Synthesis of Zwitterionic Stationary Phase Based on Hydrophilic Non-Porous Poly(glycidymethacrylate-*co*-ethylenedimethacrylate) Beads and their Application for Fast Separation of Proteins

Bolin Gong,[1,2] Chunmiao Bo,[1] Jinxia Zhu,[1] Chao Yan[2]

[1]*Key Laboratory of Energy and Chemical Engineering, Ningxia University, Yinchuan 750021, People's Republic of China*
[2]*Unimicro (Shanghai) Technologies Co., Ltd., Shanghai 201203, People's Republic of China*

Received 18 August 2007; accepted 17 September 2008
DOI 10.1002/app.29743
Published online 2 April 2009 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: A new hydrophilic strong/strong type zwitterionic stationary phase for high performance liquid chromatography (HPLC) was synthesized by chemical modification of 3.0 μm non-porous monodisperse poly(glycidylmethacrylate-*co*-ethylenedimethacrylate)($P_{GMA/EDMA}$) beads in the following steps. First, the beads were reacted with hydrochloride to obtain chlorizated beads; second, chlorizated beads were reacted with dimethylamine to obtain ammoniated beads; third, ammoniated beads were reacted with 1,3-propanesultone to obtain non-porous hydrophilic zwitterionic stationary phase. 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. The highest dynamic protein loading capacity of the synthesized zwitterionic packing for bovin serum albumin and Lys were 18.3 and 27.4 mg g^{-1}, respectively. The zwitterionic stationary phase was capable of separating two acidic and three basic proteins simultaneously in less than 2.5 min by the flow-rates of 3.0 mL min^{-1}. The zwitterionic resin was also used for rapid separation and purification of recombinant human interferon-r (rhIFN-r) and human granulocyte colony-stimulation factor (hG-CSF) from the crude extract solution. The satisfactory results were obtained.
 J Appl Polym Sci 113: 984–991, 2009

Key words: monodisperse non-porous polymeric beads; zwitterionic stationary phase; protein separation

INTRODUCTION

Zwitterionic separation materials are characterized by carrying both positive and negative charges on the material surface. Separation materials with zwitterionic functionalities have been developed for many years.[1] The zwitterionic functionality has attracted more interest in recent years, and many different zwitterionic stationary phases, including amino acids, "zwittergent" coated and covalently boned types, have been developed on the basis of silica and polymeric materials and their retention properties have been investigated.

The concept of the separation mode termed zwitterions-pair chromatography was introduced by Knox and Jurand[2] in the early 1981. Their technique makes use of long-chain amino acid (11-aminoundecanoic acid) as zwitterion-pair agents, which adsorb onto the stationary phase by hydrophobic interaction and form quadrupolar ion pairs with zwitterionic compounds in the mobile phase. Yu et al.[3,4] initially synthesized silica-based materials with covalently boned zwitterionic exchange sites of both weak/weak and strong/strong types, which could separate inorganic anions and cations simultaneously. In 1993, Hu et al.[5–8] reported a new form of ion chromatography in which a silica C_{18} column was permanently coated with a zwitterionic reagent and pure water served as the mobile phase for the separation of inorganic anions and cations. However, silica-based zwitterionic stationary phases are less stable under high conditions and exist high irreversible adsorption. In most cases, polymer-based packings can be employed for biopolymer separations even in the pH range from 1 to 14, and have a number of advantages such as their high ligand flexibility, which allows a maximum interaction between the molecule and the stationary phase and avoids denaturing of the protein. In 1999, Jiang and Irgum[9] synthesized a strong/strong type of zwitterionic exchange resin based on commercial Separon HEMA

Correspondence to: B. Gong (gongbl@nxu.edu.cn or gongbolin@163.com).
Contract grant sponsor: International Cooperation Foundation of Minister of Science and Technology; contract grant number: 2006DFA33690.
Contract grant sponsor: Program for New Century Excellent Talents in Chinese University; contract grant number: NCET-04-0986.

S300 particles for simultaneous separation of inorganic cations and anions. In 2002, Jiang and Irgum[10] prepared a strong/strong type of zwitterionic stationary phase by graft polymerization of the zwitterionic monomer of 3-[*N*,*N*-dimethyl-*N*-(methacryloyloxyethyl)ammonium] propanesulfon -ate onto the surface of porous silica particles and used for separation of inorganic cations, anions, and proteins. In 2004, Zou and coworkers[11] has reported a capillary electrochromatography (CEC) monolithic column with weak acid and weak base type of zwitterionic stationary phase, and used for separation of various types of polar compounds including phenols, anilines, and peptides.

