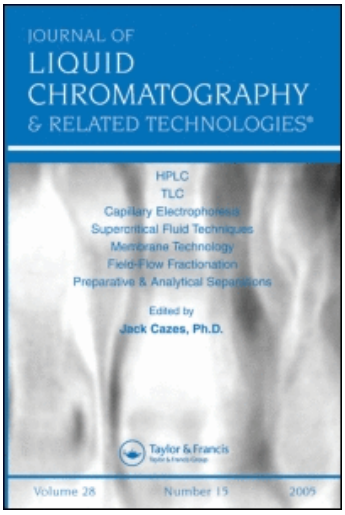

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Preparation of Weak Anion Exchange Chromatographic Packings Based on Monodisperse Polymer Beads and their Application in the Separation of Bioploymers

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Abstract: Based on the 8.0 μm monodisperse, macroporous poly-(glycidylmethacrylate-co-ethylene-dimethacrylate) beads ($P_{GMA/EDMA}$), a new hydrophilic weak anion-exchange (WAX) stationary phase for HPLC was synthesized by a new chemically modified method. The stationary phase was evaluated in detail to determine its ion-exchange properties, separability, reproducibility, hydrophilicity, and the effect of column loading and pH on the separation and retention of proteins. It was found to have an ion-exchange chromatographic (IEC) retention mechanism. The dynamic protein loading capacity of the synthesized SAX packings for BSA was 27.4 $mg \cdot g^{-1}$. Four proteins were separated within 6.0 minutes using the synthesized WAX resin. The WAX resin was also used for the rapid separation and purification of recombinant human granulocyte colony stimulating factor (rhG-CFS) from the coarse extract solution with only one step. The purity of the purified rhG-CFS was more than 95%.

Keywords: Monodisperse poly(glycidyl methacrylate-co-ethylenedimethacrylate) resins, Weak anion exchange chromatography, Protein separation, Recombinant human granulocyte colony stimulating factor

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INTRODUCTION

Ion-exchange chromatography (IEC) is of increasing interest to separation scientists for protein chromatography. Because of the nature of the mechanism by which separations are carried out,[1] IEC provides a more gentle environment for solutes than reversed-phase chromatography. This can be particularly important for biologically active compounds, which need to maintain their native forms. Because IEC can be conducted near physiological conditions, it causes less damage to sensitive biological macromolecules such as proteins.

As in all modes of chromatography, ion-exchange columns packed with good packing materials are essential for the separation. In general, ideal ion-exchange packing materials for HPLC are mechanically stable, chemically inert, hydrophilic with no irreversible adsorption, and highly efficient. Silica based packing materials are most widely used in HPLC, because of their mechanical stability, wide variety of derivatization, and relatively higher column efficiency. Unfortunately, silica based packings are less stable under high pH condition, sometimes it can not satisfy the request for the separation of biopolymers.[2,3] In most cases, polymeric stationary phases have gained considerable popularity in HPLC due to their stability in the entire pH range, broad variety of surface groups, chemistries, and polarities. Styrene-divinylbenzene (PS-DVB) copolymers are the most often used polymeric stationary phases.[4,5] Their highly hydrophobic surface accounts for their extensive use in reversed-phase chromatography and size-exclusion chromatography in non aqueous media. A search for more hydrophilic stationary phases is still in progress in order to develop polymeric media for the separation of water soluble hydrophilic compounds and proteins without adversely affecting their biological activity.[6]

Most polymer separation media are still prepared by a classical suspension polymerization. This technique affords beads that have rather broad particle size distributions. In the search for uniformly sized polymer beads as chromatographic stationary phases, Ugeltad et al.[7] developed a technique named "activated multi-step swelling and polymerization" method. Uniform beads are prepared by Ugelstad's method from a great variety of monomers.[8–13] This method is an excellent one, but it seems rather complex because at least two steps are needed in the swelling process.

