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Urea–formaldehyde resin monolith as a new packing material for affinity chromatography

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Abstract

A urea–formaldehyde resin (UF) continuous bed has been prepared through in-situ condensation polymerization in a confined tube. The monolith is an agglomerate of 2-μm irregular particles. Nitrogen adsorption shows that the monolith has a bimodal pore size distribution. It has low resistance to flow. A dyed monolith is obtained through modification of the UF monolith with Cibacron blue F3GA. Although its dye concentration and dynamic capacity are low compared to Sepharose type affinity media, the dyed monolith can separate some proteins in the affinity mode of liquid chromatography.

Keywords: Urea–formaldehyde resin monolithic column; Monolithic column; Stationary phases, LC; Affinity adsorbents; Cibacron blue F3GA; Proteins

1. Introduction

Polymer packings play an important role in the chromatographic separation and purification of biopolymers. The inherent limitations of an HPLC chromatographic column packed with typical polymeric particles include the large void volume between the packed particles and the slow diffusional mass transfer of solutes into the stagnant mobile phase present in the pores of the separation medium. These limitations contribute to peak broadening and, therefore, decreasing column efficiency.

In 1989 Hjertén et al. developed the continuous-bed chromatographic column, which was synthesized in one step by copolymerization of water-soluble monomer (e.g. methacrylamide), cross-linker and ligand monomer in the presence of salt [1] (reviewed by Liao [2]). The resulting polymer bed is hydrophilic and spongy. According to the different ligand monomers, the continuous-bed can be used for ion-exchange, hydrophobic-interaction, reversed-phase, chiral-recognition and affinity chromatography, etc.

In 1992 Svec and Frechet prepared the rigid polymeric continuous-bed column [3]. It is an integral monolith prepared with water-insoluble monomer, solvent and initiator in confined conditions. Compared with polymeric bead packing, the monolith provides many advantages such as fast diffusional mass transfer of solutes, high efficiency of the column space utilization and low column pressure [4]. At present, the materials mostly used are cross-linked polystyrene and cross-linked polymethacrylate. The rigid monoliths can be used in reversed-phase [5–10], ion-exchange [10–13], hydrophobic-interaction [14], affinity [15] and precipi-

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tation-redissolution [16] chromatography for separation of proteins, alkyl benzenes, polypeptides and polymers. Silica monoliths were also prepared and commercialized [17,18]. The two used polymers are very hydrophobic, and silica has silanol surface. They all have strong non-specific adsorption with biopolymers, which limits their application in bioengineering and biotechnology. Thus there is an urgent need to find rigid and hydrophilic polymer materials as new chromatographic supports.

Biomimetic affinity chromatography is a well known technique for purification of biopolymers. The triazine dye Cibacron blue F3GA is a typical example for making the stationary phase of biomimetic affinity chromatography [19]. Triazine dyes have been immobilized to a wide variety of support matrices, including agarose, dextran, polyacrylamide, agarose-polyacrylamide copolymer, silica [20], poly(vinyl alcohol)-coated poly(styrene–divinylbenzene) [21], poly(ethylene glycol dimethacrylate–co-2-hydroxyethylmethacrylate) [22], cellulose and glass. Recently there has been a pronounced trend to utilize mechanically stable matrices in place of the conventional soft polymer gel systems in most areas of affinity chromatography.

In this article, a new continuous-bed medium of urea–formaldehyde resin (UF) is prepared in a confined column by in-situ polymerization under acid conditions. The pore size distribution of the monolith is determined by nitrogen adsorption. It is the first continuous-bed medium prepared through condensation polymerization. This medium has a good hydrophilic property and a highly cross-linked structure that provides a good mechanical property. The monolith is further modified with Cibacron blue F3GA. The dyed monolith can be used to separate and purify some proteins in the affinity mode of liquid chromatography.

2. Experimental

2.1. Materials

Cibacron blue F3GA was obtained from Sigma. Other chemicals, including human serum albumin (HSA), lysozyme, ovalbumin and newborn calf serum, were purchased in China. Formaldehyde solution was 37% content. All the chemicals were of analytical grade.

2.2. Preparation of the continuous UF column

Urea (10 g) was dissolved in 10 ml distilled water, and the pH value of this solution was adjusted to 2.0–3.0 with concentrated hydrochloric acid. Formaldehyde solution (37%, 20 ml) was also adjusted to pH 2.0–3.0. The two solutions were mixed and stirred to a homogeneous solution quickly. A stainless steel tube (100 mm×10 mm I.D.) with one end sealed with a plug was filled with the above mixture at room temperature. After ~5 min, the solution in the tube solidified to a white UF monolith. Then the other end of the tube was sealed, and the column was heated in a drying oven at 60°C for 8 h to complete the polymerization. After this, the plugs were replaced by the column end fittings, and the column was attached to a HPLC system. More than 200 ml distilled water was pumped through the column at a flow-rate of 1 ml/min to remove unreacted substances.

