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Research Article

Metal-Ion Assisted Imprinted Hydrogels For Recognition Of Lysozyme

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ABSTRACT

Hydrogels exhibiting selectivity towards lysozyme were produced by metal-ion assisted-imprinting technology. A metal-chelate monomer N-vinyl-2-pyrrolidone is pre-assembled with the template molecule via assistance of Cu(II) ions and co-polymerized with 2-hydroxyethyl methacrylate. Lysozyme imprinted hydrogels were characterized by Fourier transform infrared spectroscopy, swelling tests, scanning electron microscopy. The conditions for the optimum adsorption capacity of the hydrogels towards lysozyme were found out by investigating the effects of initial concentration of lysozyme, medium pH, contact time and ionic strength. Maximum adsorption of lysozyme on poly(hydroxyethyl methacrylate-co-N-vinyl pyrrolidone) hydrogel was found to be 12.25 mg/g for 1.0 mg/mL initial concentration of lysozyme at 25.0°C with an optimal pH of 7.0. After ten adsorption-desorption cycles with the same hydrogel, the lysozyme adsorption capacity decreased by 13.80%.

Keywords: *Hydrogels, lysozyme, metal ion coordination, molecular imprinting*

Lizozim Tanımada Metal İyon Destekli Baskılanmış Hidrojeller

ÖZET

Lizozime karşı seçicilik sergileyen hidrojeller, metal iyon aracılı baskılama teknolojisi ile üretildi. Metal şelat monomeri olarak N-vinil-2-pirolidon, Cu(II) iyonlarının yardımıyla kalıp molekül ile önceden kompleksleştrildi ve 2-hidroksietil metakrilat ile birlikte polimerleştirildi. Lizozim baskılanmış hidrojeller, Fourier dönüşümü kızılötesi spektroskopisi, şişme testleri, taramalı elektron mikroskobu ile karakterize edildi. Hidrojellerin lizozime karşı optimum adsorpsiyon kapasitesi için en uygun koşullar, lizozimin başlangıç derişiminin, ortam pH'sının, adsorpsiyon süresinin ve iyonik kuvvetin adsorpsiyon kapasitesine etkileri araştırılarak bulundu. Lizozimin poli(hidroksietil metakrilat-ko-N-vinil pirolidon) hidrojel üzerinde maksimum adsorpsiyonu, 25.0°C'de 1.0 mg/mL başlangıç lizozim derişimi optimum pH değeri (7.0) için 12.25 mg/g olarak bulundu. Aynı hidrojel ile on adsorpsiyon-desorpsiyon döngüsünden sonra, lizozim adsorpsiyon kapasitesi %13.80 azaldı.

Anahtar Kelimeler: *Hidrojeller, lizozim, metal iyon koordinasyonu, moleküler baskılama*

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I. INTRODUCTION

Lysozyme (N-acetylmuramide glyconohydrolase) is a compact enzyme (14 kDa) consisting of 129 amino acid residues folding into a globular structure [1]. Since it exists in bodily secretions such as tears, milk, and saliva and catalyzes a reaction as breaking the β1–4 bond found in peptidoglycan residues of bacterial cell walls between N-acetylmumaric acid and N-acetylglucosamine, it is called as body's own antibiotic [2]. Lysozyme has been utilized in various areas including the extraction of bacterial intracellular products (as a cell-disrupting agent), treatment of ulcers and infections, in milk products (as a food additive) and so on [3]. Due to importance of Lys, numerous studies on the adsorption of Lys have been performed using different materials as adsorbents including nanofiber membranes, mesoporous organic silica, porous organic cages, cryogels, monolith immobilized with aptamer, silica nanoparticles and so on [4]–[9].

Hydrogels are three-dimensional polymeric networks that capable of absorbing and keeping large amounts of water in their hydrophilic structure. Hydrogels can be formed by cross-linking of both natural and synthetic polymers and polymerization of monomers using cross-linking agents [10], [11]. Because hydrogels exhibit some outstanding properties such as versatile fabrication methods, simple modification processes and similar flexibility with natural tissues, a wide variety of examples are available in the fields of biomedical engineering and biotechnology, ranging from tissue engineering and drug delivery to chromatography [12]–[16].

