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Preparation of bovine serum albumin imprinting sensitive hydrogels using ionic liquid as co-monomer and stabilizer

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ABSTRACT

Through consulting the Hofmeister series, a novel biocompatible and polymerizable ionic liquid (IL) was designed and used as stabilizer and co-monomer to prepare bovine serum albumin (BSA) imprinted hydrogels. N-isopropylacrylamide (NIPA) was chosen as the assistant monomer for imparting environmental sensitivity to the hydrogels. The stabilizing effect of the IL was verified by circular dichroism. Several parameters, such as the mass ratio of the template protein, IL and crosslinker, the drying method of hydrogels and the elution method of MIHs that could affect the performance of molecular imprinted hydrogels (MIHs) were investigated. The optimum mass ratio of BSA, IL and crosslinker was found to be 200:30:6. The best drying and preferred elution method for the MIHs was achieved by slowly evaporating and washing with 0.5 M NaCl solution at 15 °C, respectively. The MIHs prepared under optimized conditions were subsequently used in the adsorption isotherm, adsorption dynamics, adsorption selectivity, and competition test. The adsorption isotherm revealed that the MIHs showed the best imprinted effect at a BSA concentration of 0.2 mg mL^{-1} and their imprinting factor at 2.66. The adsorption dynamic studies revealed that the adsorptive rate of the MIHs was much faster than the non-imprinted hydrogels (NIHs), and both of them could be equilibrated in 1 h. The adsorption selectivity and competition tests were conducted to estimate the specific recognition property of the MIHs for BSA. The MIHs showed excellent selectivity and recognition ability to BSA. The strategy of applying biocompatible and polymerizable ILs to imprinting technology may provide a new approach for effective biomacromolecular imprinting.

1. Introduction

Molecular imprinting is a technique to create artificial receptors by copolymerizing functional monomers and crosslinkers in the presence of a template molecule. In the mixture of prepolymer, functional monomers interact with a template in a certain spatial arrangement by hydrogen bonding, electrostatic or hydrophobic interactions. After the polymerization of the monomers and crosslinkers, the spatial arrangement around the template is fixed. The removal of the template leaves the complementary binding sites with specific recognition ability. Although the first study on molecular imprinted polymer (MIP) was published 80 years ago [1], the prosperous development of molecular imprinting began only in the 1980s and it has been widely applied in sensor, catalysis, chromatographic separation and solid phase extraction (SPE) field. Haupt et al. reported a versatile fiber-optic fluorescence sensor based on MIP for herbicide [2], and its sensitivity was significantly improved by adding a signal enhanced material. Their ingenious method of using a signaling monomer in MIP has extensively simplified the detection procedure. Guo has demonstrated a new strategy of reactant–product-dual-template imprinted capsule for the simultaneous degradation and elimination of pesticide [3]. The novel strategy can effectively enhance the catalytic efficiency of the molecular imprinting catalyst by simply combining the characteristics of the imprinting reactant and product, and could be widely used in environmental fields. Manesiotis et al. reported the preparation of MIPs against S-ibuprofen [4], which was used as the stationary phase, for the complete resolution of racemic ibuprofen in predominantly aqueous mobile phases. The SPE of ibuprofen from tablets, using MIP as sorbents, resulted in 92.2% recovery.

Low molecular weight MIPs have been successfully used for imprinting [5–7]. However, the imprinting of larger and more complex molecules, such as proteins, enzyme, DNA and viruses, has been very difficult [8]. In a highly crosslinked MIP, the removal of the template is a very important issue for both micro- and macromolecules. However, macromolecules are more difficult to

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remove and transport compared to micromolecule in the tough MIP matrix. Furthermore, the imprinting process must be carried out in a stable and gentle aqueous environment because of the sensitive structural nature of biomacromolecules. Moreover, hydrogen bonding interaction is usually applied in organic solvents to obtain the good imprinted effect, but the situation is inapplicable in aqueous phase. Therefore, biomarcromolecules imprinting technology needs to be modified to overcome the above mentioned difficulties.

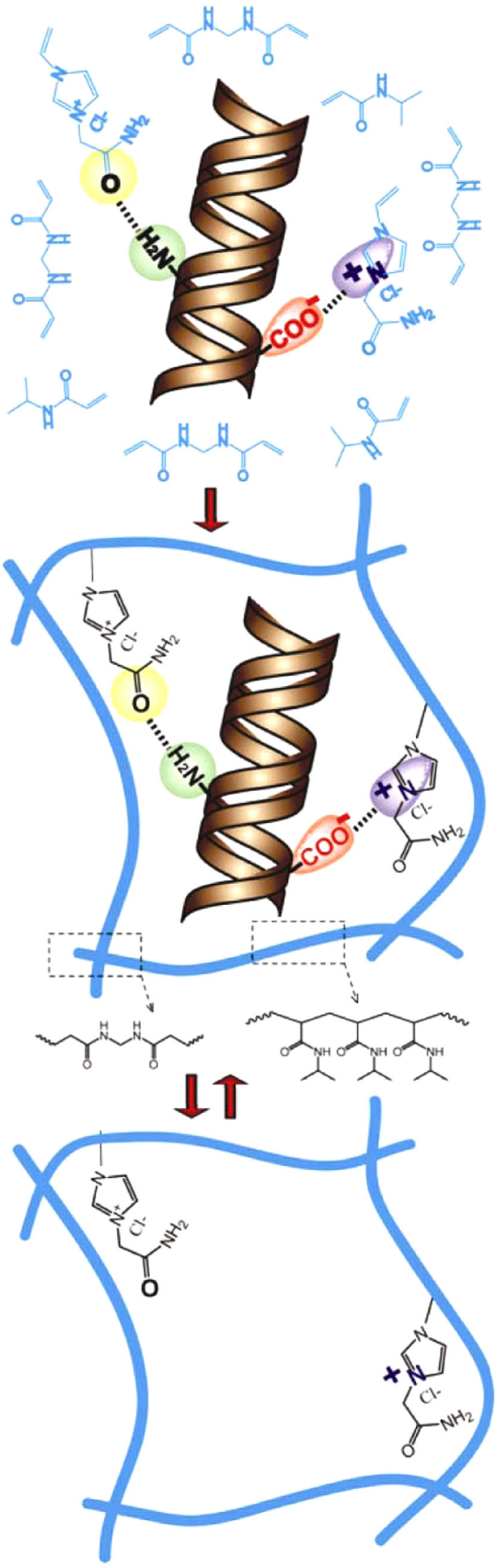
Sensitive hydrogels are those which can reversibly change their dimension by altering external environmental conditions, such as temperature, ionic strength, pH, etc. Due to their large volume change, sensitive hydrogels can be advantageously applied in macromolecule imprinting. Until now, several studies have been reported on biomacromolecule imprinted sensitive hydrogel [9,10], especially for imprinting proteins. Hua et al. utilized N-[3-(dimethylamino) propyl]-methacrylamide as the charged functional monomer and NIPA as the sensitive assistant monomer to prepare a stimuli-responsive protein imprinted hydrogel [11], and their template protein could be easily removed by controlling ionic strength. They also reported the preparation of MIH based on NIPA monomer with specific protein sensitivity compared to NIH [12]. Ran et al. reported a new method for the preparation of sensitive MIH based on NIPA at two different temperatures (25 and −20 °C) [13]. The results showed that MIH produced at −20 °C, exhibited good structural regularity and adsorption selectivity. Although there has been significant development in MIH for biomacromolecular imprinting, the focus on the structural integrity and stability of the protein in the pre-polymerization system has not been studied yet. Even though the buffer solutions have been used as the stabilizing reagent for the template, the protein could be damaged by the strong interaction with functional monomers in pre-polymerization and polymerization processes. Therefore, for biomacromolecule imprinting, it is necessary to maintain the protein nature unfolding structure and stability in a chemical reaction by monitoring its configuration state and preferred pathways for the stabilization.

