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Charged groups synergically enhance protein imprinting in amphoteric polyacrylamide cryogels

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ABSTRACT: Proteins are amphoteric biopolymers with unevenly charged exterior surfaces. Taking this point fully into account could accomplish ingenious recognition systems for the biological macromolecues. Molecularly imprinted polymers (MIPs) are good tools to study the interactions between polymeric matrices and template molecules. Here different protein imprinted cryogels were prepared. Imprinting factors (IFs) were determined with bovine serum albumin (BSA) as the template. The IF of the polymeric cryogel made from only acrylamide (AM) and N,N′-methylenebisacrylamide (BisAM) is about 1.38. The introduction of charged monomers, either acrylic acid or diallylamine, would increase IFs obviously. One of the basic cryogels gave the maximum IF (about 2.0) of that type. As both acrylic acid and diallylamine were involved, IFs were further increased. An amphoteric cryogel with a suitable acid-base ratio gave a high IF of about 3.7. Whatever used alone or both, too many added acidic or basic monomers resulted in IF reduction. Taking full advantage of charged groups in MIPs could be a good way to manipulate protein–polymer interactions. © 2016 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2016**, *133*, 43851.

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INTRODUCTION

Protein-imprinted polymers have evoked extensive interests to mimic molecular recognition systems in the nature, including antibodies, enzymes, viruses, and cells.[1] Scientific and technical advancements in field could lead to interesting applications in proteomics, biomarker discovery, illness diagnosis, environmental analysis, and so forth. Due to the big molecular sizes and fragile conformations of proteins, incompatibility as well as inefficient diffusion was usually observed when studying the interactions between rigid polymers and protein templates. As a result, protein-imprinted polymers were often subjected to surface-imprinted or nanomaterials.[2,3]

Cryogels are synthesized at temperatures lower than the freezing points of the monomer solutions.[4–6] In such a way, frozen solvents (e.g., ice crystal) exclude the originally diluted monomers, concentrating and forcing them to polymerize in a small space. This squeezing effect leads to macroporous structures, greatly enhancing mass transfer in the resulted polymers. Polyacrylamide based cryogels were widely utilized to imprint proteins because of their distinct biocompatibility. It's easy to synthesize these materials under mild conditions. Additionally numerous commercially available acryl-monomers make it facile to introduce new functional groups to study various protein–polymer interactions.

Weak acting forces such as hydrogen bonding,[7–11] hydrophilic,[12–14] and hydrophobic[15–18] interactions universally exist in protein–polymer interacting systems. As large numbers of functional monomers involved, the combination of these nonvalent forces could manifest a considerable interaction. As a result, those multivalent interactions must be taken into account when synthesize protein imprinted polymers. Different monomers should be rationally chosen and allocated to manipulate molecularly imprinted polymer (MIP)–protein interactions.

Compared with above mentioned weak interactions, metal coordination[19] and electrostatic forces[20,21] were stronger chemical bonds. Well-designed polymeric architectures could take full advantage of these forces, to flexibly regulate and precisely control molecular recognitions between the polymers and the templates. In this article we focused on examining the influence of charged functional groups, that is, carboxyl and amino, in different protein-imprinted cryogels. Results indicated both the charged groups could strengthen the affinities of the polymers to bovine serum albumin (BSA). Simultaneous involvement of the two functional groups leads to a summing effect

Table I. Volumes of Charged Monomers Added to Synthesize Modified Cryogels

Polymer no	1	2	3	4	5	6	7	8	9	10	11	Added monomers
Basic	5	10	15	20	25	30	35	40	45	50	55	Diallylamine/μL
Amphoteric	5	10	15	20	25	30	35	40	45			
	45	40	35	30	25	20	15	10	5			Acrylic acid/μL
Acidic	5	10	15	20	25	30	35	40	45			

and obviously increases the imprinting factors (IF) of different cryogels.

EXPERIMENTAL

Materials

Ovalbumin (OVA), lysozyme (LYS, from egg white), bovine hemoglobin (BHb), and BSA (>96%) was purchased from Adamas (Shanghai, China), Acrylic acid, diallylamine, acrylamide, and N,N′-methylenebisacrylamide (BisAM) were purchased from Haopeng Chemical Plant (Jinan, Shandong, China). Sodium hydrogen sulfite (SHS), sodium dodecylsulfate (SDS), and ammonium peroxydisulfate (APS) were purchased from Sinopharm Chemical Reagents Co., Ltd (Shanghai, China). Other reagents were of analytical grade.