Non-porous sorbents in the micron size range have gained great interest for the rapid separation of bimolecular, especially proteins, since the mid 1980.[12] Non-porous of small particle diameter have been gaining in interest in recent years for protein chromatography using high-performance liquid chromatography (HPLC).[13] The main advantage of such packing is that stagnant mobile-phase mass transfer, which leads to band broadening and a consequent loss of efficiency and resolution has been eliminated.[14] In a recent article,[15] we briefly described the preparation of 5.0 μm Non-porous monodisperse, hydrophilic poly(glycidylmethacrylate-*co*-ethylenedimethacrylate)($P_{GMA/EDMA}$) beads and chemical modification of the beads for preparation stationary phase of weak cation exchange (WCX) chromatography. In the following work,[16] we have prepared strong cation exchange (SCX) packing based on 3.0 μm non-porous hydrophilic $P_{GMA/EDMA}$ beads, and used it for rapid separation of biopolymers. In this article, we report the synthesis of a hydrophilic zwitterionic stationary phase for HPLC by using chemical modification of 3.0 μm non-porous monodisperse $P_{GMA/EDMA}$ beads. The chromatographic properties of the zwitterionic stationary phase for biopolymer separation are discussed in detail. The zwitterionic resin was also used for rapid separation and purification of recombinant human interferon-r (rhIFN-r) and human granulocyte colony-stimulation factor (hG-CSF) from the crude extract solution. The purity of the purified rhG-CFS and rhIFN-γ are more than 92.2% and 93.0%, respectively.

EXPERIMENTAL

Materials

Glycidyl methacrylate (GMA) (Aldrich) was distilled under vacuum. Ethylene dimethacrylate (EDMA) (Aldrich) was extracted three times with 10% sodium hydroxide aquoeous solution and distilled water. Azobisisobutyronitrile (AIBN), 1,3-propanesultone and dimethylaminne (33%) were bought from Shanghai Chemical Reagent Co. (Shanghai, China). Polyvinyl alcohol (PVA) and sodium dodecyl sulfonate (SDS) were obtained from Beijing Chemical Reagent Co. (Beijing, China). Myoglobin (Myo), ribonuclease A (RNase-A), α-chymotrypsinogen A (α-chy A), cytochrome C (Cyt-C), lysozyme (Lys), bovin serum albumin (BSA), β-Lactoglobulin (β-Lact), ovalbumin (OVA), and Insulin (Ins) were purchased from Sigma (St. Louis, MO). All chemical reagents were of analytical grade.

Instrumentation

All Chromatographic tests were carried out by using a LC-10A chromatographic system (Shimadzu, Japan) including two pumps and a multiple-wavelength UV detector. Samples were injected through a Rheodyne 7725 valve and detected at 280 nm.

FT-IR spectra of the packings were recorded on a Shimadzu FTIR-8400S (Japan) FT-IR spectrophotometer.

The elemental analyses were carried out by using a CHNS/O Analyzer (PE 2400II). The synthesized packings were burned in the pure oxygen to transform CO_2, H_2O, and SO_2, detected by heat conductivity detector. The concentrations of C, H, N, and S in the packings were obtained.

Preparation of uniform non-porous $P_{GMA/Edma}$ beads

The 3.0 μm non-porous monodisperse $P_{GMA/EDMA}$ beads were synthesized by us recently.[16] The 1.7 μm monodisperse polystyrene seed beads with low molecular weight were prepared by dispersion polymerization. First, the 1.7 μm polystyrene seed beads were dispersed in 0.1% SDS (w/w) aqueous solution. The mixture of GMA, EDMA, and AIBN initiator in terms of the total monomers were added into aqueous solution of 0.1% SDS (w/w) and 1.0% PVA (w/w) and then emulsified under ultrasonic condition until the size of oil drops became, at most of 0.5 μm. Then emulsion was sequentially added into the dispersion solution of the seed beads. The mixture was stirred for 4–6 h at room temperature so that all the emulsified organic phase was completely absorbed by the polymer seeds. The mixture was degassed by purging with nitrogen for 20 min. The polymerization was carried out at 70°C for 24 h with continuously stirring. The beads obtained were washed by hot water and methanol and then dried in air. The beads size is 3.0 μm; single point surface area is 10.26 $m^2\ g^{-1}$, and epoxide group is 0.36 mmol g^{-1}.

Preparation of strong/strong type zwitterionic stationary phase

Non-porous $P_{GMA/EDMA}$ beads (3.0 g of 3.0 μm) were suspended in 100 mL of 0.1 mol L^{-1} hydrochloride,

stirred and kept at 30°C for 2.5 h. After that, the beads were filtered, washed with water until neutral, and dried under vacuum condition, obtaining chlorizated beads.

Chlorizated beads (2.8 g) were placed in a 250-mL round-bottom flask to which 100 mL of 33% dimethylamine solution was added, and allowed to react under refluxing condition for 18 h at 60°C, then filtered, washed with water until neutral, dried under vacuum condition, obtaining ammoniated beads.

Dry ammoniated beads (2.5 g) were added into a 250-mL round-bottom flask, to which mixture containing 3.0 g of 1,3-propanesultone, 2.0 mL of nitrobenzene, and 100 mL of acetonitrice was added, heated to 80°C and allowed to react under refluxing and stirring conditions for 48 h. The beads were finally washed with ethanol, acetone and dried. Thus a new non-porous zwitterionic stationary phase was obtained. Figure 1 shows the chemical modification scheme for the preparation of the zwitterionic packings in this article. The "P" in the scheme donotes the polymer frame.