2.3. Characterization of the UF monolith pore properties

The morphology of a cross-section of the dried UF monolith was investigated by scanning electron microscopy (Hitachi S-450). The specific surface area and the pore volume of the UF monolith were calculated from the BET isotherm of nitrogen; the pore size distribution in the dry state was determined from the adsorption curve. An instrument (ASAP 2000, Micromeritics) was used for the purpose.

2.4. Preparation of the UF monolith immobilized with Cibacron blue F3GA

A 1-g sample of Cibacron blue F3GA was dissolved in 200 ml water, followed by addition of 2 g NaOH to make a basic blue solution. At room temperature, the blue solution was recirculated through the above UF rod-like column for 24 h at a flow-rate of 0.5 ml/min. The column was then removed from the chromatographic system, sealed at both ends with plugs and heated at 60°C for 6 h. The

modified column was reattached to the chromatographic system and washed at a flow-rate of 1 ml/min with distilled water, 1 *M* NaCl solution, 4 *M* urea solution and distilled water consecutively to remove the unreacted dye until the elution was colorless.

2.5. Frontal analysis

The dynamic loading capacity of the UF continuous column immobilized with Cibacron blue F3GA was determined using frontal analysis [21], which entailed pumping a lysozyme solution (0.2 mg/ml, 50 m*M* phosphate buffer, pH 7.0) through the column at a flow-rate of 1 ml/min until the absorbances (at 254 nm) of the output and input streams were identical. The column was then eluted with phosphate buffer (50 m*M*, pH 7.0) containing 1 *M* NaCl until the absorbance of the outlet returned to baseline. Dynamic capacity was calculated at 5% breakthrough (the outlet absorbance at 254 nm being 5% that of the inlet).

2.6. Determination of the dye concentration and Fourier transform (FT)-IR analysis

After all the chromatographic experiments had been completed, the dyed bed was washed with water, the bottom column fitting was removed and the dyed UF monolith was pushed out of the tube by pressure of the mobile phase. The blue monolith was dried at 60°C and cut into small pieces. Immobilized dye concentration was measured by spectrophotometric means [19]: ~100 mg dried blue UF matrix was dissolved in 10 ml 2 *M* sulfuric acid or 4 *M* hydrochloric acid solution at 80°C to release the dye. The absorbancy of the solution was measured by spectrophotometric measurements at 620 nm. Then the dye concentration was calculated.

The FT-IR spectra of the dried UF and blue UF matrixes were obtained from KBr pellets on a Bruker FT-IR (Equinox 55/S) instrument.

2.7. Determination of the negatively charged groups

Negatively charged groups in the UF and blue UF monoliths were determined as follows referring to Ref. [23]. Approximately 5 g of the wet UF or blue UF gels (monoliths were ground into powder) were washed on a glass filter, first with water and then with 25 ml of 1 *M* hydrochloric acid. The gel was then washed with distilled water until it was neutral and the washings were chloride free (tested with silver nitrate). The gel was weighed in the titration vessel, together with 5 ml of 2 *M* potassium chloride, and titration was performed with 0.01 *M* sodium hydroxide using a pH meter. The titration curve was drawn, and the estimated inflection point was taken as the equivalence point.

2.8. Affinity chromatography and sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis

A Shimadzu HPLC system consisting of a pump, an injection valve sampler (Rheodyne 7125) and a UV detector (UVD-2, 254 nm) was used to carry out all the affinity chromatographic experiments. The column was equilibrated in phosphate buffer (50 m*M*, pH 7.0) or Tris–HCl buffer (50 m*M*, pH 7.0) and eluted with pH 9.0 buffer or pH 7.0 buffer containing salt, for example 1 *M* NaCl. For some proteins the recovery was measured by the UV adsorption of the elution fractions.

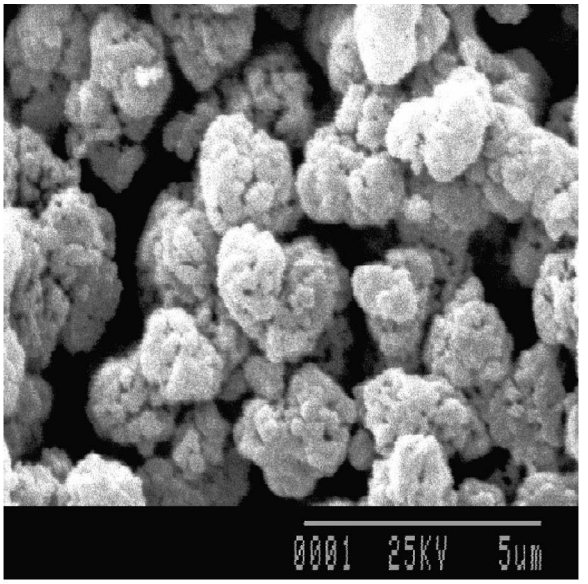
When the sample was newborn calf serum, the fractions were collected by hand and analyzed by SDS–PAGE.