Molecularly imprinted polymers (MIPs) are the materials having specific recognition ability for the target molecule [17]–[19]. In the design of MIPs, the appropriate orientation of the target analyte and functional monomer(s) is ensured, followed by polymerization using a cross-linker [20]. After the polymerization step, the target analyte, the so-called imprint molecule or template molecule, is removed resulting in cavities specific to the target analyte [21]. The interactions between functional monomer and imprint molecule can be provided through covalent bonding, non-covalent interactions, metal-ion coordination and so on. Among them, metal-ion assisted imprinting is may be a good candidate due to directionality of coordinate bonds and strength against a wide range of solvent environment [22]–[24].

Herein, we report molecularly imprinted poly(hydroxyethyl methacrylate-co-N-vinyl pyrrolidone) [poly(HEMA-co-NVP)] hydrogel for lysozyme (Lys) recognition through metal ion coordination. The structure and interior morphology of the hydrogels were characterized by Fourier transformation infrared spectroscopy (FT-IR), and scanning electron microscopy (SEM). Sol and gel fractions and equilibrium swelling ratios were also studied. Adsorption capacities of the hydrogel were investigated by changing medium adsorption time, pH, initial Lys concentration and ionic strength. Selectivity and reusability of the hydrogels were also examined.

II. MATERIAL AND METHODS

A. MATERIALS

HEMA, Lys (chicken egg white, EC 3.2.1.17), NVP, N,N′-methylenebis(acrylamide) (MBAAm), ammonium persulfate (APS), bovine serum albumin (BSA), N,N,N',N'-tetramethyl ethylene diamine (TEMED) and cytochrome C (Cyt C) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

B. PRODUCTION OF HYDROGELS

Lys imprinted poly(HEMA-NVP) hydrogel (MIH) was produced as described below. In the first step, NVP (0.1 mmol) and $Cu(NO_3) \cdot 2.5H_2O$ (0.1 mmol) were added into 1.0 mL of HEPES buffer at pH 7.0 at room temperature (RT) and the solution was allowed to be mixed for 3 h. Then, the template (i.e. Lys) was also added to the solution and mixed for 1 h for preorganization of the NVP–Cu(II) and Lys. In the

second step, 0.260 g of MBAAm was dissolved in 13.2 mL of deionized water (DW). 1.3 mL of HEMA and 0.2 mL of the complex mixture was added into this solution. After stirring the mixture, APS (20 mg) and TEMED (25 µl) were added and the mixtures are poured into disposable syringes (5 ml) with closed ends. Hydrogelation was carried out at 25 °C for 24 h. Hydrogels were taken from the syringes, washed with 200 mL of deionized water (DI) and cut into disk shape (3.5 mm in thickness). Removal of the imprint molecule (i.e., Lys) from the hydrogels was performed using 1.0 M NaCl until observing no Lys in the washing solution via a UV-spectrophotometer at 280 nm. Then the hydrogels were washed with 50 mM NaOH and DI at RT for 12 h. Non-imprinted hydrogels (NIHs) and poly(hydroxyethyl methacrylate) (PHEMA) hydrogels were also prepared without adding the Lys and the complex mixture, respectively.

C. CHARACTERIZATION STUDIES OF HYDROGELS

Gelation yield (G) and equilibrium swelling ratio (*ESR*) of the hydrogels were found out by the Equations 1 and 2, respectively:

$$G\ (\%) = \frac{W_0}{W_r}\ x\ 100 \quad (1)$$

$$\text{ESR} = \frac{W_1 - W_0}{W_0} \quad (2)$$

where W_r is total mass (g) of the reactants utilized for the hydrogelation while W_0 and W_1 are the weights (g) of dried and sufficiently swollen (with DW) hydrogels, respectively.