Preparation of BSA-Imprinted and Non-Imprinted Cryogels

The synthesis of cryogel MIPs was similar to the operation described previously.[22] In brief, acrylamide (200 mg), N,N′-methylenebisacrylamide (100 mg), BSA (40 mg), and SHS (10 mg) were dissolved in 8 mL of phosphate buffer solution (PBS, 10 mM, pH 7.0). The solution was ultrasonically degassed for 5 min both before and after the addition of APS (30 mg). Cryogenic polymerization was carried out by putting the monomer solution in a refrigerator at −20 °C for 24 h. The resulted polymer was put in a water bath at 60 °C for 2 h before removing the templates and unreacted monomers. In the absence of acrylic acid and diallylamine, the polymer was a non-modified polyacrylamide cryogel. Modified cryogels were prepared by adding different volumes (Table I) of acrylic acid, diallylamine or both in the monomer solutions. For each MIP, a related non-imprinted polymer (NIP) was made from a similar solution just without BSA.

To remove the imprinted BSA, the MIPs were put in a shaker and washed with 1 *M* NaCl (containing 10 g/L SDS) for four times (30 min each), till the absorbance of the scrubbing solution was stable at 280 nm.

All the MIPs and NIPs were dried at 80 °C for 12 h before use.

Conductometric Titrations of the Modified Cryogels

Three cryogels (*Basic 7*, *Amphoteric 7*, and *Acidic 3* in Table I) were chosen and titrated to measure their functional group densities because they gave the highest adsorption of BSA, presenting the respective type of the cryogels.

About 0.100 g of the acidic cryogel (*Acidic 3* in Table I) was put in 10 mL deionized water with a magnetic stirring bar. A standard solution of NaOH was used as the titrant. The conductivity of the polymer-contained solution was detected by a DDS-11A conductometer (Frando Technology Development Co., Ltd, Chongqing, China). Because the titration must be carried out slowly to ensure a complete reaction between the polymer and the titrant, after the addition of each batch of NaOH droplets, the polymer should be stirred sufficiently for at least 2 min. The polymer was washed three times (10 mL water for each) then 10.00 mL water was added to read the conductivity values. For the amphoteric and basic (*Basic 7*, *Amphoteric 7* in Table I) cryogels, 0.100 g of each polymer was completely acidified by 10 mL HCl (30 m*M*). The titrations were performed similarly after washing off excessive HCl.

RESULTS AND DISCUSSION

It was found that the surface charges of proteins were important not only to the biological functions,[23] but also to the interactions between proteins and materials.[24–26] Likewise the charged groups on a polymeric matrix should be taken into account to better understand the interaction mechanism therein, or to design specific functional materials.

Morphologies of Cryogels

Polyacrylamide-based amphoteric cryogels were macroporous polymers suitable for protein imprinting[22] and depletion.[27] Meanwhile modified cryogels, whatever basic, acidic, or amphoteric, were catalytically active for transesterification.[28]

As charged functional groups introduced, they caused chemical modifications and changed the morphologies of the cryogels.

Compared with the non-modified cryogel just made from acrylamide and BisAM [Figure 1(a)], the carboxyl-modified cryogel (*Acidic 3* in Table I) possesses smaller pores [Figure 1(b)]. The involvement of amino groups resulted in a basic cryogel (*Basic 7* in Table I). When modified by both amino and carboxyl groups, the cryogel (*Amphoteric 7* in Table I) became simultaneously positively and negatively charged. Either of these chemical modifications strengthened intramolecular interactions within the polymers and further reduced the pore sizes. Consequently agglomeration was observed in the two cryogels [Figure 1(c,d)]. Nevertheless no negative effect was observed for protein imprinting.

It is accepted that mass transmission resistance is lower in cryogels compared with that in other non-macroporous materials. As showed in Figure 2, the adsorption saturation was reached within 20 min for the NIP cryogel. In the case of the MIP, it took more time for the template to fit the recognizing sites (about 50 min in Figure 2).