Packing the zwitterionic packings

The synthesized non-porous beads were packed into a stainless steel column using the slurry method with distilled water under 30 MPa. The size of the column was 5 cm × 0.46 cm I.D.

Mass recovery

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

Separation and purification of rhIFN-γ and hG-CSF by the zwitterionic column

The inclusion bodies of rhIFN-γ were disrupted with buffer consisting of 20.0 mmol L^{-1} phosphate + 1.0 mmol L^{-1} EDTA + 0.2 mg mL^{-1} Lys (pH = 7.4), then the inclusion bodies were washed three times. Finally, the inclusion bodies were 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 rhIFN-γ of was obtained by centrifuging it at 18,000 r min^{-1}. The method for disposal of inclusion bodies of rhG-CSF is same as that of rhIFN-γ.

The diluted rhIFN-γ or rhG-CSF solution was directly injected into the 5.0 cm × 0.46 cm I.D. zwitterionic column. Then the column was washed with a salt gradient and the fractions were collected and assayed.

RESULTS AND DISCUSSION

Chemical modification of the non-porous $P_{GMA/Edma}$ beads for the zwitterionic stationary phase

Many reactions can be used for the chemical modification of the epoxide groups existed on the surface of the 3.0 μm non-porous $P_{GMA/EDMA}$ beads. Figure 1 shows the reaction path designed for the preparation of zwitterionic packings includes the following three steps.

First, the beads were reacted with hydrochloride to obtain chlorizated beads. Second, chlorizated beads were reacted with dimethylamine solution to obtain ammoniated beads. Third, ammoniated beads were finally sulfopropylated in a quaternizing reaction with 1,3-propanesultone to obtained the designed zwitterionic stationary phase.

Figure 2 shows the IR spectra of the non-porous beads, ammoniated beads, and zwitterionic packings. Comparable with that of the non-porous $P_{GMA/EDMA}$ beads, the IR spectra of the ammoniated beads exhibited a clear adsorption peak at 2782 cm^{-1} corresponding to N—CH_3. The IR spectra of zwitteionic packings exhibited adsorption peak at 1037 and 1380 cm^{-1} corresponding to S=O and C—N bands, respectively, and the N% and S% determined by elemental analysis are 1.82 and 1.93, respectively. These results prove that the quaternary amine and sulfonic acid groups

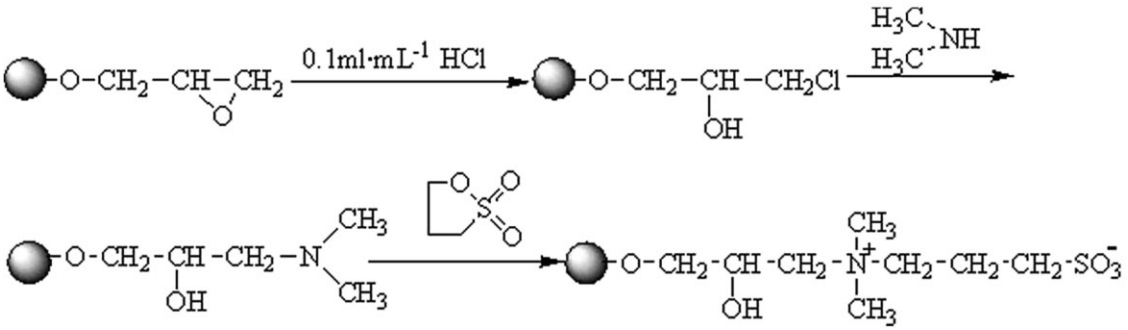

Figure 1 Chemical modification scheme for preparation of the zwitterionic packing.

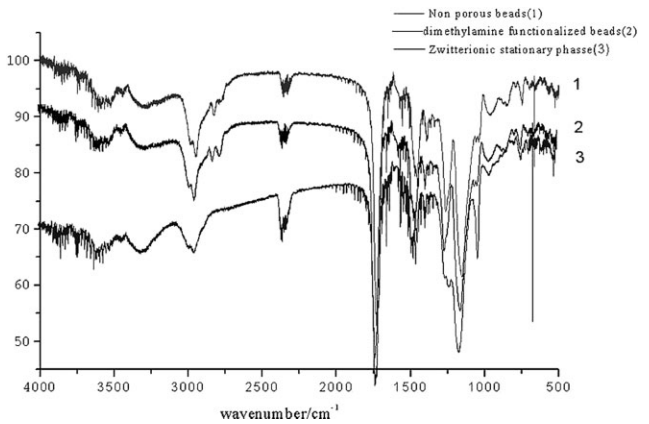

Figure 2 IR spectra of non-porous beads, dimethylamine functionalized beads, and zwitterionic packing.

were really bonded to the surface of the prepared polymer.

