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A novel antibody immobilization and its application in immunoaffinity chromatography

Shuoyao Zhang[a], Junqing Wang[a], Dawei Li[a], Juan Huang[b], Hong Yang[b], Anping Deng[a,*]

[a] College of Chemistry, Sichuan University, Chengdu, 610064, China
[b] West China School of Pharmacy, Sichuan University, Chengdu, 610041, China

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ABSTRACT

A novel antibody immobilization and its application in immunoaffinity chromatography (IAC) were presented. Using acrylamide (AM) as monomer, ethylene glycoldimethacrylate (EGDMA) as cross-linker and bulk polymerization as synthetic method, we prepared a polymer in which the Cu(II) was embedded. The Cu(II)-embedded polymer was tested for its binding with protein. It was found that Cu(II)-embedded polymer displayed a strong binding with bovine serum albumin (BSA). At 80% of methanol, no BSA was released from Cu(II)-embedded polymer. The Cu(II)-embedded polymer was then used as a novel solid support for antibody immobilization. IAC column was prepared by immobilizing polyclonal antibody (pAb) against clenbuterol (CL) on Cu(II)-embedded polymer and packing the Cu(II)-embedded polymer-pAb into a common solid phase extraction (SPE) cartridge. Under optimal extraction conditions, the IAC column was characterized in terms of maximum binding capacity for target analyte, extraction efficiency and reusability. It was revealed that, for IAC column packed with 0.1 g of solid support immobilized with antibody, the maximum capacity for CL was 616 ng; the extraction recoveries of the column for CL from three spiked food samples were 84.4-95.2% with relative standard deviation (RSD) of 9.3-15.5%; after more than 30 times repeated usage, there was not significant loss of specific recognition. The results demonstrated the feasibility of the prepared IAC column for CL extraction. The proposed antibody immobilization method exhibiting the properties of simplicity, low cost, strong binding for target analyte, no leaching of antibody, etc., would be a very useful tool applied in the field of IAC.

1. Introduction

Immunoaffinity chromatography (IAC) is a separation method that takes advantage of the specific and reversible interaction between antibody and antigen [1,2]. Antibodies raised against a target analyte (antigen) are immobilized on a solid support. The immobilized antibodies will specifically retain the antigen from a solution passed through the support. Bound antigen can then be eluted and the support regenerated for reuse. IAC offers unique and powerful technique, which enables selective extraction and enrichment of individual compounds or classes of compounds in one step. Additionally, IAC uses little organic solvents and columns may be reused and easily automated. IAC techniques have been widely used in clinical, biological, food and environmental areas for the extraction and enrichment of enzymes, peptides, hormones, receptors, viruses, drugs, mycotoxins, pesticides, organic contaminants in different matrices prior to analysis by on-line and off-line high performance liquid chromatography (HPLC), gas chromatography (GC), capillary electrophoresis (CE), or enzyme-linked immunosorbent assay (ELISA) [3–13].

The specific and affinity binding of IAC column for target analytes is mainly dependent on the quality of antibody employed for IAC preparation. Besides antibody, other factors that affect IAC binding include antibody immobilization and the solid support on which the antibody is bounded. In most IACs, antibodies are generally immobilized on the support materials such as carbohydrate-related materials (i.e. agarose [14] or dextran [15]) or synthetic organic supports (e.g. acrylamide polymers, copolymers or derivatives, polymethacrylate derivatives and polyethersulfone matrixes) [16] by a chemical covalent bonding. Agarose- and silica-based supports are the most common supports selected for both commercial and laboratory applications. However, chemical reaction frequently causes denaturation of the antibodies, thus lowering the affinity binding for the antigen. On the other hand, the commercial solid supports such as agarose are usually very expensive. Recently, non-covalent immobilization such as sol-gel method has been getting increasing attention, which has been successfully used for entrapping antibodies against PAHs [17], 1-nitropyrene [18], s-triazines [19] and TNT [20]. The sol–gel method consists in immobilizing antibodies in the pores of a hydrophilic

* Corresponding author.
E-mail address: denganping6119@yahoo.com.cn (A. Deng).