Ionic liquids (ILs) refer to the low temperature-melting organic salts that are typically composed of bulky organic cations and charge diffuse inorganic or organic anions [14]. ILs have many favorable properties including negligible vapor pressure, good thermal stability, a wide liquid range, low flammability, powerful dissolution ability, high ionic conductivity and designability. Thus, ILs have been widely used as the solvent, template [15], catalyst and reactive monomer in the fields of asymmetric synthesis [16,17], adsorbing material [18–20], fluorescence modified material [21], polymerization [22,23] and biomaterial separation [24,25,26]. Recently, ILs used for stabilizing and activating enzymes and proteins have drawn significant attention [27–29]. This study is focused on the stabilizing effect of ILs on the proteins and enzymes in accordance with the Hofmeister series [30], which reflect the ion-induced modifications of water's hydrogen (H)-bonded network [31]. The series of ions ranked by the protein stabilizing efficiency is expressed as follows [32]:

$$[SO_4]^{2-} > [dhp]^- > [ac]^- > F^- > Cl^- \,||\, [EtSO_3]^- > [BF_4]^- > \approx Br^- > [TfO]^- > I^- > [SCN]^- \approx [dca]^- \geqslant [Tf_2N]^-$$

$$K^+ > Na^+ > [Me_4N]^+ \,||\, Li^+ > [chol]^+ > [Et_4N]^+ \approx [C_2mim]^+ \approx [gua]^+ > [C_4mpyr]^+ > [C_4mim]^+ \approx [Pr_4N]^+ > [C_6mim]^+ \approx [Bu_4N]^+$$

The double bar indicates the crossover from stabilizing to destabilizing behavior. In the above series, the large ions of low charge density defined as chaotropes, which break the H-bond bulk structure of water, and the small ions of high charge density defined as kosmotropes, which were believed to enhance the H-bonded bulk structure of water [33]. The best stabilizing effect on protein was accomplished by the ionic combination of a



Scheme 1. The protocol for synthesis of MIH.

chaotropic cation with a kosmotropic anion. Taking advantage of this series, Fujita et al. "designed" a biocompatible IL, choline dihydrogen phosphate ([chol][dhp]), to enhance the stability of cytochrome c and metallo protein [34,35]. Therefore, it is reasonable to utilize the Hofmeister effect to design an IL for the protein stabilization.

In this study, we present the design and synthesis of a novel IL named 1-vinyl-3-aminoformylmethyl imidazolium chloride ([VAFMIM]Cl). The [VAFMIM]Cl consisted of a kosmotropic anion and a short alkyl imidazolium cation, which might act as a chaotropic cation [36], to afford good biocompatibility. Moreover, the electrostatic force generated by the imidazolium ring, and the hydrogen bond induced by the amide group would also interact with the template protein. Therefore, the [VAFMIM]Cl was not only used as a stabilizer, but also a co-monomer in MIH. NIPA was chosen as the assistant monomer providing the polymer "swelling/shrinking" controlled ability in the elution process. The protocol for the synthesis of MIH is shown in Scheme 1. The recognition ability of the resultant MIHs and NIHs were conducted in various adsorption tests and their selectivity were also investigated.

2. Experimental

2.1. Materials

N-isopropylacrylamide (NIPA) was purchased from Acros Organics N,N-methylenebisacrylamide (BIS), acrylamide (AAm), ammonium persulfate (APS), and N,N,N′,N′-tetramethylethylenediamine (TEMED) were obtained from Sigma. 1-Vinylimidazole and 2-chloroacetamide were purchased from Alfa Aesar. Tris (hydroxymethy)aminomethane (Tris) was purchased from J.T. Baker (Phillipsburg, NJ). 2-(dimethylamino)ethylmethacrylate (DMAEMA), bovine calf serum(BCS), bovine serum albumin (BSA; MW 66.4 kDa, pI 4.8), ovalbumin (OVA; MW 45 kDa, pI 4.7), hemoglobin (Hb; MW64.5 kDa, pI 6.8–7.0) and lysozyme (Lyz; chicken egg white, MW14.4 kDa, pI 10.7) were purchased from Sigma-Aldrich. Molecular low weight markers for protein were obtained from shanghai Jinsui Bio-Technology Co., Ltd. Deionized water was produced by a Millipore water system. All the chemicals used were at least of analytical grade. NIPA was recrystallized from n-hexane and other chemicals were used as received without further purification.

2.2. Instrumentation and method

The Fourier transforms infrared (FT-IR) spectra in KBr were recorded using a Vecto 22 spectrometer (Bruker). The ^{1}H nuclear magnetic resonance (^{1}H NMR) spectra were recorded using an Avance 300 MHz spectrometer. The elemental analyses were carried out using an Elementar Vario EL (Germany) to determine the chemical compositions. The ultraviolet (UV) spectrum was measured using an ultraviolet spectrophotometer (Varian, Cary-1E). The scanning electron microscope (SEM) images were recorded by Inca Oxford (Oxford). The circular dichroism (CD) experiments were carried out by Applied Photophysics Chirascan instrument. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a DYY-6C electrophoresis system (Beijing Liuyi instrument plant).