Column compressibility

With the use of non-porous packings, the backpressure-flow rate is one of the most important factors in column performance. It can be seen from Figure 3, the backpressure of the column with non-porous zwitterionic packings is only 25 MPa at a flow rate of 5.0 mL min^{-1}, which is lower than that on silicon-based packings,[18] and also shows good linearity. This indicates that the synthesized resins are monosized, which are very much favorable for fast separation.

Independent and simultaneous separation of acidic and basic proteins by the zwitterionic column

To compare the chromatographic properties of this newly synthesized zwitterionic packings with the cation-exchange packing, we carried out separation using chromatographic conditions (using linger gradient with increasing salt concentration in 20 mmol L^{-1} phosphate buffer) that were same to those used before.[15,16] Experiments were performed to examine the basic proteins of which the isoelectric points (p*I*) are more than 7.0, the protein mixture consisting of Myo (p*I* 7.3),α-Chy-A (p*I* 9.0), Cyt-C (p*I* 9.4), and Lys (p*I* 11.0). As Figure 4(a) shows that four basic proteins are completely separated within 8 min under linear gradient elution. Elution order of the proteins depends on their p*I*s, which accords with the cation-exchange retention mechanism. To test the anion exchange properties of the synthesized zwitterionic packings, experiments were also performed to examine the basic proteins of which the isoelectric points (p*I*) are less than 7.0, the proteins mixture consisting of OVA (p*I* 4.7), β-Lact (p*I* 5.1), and Ins (p*I* 5.3). As Figure 4(b) shows that three acidic proteins are completely separated within 6 min under linear gradient elution.

The most advantage of zwitterionic stationary phase is simultaneous separation of basic and acidic proteins. As Figure 4(c) shows, this zwitterionic column was achieved due to simultaneous separation of two acidic and three basic proteins in a single run, using gradient elution in 8 min, which is comparable with that of porous silica grafted polymer zwitterionic column.[10]

Effect of flow-rate on protein separation

Factors that influenced the efficiency of HPLC columns include eddy diffusion of mobile phase mass transfer between and within particles, and stationary phase mass transfer. The column efficiency is greatly improved by using non-porous particles, since intraparticulate diffusion, stagnant mobile phase mass transfer is eliminated. Consequently, separation on non-porous packing is characterized by very narrow bandwidths, and their efficiency is retained over a broader range of flow-rates. High speed is important in application where a large number of samples must be analyzed in a short period of time. In addition, short elution times are conducted to high protein recovery.[19]

In this article, the zwitterionic stationary phase was synthesized based on non-porous beads, so we investigated the effect of flow-rate on protein separation. Figure 5(a–c) shows the chromatograms of protein separation by the flow-rate of 1.0, 2.0, and 3.0 mL min^{-1}, respectively. In Figure 5(c), five standard proteins can be separated baseline in less than 2.5 min by the flow rate of 3.0 mL min^{-1}. In comparison with Figure 5(a) using the flow-rate of 1.0 mL min^{-1}, the column efficiency is almost the same. This indicated that the synthesized non-porous zwitterionic packing have the advantages of high performance and fast transfer, and provide superior resolution when high-rates and shorter gradient times were used.

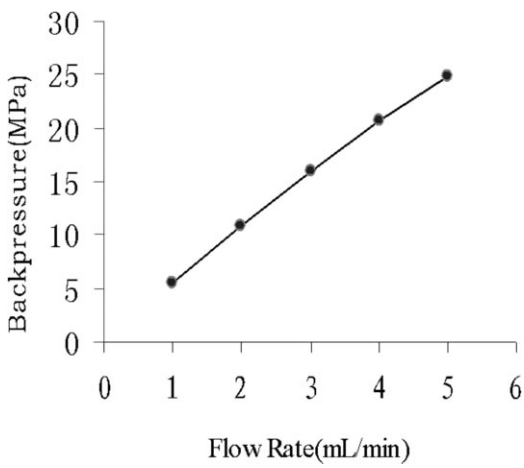

Figure 3 Effect of flow rate on column backpressure.