We have reported a preparation of 8.0 μm monodiserpse $P_{GMA/EDMA}$ beads with macroporous by a single step swelling and polymerization method in the presence of solvents, linear polystyrene as porogens, and new chemical modification of $P_{GMA/EDMA}$ for the preparation of stationary phases of hydrophobic interaction chromatography,[14] strong cation exchange chromatography[15] and affinity chromatography,[16] and their application for separation of biopolymers. Anion exchange chromatographic packings are, however, the most promising materials for chromatographic applications in the study of biopolymers. In this paper, we report the

synthesis of a hydrophilic WAX stationary phase for HPLC by use of a new chemically modification of monodisperse $P_{GMA/EDMA}$ beads with macroporous. The chromatographic properties of the WAX stationary phase for biopolymer separation are discussed in detail. The WAX resin was also used for the rapid separation and purification of rhG-CFS from the coarse extract solution with only one step. The purity of the purified of rhG-CFS was more than 95%.

EXPERIMENTAL

Materials

Monodisperse, poly-(glycidylmethacrylate-co-ethylenedimethacrylate) ($P_{GMA/\ EDMA}$) macroporous beads, 8.0 μm, (median pore diameter by gel permeation chromatography, 89.1 nm; epoxide groups, 2.8 $mmol \cdot g^{-1}$) were synthesized according to reference [14]. Epichlorohydrin, and N-2-chloroethyl-N, N-diethylammonium hydrochloride were purchased from Beijing Chemical Company in China. Myoglobin (Myo), carbonic anhydrase (CA), conalbumin (CON), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, USA). All chemical reagents are analytical grade.

Instrumentation

All Chromatographic tests were carried out by using a LC-10A chromatographic system (Shimadzu, Japan) including a pump and a Multiple-wavelength UV detector. Samples were injected through a Rheodyne 7125 valve and detected at 280 nm.

Preparation of Weak Anion Exchange Chromatographic Media

Beads of $P_{GMA/EDMA}$, 5.0 g, were suspended in 60 mL of 0.1 $mol \cdot L^{-1}$ sulfuric acid, stirred, and kept at 60°C for 10 h. The beads were then filtered, washed with water until neutral, and dried under vacuum to obtain the hydrolyzed beads.

The hydrolyzed beads (4.0 g dry) were then dispersed in 20 mL of water and stirred with a magnetic bar for 16 h. The excess water was removed by a fritted-glass filter, and the beads were redispersed in 20 mL 50 wt % aqueous potassium hydroxide and stirred for 1 h. The liquid was removed, and the beads were transferred to 40 mL of an epichlorohydrin-water (1 : 1) mixture and stirred at room temperature for 3 h. The product was washed with water and acetone and dried to give beads containing 2.0 $mmol \cdot g^{-1}$ epoxide groups. The beads were then hydrolyzed and worked up using the same procedure as described above to afford beads with diol groups.

The dry beads of 3.0 g with diol groups were dispersed in 50 mL of 2.0 $mol \cdot L^{-1}$ NaOH solution, 5.0 g of N-2-chloroethyl-N, N-diethylammonium

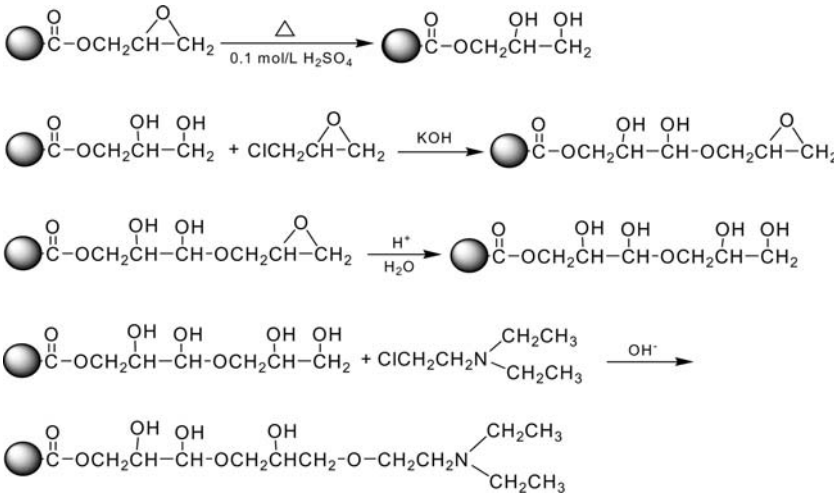

Figure 1. Synthetic route for preparation of the WAX packings.

hydrochloride were added, and allowed to react for 8 h at 60°C. The beads were finally washed with water, acetone, and dried to give the new WAX stationary phase for HPLC. Figure 1 shows the chemical modification scheme for the preparation of the WAX packings in this paper. The "P" in the scheme denotes the polymer frame.