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glass matrix. The entrapment of antibody is a simple and rapid two-step procedure in which hydrolysis is followed by polymerization of tetramethoxysilane. Although the entrapped antibodies can retain their affinity and specificity, however some extent of antibody leaching is unavoidable, which make the IAC column prepared by sol–gel method to be limited for the extraction of trace analyte.

Transitional metal ions (such as copper, zinc, iron ions, etc.) are well known to form rather stable complexes with amino acid residues (such as histidine, homocysteine, tryptophan, etc.) in nearly neutral aqueous solution. A support material with fixed transition metal ions may interact with surface-exposed amino group and thiol group on the surface of enzyme or other proteins. It is reported [21,22] that, among four transitional metal ions (Cu, Zn, Ni and Mn), Cu exhibits the highest affinity binding for proteins. Immobilized-metal-ion affinity chromatography (IMAC) proposed by Porath et al. [21], has been as a useful tool for detailed studies of proteins and for separation of proteins [23–29]. However, up to now, there is no report for IMAC for the separation and extraction of small compounds in food and environmental analysis.

Clenbuterol (CL) is a synthetic β-adrenergic agonists widely used in the treatment of pulmonary diseases such as asthma bronchiale or bronchial hyperreactivity for human and animals. CL could sharply increase the promotion of muscle growth and the protein to fat ratio, therefore, it was often abused as a growth promoting agent in animal feeds to enhance lean meat to fat ratio for livestock and as a doping drug to enhance the performance of human athletes. CL had been prohibited as therapeutic or prophylactic medicine in food-producing animals in many countries.

Taking the advantage of strong adsorption of Cu(II) for protein, in this study, we proposed a novel antibody immobilization and applied it in IAC for the first time. We synthesized a polymer in which the Cu(II) was embedded. The Cu(II)-embedded polymer was then used as a solid support for antibody immobilization (herein polyclonal antibody against CL was utilized). The novel antibody immobilization method exhibits the properties of simplicity, low cost, strong binding for target analyte, no leaching of antibody, etc., which make it to be a useful method for the preparation of IAC.

2. Experiment

2.1. Chemicals and apparatus

Acrylamide (AM) and ethylene glycoldimethacrylate (EGDMA) were purchased from Fluka (Buchs, Switzerland). Methanol and acetonitrile of HPLC grade were purchased from Fisher Scientific (Pittsburgh, PA). Bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO). Clenbuterol (CL) was obtained from Yabang Chemical Industry Cooperation (Changzhou, China). Copper acetate, methanol (analytical grade), 2,2-azoisobutyronitrile (AIBN), n-hexane and hydrochloric acid were purchased from Chang Zhen Chemical Company (Chengdu, China). The other reagents are all analytical grade.

Ultraviolet visible spectrophotometer (UV-2300) was purchased from Techcom Com. (Shanghai, China). Electronic balance BS 124S and pH meter PS-10 were bought from Sartorius Com. (Gottingen, Germany). HPLC system with a C18 column (250 mm × 4.6 mm, 5.0 μm of particle in size) and the HPLC work station software used for instrumental control and data analysis were bought from Alltech Associates, Inc. (Deerfield, IL). The deionized RO water supply system (DZG-303A) was purchased from AK Company (a conjunctive company between Chengdu and Taiwan, Chengdu, China). The constant temperature oscillator (SHZ-88) was bought from Jiangsu Experimental Instruments Factory (Taichang, China). The thermostatic water bath (DF-101S) was bought from Gongyi Experimental Instruments Factory (Henan, China).

2.2. Preparation of Cu(II)-embedded polymer

Using AM as monomer, EGDMA as cross-linker, 2, 2-azoisobutyronitrile (AIBN) as initiator, based on different amount of copper acetate added, six types of Cu(II)-embedded polymers were prepared by bulk polymerization method [30,31]. Briefly, 0.6084 g (8.0 mmol) of AM and different amount of copper(II) acetate (the initial amount was 0, 5, 10, 15, 20 and 30 mg, respectively) were dissolved in 8.0 mL of acetonitrile in a 25 mL glass flask. After the mixture solution was placed into the refrigerator at 4 °C for 3 hours, 20.0 mmol of EGDMA and 0.24 mmol of initiator AIBN were added. The flask was degassed in a sonicating bath for 15 min, and then purged with nitrogen for 10 min and sealed. The polymerization was carried out in a constant water bath at 58 °C overnight under light protection. After being dried, the polymers were mechanically grounded in a mortar and sieved to select particles between 75 μm and 35 μm in size.