2.3. Synthesis and characterization of [VAFMIM]Cl functional monomer

A 250 mL three necked round bottom flask was equipped with a magnetic stirrer and a thermometer. 2-Chloroacetamide (5 g, 53.47 mmol) was dissolved with 120 mL acetone in the three necked round bottom flask at 50 °C, and then 1-vinylimidazole (5.03 g, 53.47 mmol) was dripping slowly into the three necked round bottom flask by using constant pressure funnel at 50 °C. The reaction mixture was stirred for 18 h to afford 4.13 g of white solid, which was further purified by washing with small amounts of acetone and freeze-dried by lyophilization.

^{1}H-NMR (DMSO-d_6, δ)□9.62(s, 1H, NC*HN), 8.26(s, 1H, CH_2=CHNC*H=CH), 7.89(s, 1H, CH=C*HNCH$_2$), 8.12(s, 1H, N*H$_2$), 7.58(s, 1H, N*H$_2$), 7.42(dd, 1H, CH2=C*HN), 6.02(d, 1H, C*H$_2$=CH), 5.44 (d, 1H, C+H$_2$=CH). IR (KBr, cm^{-1}): 3374, 3124, 3082, 3048, 2980, 2914, 1671, 1594, 1569, 1545, 1405, 1315, 1183. Elemental analysis calcd. (%) for $C_7H_{10}N_3OCl$ (187.5): C 44.80, H 5.33, N 22.40. Found: C 44.12, H 5.46, N 21.93.

2.4. Preparation of molecular imprinted hydrogel (MIH) and non-imprinted hydrogel (NIH)

The reactant components of MIHs and NIHs were listed in Table S1 of the Supporting information. A typical MIH synthesis procedure is as follows: NIPA (200 mg, 1.769 mmol), [VAFMIM]Cl (30 mg, 0.159 mmol), BIS (6 mg, 0.039 mmol), 10%APS (20 μL) and template protein BSA (200 mg, 0.003 mmol) were dissolved in Tris–HCl buffer solution (3 mL,10 mM, pH 7.0) in a 40 mm × 25 mm (diameter × height) weighing bottle. The solution was pre-polymerized at 25 °C for 6 h and subsequently deoxygenated by purging with nitrogen for 15 min. Before weighing bottle sealed, a volume of 5 μL TEMED was added into the solution promptly. The polymerization was carried out at 25 °C for 3 h. After the reaction, the resultant hydrogels were cut into disks with a diameter of 10 mm and a thickness of 2 mm using a puncher.

The washing procedure was accomplished by dipping the hydrogel disks in eluant. The disks were washed with different concentrations (0.3–1.0 M) of NaCl solution at varied temperature (5–25 °C) for 3 h as one time elution. Each time of elution would use 100 mL NaCl solution. The removal of 95% BSA was regarded as the complete elution. The least times of elution was considered to be the best elution method. After the protein removal, 1 L of deionized water was used to remove traces of NaCl. The removal of BSA from MIH was confirmed using a UV spectrophotometer at 278 nm. After washing, the disks were dried either by evaporation at 25 °C for 3 days or directly in vacuum at 50 °C for 3 h. The corresponding NIHs were prepared in the same way, but in the absence of the template protein.

2.5. Characterization of the molecular imprinted hydrogel

Scanning electron microscopy was carried out to analyze the detailed morphology of hydrogels. The dried hydrogel disks were mounted on metal stubs and at a low vacuum degree (~10^{-3} atm).

The CD experiments were carried out to estimate the effect of [VAFMIM]Cl on the stability of BSA. The spectrophotometer was purged with sufficient nitrogen before starting the instrument. A quartz cuvette of 1 cm path length was used. The spectra were scanned in the range of 195–250 nm with 1 nm resolution. Baseline correction was applied to all the spectra, and no contribution of [VAFMIM]Cl was found in the CD-spectra in the wavelength region 190–300 nm. All the test samples used the same compositions as the pre-polymerized solutions, which contained a certain mass ratio of BSA and [VAFMIM]Cl in Tris–HCl buffer solution (3 mL, 10 mmol L^{-1}, pH 7.0). In order to observe the obvious difference in the change of the protein structure, the pre-polymerized solutions were incubated for 48 h at 25 °C, and then the solutions were diluted to 0.04 mg mL^{-1}for the subsequent CD experiments.

The mean residue ellipticity (MRE) in deg cm^2 $dmol^{-1}$ is expressed as follows:

$$\mathrm{MRE} = \frac{\text{observed CD}(m\ \text{deg})}{C_p n l \times 10}$$

Where C_p is the concentration of BSA solution, n is the amount of amino acid residue of BSA, and l is the path length of quartz cuvette.

The α-helix structure content of BSA was calculated from the MRE values at 208 nm by using the following equation [37]:

$$\alpha - \mathrm{helix}(\%) = \frac{-\mathrm{MRE}_{208} - 4000}{33,000 - 4000} \times 100$$

For the determination of equilibrium swelling ratio, about 10 mg of the dried gel disks were incubated in Tris–HCl buffer solution (10 mM, pH 7.0) within the temperature range 15–45 °C for 24 h. After equilibration, the hydrogels were removed from the buffer solution, and the excess water on the surface was wiped off using a filter paper. The mass of the gels was determined, and the swelling ratio was calculated from the following equation [38]:

$$\mathrm{Swelling ratio} = \frac{(W_s - W_d)}{W_d}$$

Where W_s and W_d are the weights of the swollen and dry hydrogels, respectively.

The adsorption experiments involve the adsorption isotherm, selective adsorption, competitive adsorption and adsorption dynamic. In these experiments, about 10 mg MIH or NIH was incubated in 8 mL protein solution within a range of concentrations. After the incubation, the protein solution was collected, and the concentration was determined using a UV spectrophotometer. The amount of the protein adsorbed on MIH or NIH was calculated according to the following equation:

$$Q = \frac{(C_0 - C_e)V}{m}$$

Where C_0 (mg mL^{-1}) and C_e (mg mL^{-1}) are the initial and final concentrations of the protein solution, respectively. V (mL) is the volume of the protein solution, whereas m (g) is the mass of the hydrogel.

The specific recognition property of MIH was evaluated by imprinting factor (IF), which is defined as $IF = Q_{MIH}/Q_{NIH}$, where Q_{MIH} and Q_{NIH} are the adsorption capacities of the template or the analog of MIH and NIH, respectively.

The selectivity factor (β) is defined as $\beta = IF_{temp}/IF_{ana}$, where IF_{temp} and IF_{ana} are the imprinting factors for the template molecule and for the analog, respectively.