Determination of Epoxy Groups

The $P_{GMA/EDMA}$ beads were dispersed in $0.1\,mol \cdot L^{-1}$ tetraethylammonium bromide in acetic acid solution and titrated with $0.1\,mol \cdot L^{-1}$ perchloric acid solution until the crystal violet indicator changed to blue-green.

Mass Recovery

According to the Bradford method,[17] Coomassie Blue G250 was used as the development reagent to measure the absorbance at 595 nm, using pure BSA as the calibration curve, for the determination of protein concentration and calculation of mass recovery.

Separation and Purification of rhG-CFS by the WAX Resin

The inclusion bodies of rhG-CFS were disrupted with buffer consisting of $20\,mmol \cdot L^{-1}$ phosphate $+ 1.0\,mmol \cdot L^{-1}$ EDTA $+ 0.2\,mg \cdot mL^{-1}$ lysozyme (pH = 7.4). The inclusion bodies were then washed three times and finally

dissolved in 7.0 mol · L^{-1} guanidine hydrochloride (Gu · HCl) solution. After incubation at 4°C for 24 h with full agitation, the supernatant of rhG-CFS was obtained by centrifuging it at 20000 r · min^{-1}.[18]

The supernatant contains only approximately 37% rhG-CFS. The diluted rhG-CFS solution was directly injected into the 10.0 × 0.46 cm I.D. WAX column. The column was then washed with a salt gradient and the fractions were collected and assayed. Figure 4 shows the chromatogram of the separation and purification of the diluted rhG-CFS extract solution. SDS-PAGE analysis shows one main band of purified rhG-CFS extract, and the purity of the purified rhG-CFS is more than 95.0% with only one step and 25 min.

RESULTS AND DISCUSSION

Chemical Modification of $P_{GMA/EDMA}$ Beads

Because of the hydrophobicity of the surface of the macroporous $P_{GMA/EDMA}$ resin, it is difficult to use it for separation of biopolymers. With the chemical modification of the hydrophobic surface by using a hydrophilic reagent, the irreversible adsorption on the surfaces of the beads and changes in the molecular conformation of protein could be avoided or diminished. Many reactions can be used for chemical modification of the epoxide groups present on the surface of the $P_{GMA/EDMA}$ resin. Figure 1 shows that the reaction procedure designed for the preparation of the WAX packings includes an additional hydrophilization step consisting of the reaction of the hydroxyl groups of diol beads with epichlorohydrin followed by another hydrolysis of the newly introduced epoxide groups. This additional hydrophilization step results in a better shielding of the hydrophobic main chains of the polymer, preventing their contact with the protein molecules.[11]

Elemental analysis revealed the nitrogen content to be 4.23%. The IR spectrum of the modified polymer exhibited a large broad adsorption peak at 4342 m^{-1} and 1240 cm^{-1} corresponding to the OH and C–N band. These results prove that the ion exchange groups were really bonded to the surface of the $P_{GMA/EDMA}$ beads.

Separation of Biopolymer by WAX Column

In order to test the resolution property of the synthesized WAX column, an experiment was performed to resolve acidic proteins with differences in their isoelectric points (pI). The protein mixture consisting of Myo (pI 7.3), CA (pI 6.2), CON (pI 5.88), and BSA (pI 4.98) was chromatographed on the column, which was shown in Figure 2 at a flow rate of 1.0 mL/min. When the experiment was done at a flow rate of 4 mL/min, a baseline separation of these proteins was shown in Figure 3, which demonstrated that the

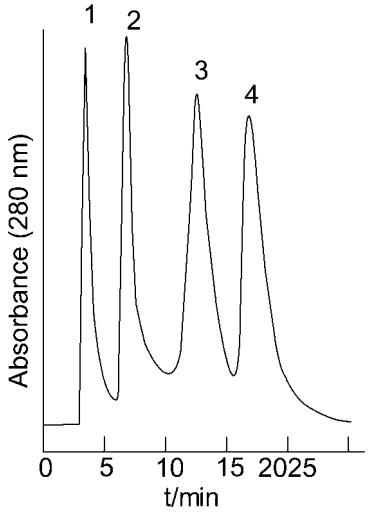

Figure 2. Chromatogram of standard proteins separated by the WAX column (10 × 0.46 cm I.D.). The linear gradient elution was from 100% solution A (20 mmol/L of phosphate, pH 6.5) to 100% solution B (20 mmol/L of phosphate + 1.0 mol/L NaCl, pH 6.5) at a flow rate of 1.0 mL/min for 25 min, with a delay for 5 min. AUFS, 0.08, UV detection at 280 nm. Proteins: 1, solvent + Myo; 2, CA; 3, CON; 4, BSA.