2.3. Binding of the Cu(II)-embedded polymer for protein

2.3.1. Binding of different types of Cu(II)-embedded polymers for protein

Before immobilizing antibody on the Cu(II)-embedded polymer, a model protein, bovine serum albumin (BSA), was employed for binding test. The binding of different types of Cu(II)-embedded polymers for BSA was carried out as follows. Six types of the Cu(II)-embedded polymers prepared in *2.2* at the weight of 20 mg were placed into six 10 mL of colorimetric tubes with stopper, and then 2 mL of BSA standard solution prepared in pure water at concentration of 2.00 mg mL^{-1} (total BSA: 4 mg) was individually added to the tubes. The tubes were shaken in a 25 °C bath overnight and then filtrated with 0.45 μm filter. The supernatant was doubly diluted with pure water and detected by UV spectrophotometer at 280 nm. The amount of BSA bound on the polymers was obtained by subtracting the free concentration from initial BSA added. The Cu(II)-embedded polymer with the biggest binding capacity for BSA was selected for further experiment.

2.3.2. Cu(II)-embedded polymer for protein binding in different binding solution

Binding solution with different ionic strength and pH value is an important factor affecting the ability of Cu(II)-embedded polymer for BSA. The binding of the Cu(II)-embedded polymer for BSA was respectively carried out in following four binding solutions, they are: (1) water; (2) 0.01 mol L^{-1} phosphate buffered saline (PBS), pH 7.4, containing 0.15 mol L^{-1} NaCl; (3) 0.02 mol L^{-1} PBS, pH 8.0, containing 0.05 mol L^{-1} NaCl; and (4) 0.05 mol L^{-1} carbonate buffer, pH 9.6. To four 10 mL colorimetric tubes with stopper, 20 mg of polymer selected in *2.3.1* were placed, and then 2 mL of BSA standard solution prepared with the above four solvents at concentration of 2.00 mg mL^{-1} (total BSA: 4 mg) was individually added to the tubes. The tubes were shaken in a 25 °C bath overnight and then filtrated with 0.45 μm filter. The supernatant was doubly diluted with pure water and detected by UV spectrophotometer at 280 nm. The amount of BSA bound on the polymers was obtained by subtracting the free concentration from initial BSA added.

2.3.3. Cu(II)-embedded polymer for protein binding in different binding time

The amount of BSA bound on the Cu(II)-embedded polymer in different binding time was tested as follows. To ten 10 mL colorimetric tubes with stopper, 20 mg of polymer selected in *2.3.1* were placed, and then 2 mL of BSA standard solution prepared with pure water at concentration of 2.00 mg mL^{-1} (total BSA: 4 mg) was individually added to the tubes. The tubes were shaken in a 25 °C bath continuously. After every 1 to 2 hours, one tube was taken and the

mixture was filtrated with 0.45 μm filter. The supernatant was doubly diluted with pure water and detected by UV spectrophotometer at 280 nm. The amount of BSA bound on the polymers in different period of time was obtained by subtracting the free concentration from initial BSA added.

2.3.4. Maximum binding of the Cu(II)-embedded polymer for protein

The maximum binding of the Cu(II)-embedded polymer for protein was tested in a series of BSA standard solution. Briefly, to eleven 10 mL colorimetric tubes with stopper, 20 mg of polymer selected in *2.3.1* were placed, and then 2 mL of BSA standard solution prepared with pure water at concentration of 0.25, 0.50, 0.70, 0.85, 1.00, 1.15, 1.50, 2.00, 2.50, 2.60 and 3.00 mg mL^{-1} (total BSA: .0.5∼6.0 mg) was individually added to the tubes. The tubes were shaken in a 25 °C bath overnight and then filtrated with 0.45 μm filter. The supernatant was doubly diluted with pure water and detected by UV spectrophotometer at 280 nm. The amount of BSA bound on the polymers in different concentration of BSA was obtained by subtracting the free concentration from initial BSA added.