For FT-IR spectra of hydrogels, the samples were firstly dried and then placed on the ATR probe. FT-IR spectra of the hydrogels were obtained in the range of 4000–400 cm^{-1} using a FTIR-ATR spectrophotometer (Nicolet iS20 ATR-FTIR, Thermo Scientific, USA). Microstructure and wall morphology of the freeze-dried hydrogels were analyzed using SEM (Hitachi, SU1510, Tokyo, Japan) Before SEM analysis, the hydrogels were gold-coated for 2 min.

D. ADSORPTION STUDIES

Effects of medium pH (in the range of 6.0 and 9.0) and adsorption time, initial Lys concentration (in the range of 0.1-2.0 mg/mL), and ionic strength (in the range of 10-100 mM of NaCl) on the Lys adsorption capacity of the imprinted hydrogels were studied in a batch system and the adsorption amounts were found out spectrophotometrically at 280 nm. The experiments were done in replicates of three. The amounts of Lys absorbed by the hydrogels were found using the following formula:

$$Q = \frac{C_i - C_f}{m}\ x\ V \quad (3)$$

where Q (mg/g) is the mass of Lys adsorbed by a unit mass of dry hydrogels, C_i (mg/mL) and C_f (mg/mL) represent the initial and final concentrations, respectively, m is the weight of the hydrogels utilized ($W_{dried\ hydrogels}$ = 55.00 ± 1.95 mg) and V (mL) is solution volume.

E. SELECTIVITY STUDIES

BSA and Cyt c were utilized as competitor proteins (prepared separately with initial concentrations of 1.0 mg/mL) to investigate the Lys selectivity of the imprinted hydrogels. MIHs and NIHs were performed separately in the aqueous solutions of proteins at the batch system for 3 h and initial and final concentrations were measured using a UV spectrophotometer. The terms given below were calculated with the following equations respectively in order to examine the selectivity of the hydrogels for Lys:

$$Distrubition\ coefficient\ (K_d) = \frac{C_i - C_f}{C_f}\ x\ \frac{V}{m} \quad (4)$$

$$Selectivity\ coefficient\ (k) = \frac{K_{d\ (Lys)}}{K_{d\ (competitor\ protein)}} \quad (5)$$

$$\text{Relative selectivity coefficient } (k') = \frac{k_{imprinted}}{k_{non-imprinted}} \quad (6)$$

F. REUSABILITY PERFORMANCE OF THE HYDROGELS

The reusability of the hydrogels was investigated by performing ten cycles of adsorption-elution-regeneration process with the same hydrogels.

III. RESULTS AND DISCUSSION

A. CHARACTERIZATION STUDIES

In literature, it is showed that Cu(II) ions interacts with NVP through its carbonyl group and the lone pair of the nitrogen atom [25]. FT-IR spectra of the hydrogels were given in Figure 1. For the spectrum of PHEMA, a broad band around 3300 cm^{-1} was due to O-H group. The peak at 1718 was associated with ester carbonyl group. The peaks at 1150 and 1070 cm^{-1} were attributed to the stretching vibrations of C-O [26]. Since amount of HEMA used in reaction mixture is 100 times higher in mole than NVP, the peaks of NVP are not so clear. However, higher intensity for the peak around 1665 cm^{-1}, might be the contribution of the carbonyl stretching banding of NVP [26].