The adsorption isotherm studies were conducted with 10 mg dry hydrogel at various concentrations. The MIH or NIH samples were firstly equilibrated in Tris–HCl buffer (10 mM, pH 7.0) at 25 °C for 24 h, and then the equilibrium solution was replaced by 8 mL of the same buffer solution with varying initial concentration of BSA in the range 0–3 mg mL^{-1} for 1 h. The amount of the protein adsorbed by the hydrogels can be determined as described above.

The selectivity of the MIHs or NIHs toward proteins was investigated to compare their adsorption on the template protein with those of the reference proteins. A known weight of dry hydrogel sample was firstly equilibrated in Tris–HCl buffer (10 mM, pH 7.0) at 25 °C for 24 h, and then the equilibrium solution was replaced by 8 mL of the same buffer solution containing 0.2 mg mL^{-1} of the template proteins or reference proteins. The solution was shaken for 1 h at 25 °C. The amount of the protein adsorbed by the polymers can be determined as described above. The UV detection wavelengths were fixed at 278 nm for BSA, OVA and Lyz, and at 404 nm for Hb, respectively.

The competitive adsorption experiment was performed with a protein mixture containing 0.2 mg mL^{-1} BSA and 0.2 mg mL^{-1} OVA in Tris–HCl buffer (10 mM, pH 7.0) at 25 °C. First, 10 mg of dry hydrogel was conditioned in Tris–HCl buffer (10 mM, pH 7.0) at 25 °C for 24 h, and then the equilibrium solution was replaced by 5 mL of the protein mixture solution as mentioned above at the same temperature for 1 h. The solutions before and after the adsorption were concentrated to five-fold. Ten μL of each protein solution was measured by SDS-PAGE using 12.5% polyacrylamide separating gel and 5% polyacrylamide stacking gel.

The imprinted hydrogels were applied to purify BSA from real sample of bovine calf serum (BCS). The serum was ten-fold diluted with Tris–HCl buffer (10 mM, pH 7.0) at 25 °C. About 20 mg of the dry hydrogels was conditioned in Tris–HCl buffer (10 mM, pH 7.0) at 25 °C for 24 h, and then the equilibrium solution was replaced by the diluted serum for 1 h. The hydrogels were then treated with Tris–HCl buffer (10 mM, pH 7.0) containing 5 mM NaCl to remove the non-specifically adsorbed proteins, and then with 0.5 M NaCl solution to elute the specifically adsorbed proteins. The eluate was desalted and concentrated to twenty-fold. Then 10 μL of eluate was measured by SDS-PAGE using 12.5% polyacrylamide separating gel and 5% polyacrylamide stacking gel.

The adsorption dynamic studies were carried out for MIH and NIH to investigate the rate of adsorption. First, 10 mg of the dry hydrogel was incubated in Tris–HCl buffer (10 mM, pH 7.0) at 25 °C for 24 h, and then the equilibrium solution was replaced by 8 mL of the same buffer solution containing 0.2 mg mL^{-1} of BSA at the same temperature. The concentration of residual BSA solution was measured by UV at different incubation time.

3. Results and discussion

The hydrogels based on NIPA monomer can undergo a reversible volume transition between shrinking and swelling, which are controlled by external temperature. Fig. 1 shows the effect of temperature on the degree of swelling of the MIH and NIH. For the temperature responsive MIH and NIH, the degree of swelling drastically decreased with increasing temperature above the lower critical solution temperature (LCST). Although the LCST of PNIPA was about 32 °C [39], the LCST of both the MIH and NIH increased to approximately 38 °C resulting from the copolymerization of the hydrophilic monomer [VAFMIM]Cl. The involvement of the hydrophilic monomer would directly influence the hydrophilic and hydrophobic properties of the hydrogels by hindering the dehydration of the polymer chains [11]. Consequently, the adsorption

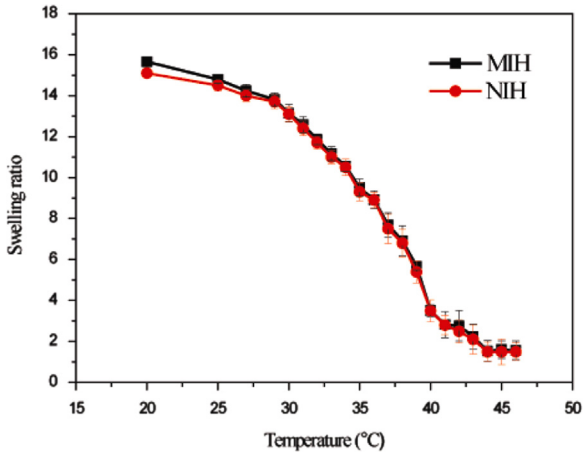


Fig. 1. Effect of temperature on the swelling ratio of MIH and NIH in Tris–HCl buffer (10 Mm, pH 7.0).

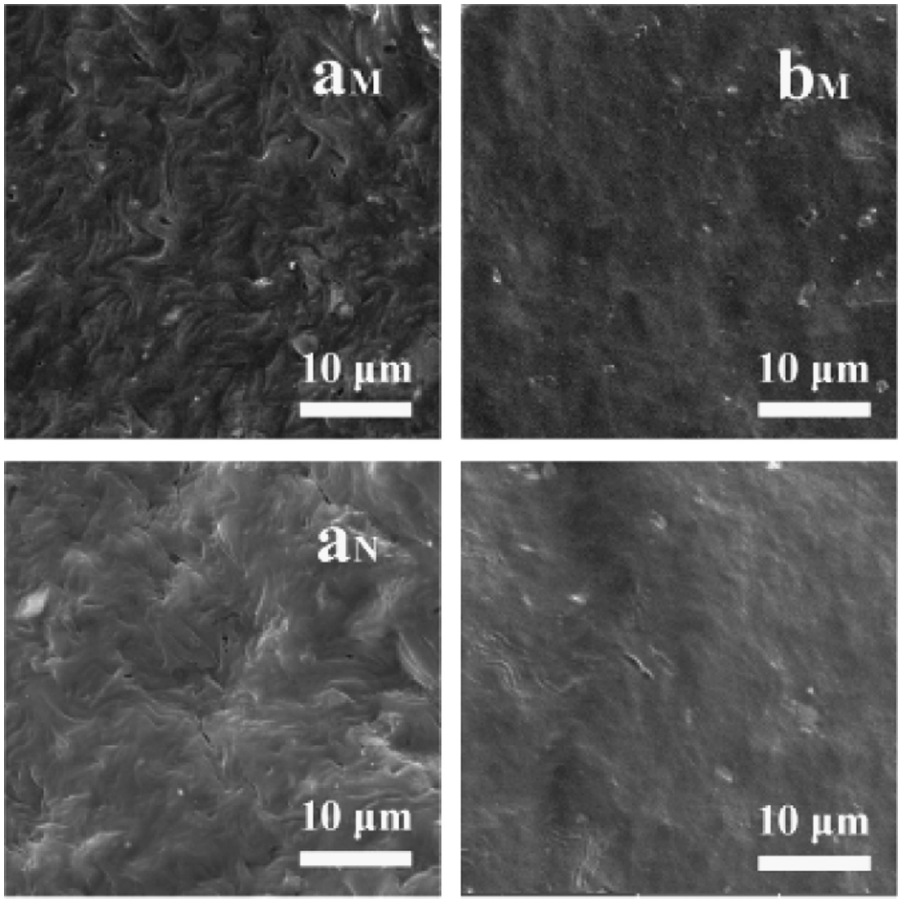


Fig. 2. SEM Images of (a) MIH and NIH dried by vacuum; (b) MIH and NIH dried by evaporation.

and elution for both the MIH and NIH were found to be advantageous below 38 °C.