2.3.5. Stability of the Cu(II)-embedded polymer-BSA

After immobilizing BSA on the Cu(II)-embedded polymer, the stability of the formed Cu(II)-embedded polymer-BSA was tested in four eluting solutions, e.g. 50% methanol, 80% methanol, 50% methanol (pH = 2) and 80% methanol (pH = 2), which are the solutions containing high concentration of organic solvent or low pH value. To four 10 mL colorimetric tubes with stopper, 20 mg of polymer selected in *2.3.1* were placed, and then 2 mL of BSA standard solution prepared with pure water at concentration of 2.00 mg mL^{-1} (total BSA: .4.0 mg) was individually added to the tubes. The tubes were shaken in a 25 °C bath overnight and then centrifuged. The supernatant was doubly diluted with pure water and detected by UV spectrophotometer at 280 nm. The amount of BSA bound on the polymers was obtained by subtracting the free concentration from initial BSA added. On the other hand, the precipitate, e.g. the Cu(II)-embedded polymer-BSA was washed three times with pure water (3 × 2 mL). All the washed solutions were collected and detected by UV spectrophotometer at 280 nm. After washing, the Cu(II)-embedded polymer in four tubes was suspended with 2 mL of above eluting solutions, respectively, and shaken in a 25 °C bath for 30 min. Finally, the tubes were centrifuged again, and then 1 mL of the supernatant was taken for UV detection at 280 nm.

2.4. Preparation of IAC column

In this study, for the preparation of IAC column, polyclonal antibody (pAb) against clenbuterol (CL) raised from rabbit [32] was selected to be bound on Cu(II)-embedded polymer.

The IAC column was prepared by immobilizing pAb on Cu(II)-embedded polymers and packing the Cu(II)-embedded polymer-pAb into a common SPE cartridge. Briefly, 100 mg Cu(II)-embedded polymer was weighed into a conical flask, and then 2 mL of pure water and 2 mL of pAb (∼18.4 mg) were consecutively added. The pAb immobilization was carried out in the incubator shaker at 25 °C overnight. The resultant Cu(II)-embedded polymer-pAb was transferred to a common SPE cartridge with frits placed on both top and bottom. The IAC column was washed extensively with 0.01 mol L^{-1} PBS (pH 7.4, containing 0.05 mol L^{-1} NaCl) and stored in 0.01 mol L^{-1} PBS containing 0.02% NaN_3 at 4 °C until use.

2.5. Optimization of extraction condition for IAC column

To obtain high extraction efficiency, the extraction conditions of IAC column such as loading, washing and eluting solutions should be optimized. In this study, two loading solutions (pure water and 0.01 mol L^{-1} PBS), two washing solutions (pure water, 5% methanol) and five eluting solutions containing 40%, 50%, 60%, 70% and 80% of methanol/water (v/v, %) were examined. 1 mL of CL standard solution prepared in either pure water or 0.01 mol L^{-1} PBS at the concentration of 100 ng mL^{-1} (total CL: 100 ng) was loaded onto the IAC column. Then 3 mL of washing solution (pure water or 5% methanol) was applied to the column to remove the unspecific binding. Finally, 3 mL of eluting solution was loaded onto the column to elute the target analyte specifically bound on the column. All fractions in the loading, washing and eluting steps are collected and evaporated to dryness by low-pressured rotation evaporator at 45 ◦C. The residues were dissolved in 0.2 mL mobile phase and detected by HPLC.

2.6. Maximum capacity of IAC column for target analyte

The maximum amount of CL to be retained by immobilized pAb in IAC column was determined by continuously loading CL standard solution onto the IAC column so that excess amount of CL would saturate all accessible sites of the IAC. Under optimal extraction conditions, 10 mL of CL standard solution at the concentration of 100 ng mL^{-1} (total CL: 1000 ng) was loaded onto the column. The fractions in loading, washing and eluting step were collected and evaporated to dryness by low-pressured rotation evaporator at 50 °C. The residues were dissolved in 0.2 mL of mobile phase and analyzed by HPLC.