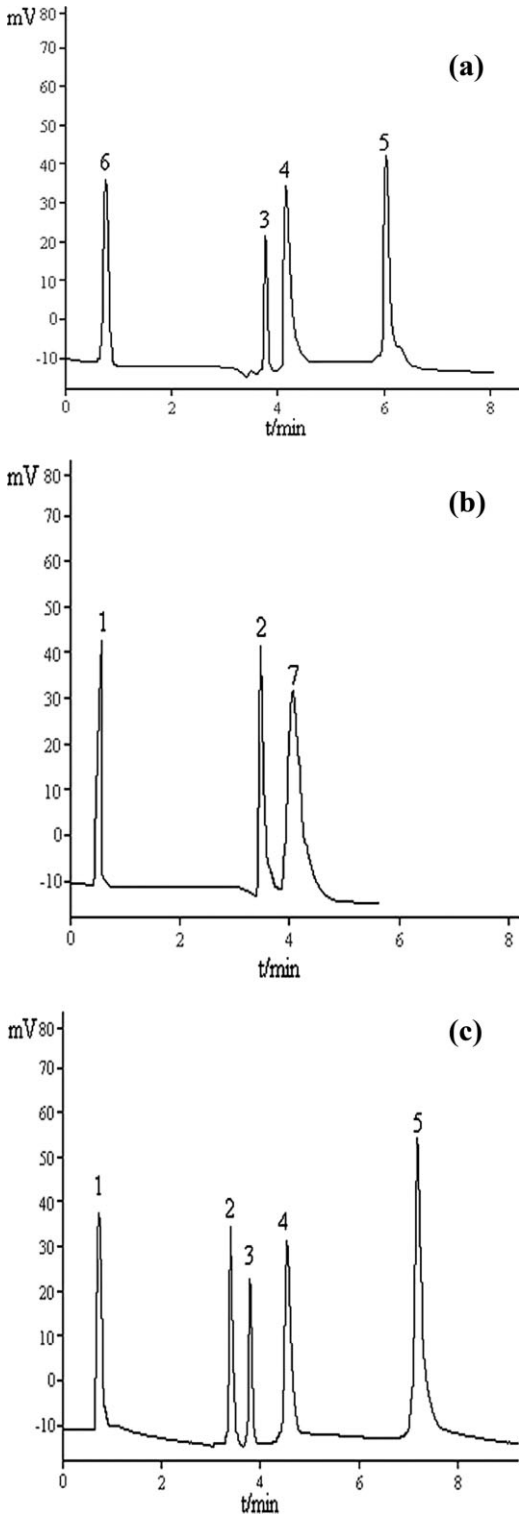

Figure 4 Chromatogram of independent and simultaneous separation of acidic and basic proteins by the zwitterionic column. (a) separation of basic proteins; (b) separation of acidic proteins; (c) simultaneous separation of acidic and basic proteins. The linear gradient elution was from 100% solution A (20 m*M* of phosphate, pH 5.5) to 100% solution B (20 m*M* of phosphate + 1.0 mol L^{-1} NaCl, pH 5.5) at a flow-rate of 1.0 mL min^{-1} for 10 min with a delay for 5 min. AUFS 0.08, UV detection at 280 nm. Proteins: 1, OVA; 2, β-Lact; 3, α-chy A; 4, Cyt-c; 5, Lys; 6, Myo; 7, Ins.