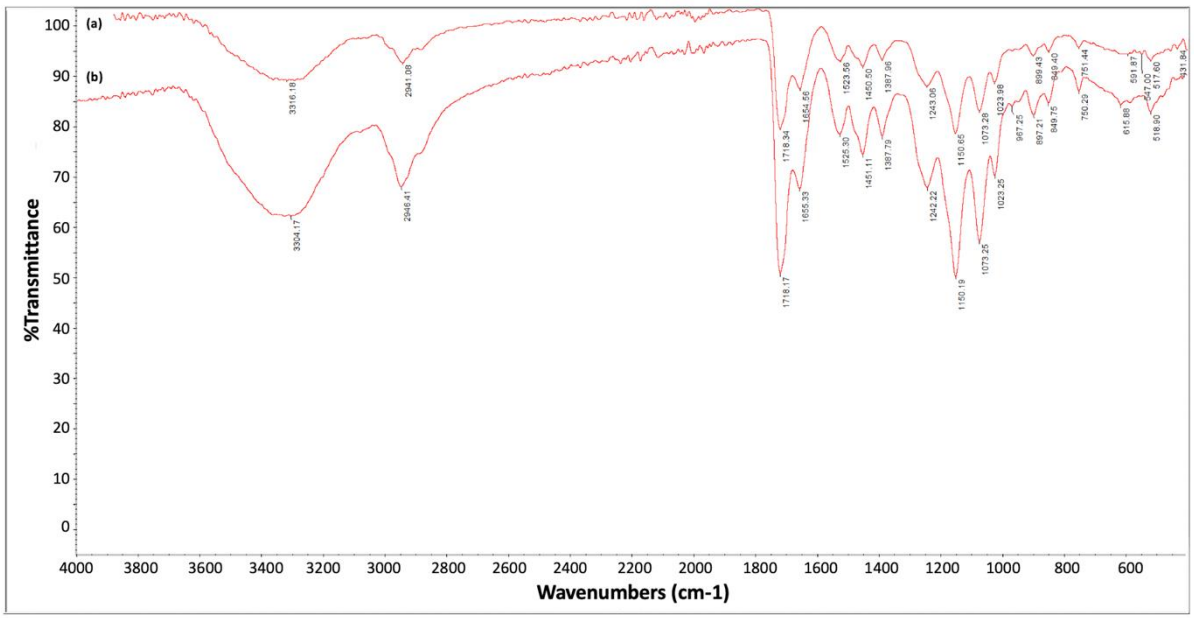

Figure 1. *FT-IR spectra of* ***(a)*** *P(HEMA-NVP) and* ***(b)*** *PHEMA hydrogels.*

Gelation yields of PHEMA and P(HEMA-NVP) hydrogels were calculated as 86.82% and 83.41%, respectively. Equilibrium swelling ratio of PHEMA and P(HEMA-NVP) hydrogels were found to be

8.78 and 9.73 g water/g hydrogel, respectively. This is an expected situation since NVP has more hydrophilic character than HEMA [27].