The detailed morphology of the hydrogels and the effects of two types of drying method on the MIHs and NIHs were studied by SEM. The SEM images shown in Fig. 2 clearly indicate that both MIH and NIH have identical rough surface, which is favorable for the adsorption of the template protein. The hydrogels, which were directly dried in vacuum at 50 °C, possessed micrometer sized cracks on their surfaces as shown in Fig. 2a probably because of the heterogeneous shrinking in a short time, which forms the cracks on the surface leading to the defective mechanical behavior in the hydrogels. In contrast to the drying method under vacuum, the hydrogels dried by the slow evaporation method showed a compact morphology (Fig. 2b), which resulted in good mechanical strength. Therefore, considering the reuse and regeneration of the hydrogels, slow evaporation was selected as the standard drying method.

The stability of BSA in the presence of [VAFMIM]Cl was studied using CD of the pre-prolymerized solutions containing only BSA and [VAFMIM]Cl in a 3 mL of Tris–HCl buffer (10 mM, pH 7.0). The pre-polymerized systems of M-v10b6p200, M-v30b6p200 and M-v50b6p200 were selected for the tests. In the far UV region, the CD spectrum is an effective probe for the study of the protein secondary structure such as α-helix and β-sheet. In the case of BSA, the region at 208 and 222 nm provides information on the α-helix content. Fig. 3 and Table S2 (supporting information) show that after 48 h incubated at 25 °C, the α-helix content of the pure BSA solution decreased to 40.8%, whereas the CD spectrum of BSA clearly showed an increase in the presence of [VAFMIM]Cl. Interestingly, with the increase in the IL content, it was found the best stabilizing effect on BSA with an α-helix content up to 56.4%.

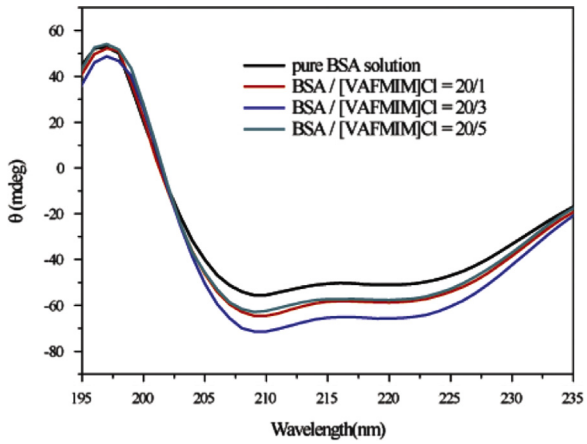


Fig. 3. Effect of [VAFMIM]Cl on the stability of BSA.

However, further increase in [VAFMIM]Cl reduced the α-helix content to 47.9%.

As mentioned above, the ion effect of ILs on BSA could be explained by the Hofmeister effect [24]. It was well known that the effect of anion on stability of protein is more pronounced compared to the cation in the ILs [40]. In [VAFMIM]Cl, Cl^- which is a kosmotropic anion, could compete for the water molecules originally associated with the molecular protein, together from being excluded from the protein surface. This minimized the surface area of the protein, which was exposed to the solvent, and this behavior contributed to its native configuration [41]. Therefore, the incubation of BSA with [VAFMIM]Cl for long time could lead to a more

integrated configuration compared to the BSA solution alone. Moreover, as it was also a co-monomer, the amide group and imidazolium ring with a positive charge could interact with the functional groups on the surface of BSA, resulting in the distortion in the BSA configuration. Therefore, with the increase of ILs concentration, the predominant effect of Cl^- ion on stabilizing BSA would gradually reach a saturation point, and further increase in the cations in the ILs may lead to the decrease in the protein stability. Consequently, 30 mg of [VAFMIM]Cl was found to be the optimal quantity in stabilizing 200 mg BSA in the pre-polymerized solution.

To compare the stabilizing effects of the IL with conventional monomers, DMAEMA, which have a positive charge in neutral solution [42], was chosen as the reference. Both [VAFMIM]Cl and DMAEMA were used in equimolar ratio in the hydrogels. Their adsorption performances and the CD spectrum of pre-polymerized solution are shown in Fig. 4, indicating that the MIH and NIH based on [VAFMIM]Cl absorbed 54.1 mg g^{-1} and 20.3 mg g^{-1}, respectively, at an initial BSA concentration of 0.2 mg mL^{-1}. Therefore, the IF value of MIHs based on [VAFMIM]Cl could reach 2.66, whereas, the MIH and NIH consisting of DMAEMA absorbed 30.1 mg g^{-1} and 19.2 mg g^{-1} respectively, with a relatively lower IF value of 1.56, indicating that the hydrogels composed of stabilizing IL exhibited better recognition ability compared to the conventional ones. This is probably because of the stabilizing effect of the IL on the template protein in the process of pre-polymerization and polymerization, which retained their structural integrity. With the more intact protein in the polymerization, the more imprinted cavities were created, and the better recognition ability was achieved. To support the above hypothesis, their pre-polymerized solutions were investigated via CD spectrum (the black and red plot in Fig. 4 insert). The pure BSA solution was used as the reference under the same conditions (the green plot in Fig. 4 insert). After 6 h incubation at 25 °C, the CD plot of the pure BSA solution at 208 nm was almost identical to the pre-polymerized solution with the IL. The ellipticity at 222 nm showed a weak decrease, indicating a small change in the protein configuration. However, the ellipticity at both 208 and 222 nm decreased in the pre-polymerized solution with DMAEMA, indicating the adverse effect of DMAEMA on the protein structure. The result further demonstrated that [VAFMIM]Cl was a biocompatible and stabilizing reagent, maintaining the structure of template protein during the reaction.