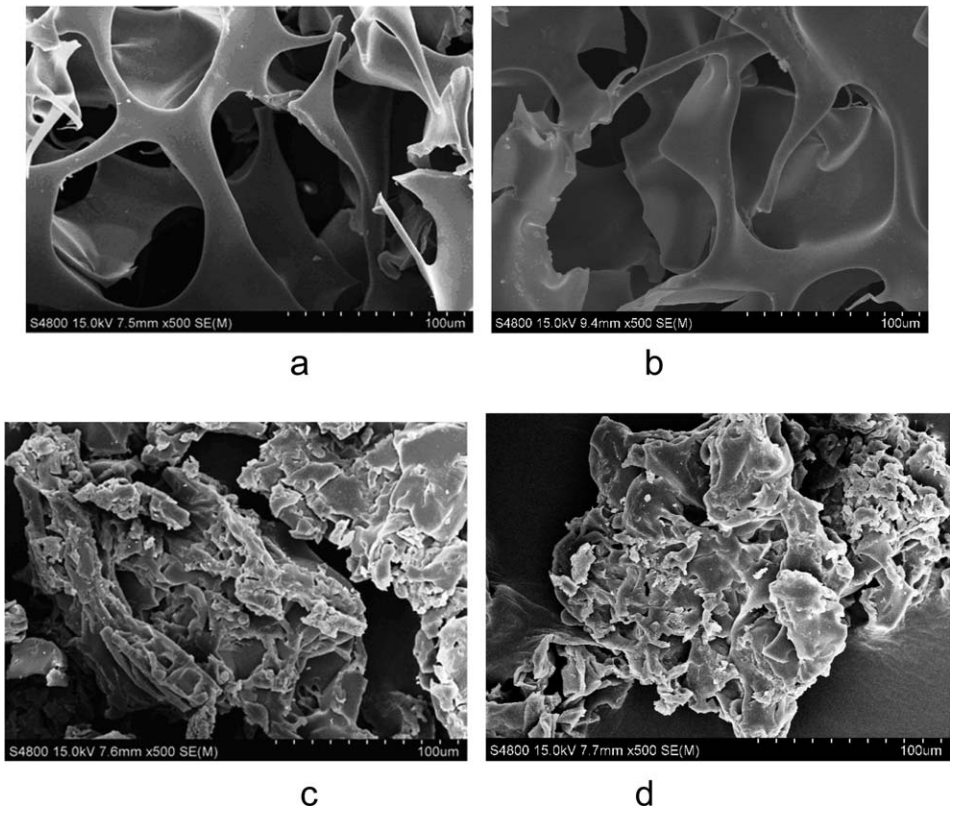

Figure 1. SEM of (a) nonmodified, (b) acidic, (c) basic, and (d) amphoteric cryogels.

Quantification of Carboxyl and Amino Groups on Different Cryogels

The titration reactions are presented as:

$$R-CO_2H+OH^- \rightarrow R\text{-}CO_2^- + H_2O \quad (1)$$

$$R-N^+H_3+OH^- \rightarrow R\text{-}NH_2+H_2O \quad (2)$$

$$H_3N^+-R-CO_2H+OH^- \rightarrow H_3N^+-P-CO_2^- + H_2O$$
$$H_3N^+-R-CO_2^- +OH^- \rightarrow H_2N-P-CO_2^- + H_2O \quad (3)$$

For the titration of the acidic cryogel [eq. (1)], the reaction is equivalent to a gradual ionization of the polymer bearing carboxyls. Firstly the conductivity increased slightly [Figure 3(a)]. When the carboxyl protons were exhausted, the titration curve becomes a horizontal line and the inflection point is regarded as the stoichiometric point.

The protonated amino groups of the basic cryogel were gradually neutralized [eq. (2)], showing a declining curve of conductivity versus the volume of the titrant [Figure 3(b)]. In the case of the amphoteric cryogel, the reaction includes consecutive neutralization of the carboxyl and the protonated amino groups [eq. (3)], giving two stoichiometric points at the top and the bottom inflection points, respectively [Figure 3(c)].

Charged group densities on the cryogels were calculated and illustrated in Table II. These values are one order of magnitude lower than that on a cryogel catalyst.[28] In fact too many charged groups will weaken the affinities toward BSA, indicating lower adsorption of the template.

