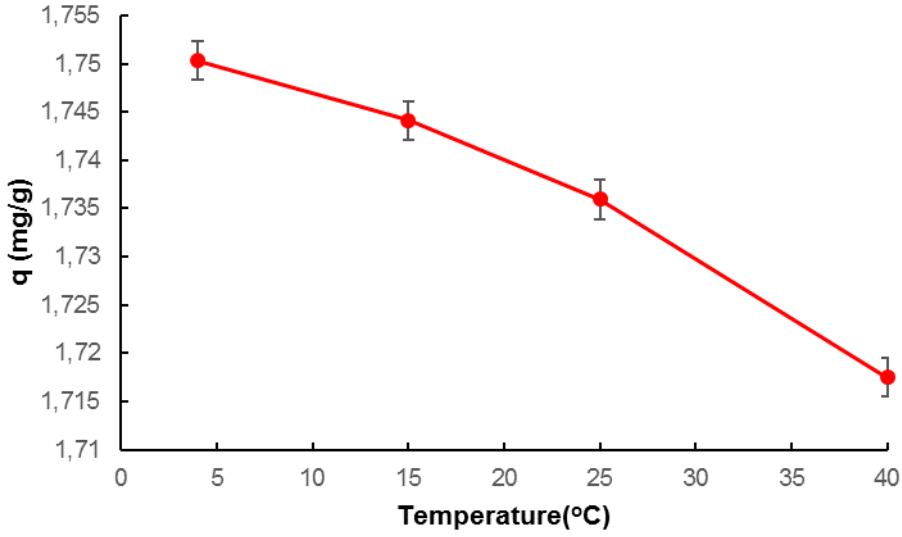

c

Figure 4. The effect of a) Interaction time. $C_{Calmodulin}$: 5 mg/L; temperature: 25°C. b) Calmoduline initial concentration. Interaction time: 20 min.; temperature: 25°C. c) Temperature. $C_{Calmodulin}$: 5 mg/L; interaction time: 20 min. on the adsorption process. All experiments were repeated three times applying 95% confidence interval for calculating mean values reported.

To test the reusability properties of the poly(HEMA-GMA)@Nic-Ca(II) cryogels, the same cryogel was used 5 times for the adsorption-desorption process. There was no significant decrease was observed in the adsorption capacity of cryogel at the end of the 5 cycles (from 1.753 to 1.7391) (Figure 5). This result has shown that the reusability performance of poly(HEMA-GMA)@Nic-Ca(II) cryogels are significantly high.

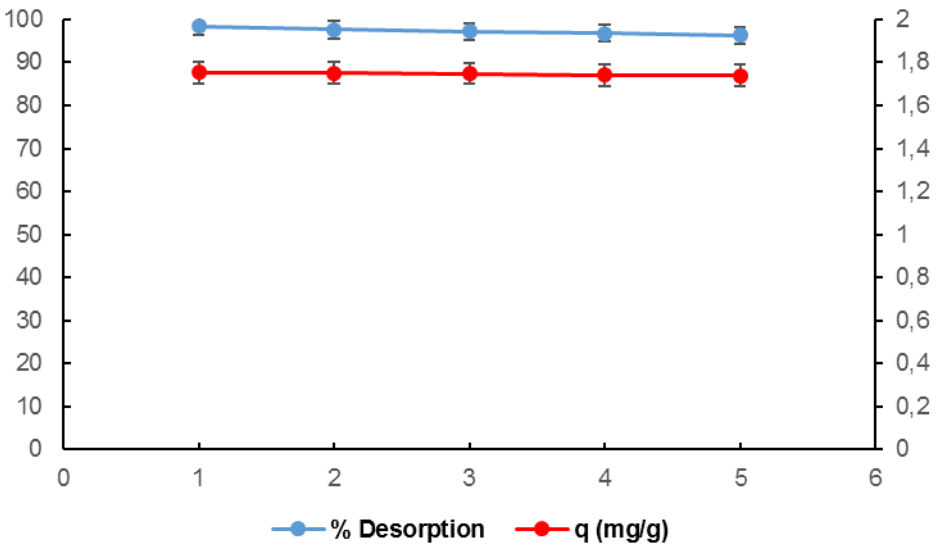

Figure 5. The reusability of poly(HEMA-GMA)@Nic-Ca(II) cryogels.