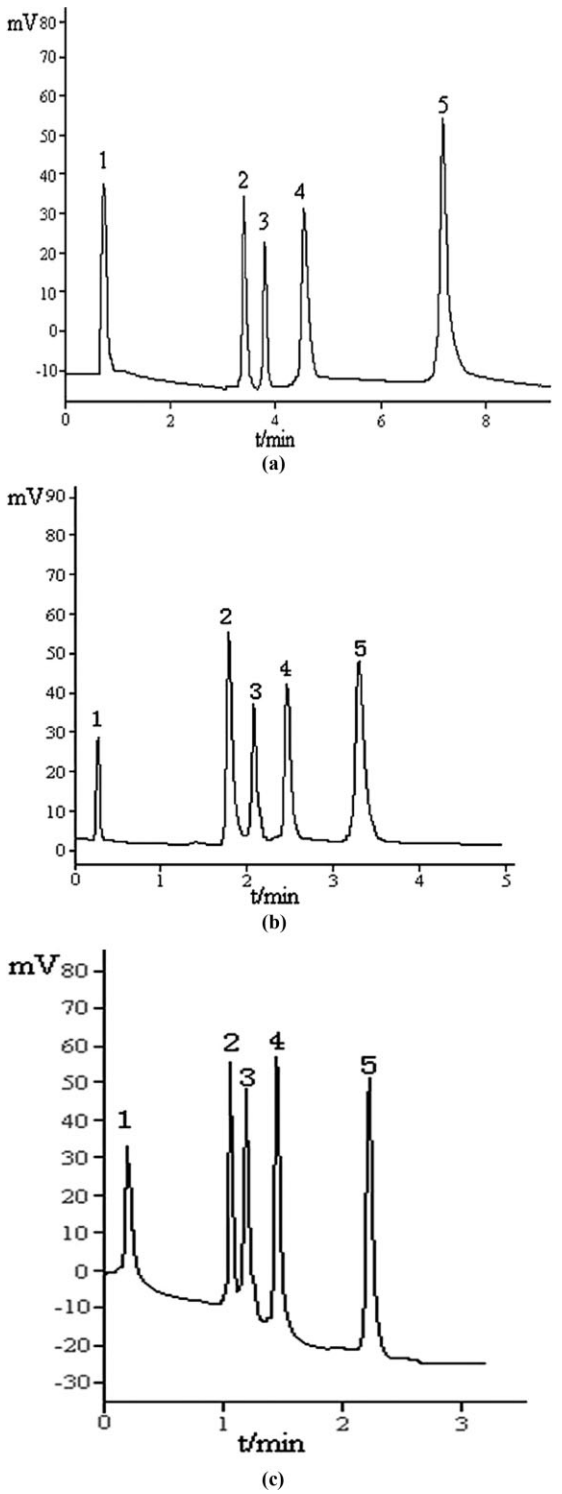

Figure 5 Chromatogram of five standard proteins separated by zwitterionic column by the different flow rates. (a) 1.0 mL min^{-1}, (b) 2.0 mL min^{-1}, (c) 3.0 mL min^{-1}. The linear gradient elution was from 100% solution A (20 mmol L^{-1} of phosphate, pH 5.5) to 100% solution B (20 mmol L^{-1} of phosphate + 1.0 mol L^{-1} NaCl, pH 5.5) at a flow-rate varied from 1.0 to 3.0 mL min^{-1} for 10–3.0 min with a delay for 5 min. AUFS 0.08, UV detection at 280 nm. Proteins: 1, OVA; 2, β-Lact; 3, α-chy A; 4, Cyt-C; 5, Lys.

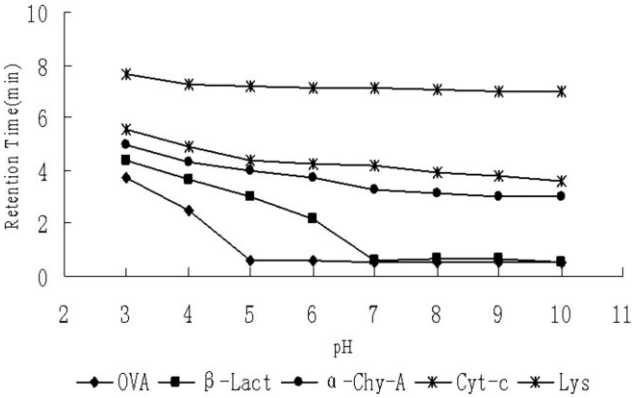

Figure 6 Effect of pH of mobile phase on the protein retention.

Effect of pH of mobile phase on the acidic and basic proteins retention

One of the more attractive features of separation proteins by ion exchange is the ability to predict chromatographic behavior with respect to the protein's isoelectric point (p*I*). As shown in Figure 6, the pH value of the mobile phase has an important influence on the two acidic proteins and three basic proteins retention in the zwitterionic column. The retention times of proteins decreased with increasing pH in the range of 3.0–10.0. OVA and β-Lact lacked retention at pH above 5 and 7, respectively, which is consistent with the phenomenon observed by Knut Irgum et al. in the literature.[10]

Effect of organic solvent on protein elution

Isopropanol (IPA) was used as an additive in the mobile phases to detect any hydrophobic adsorption between the solutes and the packing material. If hydrophobic interactions exist, adding a few percent IPA to the mobile phase should reduce the retention significantly.

In this article, the effect of hydrophobicity on the biopolymer retention was investigated by adding 5% (v/v) IPA into the mobile phase. Figure 7 shows that compared with the absence of any organic solvent in the mobile phase, the retention of two acidic proteins and three proteins was almost same with and without IPA added. This fact indicated that hydrophilicity of the zwitterionic stationary phase was increased greatly after the chemical modification.

Protein recovery

High yields in the separation of proteins is an essential requirement for industrial downstream processing. The mass recoveries of five proteins with three continuous individual measurements obtained from the zwitterionic column are listed in Table I. It is apparent that all mass recoveries are greater that 95%. The relative standard deviations of recoveries of the five proteins in three parallel tests are all less than ± 4%. This result further shows that a high mass recovery of proteins by using the zwitterionic column was obtained in this study.

Dynamic capacities the zwitterionic column

Breakthrough curves provide valuable information for evaluation of the dynamic binding capacity of the separation medium. The capacities of the zwitterionic packings for adsorption of acidic protein of BSA and the basic protein of Ly was determined by the dynamic method.[20] A 5.0 cm × 0.46 cm I. D. zwitterionic column was used; the dynamic capacities of the column for BSA and Lys were 18.3 mg g^{-1} and 27.4 mg g^{-1}, respectively. The capacities are much higher than those of common non-porous cation column for Lys[15] and small pore anion exchange column for BSA.[21]

Stability and reproducibility

After the zwitterionic column was washed with 1000 mL of 1.0 mol L^{-1} NaOH and 1000 mL of