As protein frequently aggregate or precipitate at high concentrations which lead MIH to nonspecific adsorption [43], the effect of BSA content in MIH preparation on the selectivity was investigated. The MIHs were prepared with 100 mg, 200 mg and 300 mg

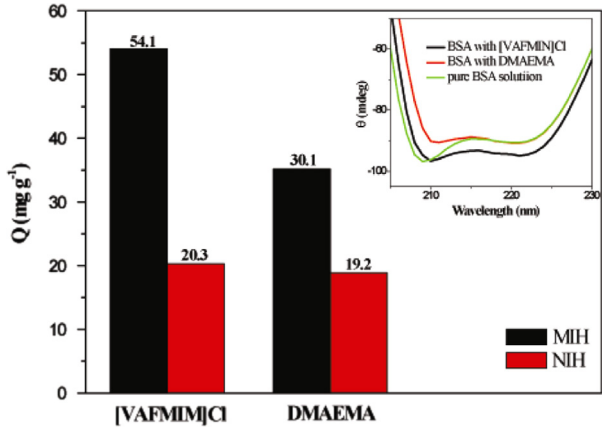


Fig. 4. The adsorption performance of [VAFMIM]Cl and DMAEMA based hydrogels. The inset is the CD spectrum of their pre-polymerized solution.

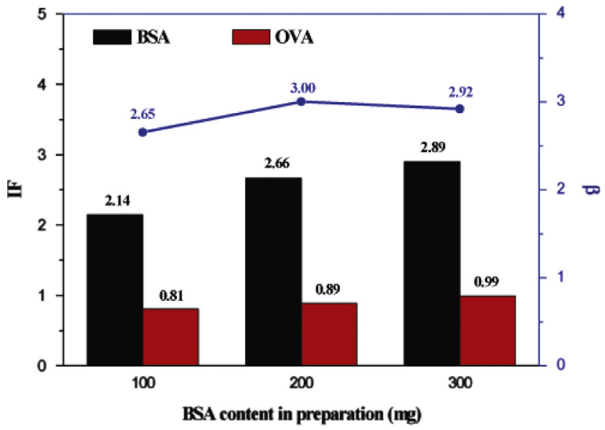


Fig. 5. Influence of BSA content in preparation on selectivity of MIH.

BSA, and their reference NIHs were selected as the experimental samples (Fig. 5). The left Y-axis of IF represents the specific recognition ability of MIHs against NIHs, whereas the right Y-axis represents the selectivity of MIHs to BSA against OVA. It is clear that the value of IF_{BSA} increased with increasing amount of the template protein. It seemed like the more template, resulted in better recognition. However, it was also observed that the IF_{OVA} value increased with increasing template protein in the reaction. Therefore, the maximum β value was obtained, when the MIHs were prepared using 200 mg of BSA. This phenomenon might be explained by the weak aggregation behavior of BSA in the high protein concentration, which led to the nonspecific adsorption, and was negative for the accurate recognition of the imprinted polymer. Thus, 200 mg of BSA was employed in the hydrogel preparation.

Functional monomers play an important role in the adsorption performance of the MIPs. In this study, ionic liquids based on 1-vinylimidazole were also used as a co-monomer in the molecular imprinted process to provide the electrostatic and hydrogen bonding interactions with BSA. The effect of the amount of [VAFMIM]Cl on the IF value was also analyzed. The adsorption capacity of both MIH and NIH significantly increased with increasing amount of [VAFMIM]Cl from 10 mg to 30 mg (Fig. 6) because of the increasing binding sites in the hydrogels. However, a higher content of [VAFMIM]Cl, such as 50 mg, led to only a slight increase in the IF value probably because of the increased number of nonspecific binding sites and the weakened stability of BSA. Finally, 30 mg of [VAFMIM]Cl was chosen as the optimum amount in the hydrogel preparation in the view of the specific recognition and the stabilizing effect on BSA.

The crosslinker often affect the network structures of the hydrogels which can control the transportation of the protein and the production of the imprinted cavity. In this study, three types of hydrogels with different crosslinker content were prepared to investigate the best recognition effect and adsorption capacity. As shown in Fig. 7, the adsorption capacity of both the MIH and NIH were successively reduced with the increasing BIS content. It might be due to the intensive network structure which was generated by the high content of the crosslinker. However, the value of IF increased with increasing BIS content, indicating that a large amount of the crosslinker benefited the construction of the imprinted cavity of the template. The optimum amount of BIS in the preparation of the MIH and NIH was found to be 6 mg, considering the moderated network sizes and good specific recognition effects of the hydrogels.

The elution of BSA in high crosslink densities imprinted polymer is very difficult, and that may lead to a poor performance of the imprinted polymer in the adsorption process. Thermo-sensitive and

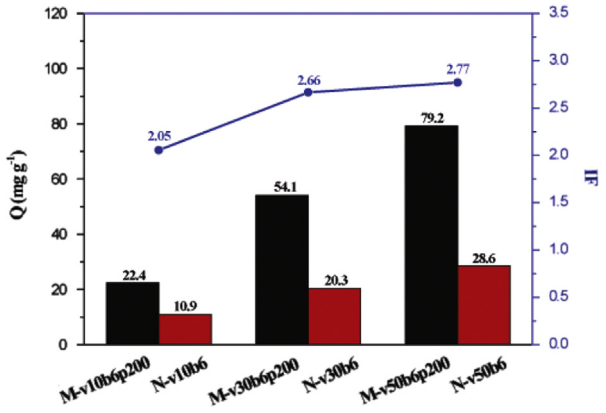


Fig. 6. Influence of the amount of [VAFMIM]Cl on specific recognition property of MIH.

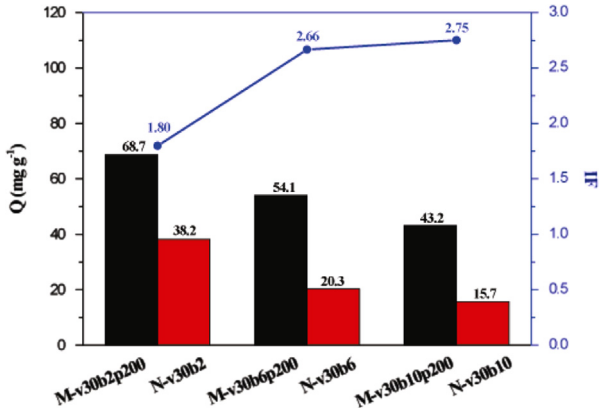


Fig. 7. Influence of BIS content on the specific recognition ability and adsorption capacity of MIH.