3. Results and discussion

3.1. Preparation and properties of the UF monolith

A cross-linked UF monolith can be obtained through condensation polymerization between urea and formaldehyde under acid conditions (pH 2.0–3.0) in a confined tube. The pH value of the reacting mixture and reaction temperature are two important controlling factors. If the pH value is below 2.0, the reaction in the tube is too fast to control: the abrupt reaction in the confined tube releases too much heat, resulting in large voids in the monolith. When the pH value is above 3.0, the reaction is so slow that the produced particles are small. Temperature has an

effect similar to the pH value, i.e. fast reaction at high temperature, and slow reaction at low temperature. In this work, room temperature is appropriate to carry out the preparation, but the monolith was finally kept at 60°C to complete the reaction. The method is simple, low cost and time saving. Additionally, in the process no other organic chemicals are used that can cause pollution and difficult recovery.



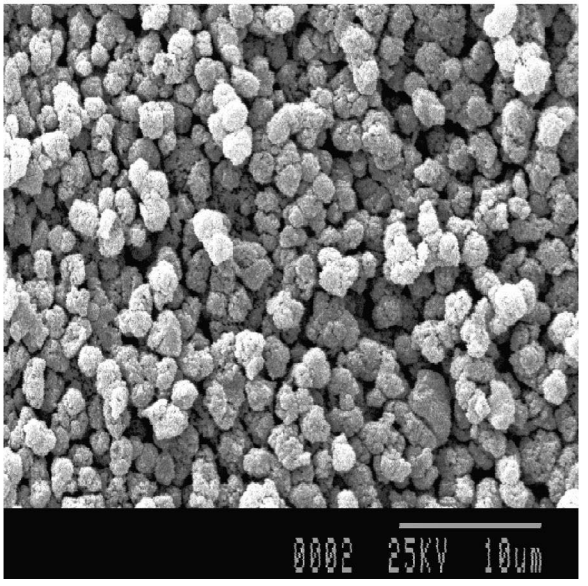


Fig. 1. SEM micrograph of the inner part of the UF monolith.

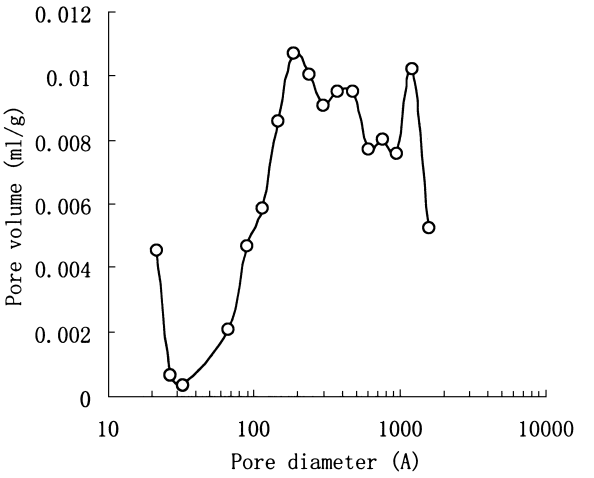


Fig. 2. Differential pore size distribution curve of the UF monolith measured by nitrogen adsorption.

Scanning electron micrographs of a cross-section (Fig. 1) reveal clusters of particles separated by large pores in the UF monolith. The particles are ~2 μm in size and irregular. The size of the large pores between clusters is ~1 μm. There are also many pores whose diameter is ~200 nm on the surface of

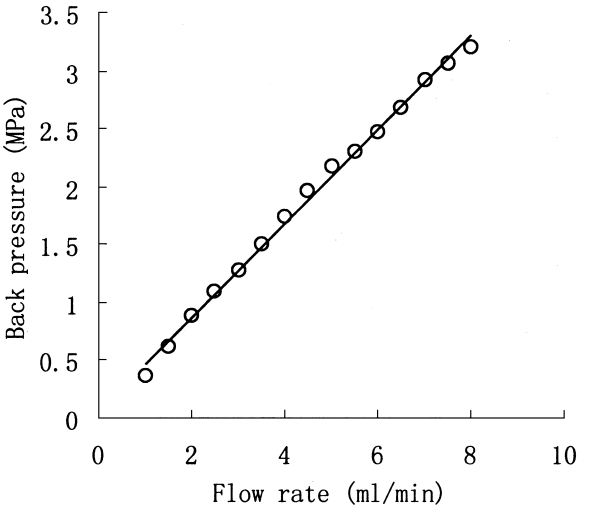


Fig. 3. Effect of flow-rate on back pressure in the UF column. Column: 100 mm×10 mm I.D.; mobile phase: water.

the particles. So the UF monolith has good flow characteristics.