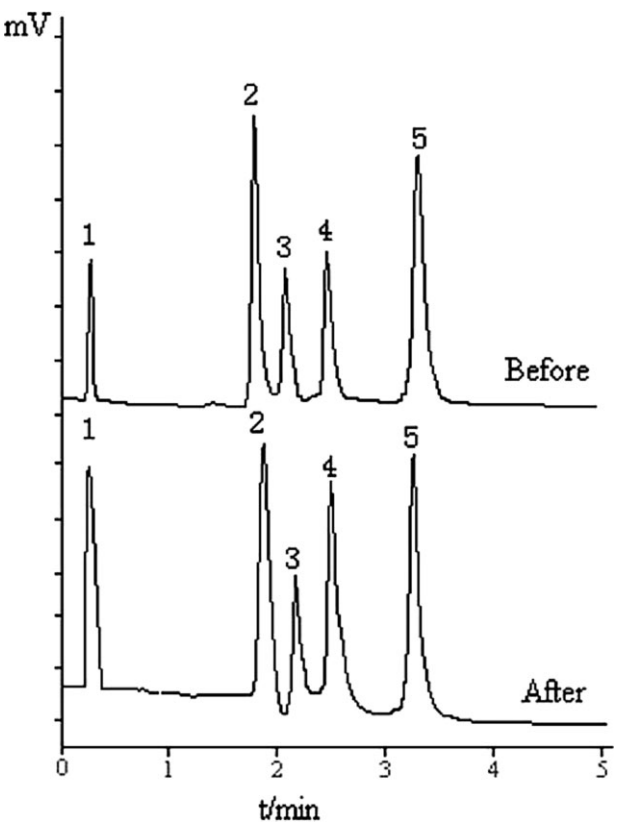

Figure 7 Effect of organic solvent in the mobile phase on protein elution. Before: IPA was without added both to solution A and B. After: 5% IPA was added to both solution A and B. Flow-rate: 2 mL min^{-1}; other chromatographic conditions and proteins are the same as indicated in Figure 4.

TABLE I
Mass Recovery of Four Proteins by Using the Synthesized Zwitterionic Column[a]

Proteins	Recovery (%)
OVA	95.9 ± 3.8
β-Lact	95.2 ± 3.3
α-chy-A	96.8 ± 2.8
Cyt-C	96.3 ± 2.4
Lys	97.3 ± 2.6

[a] The linear gradient elution was from 100% solution A (20 mmol L^{-1} of phosphate, pH 5.5) to 100% solution B (20 mmol L^{-1} of phosphate + 1.0 mol L^{-1} NaCl, pH 5.5) at a flow rate of 1.0 mL min^{-1} for 25 min with a delay for 5 min. AUFS 0.2, UV detection at 280 nm.

0.5 mol L^{-1} H_2SO_4, and then tested by proteins separation. The results showed that the column can be used from pH 1 to 14 and its resolution does not change. When the zwitterionic column was used repeatedly (up to 60 times) for the separation of biopolymers, its separation efficiency did not find decrease. After 6 months, the zwitterionic column was again used for protein separation. We found that there was no significant difference in retention times or resolution from 6 months ago.

APPLICATION

Separation and purification of rhG-CSF and rhIFN-γ by the zwitterionic column

The human granulocyte colony-stimulating factor (hG-CSF) is a 19.6 kDa glycoprotein with 174 amino acids, and p*I* is 6.0. The human interferon-r is a 17 kDa glycoprotein with 143 amino acids, and p*I* is 9.0. In this article, both the rhG-CFS and rhIFN-γ were expressed in *Esherichia coli*. Figure 8 shows the chromatogram of the purification of the rhIFN-γ course extract solution. SDS-PAGE analysis shows one main band of purified rhIFN-γ extract, the purity of the purified rhIFN-γ is more than 93% after a single-step purification by the zwitterionic column. Figure 9 shows the chromatogram of the purification of the rhG-CFS extract solution. The purity of the purified rhG-CFS is more than 92.2%.

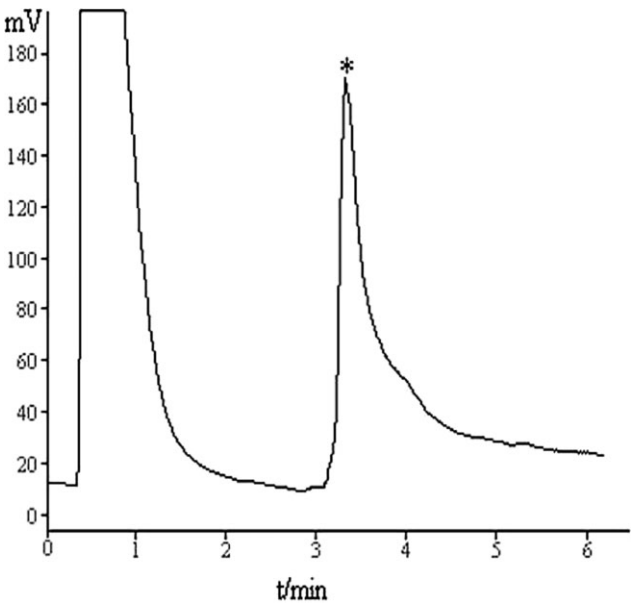

Figure 8 Chromatogram for purification of rhIFN-γ from extract solution by the zwitterionic column, *rhIFN-γ.