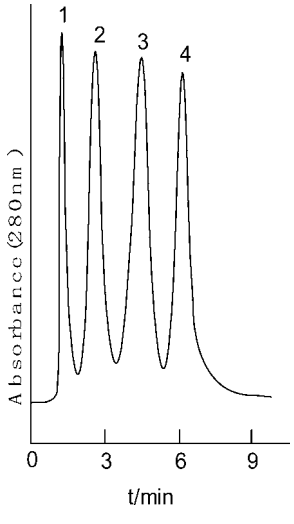

Figure 3. The effect of mobile phase flow rate on the retention of proteins. Except for the linear gradient in 6 min and flow rate of mobile phase being 4.0 mL/min, other conditions are the same as indicated in Fig. 2.

Table 1. Mass recovery of four proteins by using the synthesized WCX column[a]

Protein	Recovery (%)
Myoglobin	88.1 ± 4.0
Carbonic anhydrase	87.5 ± 3.5
Conalbumin	91.4 ± 2.6
Bovine serum albumin	94.2 ± 2.5

[a]The linear gradient elution was from 100% solution A (20 mmol/L of phosphate, pH 7.5) to 100% solution B (20 mmol/L of phosphate-1.0 mol/L NaCl, pH 7.5) at a flow rate of 1.0 mL/min for 25 min with a delay for 5 min. AUFS, 0.2, UV detection at 280 nm.

packings can be operated efficiently at high-rates. When carbonic anhydrase was used as a solute, the theoretical plate number (N) of the column obtained was more than 11,000/m at flow rate of 1.0 mL/min. This result is comparable to that for porous silica-based WAX column.[19]

The effect of hydrophobicity on the retention of biopolymers was investigated by adding 5% (*V*/*V*) 2-propanol into the mobile phase. It was found that compared with the absence of any organic solvent in the mobile phase, the retention of Myo, CA, and CON decreased by less than 4%, while that of BSA shortened by approximately 6%. This indicates that the hydrophobic interaction between protein and stationary phase is very weak, and that electrostatic interaction dominates the retention behavior of proteins, otherwise the retention of proteins would seriously decrease under the investigated conditions. The hydrophilicity of the resin was proven to increase greatly after the chemical modification. Therefore, the three dimensional structure of the separated protein molecules under these circumstances should not be changed.

The mass recoveries of the five proteins from the WAX column in three continuous individual measurements obtained are listed in Table 1. It is apparent that all mass recoveries are greater that 87%. The relative standard deviations of recoveries of the five proteins in three parallel tests are all less than ±5%. This result further shows that the surface of the synthesized WAX resin has very weak hydrophobicity, high mass recovery of proteins by use of the WAX column was obtained throughout this study.

Effect of Mobile Phase pH on the Protein Retention

As shown in Table 2, the pH value of the mobile phase has an important influence on the protein retention in WAX. The retention time of carbonic anhydrase increases gradually with increasing pH in the range of 5.0 ~ 9.0,

Table 2. Effect of pH of mobile phase on the protein retention[a]

pH	Myo	CA	CON	BSA
5.0	4.2	5.8	14.0	18.5
6.0	3.6	6.0	12.5	17.8
7.0	3.2	7.2	13.5	16.2
8.0	3.2	7.5	11.5	15.1
9.0	3.8	8.2	10.4	13.7

[a]Buffer, 20 mmol/L phosphate; linear gradient in 25 min from 0 to 1.0 mol/L NaCl, then at 1.0 mol/L NaCl for 5 min, pH (5.0–9.0), UV detection at 280 nm.

