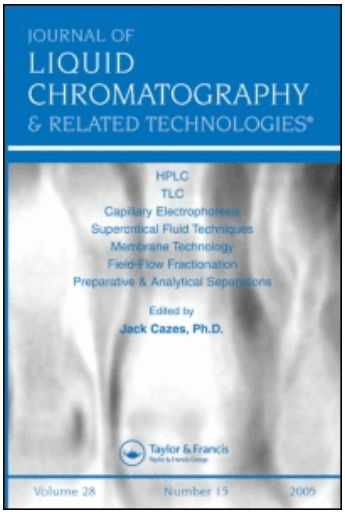

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Bolin Gong[a]; Yuehua Shen[a]; Xindu Geng[a]
[a] Institute of Modern Separation Science, Key Laboratory of Modern Separation Science, Shannxi Province, Northwest University, Xi'an, P.R. China

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Preparation of Strong Cation Exchange Packings Based on Monodisperse Poly(Glycidyl Methacrylate-co-Ethylenedimethacrylate) Particles and Their Application

Bolin Gong, Yuehua Shen, and Xindu Geng*

Institute of Modern Separation Science, Key Laboratory of Modern Separation Science, Shannxi Province, Northwest University, Xi'an, P.R. China

ABSTRACT

Monodisperse macroporous poly(glycidyl methacrylate-co-ethylenedimethacrylate) ($P_{GMA/EDMA}$) particles were synthesized by a single-step swelling and polymerization method. Based on this medium, a strong cation ion exchange resin was prepared by a new chemically modified method. It is demonstrated that the prepared resin in this study has advantages for biopolymer separation, high column efficiency, low column back pressure, and good resolution for proteins. The bioactivity recovery for lysozyme (Lys) with the resin was measured to be $102 \pm 5\%$.

*Correspondence: Xindu Geng, Institute of Modern Separation Science, Key Laboratory of Modern Separation Science, Shannxi Province, Northwest University, Xi'an 710069, P.R. China; E-mail: xdgeng@nwu.edu.cn.

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The resin was also used for the separation and purification of recombinant human interferon-γ (rhIFN-γ) to raise its purity, in the coarse extract containing 0.2 mol/L guanidine hydrochloride, from 36% to 93%.

Key Words: Monodisperse poly(glycidyl methacrylate-co-ethylene-dimethacrylate) particles; Strong cation exchange chromatography; Resin; Protein separation; Interferon-γ.

INTRODUCTION

Because silica-based packings are less stable under high pH conditions, they cannot, sometimes, satisfy the requirement for the separation of biopolymers, this led us to the development of various polymer-based resins, or packings, in high performance liquid chromatography (HPLC). In most cases, polymer-based packings can be employed for biopolymer separations even in the pH range from 1 to 14. Synthetic polymers were introduced into liquid chromatography in 1964;[1] they were mainly based on the styrene-co-divinylbenzene (PS-DVB) copolymers which are usually prepared by means of suspension polymerization. Polymer particles obtained in this way, followed by labor-intensive size classification, are never uniform in terms of size distribution.

Ugelstad et al.[2] developed a method for the preparation of uniformly sized beads, termed "activated multi-step swelling and polymerization", in 1979. Uniform particles are prepared by Ugelstad's method from a great variety of monomers, such as styrene,[3] methylmethacrylate,[4] 2-hydroxyethyl methacrylate,[5] glycidyl methacrylate,[6,7] vinylphenol,[8] and chloromethylstyrene.[9] This method is an excellent one, but it seems rather complex since, at least two-steps are needed in the swelling process; the first step is for the seed particles to be activated by the absorption of a water-insoluble compound, and a subsequent step is for the absorption of monomer, cross-linker, and diluent.

Ogino et al.[10] reported a preparation of the uniform PS-DVB particles by a single-step swelling and polymerization method in 1995. However, due to PS-DVS having strong hydrophobicity, the subsequent chemical modification of PS-DVS for protein separation is difficult.

In this paper, we present a preparation of poly(glycidyl methacrylate-co-ethylenedimethacrylate) ($P_{GMA/EDMA}$) particles by a single-step swelling and polymerization method, and a new chemically modified method for the preparation of strong cation exchange (SCX) packings. The SCX packings were tested by separating biopolymers and recombinant human interferon-γ (rhIFN-γ); a satisfactory result was obtained.