Fig. 2 shows the pore size distribution profile for the UF monolith by nitrogen adsorption analysis. Obviously, the material contains two sharply divided sizes of pores: large pores with size ranging from 50 to 1500 Å and relatively small pores under 50 Å (the limit of the instrument is 10 Å). The large pores exceeding 100 Å are the main contributors to the pore volume, representing almost 90% of the total pore volume. The smallest pores with sizes under 100 Å mainly contribute to the specific surface area of the monolith (almost 50% of the area), though their volume is only 10% of the total.

The specific surface area measured by nitrogen adsorption is found to be 42.0 m^2/g and the total pore volume (pores less than 1222 Å) is 0.11 ml/g. The average pore diameter (4 V/A) is 105 Å.

Fig. 3 shows the effect of flow-rate on back pressure in a continuous UF bed (100 mm×10 mm I.D.) when water is the eluent. The back pressure versus flow-rate dependency is a straight line up to a flow-rate of 8 ml/min, near the limit of the instrument. It is confirmed that the monolith is not compressed even at the high flow-rate. It can be seen in Fig. 3 that the flow through the UF monolith is actually satisfactory as the back pressure in the column is lower than a conventional column packed with beads. The result is similar to other continuous beds. For example, a continuous poly(styrene–divinylbenzene) monolith (50 mm×8 mm I.D.) exhibited a back pressure of 1.6 MPa at a flow-rate of 5 ml/min when acetonitrile–water (20:80, v/v) mixture was the eluent [5]. Using the same linear flow-rate (10 cm/min), a simple calculation shows that the same column of 100 mm×10 mm I.D. would have a back pressure of 3.2 MPa and a volume flow-rate of 7.8 ml/min. Under the same flow-rate the continuous UF bed with water as the eluent displays a back pressure of 3.2 MPa, which corresponds well with the above poly(styrene–divinylbenzene) monolith.

3.2. Preparation and properties of the UF monolith immobilized with Cibacron blue F3GA

3.2.1. Preparation

Compared with the IR spectrum of UF, the IR spectrum of blue UF exhibits decreased strength at 3353 and 1557 cm^{-1} (Fig. 4), which are the bands of

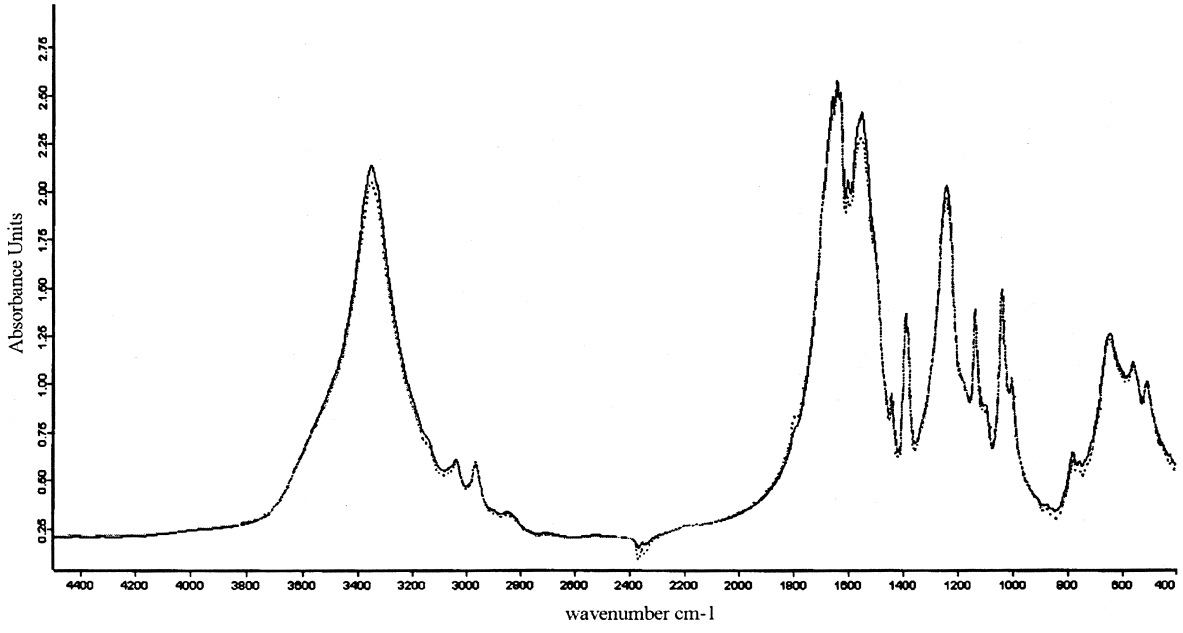


Fig. 4. FT-IR spectra of UF and the polymer modified with Cibacron blue F3GA. Solid line: UF; dotted line: blue UF.