Adsorption of BSA on the Cryogels

Charged functional groups affect protein imprinting profoundly. Modified cryogels showed increased rebinding abilities by adsorbing more BSA from the solutions.

Adsorption capacities were measured according to:

$$Q=(C_0-C_t)V/m$$

where C_0 and C_t are the concentrations of BSA before and after adsorption.

As showed in Figure 4(a), the acidic MIPs adsorbed 38–60% more BSA than the related NIPs.

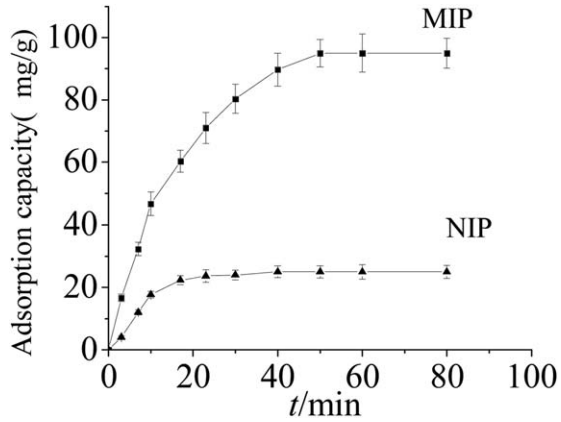

Figure 2. Binding kinetics of MIP (Amphoteric 7 in Table I) and the NIP.

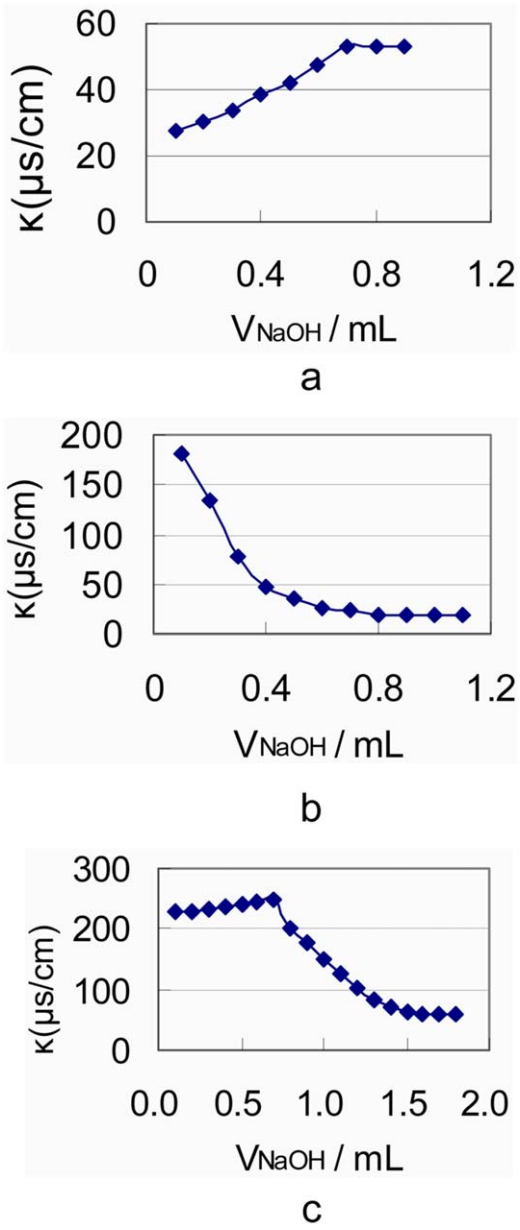

Figure 3. Conductometric titrations of (a) acidic, (b) basic, and (c) amphoteric cryogels. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

BSA itself is a slightly acidic protein, with an isoelectric point of around 4.8. As adsorption increased when proteins and polymeric adsorbents were oppositely charged.[25,26] Naturally basic cryogels adsorbed more BSA than acidic ones. The excessive adsorption of the MIPs over NIPs is from 47 to 100%

Table II. Functionality Densities (mmol/g)