salt-sensitive hydrogels, considered as modified polymers, have the ability to change their network sizes by altering the external environment, and offering easy protein elution. Table S3 in supporting information lists the effects of temperature and NaCl concentration on the elution efficiency. Evidently, washing with higher NaCl concentration, like 1.0 M, could significantly improve the efficiency of the elution because of the strong electrostatic interaction between the BSA and NaCl in the aqueous solution. Moreover, the hydrogels based on NIPA shrink at higher NaCl concentration because of its salt-sensitive property that could accelerate the elution process as well. However, the higher NaCl concentration could also result in the irreversible shrinking, which often occurred in the elution with 1.0 M NaCl at 15 °C and 25 °C, and a similar result was reported by Adrus [44]. Therefore, the elution with 1.0 M NaCl solution at 15 °C and 25 °C was excluded. From Table S3 (Supporting Information), it was also observed that elution efficiency could be improved at lower temperature. This phenomenon could be explained by the thermo-sensitive behavior of hydrogel on the temperature, and that resulted in a good transportation of the protein. Nevertheless, the strength of hydrogels at lower temperature, such as 5 °C, sharply decreased due to their high degree of swelling, which was disadvantageous for the subsequent rebinding test. Therefore, the process of elution at 5 °C was also excluded. Finally, washing with 0.5 M NaCl solution at 15 °C was regarded as the standard elution method considering the elution efficiency and sustained reuse.

M-v30b6p200 and N-v30b6 were chosen as the final test samples for the various adsorption experiments after the optimization.

The adsorption dynamic studies were carried out for MIH and NIH to investigate the rate of adsorption. The adsorption capacities of the MIH and NIH were determined as a function of time. The

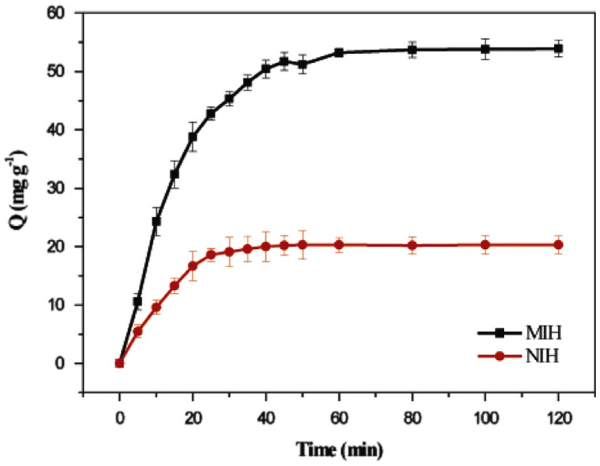


Fig. 8. Adsorption dynamic curve of MIH and NIH.

dynamic curve in Fig. 8 shows that the adsorption rate of MIH was faster than NIH in 0.2 mg mL^{-1} BSA solution within 30 mins. It implied that the interaction between the MIH and BSA was much stronger compared to the interaction between the NIH and BSA, which might be due to the formation of BSA-sized cavities in the MIH. After 30 min of adsorption, the adsorption rate in both the MIH and NIH gradually slowed down suggesting the reduction of driving force between the internal and external environment of the hydrogel. Finally, the MIH and NIH were both equilibrated in 1 h, and this rapid adsorption behavior of polymer based on IL was also reported by Luo et al. [45]. Compared to conventional MIP [12], MIP containing IL could be potentially applied in real-time detection field.

Based on the previous studies, the MIPs containing ionic liquids were defined as the anion exchange material [46,47]. Therefore, the adsorption of hydrogels confined within the ionic liquid monomer to proteins could be the result of ion exchange reaction instead of the conventional hydrogen bonding interaction. From the adsorption isotherm of the MIHs and NIHs (Fig. 9), it is easy to observe that the adsorption capacity increased with increasing BSA concentration. The adsorption capacity of the MIHs was much higher than the NIHs; in particular, in the BSA concentration of 0.2 mg mL^{-1}, the IF reached the maximum value of 2.66. However, it's interesting to find that there were two steps in the adsorption isotherm in both the MIHs and NIHs. This phenomenon also appeared in the depletion curve for Lyz adsorbed to quaternary ammonium sepharose, which was a strong anion exchange resin reported by Noh et al. [48]. However, this phenomenon was not investigated in detail in their study. We hypothesized that the adsorption phenomenon exhibited by the ILs based hydrogels would be similar to the adsorption model, which was proposed for the ion exchange resins in a solution containing the surfactants [49]. In the first step of 0∼0.4 mg mL^{-1} (Fig. 9 insert), the adsorption mainly occurred by the ion exchange at the polymer surface. Therefore, more specific recognition of MIHs will be studied in this section. When the concentration was greater than 0.5 mg mL^{-1}, the proteins binding on the hydrogel could attract others by the hydrophobic interaction resulting in a multilayer adsorption. Consequently, the specific recognition property of MIH would be weakening in this section. Even when the concentration of BSA was high enough, the specific recognition property of MIH against NIH would be neglected. The continuous lowering of the IF value with increasing concentration would probably support the hypothesis.

The selectivity experiment of MIH was carried out using three types of protein related to BSA. OVA and Hb were selected because of their similar isoelectric point (pI) and molecular

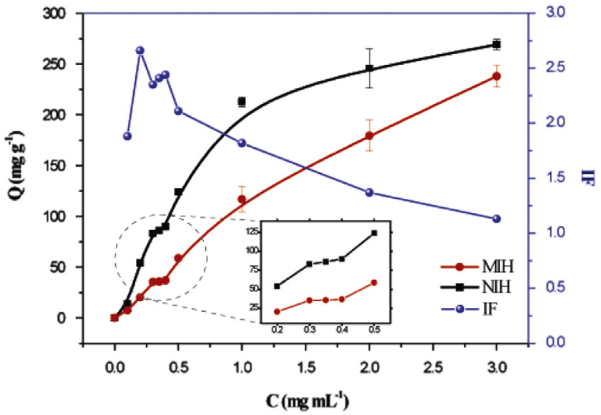


Fig. 9. Adsorption isotherm of MIH and NIH at 25 °C.

Table 1
Selectivity of MIH in the adsorption of proteins.