N–H stretching and bending vibrations, respectively. These show that the imino groups of the UF molecules are mainly involved in the reaction while the UF monolith is modified by Cibacron blue F3GA. The terminal amino groups are also able to react with Cibacron blue F3GA, because the primary, secondary and even tertiary amines are all of similar reactivity in this type of nucleophilic substitution [24]. Their number is rather small as compared to that of imino groups. Though immobilization can occur through the terminal hydroxyl groups of the UF molecules, the reacted groups are not seen by IR.

The reactive imino groups in the UF structure can be controlled by the molecular ratio between urea and formaldehyde. In the preparation this ratio was 1:1.6 mol; there should be 1.4 mol spare imino groups (corresponding to 17.5 mmol/g UF resin) left for further modification. Scheme 1 shows the reaction of UF resin and Cibacron blue F3GA. The chlorine atom in the triazine ring of the dye is reactive, and the dye is covalently coupled to the UF resin via the nucleophilic reaction between the chlorine of the triazine ring and the imino groups of the UF molecules. Thus a dyed UF monolith is prepared by in-situ modification under alkaline conditions. From the reaction scheme, it is evident that addition of NaOH in the reacting mixture can lead to a higher dye concentration [19]. It is found that increasing temperature plays the same roles as the base.

3.2.2. Dye concentration and the negatively charged groups content

After being pushed out of the tube, the dyed monolith appears homogeneous, integral and pale blue. Its dye concentration is 0.43 mg/g (0.56 μmol/g) as measured by the spectrophotometric method. The dye concentration is lower than that of dyed monodisperse silicas with no pores (1.2 mg/ml) [20] and a commercial product Blue Sepharose 6 Fast Flow (5.6 mg/ml) [25]. It is obvious that the usage of imino groups in the UF monolith is very low efficient, because 17.5 mmol amino groups per gram of UF resin will give 1.20 mmol F3GA per gram of dyed UF resin. The obtained F3GA content is 1/2000 of the theoretical value. The reasons for such low dye concentration are: (i) the UF monolith has a high cross-linking density; only the imino groups on the surface of the pores can react with F3GA; (ii) the imino groups are too close, so it is impossible that every imino group reacts with a bulk F3GA molecule; and (iii) the in-situ modification is a dynamic process.

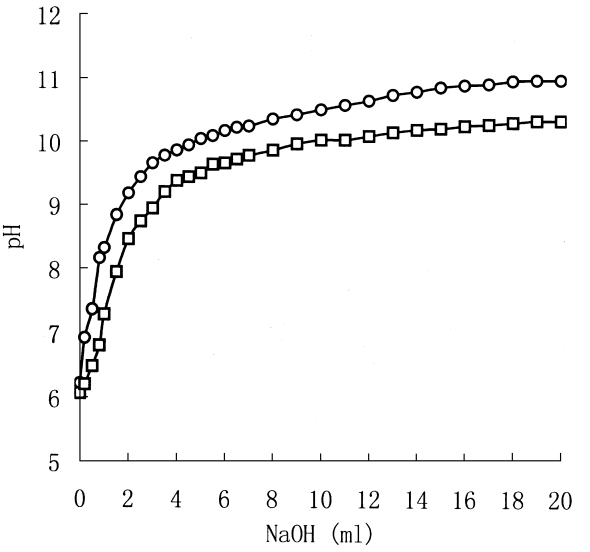
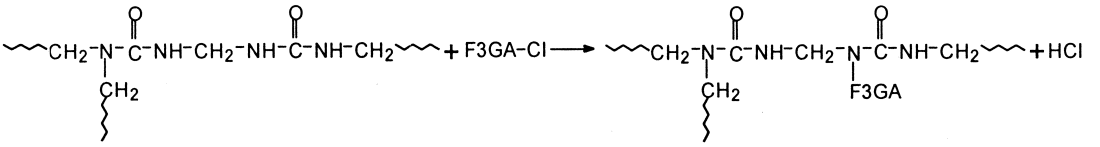


Fig. 5. Titration curves of UF and the polymer modified with Cibacron blue F3GA. Circle: UF; square: blue UF.

The residual ionizable groups can affect the specificity of the stationary phase, so it is useful to determine the presence of the negatively charged groups. From the titration curves of the UF and blue UF (Fig. 5), we can estimate that the amounts of the groups in the UF and blue UF supports are 0.48 and



Scheme 1. Reaction of UF resin and Cibacron blue F3GA

1.94 μmol/g, respectively. The UF itself contains a negligible amount of the negatively charged groups. The blue UF contains 0.56 μmol/g of F3GA; if the three sulfonic acid groups in F3GA are ionized, the total negatively charged groups content should be ~2.16 (0.48+1.68) μmol/g, which is very close to our measurement.

3.2.3. Dynamic capacity

Fig. 6 shows the results of the frontal analysis. The dynamic capacity with lysozyme calculated at 5% breakthrough is 8 mg per column. Since the column volume is 7.8 ml, the specific capacity of the dyed rod is 1.02 mg/ml. It is lower than that of some conventional dyed supports. For example, the binding capacity of the Blue Sepharose 6 Fast Flow is 18 mg human serum albumin/ml [25]. It is obvious that the low dynamic capacity is a result of the low dye concentration.