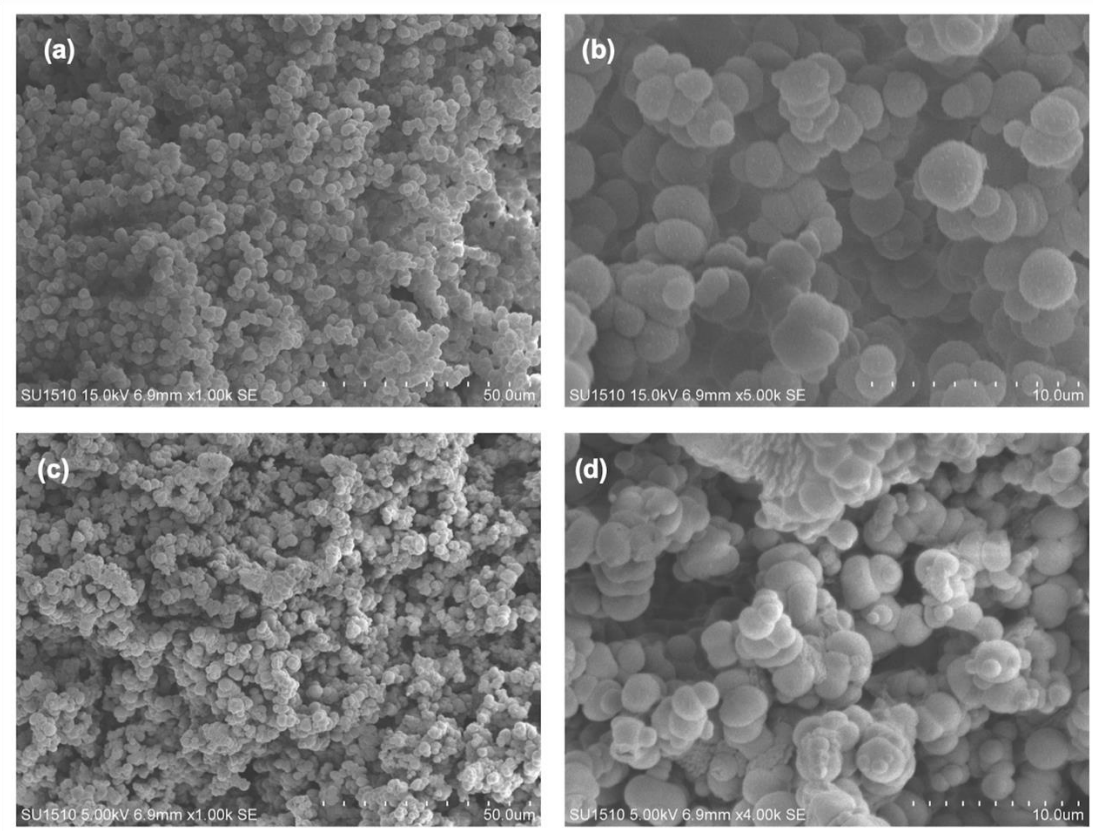

Figure 2. *SEM images of the hydrogels.* ***(a and b)*** *poly(HEMA) and* ***(c and d)*** *poly(HEMA-NVP) hydrogels. (Magnifications: 6.9 mm x 1.00k for a and c; 6.9 mm x 5.00k for b; 6.9 mm x 4.00k for d).*

Surface morphology and internal structure of the hydrogels were analyzed via SEM. In literature, it was found that hydrogels prepared in cylinder form have higher pore sizes than those prepared in film form [28]. Because higher pore size causes lower back-pressure, hydrogels were produced in cylindrical form and then cut into disc form. As shown in Figure 2, maximum pore size of the hydrogels is around 10 μm.

B. ADSORPTION STUDIES

B. 1. pH

The adsorption amount of Lys by MIHs at various pH values are shown in Figure 3. The maximum Lys adsorption capacity of MIHs was obtained as 12.25 mg/g polymer at pH 7.0 and a decrement was observed above and below of this pH value. In the hydrogelation process NVP–Cu(II)-Lys was prepared at pH 7.0. Therefore, the high binding affinity at pH 7.0 could be explained due to the memory effect of the molecular imprinting [7].

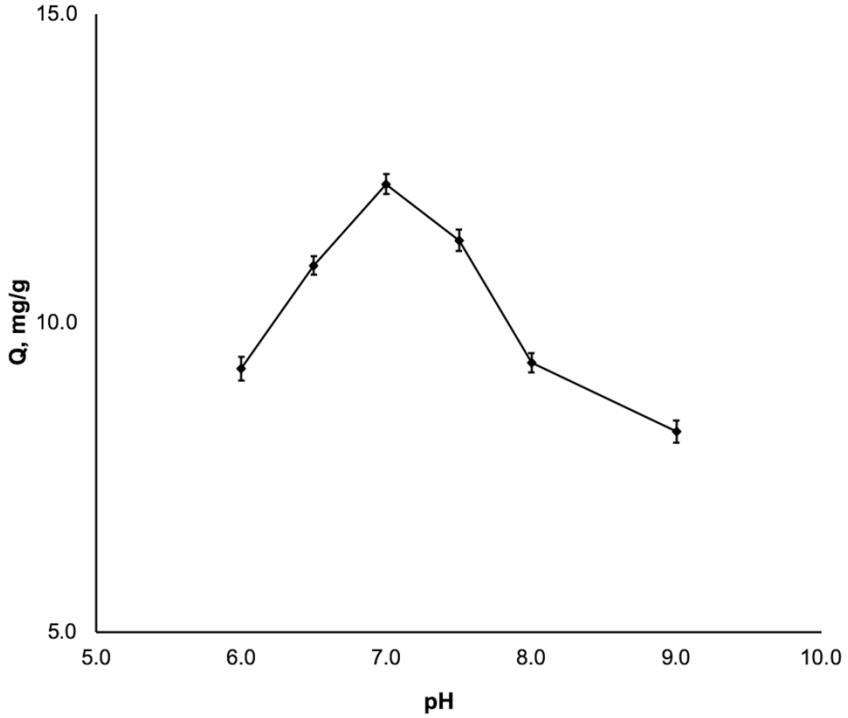

Figure 3. *Effect of pH on Lys adsorption by MIHs. $C_{i_{Lys}}$: 1.0 mg/mL; t: 3 h.*