	Amount of the proteins adsorbed(mg g^{-1} dry gel)			
	BSA	**OVA**	**Hb**	**Lyz**
MIH	54.1 ± 0.7	21.4 ± 1.7	11.7 ± 0.4	7.2 ± 0.2
NIH	20.3 ± 1.2	24.1 ± 1.8	14.9 ± 0.6	7.7 ± 0.2
IF	2.66	0.88	0.78	0.93

weight, respectively, compared to BSA. Lyz with much difference in molecular weight compare to BSA was also chosen because of its opposite charge in pH 7.0 buffered solutions. Table 1 shows the adsorption capacities of the MIHs and NIHs for the different proteins in pH 7.0 buffered solutions at a protein concentration of 0.2 mg mL^{-1}. The adsorbing capacities of NIHs were higher compared to the MIHs in the three reference proteins, suggesting the shape memory effect in the MIHs. However, the adsorption interaction to OVA which had the similar pI to BSA was much stronger than Hb and Lyz. This is probably because of the electrostatic attraction between the positively charged imidazole rings and negatively charged OVA at pH 7.0. The adsorption capacity of Hb, which was almost neutral at pH 7.0, was relatively low despite the similar molecular weight to BSA, presumably caused by the weak electrostatic interaction. In contrast to OVA and Hb, Lyz with the same charge as imidazole rings at pH 7.0 resulted in the minimum adsorption amount, which might be caused by the electrostatic repulsion. Similar selective adsorption because of electrostatic interaction was also exhibited by 1-vinyl-3-ethylimidazolium bromide based polymer [50]. Therefore, the MIHs could achieve preferable selectivity to BSA by virtue of the electrostatic and the shape memory effect.

The competitive adsorption test was carried out in a protein mixture of BSA and OVA. The OVA was considered as a reference protein because of the similar pI to BSA, and relatively high adsorption capacity for both MIH and NIH in the selectivity experiment. The SDS-PAGE analysis (Fig. 10) shows four lanes it in the gel plate corresponding to the protein molecular weight marker, the protein mixture solution before and after the adsorption, respectively. Two blue transverse lines in lane 2 represent BSA and OVA, and the depth of blue could be regarded as the content of the protein. In lane 3, the first blue line is much shallower compared to the line in lane 2, and the depth of the first blue line in lane 4 is almost the same as the lane 2. It indicated the good recognition ability of MIH to BSA. In both lanes 3 and 4, the second transverse line, which represented OVA, became slightly lighter compared to lane 2, suggesting that a small amount of OVA was also absorbed by both the MIH and NIH. In order to

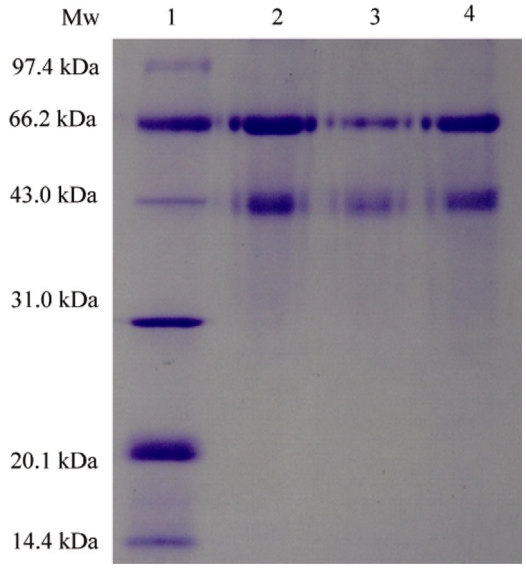


Fig. 10. The results of SDS-PAGE analysis for competitive adsorption of MIH and NIH from a mixture solution. Lane 1, protein molecular weight marker; lane 2, protein mixture solution containing BSA and OVA each with a concentration of 0.2 mg mL^{-1}; lane 3, protein mixture solution after the adsorption by MIH; and lane 4, protein mixture solution after the adsorption by NIH.

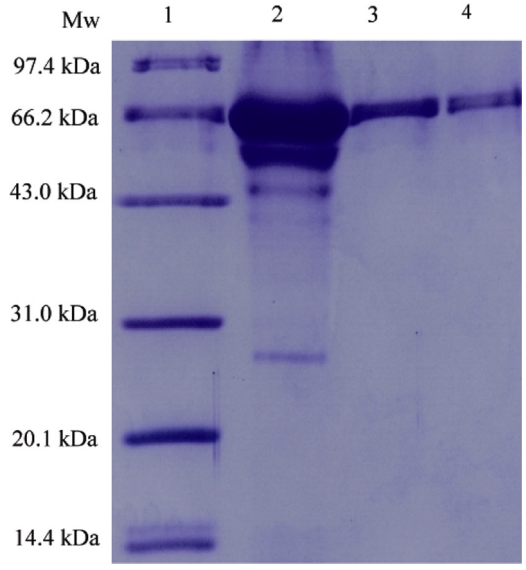


Fig. 11. SDS-PAGE analysis of the results for the isolation of BSA from BCS. Lane 1, protein molecular weight marker; lane 2, 10 μL of 10-fold dilution of BCS; lane 3, 10 μL of BSA solution (0.5 mg mL^{-1}); and lane 4, 10 μL of MIH eluate.

demonstrate the applicability of MIH, bovine calf serum (BCS) was employed to perform direct purification of BSA from real sample. Lanes 2 and 3 in Fig. 11 represent the BCS and BSA samples, respectively, and lane 4 was the eluted protein from the MIHs, which exhibited a band with a molecular mass of 66 kDa. All the other proteins in BCS were not found in lane 4, indicating that MIHs could selectively adsorb BSA from BCS. In conclusion, MIHs have excellent recognition ability to BSA and could isolate BSA from the serum.

4. Conclusion

In this study, a novel biocompatible IL was designed and used as co-monomer and protein stabilizing reagent for the preparation

of sensitive BSA imprinted hydrogel. The biocompatible IL has been proved to significantly enhance the stability of BSA in Tris–HCl buffer. The preparation and elution conditions of the MIHs were optimized to afford the best absorption capacity and selectivity. The optimized MIH were subsequently used in the adsorption dynamic, adsorption isotherm, adsorption selectivity, and competition test. The adsorption dynamic studies showed that the adsorption rate of MIHs was much faster compared to NIHs suggesting the formation of BSA-sized cavities in the MIHs. Both the MIHs and NIHs could reach equilibrium in 1 h, and this rapid adsorption effect may be applied in real-time detection field. By analyzing the adsorption isotherm experiment of MIH and NIH, we found that both of them exhibited two steps adsorption isotherm, which might be induced by the multilayer adsorption. This phenomenon was assumed to be similar to the one exhibited by the surfactants absorbed on the ion exchange resins. The selectivity and competitive tests proved that the MIHs showed excellent specific recognition ability to BSA. The purified perform of the MIH in BCS demonstrated its ability to isolate BSA from serum. The strategy of designing a biocompatible IL and its use as a stabilizer and monomer in imprinting technology would provide a new pathway for the precise and effective preparation of the biomacromolecular imprinted material.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.12.061.

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