3.3. Affinity chromatography

Careful selection of the equilibration buffer is necessary for affinity chromatography. In this work we choose the frequently used phosphate buffer (50 m*M*, pH 7.0) and Tris–HCl buffer (50 m*M*, pH 7.0) as the equilibration buffer.

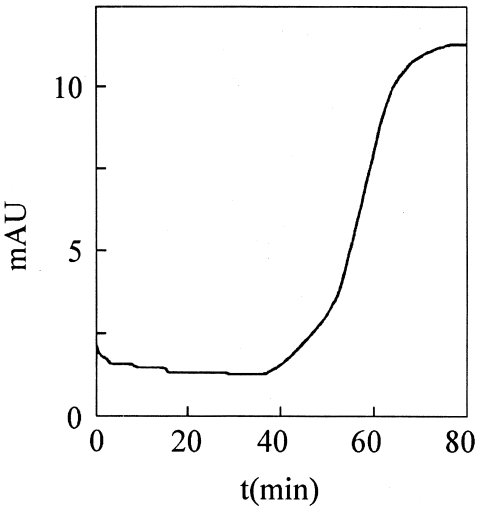


Fig. 6. Frontal analysis curve for lysozyme on Cibacron blue F3GA immobilized UF monolith. Column: 100 mm×10 mm I.D.; mobile phase: 50 m*M* potassium phosphate, pH 7.0; lysozyme concentration: 0.2 mg/ml; flow-rate: 1 ml/min; detector: UV 254 nm.

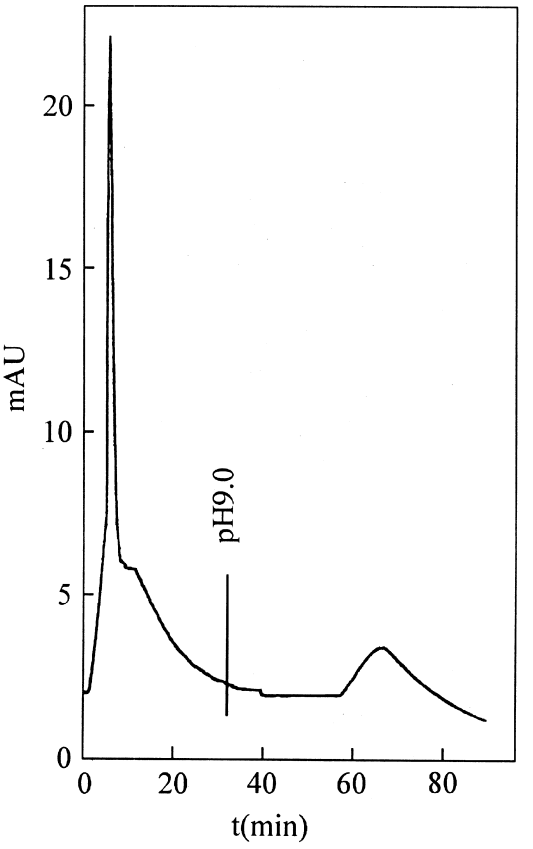


Fig. 7. Affinity elution of human serum albumin (HSA) on Cibacron blue F3GA immobilized UF column. Column: 100 mm×10 mm I.D.; detector: UV 254 nm; sample concentration: 5 mg/ml; injection volume: 20 μl; flow-rate: 1 ml/min; eluent: (1) 50 m*M* potassium phosphate, pH 7.0, (2) 50 m*M* potassium phosphate, pH 9.0, step gradient as indicated in chromatogram.

There are usually two elution methods in affinity chromatography. One is specific elution with special substrates such as NADP etc., and the other is non-specific elution with salt or pH changes. It is reported that triazine dye columns are more effectively eluted with salt or pH changes. This is cheaper than specific elution with substrates and, hence, can be important for larger scale separations [19]. Proteins tend to bind to triazine dyes more tightly at lower pH values, presumably because of a contribution from ionic interactions favored by increasing positive charges on interacting protein molecules.