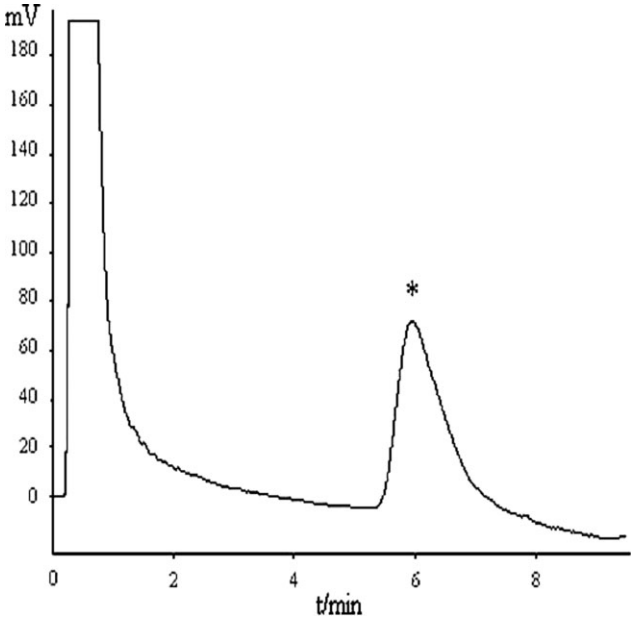

Figure 9 Chromatogram for purification of hG-CSF from extract solution by the zwitterionic column, *hG-CSF.

CONCLUSIONS

A new zwitterionic stationary phase based on 3.0 μm non-porous $P_{GMA/EDMA}$ beads was successfully synthesized in this work. It was capable of separating acidic and basic proteins independently and simultaneously in a single run using gradient elution. A better separation, sharpness of the peak shade and fast separation of five proteins indicated the synthesized zwitterionic stationary phase have higher chromatographic performance.

Compared with the WCX column[15] and SCX column,[16] which were reported previously by using the 5.0 μm beads and the 3.0 μm beads, advantages of the synthesized zwitterionic column are: (1) the zwitterionic column can be used for simultaneous separation of three basic and two acidic proteins; (2) the separation time is much shorter than the WCX column under the same flow-rate. Compared with silica-based packings,[10] it can be used over wider pH range and also with a higher column loading; its

hydrophilicity greater, this results in higher mass recovery of proteins. The zwitterionic resin was firstly used for the rapid separation and purification of rhIFN-γ and rhG-CFS from course extract solution to obtain a satisfactory results.

References

1. Nesterenko, P. N.; Hadded, P. R. Anal Sci 2000, 16, 565.
2. Knox, J. H.; Jurand, J. J Chromatogr 1981, 203, 85.
3. Yu, L. W.; Floyd, T. R.; Hartwick, R. A. J Chromatogr Sci 1986, 24, 177.
4. Yu, L. W.; Hartwick, R. A. J Chromatogr Sci 1989, 27, 176.
5. Hu, W. Z.; Takeuchi, T.; Haraguchi, H. Anal Chem 1993, 65, 2204.
6. Hu, W. Z.; Tao, H.; Haraguchi, H. Anal Chem 1994, 65, 2514.
7. Hu, W. Z.; Tominaga, M.; Cao, S. A.; Miyazaki, A. Anal Chim Acta 1994, 299, 249.
8. Hu, W. Z.; Tanaka, K.; Haddad, P. R.; Hasebe, K. J Chromatogr A 2000, 884, 161.
9. Jiang, W.; Irgum, K. Anal Chem 1999, 71, 333.
10. Jiang, W.; Irgum, K. Anal Chem 2002, 74, 4682.
11. Fu, H. J.; Xie, C. H.; Dong, J.; Zou, H. F. Anal Chem 2004, 76, 4866.
12. Chen, C. H.; Lee, W. C. J Chromatogr A 2001, 921, 31.
13. Lee, W. C.; Lin, C. H.; Ruaa, R. C.; Hsu, K. Y. J Chromatogr A 1995, 704, 307.
14. Round, M. A.; Regnier, F. E. J Chromatogr 1988, 443, 73.
15. Gong, B. L.; Zhu, J. X.; Li, L.; Qiang, K. J.; Ren, L. Talanta 2006, 68, 666.
16. Zhu, J. X.; Bo, C. M.; Gong, B. L. Chin J Chromatogr (in Chinese) 2006, 24, 129.
17. Bradford, M. M. Anal Biochem 1983, 133, 251.
18. Gao, W. H.; Yang, G. L.; Yang, J.; Liu, H. Y.; Yin, J. F.; Chen, Y. Chin J Anal Chem (in Chinese) 2004, 32, 295.
19. Kato, Y.; Kitamura, T.; Nakatani, S.; Hashimoto, T. J Chromatogr 1989, 483, 401.
20. Mihelic, I.; Koloini, T.; Podgornik, A.; Strancar, A. J High Resolut Chromatogr 2000, 23, 39.
21. Lloyd, L. L. J Chromatogr 1991, 544, 201.