Myo: myoglobin; CA: carbonic anhydrase; CON: conalbumin; BSA: bovine serum albumin.

however, the retention time of conalbumin and bovine serum albumin decreases slightly with increasing pH, which is consistent with the phenomenon observed in the literature.[20] The elution order of these proteins is directly related to their isoelectric points (pI). A protein with a lower pI is retained longer, as expected in anion-exchange mode.

Stability and Reproducibility

After the WAX column was washed with 1,000 mL of 1.0 mol $\cdot$ L^{-1} NaOH and 1,000 mL of 0.5 mol $\cdot$ L^{-1} H_2SO_4, it was then tested by protein separation. The results showed that the column can be used from pH $1 \sim 14$ and its resolution does not change. When the WAX column was used repeatedly (up to sixty times) for the separation of biopolymers, its separation efficiency did not decrease.

Dynamic Capacity of the WAX Packings

The capacity of WAX packings for adsorption of BSA was determined by the dynamic method.[21] A 5.0×0.2 cm I. D. WAX column was used; the dynamic capacity of the column for BSA is 27.4 mg $\cdot$ g^{-1}.

Separation and Purification of rhG-CFS by the WAX Column

The human granulocyte colony stimulating factor (hG-CSF) stimulates granulocyte colony formation and affects the proliferation, differentiation,

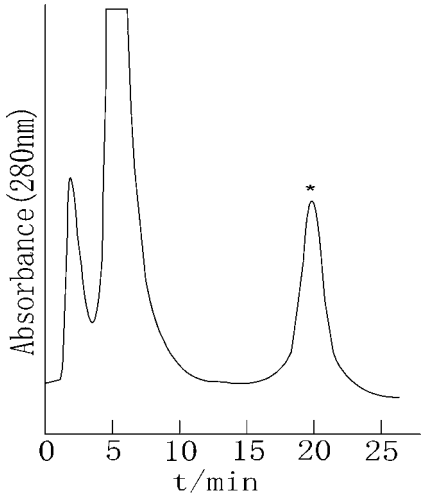

Figure 4. Chromatogram of the rhG-CFS extract solution by WAX mode on the prepared WAX column. All chromatographic conditions are the same as that indicated in Fig. 2. *rhG-CFS.

and activation of hematopoietic cells of the neutrophilic granulocyte lineage. It is a 19.6 kDa glycoprotein with 174 amino acids, and p*I* is 6.0. In this paper, the rhG-CFS was expressed in *Esherichia colin.*[22] The rhG-CFS extract solution was directly injected into the WAX column. Figure 4 shows the chromatogram of the purification of the rhG-CFS extract solution. In Figure 5, sodium dodecylsulfonate-PAGE analysis shows one main band of purified rhG-CFS from extract solution, and the purity of the purified rhG-CFS is more than 95% after a single step purification by the WAX column.

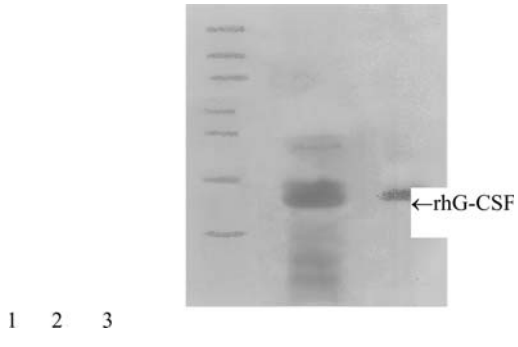

Figure 5. SDS-PAGE analysis of rhG-CFS extract solution. 1. Marker (14400, 20100, 31000, 43000, 66200, 97400 Da). 2. rhG-CFS extract solution. 3. purified rhG-CFS extract solution.

CONCLUSION

Based on the 8.0 μm monodisperse poly (glycidyl methacrylate-co-ethylene-dimethacrylate) with macroporous, one kind of WAX resin was synthesized by a new chemically modified method. Compared with silica based packings, the advantages of the synthesized WAX packings are: (1) it can be used over a wider pH range and also with a higher column loading, and as shown in Figure 2, resolution is comparable;[19] (2) it is more chemically and mechanically stable than dextran and agarose; (3) compared with PS-DVB based WAX packings, its resolution is better and its hydrophilicity greater; this results in higher mass recovery of proteins.[23]The WAX column was also used for the separation and purification of rhG-CFS from the coarse extract solution, satisfactory results were obtained.

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