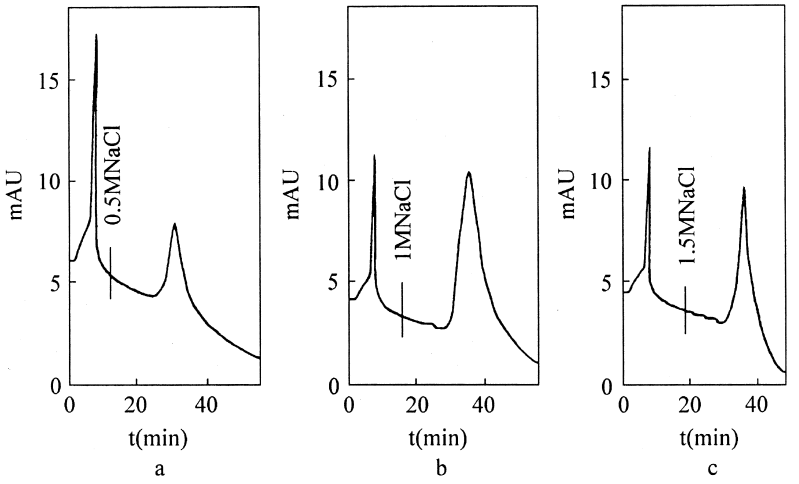


Fig. 8. Affinity elution of human serum albumin (HSA) on Cibacron blue F3GA immobilized UF column with different eluents. Column: 100 mm×10 mm I.D.; detector: UV 254 nm; sample concentration: 1 mg/ml; injection volume: 20 μl; flow-rate: 1 ml/min; eluent: (1) 50 m*M* Tris–HCl, pH 7.0, (2) 0.5 M (a), 1 M (b), 1.5 M (c) sodium chloride in (1), step gradient as indicated in chromatogram.

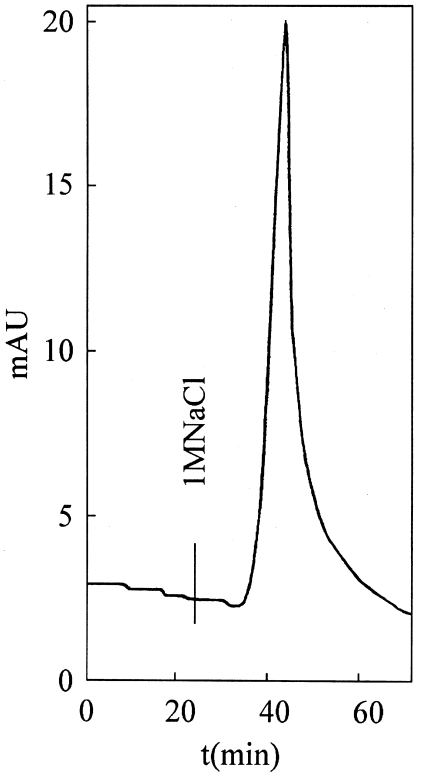


Fig. 9. Affinity elution of ovalbumin on Cibacron blue F3GA immobilized UF column. Column: 100 mm×10 mm I.D.; detector: UV 254 nm; sample concentration: 1 mg/ml; injection volume: 20 μl; flow-rate: 1 ml/min; eluent: (1) 50 m*M* Tris–HCl, pH 7.0, (2) 1 *M* sodium chloride in (1), step gradient as indicated in chromatogram.

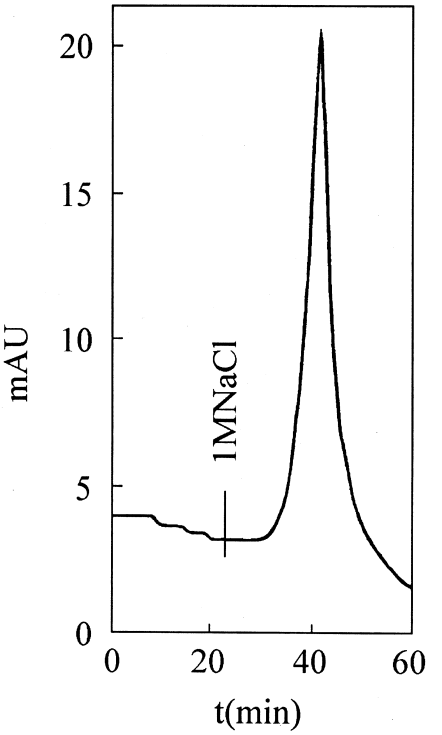


Fig. 10. Affinity elution of lysozyme on Cibacron blue F3GA immobilized UF column. Column: 100 mm×10 mm I.D.; detector: UV 254 nm; sample concentration: 1 mg/ml; injection volume: 20 μl; flow-rate: 1 ml/min; eluent: (1) 50 m*M* Tris–HCl, pH 7.0, (2) 1 *M* sodium chloride in (1), step gradient as indicated in chromatogram.

Gradients to higher pH values are successful in separating proteins, but the desorption pH is critical. The dyed adsorbents can not be operated at too low or too high pH values because dye leakage is evident under these conditions. Salts such as KCl and NaCl have been widely used to selectively elute enzymes and proteins in affinity chromatography. In this article the proteins are eluted by increasing pH value or salt concentration.

The dye containing adsorbents usually exhibit group specificity towards proteins. Blue adsorbents (e.g. Cibacron blue F3GA) function on the basis of specific interaction with serum albumin (isolation and removal of albumin from blood serum) and nucleotide-dependent enzymes. In this article HSA, ovalbumin, lysozyme and newborn calf serum are selected as the samples.