B. 2. Incubation time

Time dependence of the adsorption values of Lys by MIHs are presented in Figure 4. At the beginning, relatively faster adsorption rates were observed while it was decreased by time and reached adsorption equilibria in around 3 h.

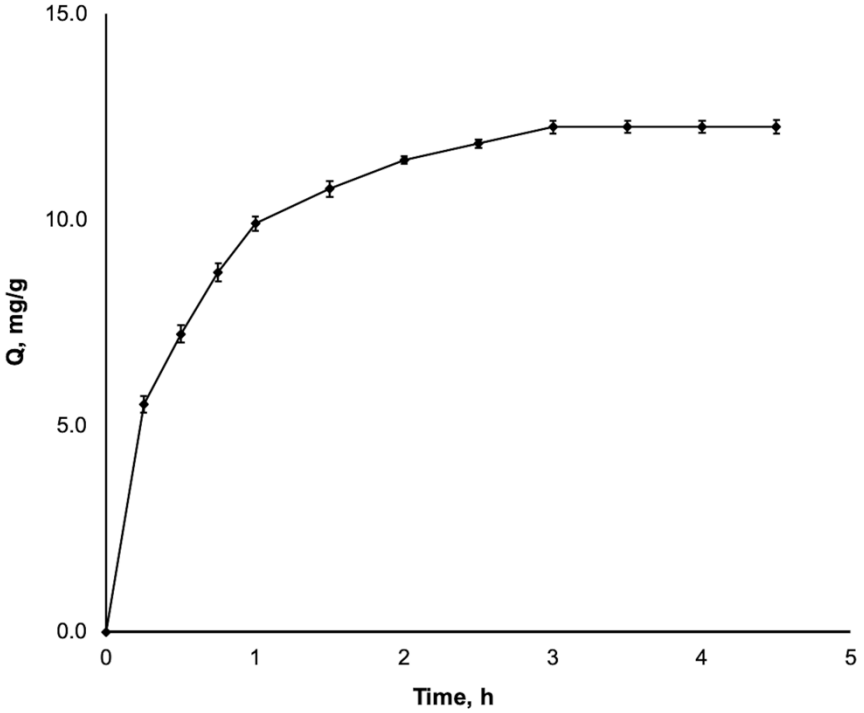

Figure 4. *Effect of incubation time on Lys adsorption by MIHs. $C_{i_{Lys}}$: 1.0 mg/mL; pH: 7.0.*

B. 3. Initial Lys concentration

The change in the adsorption capacity of MIHs by varying the initial Lys concentration ($C_{i_{Lys}}$) was investigated (Figure 5). Firstly, a drastic increase was observed and then reached to the equilibrium at $C_{i_{Lys}}$ = 1.0 mg/mL. Maximal adsorption capacity of MIHs was observed as 12.25 mg/g dry hydrogel which represents occupation of all imprinted cavities.