The calmoduline adsorption performance of poly(HEMA-GMA), poly(HEMA-GMA)@Nic and poly(HEMA-GMA)@Nic-Ca(II) cryogels were compared with each other under predetermined optimum adsorption conditions (interaction time: 20 min., calmoduline initial concentration: 5.0 mg/L). As can be seen from Figure 6, the calmoduline adsorption capacity of poly(HEMA-GMA) (0.125 mg/g) is very much lower than that of poly(HEMA-GMA)@Nic-Ca(II) cryogel (1.75 mg/g). According to this result, the incorporation of nicotinamide and Ca(II) into the structure has provided a substantial increase of calmoduline affinity of the cryogel. In the method performed before (26), the calmoduline adsorption amount of 0.2 µg was achieved per procedure, and thus the high performance of cryogels used in this study is remarkable as a conclusion of the comparison with this result.

Adsorption Isotherms

The characterization of the adsorption of calmoduline molecule onto poly(HEMA-GMA)@Nic-Ca(II) cryogels, the adsorption isotherms were examined. According to the Langmuir adsorption isotherm, the adsorption phenomena is on the surface, uniform and assumed to have occurred in one layer (27), whereas according to the Freundlich adsorption isotherm, the adsorption is not limited to a single layer and are considered to be heterogeneous (28). The following equations were used for the Langmuir and Freundlich isotherms.

$$\text{Langmuir equation: } C_{eq}/Q = 1/(Q_{max.}\, b) + (C_{eq}/Q_{max.}) \qquad \text{(Eq.2)}$$

If it is linearized, Eq. 3 is obtained:

$$1 / Q_{eq} = [1 / (Q_{max.}\ b)][\ 1 / C_{eq}] + [1 / (Q_{max})] \quad \text{(Eq. 3)}$$

Where, the y-intercept of the plot $1/C_{eq}$ vs $1/Q_{eq}$ is $1/Q_{max}$ and the slope is $1/Q_{max.}b$. Q_{eq} is the adsorbed calmoduline amount (mg/g), C_{eq} is the calmoduline concentration at equilibrium, b is the Langmuir adsorption constant (L/mg), and $Q_{max.}$ is the maximum adsorption capacity (mg/g).

$$\text{Freundlich equation: } \ln Q_{eq} = \ln K_f + (n \times \ln C_{eq}) \quad \text{(Eq. 4)}$$

Where K_f and n are the Freundlich isotherm constants. The y-intercept of the $\ln Q_{eq}$ vs $\ln C_{eq}$ plot is the $\ln K_f$ and the slope is the n value.

The necessary adsorption and correlation coefficients for both adsorption isotherms were calculated and given in Table 1.