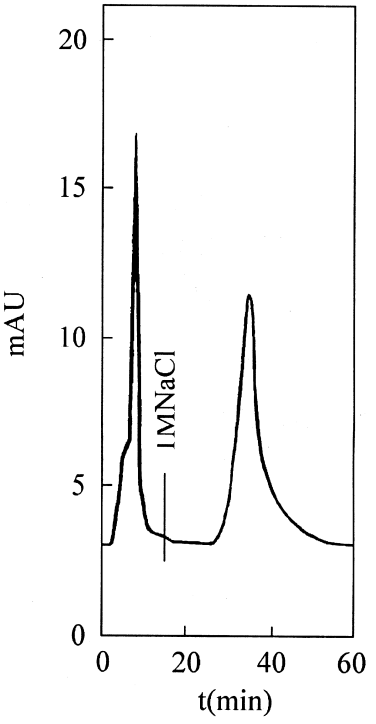


Fig. 11. Affinity separation of newborn calf serum on Cibacron blue F3GA immobilized UF column. Column: 100 mm×10 mm I.D.; detector: UV 254 nm; sample concentration: 30% (v/v, prepared with the equilibration buffer); injection volume: 20 μl; flow-rate: 1 ml/min; eluent: (1) 50 m*M* Tris–HCl, pH 7.0, (2) 1 *M* sodium chloride in (1), step gradient as indicated in chromatogram.

With phosphate buffer (50 m*M*, pH 7.0) as the equilibration buffer, protein HSA can be eluted using pH 9.0 buffer (Fig. 7). From Fig. 7, we can see that the unretained impurity proteins flow through the void volume and HSA is reserved. But the eluted peak is small. The reason is perhaps that the elution ability of the eluent (high pH mobile phase) is limited.

Fig. 8 also shows affinity elution of HSA on the dyed rod column. The equilibration buffer is Tris–HCl solution (50 m*M*, pH 7.0) and the eluent is the buffer containing different salt content. It is observed that the greater the salt content in the mobile phase, the larger and narrower the elution peak. The ability of elution increases with increasing salt content in eluent. Compared with elution with changing pH value in Fig. 7, it can be seen that when using the salt to elute, the elution of protein is more complete and the elution time is shorter. So elution with the salt is more competitive. In Fig. 8b, protein recovery is 95%. The separations of ovalbumin and lysozyme

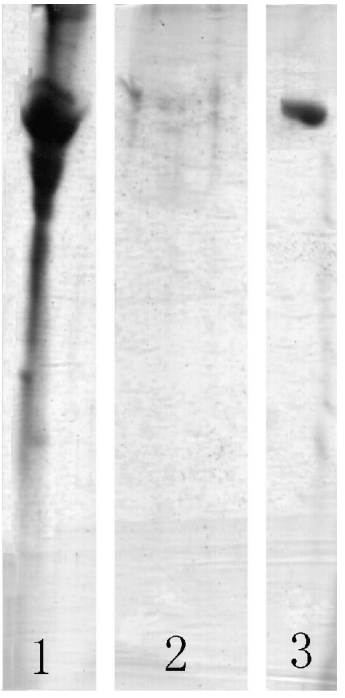


Fig. 12. SDS–PAGE analysis of fractions of newborn calf serum. Lanes 1: newborn calf serum; 2: unbound fraction; 3: bound and eluted fraction.

on the dyed monolith are shown in Figs. 9 and 10, respectively. Because they have high purity, the proteins are completely retained and there are no peaks after injection at equilibration stage. The recoveries of ovalbumin and lysozyme are 94 and 97%, respectively.

The newborn calf serum can also be separated on the dyed monolith. The eluent is the pH 7.0 Tris–HCl buffer containing 1 *M* NaCl (Fig. 11). The peak before elution is the unretained proteins and other impurity. The unbound and eluted fractions are collected by hand and analyzed by SDS–PAGE (Fig. 12). Lane 3 shows that the bound and eluted fraction contains bovine serum albumin (BSA). Lane 2 shows the unbound fractions and no BSA present in the flowthrough.

4. Conclusions

In this study, a new UF monolith is prepared through in-situ condensation polymerization in acidic aqueous environment. It has an integral structure. From SEM and nitrogen adsorption results, it can be determined that the monolith is made of 2-μm irregular particles, and has a bimodal pore size distribution. It has good flow characteristics.

A blue dyed UF monolith is prepared through reaction between Cibacron blue F3GA and the UF monolith under alkaline conditions. Although the dye concentration and dynamic capacity are low, the column can be used as a biomimetic affinity chromatographic medium to separate HSA, ovalbumin, lysozyme, and newborn calf serum. The proteins can be eluted by two methods: increasing the buffer pH value or increasing its salt content, and the latter is better. The proteins can be eluted more completely through increasing the salt content in the eluent, and the peaks are much narrower.

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