Polymer no	Amino	Carboxyl
Basic 5	0.18	
Acidic 5		0.14
Amphoteric 5	0.17	0.15

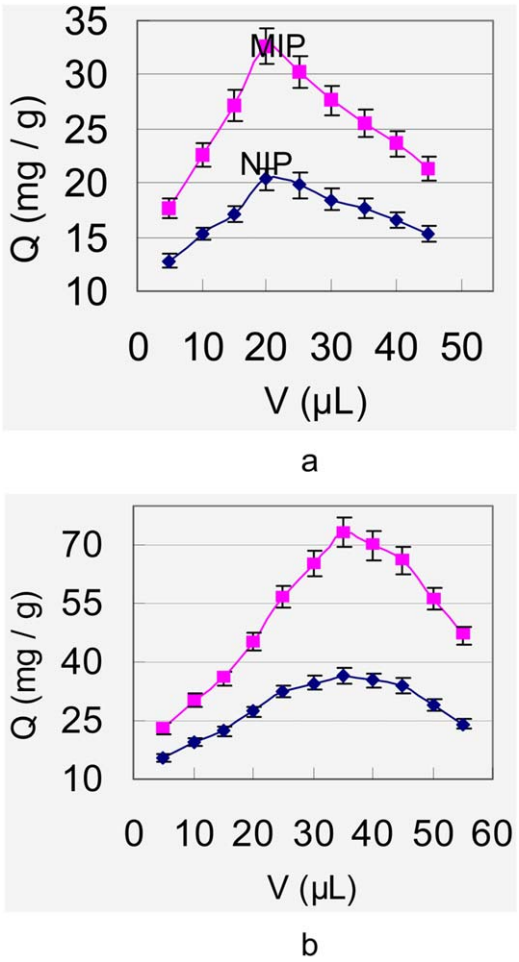

Figure 4. Adsorption of BSA on (a) acidic and (b) basic cryogels. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

[Figure 4(b)]. When acrylic acid and diallylamine were simultaneously involved to synthesize the cryogels, the adsorption capacities of BSA were further increased.

In addition, IF were calculated as the quotients of the adsorption capacities on an MIP and that on the related NIP:

$$IF = Q_{MIP} / O_{NIP}$$

The IF of the cryogel with neither acrylic acid nor diallylamine is 1.38 (point *a* on the *z* axis in Figure 4). Either acidic or basic, the introduction of charged groups in the polymers obviously increased the IF values (curve *b* and *c* in Figure 5). When both acrylic acid and diallylamine were involved, the polymers became amphoteric and showed enhanced affinities to BSA. They adsorb 1.8–3.7 folds more BSA than the related NIPs (curve *d* in Figure 5). A synergical enhancement is obvious.

However, the IFs do not increase abidingly along with the addition of charged groups. All curve *b*, *c*, and *d* (Figure 5) give a top value in their middle parts. This could be attributed to the coulombic repulsion toward the template molecule from redundant charged groups. Actually rebinding was reduced if the MIP and the template were both made positively charged.[26]

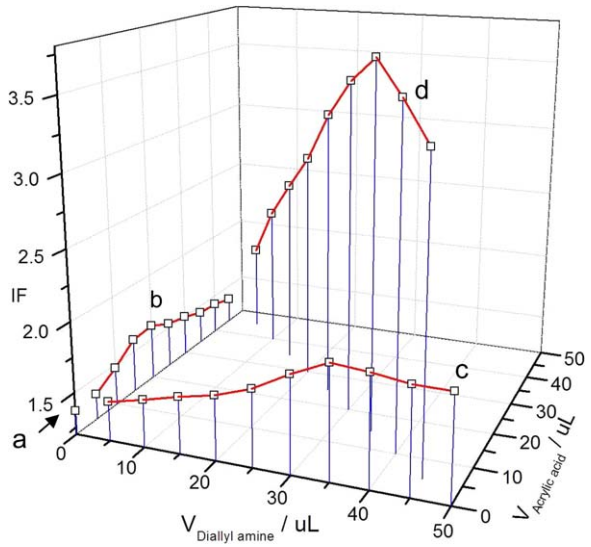

Figure 5. IFs of BSA on (a) nonmodified, (b) acidic, (c) basic, and (d) amphoteric cryogels. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Coulombic forces are exquisite manipulating tools in protein–MIP systems but need to be carefully deployed.

Ovalbumin, lysozyme, and bovine hemoglobin were used to examine the selectivity of the MIP (*Amphoteric 7*). As showed in Figure 6, Compared with the adsorption of BSA by the MIP, the adsorption capacities of the competitive proteins are quite low, either on the MIP or on the NIP. It indicates good affinity between the template and the MIP polymer.