2.7. Extraction efficiency of IAC column for target analyte from real samples

The proposed IAC was applied for the extraction of target analyte from real samples. In this study, three samples (feed, pork meat and liver) were collected from the local markets in Chengdu, China. The samples were grounded (or cut) and be intensively homogenized. 10 g of homogenized samples were put into separate glass tubes with glass stoppers. CL stock solution was added into each tube to prepare a final concentration of 10 ng g^{-1} for feed sample and 5 ng g^{-1} for pork meat and liver sample. 10 ml of 0.1 mol L^{-1} HCl solution was added for extraction. The tubes were shaken in a water bath at 25 °C for 1 h, and then kept at 4 °C overnight. On the next day, after maintaining at room temperature for 30 min, the tubes were vortexed vigorously for 5 min and centrifuged at 12000 rpm for 10 min. All supernatants were transferred to volumetric 10-mL tubes and the pH value was adjusted to 6∼8 with 1 mol L^{-1} NaOH. Depending on the samples, a precipitate might be formed after pH adjustment which was removed after centrifugation. The tubes were supplemented with pure water to yield a final volume of 10 mL and were shaken for 30 seconds. 5 mL of above samples extract was loaded to the IAC column. After loading, the column was washed with 3.0 mL of pure water and eluted with 3.0 mL of 60% of methanol/water (v/v, %). The eluting solution was evaporated to dryness by low-pressured rotation evaporator at 50 °C. The residues were dissolved in 0.2 mL of mobile phase and analyzed by HPLC. For each sample, three separate extractions were performed. Unspiked samples were extracted in the same way and used as blanks.

2.8. HPLC analysis

A HPLC system from Alltech Associates, Inc. (Deerfield, IL, USA) with a C18 column (250 mm × 4.6 mm, 5.0 μm particle size) and the HPLC work station software used for the instrument control and data analysis was employed for detection of CL. The detection wavelength was 240 nm. Methanol-0.01 mol L^{-1} NaH_2PO_4 (45/55, v/v) was used as mobile phase at a flow-rate of 1 mL min^{-1}. The

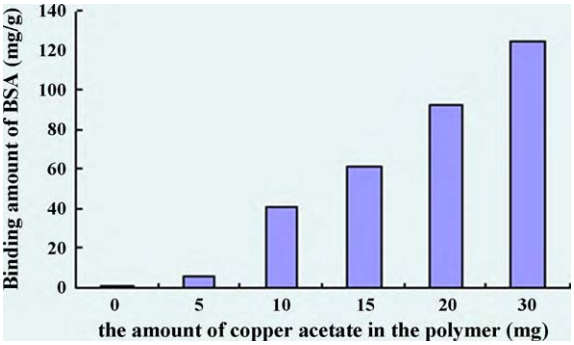

Fig. 1. The effect of amount of copper ion in the polymer on binding amount of BSA. Six types of polymers were prepared by adding 0, 5, 10, 15, 20 and 30 mg copper acetate in the polymerization.

injected volume of standard or extract was 20 μL. The calibration curve for CL was constructed with CL standards of 0.1-20 μg mL^{-1}.

3. Results and discussions

3.1. Preparation of Cu(II)-embedded polymer

In this study, the Cu(II)-embedded polymer was prepared by bulk polymerization method, which was often used for the synthesis of molecularly imprinted polymers (MIPs). In MIPs, the template molecule was added, and after polymerization, the MIPs particles should be extensively washed with a lot of organic solvent to remove the template molecule entrapped inside. Compared to MIPs, the preparation of Cu(II)-embedded polymer herein is very simple. During polymerization, copper acetate instead of template molecule was added, and after polymerization there was no need to remove template molecule. Six types of Cu(II)-embedded polymers were prepared by bulk polymerization, in which the initial amount of copper acetate added was 0, 5, 10, 15, 20 and 30 mg, respectively.

3.2. Binding of the Cu(II)-embedded polymer for protein

3.2.1. Binding of different types of Cu(II)-embedded polymers for protein

To test the binding of the Cu(II)-embedded polymers for BSA, 20 mg of different types of the polymer were placed into six 10 mL of colorimetric tubes and 4 mg of BSA was individually added. After shaking at 25 °C bath overnight, the maximum binding of BSA on Cu(II)-embedded polymers (in form of mg/g) were illustrated in Fig. 1. It was seen that the amount of BSA bound on Cu(II)-embedded polymers was increased with the amount of added copper acetate in the polymer increase, which clearly demonstrates that the binding of protein on the Cu(II)-embedded polymer is mainly dependent on the complex reaction between Cu(II) ions on the surface of polymer and the BSA in the solution. The higher the amount of Cu(II) on the surface, the more the BSA bound on the polymer. It was also observed that when 40 mg of copper acetate was used, the corresponding Cu(II)-embedded polymer was brittle. Therefore, in this study, the biggest-BSA binding Cu(II)-embedded polymer in which the initial amount of copper acetate added was 30 mg was selected for further experiment.