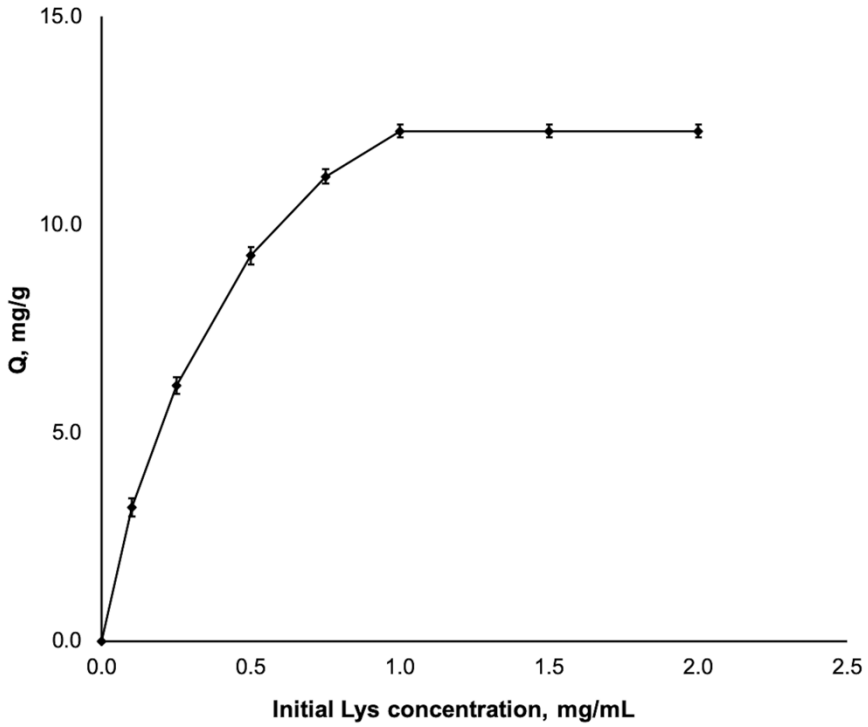

Figure 5. *Effect of $C_{i_{Lys}}$ on Lys adsorption by MIHs. t: 3 h; pH: 7.0.*

B. 4. Ionic strength

As seen in Figure 6, amount of adsorbed Lys by MIHs was decreased by increasing NaCl concentration. The reason behind the decrease in the adsorption capacity by increasing salt concentration could be that the salt ions mask the binding sites for Lys [29]. NaCl at higher concentrations may promote anions to occupy the free coordination sites and leading lower adsorption capacity [30].

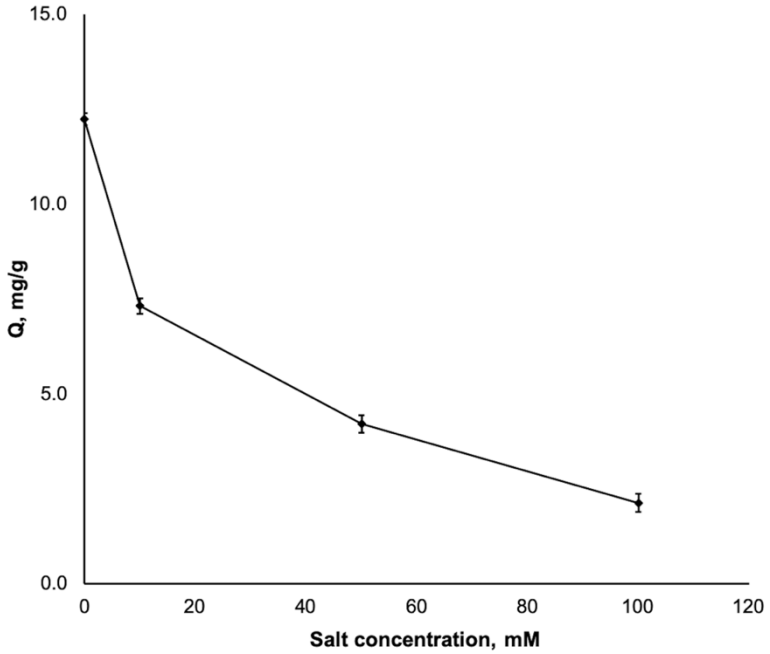

Figure 6. *Effect of ionic strength on Lys adsorption by MIHs.* $C_{i_{Lys}}$*: 1.0 mg/mL; t: 3 h; pH: 7.0.*

C. SELECTIVITY STUDIES

Selective recognition of the target molecule is a significant feature of the systems based on molecular imprinting technology. The MIHs exhibited a high specificity degree towards Lys compared with the other proteins (K_d for Lys is 5.27 while K_d for BSA and Cyt c are 3.88 and 2.77, respectively). Values of k' of MIHs for Lys/BSA is 1.92 times and Lys/Cyt c is 2.15 times bigger than the NIHs. According to results, high selectivity came from the rigid structure of the cavities and the nature of the between the metal ion mediated pre-polymerized complex of Lys and NVP.