Graphical Representation and Explanation of the Synergical Effects of Charged Groups

To realize sufficient affinities, a shape complementary cavity should be formed in the MIP to emplace the template. In those complementary cavities, weak forces like hydrogen bonding, electrostatic, and hydrophobic interactions are commonly functionating. The cumulative effects of these weak forces play important roles to achieve multisite interacting systems between the polymer and the template.

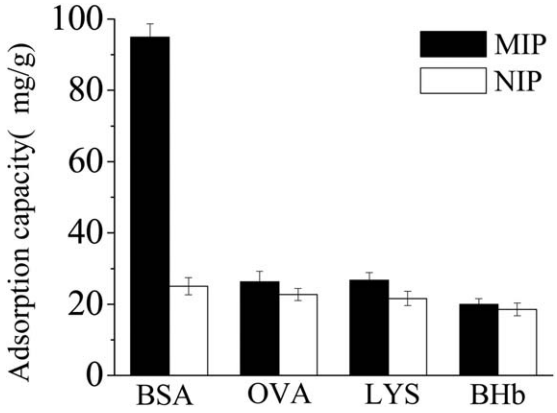

Figure 6. Selectivity test of BSA and other proteins analogues on MIP and NIP.

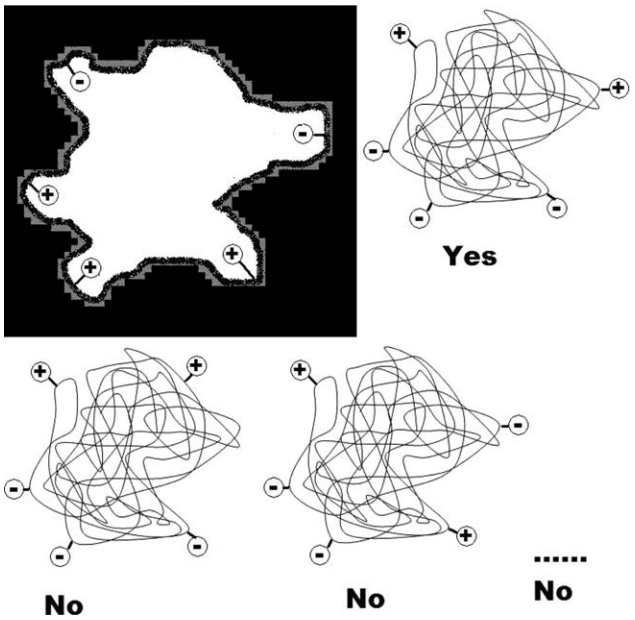

Figure 7. Schematic presentation of steric affinity directed by oppositely charged groups.

Ionic or coordinate bonds are magnitudes order stronger than hydrogen bonds, hydrophilic, or hydrophobic interactions. As a result they could be powerful ways to adjust the matrix–template interactions.[19–21]

To clarify the synergical effect of carboxyl and amino groups, a schematic diagram is expressed in Figure 7.

Carboxyl and amino groups are not distributed uniformly and symmetrically within the inner surface, making the imprinted cavity a directional or polarized recognizing pocket. Now a good template should possess not only complementary molecular shape and size, but also right charge number and spatial layout. In Figure 7, just the "right" protein adapts itself to the imprinted cavity, by both spatially complementary shape and exactly attractive charged group pairs respectively from the template and the polymer. If a different protein is placed in the pocket, steric hindrance, or electrostatic repulsion will be produced inevitably. This adequately ensures a high affinity between the MIP and template.

CONCLUSIONS

A desirable recognizing system between proteins and MIPs should be specific, flexible and mutually compatible. Meanwhile it's important to enable expedite diffusion of solvents and analytes into and out of a three-dimensional polymeric network. Cryogels based on amphoterically modified polyacrylamide were testified to be good materials for protein imprinting. Besides universally existing non-covalent forces, stronger interactions especially electrostatic attraction can be applied to build spatially specific binding sites in MIPs for proteins. In this way, hierarchical recognizing systems could be realized between proteins and polymeric cryogels. This is a useful protocol to immobilize, manipulate and control proteins or microorganisms.

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