3.2.2. Cu(II)-embedded polymer for protein binding in different binding solutions

The binding of Cu(II)-embedded polymer for BSA was tested in four binding solutions with different ionic strength and pH value. As shown in Fig. 2, among four binding solutions, the amount of BSA bound on Cu(II)-embedded polymer performed in pure water is 3∼6 time higher than that in other binding solutions. The highest

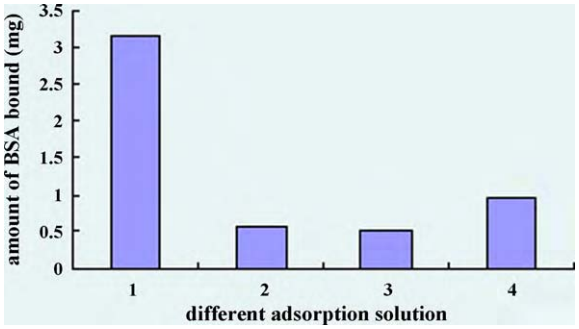

Fig. 2. The effect of binding solutions on binding amount of BSA: 1, pure water; 2, 0.01 mol L^{-1} phosphate buffered saline, pH 7.4, containing 0.15 mol L^{-1} NaCl; 3, 0.02 mol L^{-1} phosphate buffered saline, pH 8.0, containing 0.05 mol L^{-1} NaCl; 4, 0.05 mol L^{-1} carbonate buffer, pH 9.6.

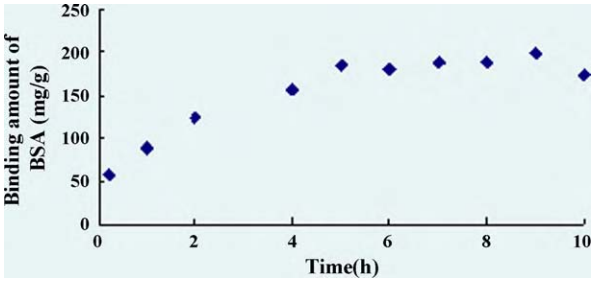

Fig. 3. The effect of binding time on binding amount of BSA.

BSA binding in pure water may be ascribed to the neutral aqueous solution and very small ionic strength. Therefore, pure water was chosen as protein binding solution.

3.2.3. Cu(II)-embedded polymer for protein binding in different binding time

The binding amount of BSA on Cu(II)-embedded polymer for BSA in pure water was tested in every 1 to 2 hours. As shown in Fig. 3, the amount of BSA bound on the Cu(II)-embedded polymer was gradually increased in the binding time less than 5 hours and after 5 hours the Cu(II)-embedded was saturated with BSA. In this study, overnight (14∼20 hours) was selected as binding time to ensure that the BSA binding was at saturated equilibrium.

3.2.4. Maximum binding of the Cu(II)-embedded polymer for protein

The maximum binding of 20 mg Cu(II)-embedded polymer for BSA was tested in different concentration of BSA solution. As illustrated in Fig. 4, the amount of BSA bound on Cu(II)-embedded polymer was gradually increased when the initial amount of BSA was in the range of 0.5-2.0 mg. Maximum amount of BSA bind-

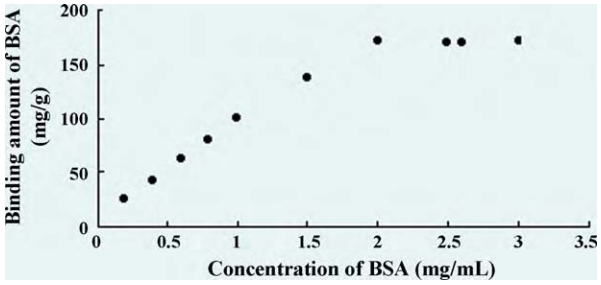

Fig. 4. The effect of initial BSA concentration in binding process on maximum binding of BSA. BSA concentration: 0.2-3.0 mg mL^{-1}; BSA solution volume: 2.0 mL; binding time: overnight.