Table 1. K_d, k and k' coefficients for MIHs and NIHs.

	MIHs		**NIHs**		
Protein	K_d(mL/g)	k	K_d (mL/g)	k	k'
Lys	5.27	–	3.72	–	–
BSA	3.88	1.36	5.25	0.71	1.92
Cyt c	2.77	1.90	4.21	0.88	2.15

D. DESORPTION AND REUSABILITY

Since reusability of a material is a significant factor in terms of environment and cost, retained Lys adsorption capacity and reusability of the MIHs were investigated in a batch system. Same MIHs were used in 10 times adsorption-desorption cycles under the optimal adsorption conditions. As seen in Figure 7, after the tenth cycle, adsorbed amount of Lys was found to be 10.56 mg/g and 86.20% of its capacity was retained. According to findings, MIHs can be utilized several times for Lys adsorption with no significant decrease in adsorption capacity.

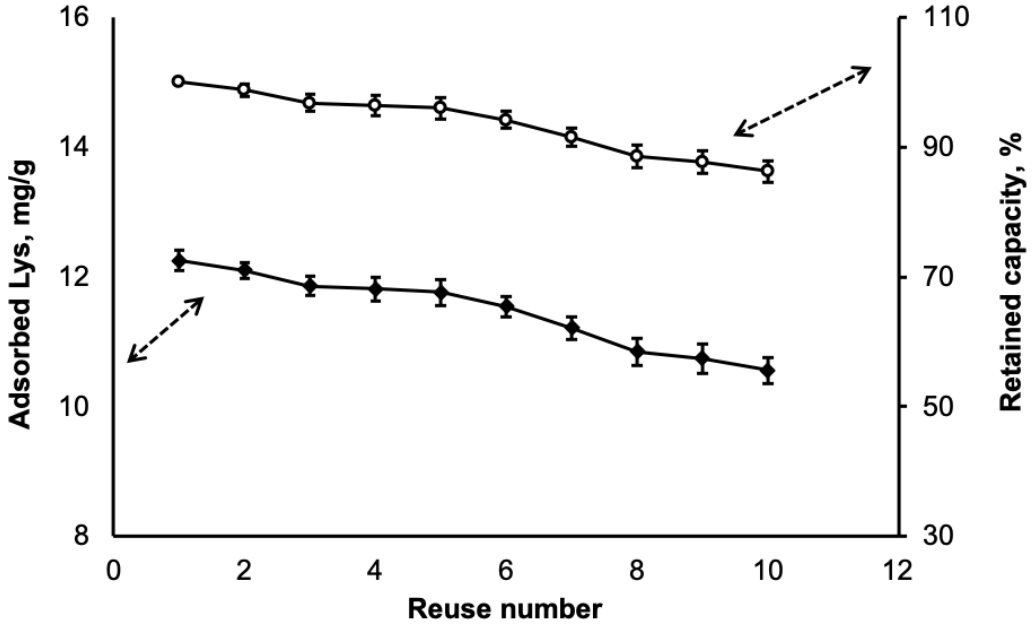

Figure 7. *Retained capacity and reusability of the MIHs. Adsorption pH 7.0;* $C_{i_{Lys}}$*: 1.0 mg/mL; T: 25 °C.*

IV. CONCLUSION

Adsorption and isolation of Lys have been getting importance due to its utmost features such as being a naturally occurring antibacterial enzyme with high activity. In this study, hydrogels that can recognize Lys were designed by combining the easy-preparation and selectivity of molecular imprinting and higher stability of metal ion coordinated interactions. Imprinted P(HEMA-NVP) hydrogels exhibited a higher affinity towards the target molecule. The hydrogels also exhibited a good reusability performance with negligible loss in Lys adsorption capacity. Considering all these results, MIHs could be a good candidate for Lys recognition.

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