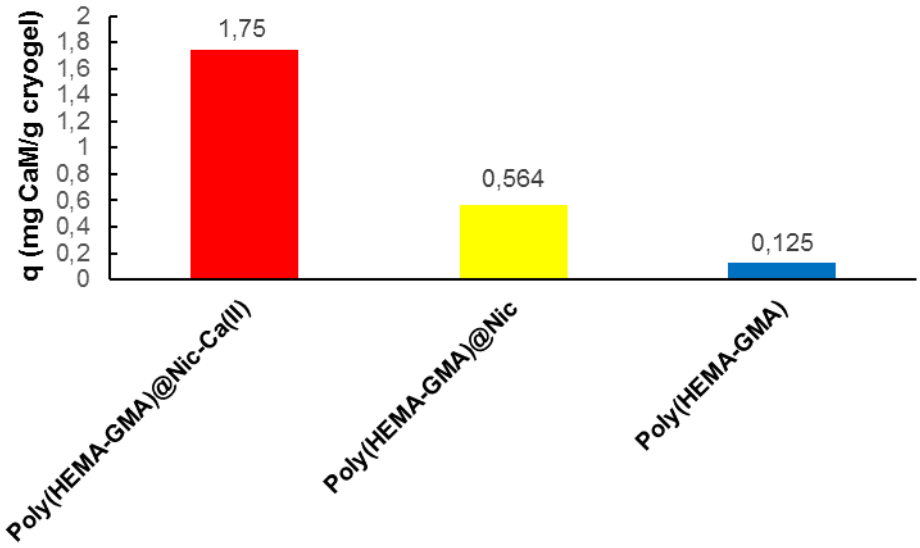

Figure 6. The comparison of the calmoduline adsorption capacities of poly(HEMA-GMA), poly(HEMA-GMA)@Nic and poly(HEMA-GMA)@Nic-Ca(II) cryogels.

Table 1. The parameters obtained from adsorption isotherms.

	Langmuir Constants			Freundlich Constants			
$Q_{exp.}$ (mg/g)	$Q_{max.}$(mg/g)	b (L/mg)	R^2	K_f	n	1/n	R^2
1.812	1.821	7.358	0.9349	1.74	0.0133	75.19	0.925

According to the experimental data obtained, the correlation coefficient of the Langmuir isotherm is higher and the Q_{max} value corresponds to the value obtained in the experiment.

CONCLUSIONS

It was determined that poly(HEMA-GMA)@Nic-Ca(II) cryogel is a suitable adsorbent for the adsorption of calmoduline molecule. It is thought that the interaction occurs between Ca(II) ion within the structure of cryogel and the charged groups on the calmoduline molecule. The decrease in the amount of adsorption with increasing temperature and ionic strength is an indicator of the electrostatic interaction. The interaction between the cryogel and calmoduline fits well the Langmuir adsorption model. Therefore, according to this model, the calmoduline molecules are adsorbed onto the poly(HEMA-GMA)@Nic-Ca(II) cryogels on a monolayer homogeneously.

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Türkçe Öz ve Anahtar Kelimeler

Nikotinamid Tutuklanmış poli(HEMA-GMA) Kriyojelleri ile Kalmodulinin Adsorpsiyonu

Kadir Erol

Öz: Önemli bir biyomolekül olan kalmodulinin ayrılması ve saflaştırılması son derece önemlidir. Adsorpsiyon tekniği oldukça popülerdir, makro-gözenekli yapıları ve içinde barındırdığı akış kanalları ile kriyojellerin adsorban olarak kullanıldığı görülmektedir. Bu çalışmada, kalmodulinin Ca(II) tutuklanmış poli(2-hidroksietil metakrilat-glisidil metakrilat, poli(HEMA-GMA)) ile kalmodulinin adsorpsiyonu çalışılmış ve etkileşim süresi, kalmodulinin ilk derişimi ve sıcaklık değiştirilerek deneyler yapılmıştır. Kriyojellerin karakterizasyonu için, şişme testi, Fourier Dönüşüm Kızılötesi Spektroskopisi (FT-IR), Taramalı Elektron Mikroskopisi (SEM), yüzey alanı (BET), elementel analiz ve ICP-OES yöntemleri kullanılmıştır. Nikotinamid molekülü, Ca(II) için kelatlama aracı olarak kullanılmış ve 1,812 mg kalmodulin/g kriyojel olarak, adsorpsiyon kapasitesi bulunmuştur. Sürecin adsorpsiyon modelleri Langmuir ve Freundlich izoterm modellerine göre incelenmiştir.

Anahtar kelimeler: Adsorpsiyon; kalmodulin; kriyojel; nikotinamid.

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