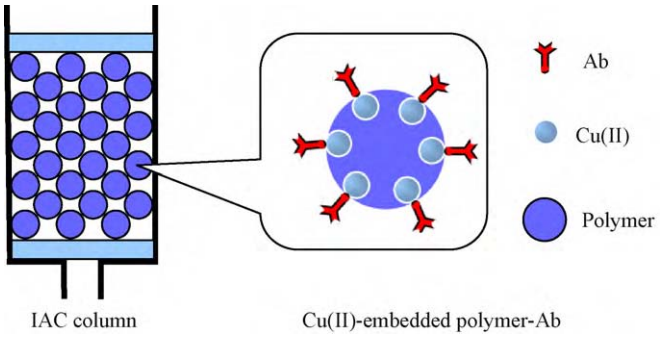

Fig. 5. The schematic diagram of IAC column packed with Cu(II)-embedded polymer-Ab.

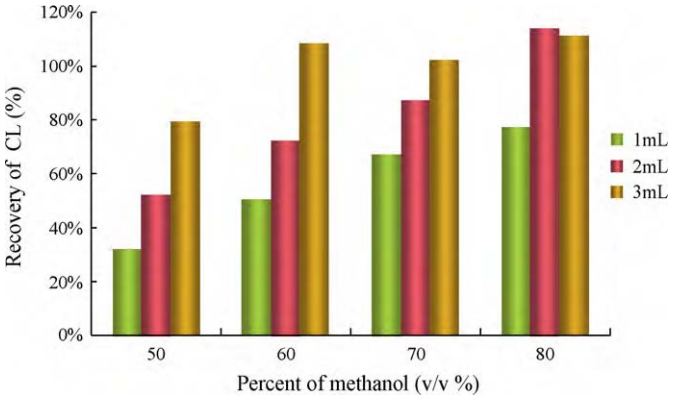

Fig. 6. CL recovery in the fractions of eluting step when 50%, 60%, 70% and 80% of methanol/water (v/v, %) in volume of 1-3 mL were used as eluting conditions. 100 ng of CL was loaded onto the IAC column.

ing was reached after the initial amount of BSA was over 2.0 mg, indicating the all binding sites on the surface were saturated with BSA and no more BSA will be bound on the polymer even more amount BSA was added in the solution. This phenomenon also further demonstrates that the BSA binding on the Cu(II)-embedded polymer is resulted from specific binding of the embedded Cu (II) for protein. From Fig. 4, the maximum amount of BSA bound on the polymer was found to be 172 mg g^{-1}. It was also observed that at optimal experimental conditions, three batches of the synthesized Cu(II)-embedded polymers exhibited the similar amount of the maximum binding for protein with relative standard deviation (RSD) less than 5%.

3.2.5. *Stability of the Cu(II)-embedded polymer-BSA*

The stability of the formed Cu(II)-embedded polymer-BSA was tested in four eluting solutions with high concentration of organic solvent or low pH value. After immobilizing BSA on the Cu(II)-embedded polymer, the formed Cu(II)-embedded polymer-BSA was suspended with 2 mL of eluting solution and shaken in a 25 °C bath for 30 min. It was found that, either in 50% or 80% methanol, there was no BSA released from Cu(II)-embedded polymer, indicating that the affinity binding of BSA on Cu(II)-embedded polymer is very strong and the Cu(II)-embedded polymer-BSA is very stable even in the 80% methanol. However, it was observed that in 50% of methanol (pH = 2) and 80% of methanol (pH = 2), about 80% of BSA was released from Cu(II)-embedded polymer, which indicated that the pH value played a more important role in lowering the complex reaction between Cu(II) and protein.

3.3. *Optimization of extraction condition for IAC column*

The IAC column was prepared by immobilizing pAb against CL on Cu(II)-embedded polymer and packing the formed Cu(II)-embedded polymer-pAb into a common SPE cartridge. The schematic diagram of IAC column packed with Cu(II)-embedded polymer-Ab was shown in Fig. 5. Before applying the IAC column for the extraction of target analyte, the extraction conditions of IAC column should be optimized. In loading step, all target analyte should be specifically bound on the column, while in the washing step, the possibly bound nonspecifically adsorption should be removed completely, and moreover, in the eluting step, all the bound analyte should be eluted and the Ab activity should be remained as much as possible.

When total 100 ng of CL was loaded onto the IAC column, two loading solutions (pure water and 0.01 mol L^{-1} PBS), two washing solutions (pure water, 5% methanol) and five eluting solutions containing 40%, 50%, 60%, 70% and 80% of methanol/water (v/v, %) were examined. It was found that when pure water was used as loading solution, almost all analyte was specifically bound on the column. Therefore, pure water was selected as loading solution. In washing step, it was observed that either with pure water or 5% methanol to wash the column, there was no CL in the effluent fractions, e.g. no CL was removed from the column, so either pure water or 5% methanol could be used as washing solution. In most off-line IAC procedures, eluting the analyte bound on the column was achieved with a high percentage of an organic solvent mixed with water [33,34]. The effect of methanol concentration and elution volume on extraction efficiency was illustrated in Fig. 6. It was noticed that 3 mL 60%, or 3 mL 70%, or 2-3 mL 80% methanol was able to elute the bound CL completely. Considering the high concentration of methanol might be more harmful to the antibody, decreasing the stability of the column, therefore, 3 mL of 60% methanol was chosen as eluting condition. It should be mentioned that such eluting condition is able to elute the target analyte from IAC column, e.g. it can break the affinity binding between Ab and antigen, but it can be expected that such eluting solution will not remove the Ab away from the Cu (II)-embedded polymer (see *3.2.5.*)

3.4. *Maximum capacity of IAC column for target analyte*

The maximum of IAC column for target analyte was measured by breakthrough volume test. 10 mL of CL standard solution at concentration of 100 ng mL^{-1} (total CL: 1000 ng) was loaded on the column. It was found that with the increase of CL loaded on the column, the CL content specifically bound on the column increased. When loading amount of CL reached 616 ng, the column was saturated with CL. Further loading of CL did not increase the amount of CL on the IAC column. Therefore, the maximum binding capacity of the IAC column for CL was estimated to be 616 ng.

3.5. *Extraction efficiency of the IAC column for target analyte from real samples*

Three food samples collected from the local market were spiked with CL and extracted with 0.1 mol L^{-1} HCl solution. After pH adjustment to 6~8, the extracts were loaded on IAC. The target analyte specifically bound on the column was eluted with eluting solution and detected by HPLC. The results of the spiking experiment were listed in Table 1. It was found that there was no

Table 1
The extraction recoveries of the IAC column for CL from three spiked food samples

Sample	CL content spiked (ng/g)	CL content measured (ng/g)	Recovery (%)	RSD (%, n=3)
meat	5	8.48	84.8%	15.5
liver	5	4.76	95.2%	10.7
feed	10	4.57	91.4%	9.3

detectable CL in three blank samples and the recoveries of CL in three spiked samples were within 84.4-95.2% with relative standard deviation (RSD) of 9.3-15.5%, indicating the applicability of the proposed IAC column for the extraction of target analyte from real samples.

3.6. Reusability of the IAC column

The antibodies are normally difficult to obtain and are very expensive. Therefore, the prepared IAC columns are expected to be used for many times. Actually most antibodies are tolerant to some extent of organic solvent without significant loss of activity and the IAC column can be easily regenerated with PBS solution. The IAC column with the maximum capacity of 616 ng CL was tested for its stability. It was observed that after 30 times usage, about 80% of the maximum capacity was still remained, indicating high stability of the IAC column.

4. Conclusions

Using acrylamide (AM) as monomer, ethylene glycoldimethacrylate (EGDMA) as cross-linker and bulk polymerization as synthetic method, a polymer embedded with Cu(II) was prepared. The Cu(II)-embedded polymer displayed a strong binding with protein. Therefore it can be used as a solid support for antibody immobilization. An IAC column was prepared by immobilizing pAb against clenbuterol to Cu(II)-embedded polymer and packing the Cu(II)-embedded polymer-pAb into a SPE column. The IAC column was characterized in terms of maximum binding capacity for target analyte, extraction efficiency and reusability. The results demonstrated the feasibility of the prepared IAC column for CL extraction. This study provides an alternative and useful antibody immobilization method in IAC

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