EXPERIMENTAL

Equipment and Materials

All of the chromatographic experiments were carried out with a Shimadzu LC-6A HPLC system (Shimadzu Co., Kyoto, Japan) consisting of two HPLC pumps, SPD-6AV UV–VIS spectrophotometric detector, SCL-6A system controller, and C-R3A Chromatopac.

Glycidyl methacrylate (GMA) (Aldrich, USA) was distilled under vacuum. Ethylene dimethacrylate (EDMA) (Aldrich, USA) was extracted three times with a 10% aqueous solution of sodium hydroxide and distilled water, and then dried over anhydrous magnesium sulfate. Poly(vinylpyrrolidone, k-30) (PVP, k-30) was purchased from Aldrich (USA). Azobisisobutyronitrile (AIBN), dibutyl phthalate, and cyclohexanol were purchased from Shanghai Chemical Reagent Co. Ltd. (Shanghai, China). Polyvinyl alcohol (PVA) and sodium dodecyl sulfonate (SDS) were obtained from Beijing Chemical Reagent Co. Ltd. (Beijing, China). Benzyl peroxide (BPO) was obtained from Xi'an Chemical Reagent Co. Ltd. (Xi'an, China). All chemicals were purified by normal standard methods.

Lysozyme (chicken egg white, Lys), ribonuclease A (bovine pancreatic, RNase-A), myoglobin (horse skeletal muscle, Myo), α-chymotrypsinogen A (bovine pancreatic, α-Chy-A), lactoperoxidase (Lac) and cytochrome C (horse heart, Cyt-C) were purchased from Sigma, (St. Louis, USA).

Dispersion Polymerization

According to the method reported by Pain, et al.[11] monodisperse polystyrene seed particles of low molecular weight were prepared by dispersion polymerization of styrene in alcohol medium in the presence of the inhibitor, AIBN, and the stabilizer, PVP, under a nitrogen atmosphere. After centrifugal separation, the seed particles obtained were dispersed in an aqueous solution containing 1.0% (w/w) PVA and made the content to be 0.1 g/mL. The size of the prepared particles was measured to be 3.5 μm by scanning electron microscopy. Table 1 shows the conditions for preparing the seed particles.

Preparation of Uniform Porous Beads

Exactly 1.0 g dispersed seed and 60 mL of 1.0% PVA (w/w) of aqueous solution were placed in a 500-mL flask and slowly stirred by a mechanical stirrer. Then, 30 g of the mixture, consisting of 6.3 g glycidyl methacrylate, 4.2 g ethylene dimethacrylate, 14.0 g cyclohexanol, 5.5 g dibutyl phthalate,

Table 1. The conditions for preparing the seed of polystyrene particles.[a]

Substances	Concentrations,[b] % (w/w)	Amount (g)
Styrene	20%	5.0
Alcohol	80%	20.0
PVP, k-30	2%	0.5

[a]Polymerization temperature, 70°C; polymerization time, 24 h; initiator, AIBN; 0.15 g (3%, w/w, based on styrene).
[b]Based on total recipe (25 g).

and 2% (w/w) (based on the total monomers) of AIBN initiator were added to 300 mL of an aqueous solution of 0.1% (w/w) SDS and 1.0% (w/w) PVA and then emulsified under ultrasonic conditions until the size of the oil drops became, at most, 0.5 μm (observed by optical microscope). The emulsion was sequentially added into the dispersion solution of the seed particles. The mixture was stirred for 6–10 h at room temperature so that all the emulsified organic phase was absorbed by the polymer seeds. The whole process was monitored by an optical microscope until the organic liquid drops completely disappeared. 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 with water and methanol; the beads were then extracted with toluene for 48 h in a Soxhlet apparatus to remove the porogens. The beads were washed with methanol again and dried in air.

Modification of the Particles of Strong Cation Exchange Medium

Exactly 3.0 g of particles of $P_{GMA/EDMA}$ were suspended in 60 mL of 0.1 mol/L sulfuric acid, stirred and kept at 60°C for 10 h. After that, the particles were filtered, washed with water until neutral, and dried under vacuum condition, yielding the hydrolyzed particles.

Then, 3.0 g hydrolyzed particles were put into a 100-mL beaker, and 25 mL 1.4-dioxane, 7.0 mL epichlorohydrin, 0.5 mL boron trifluoride-ethyl complex were added, stirred, and kept at 80°C for 3.0 h. After the particles were washed with ethanol and acetone, 80 mL of a 10% aqueous solution of sodium hydrogen sulfite were added and allowed to react for 24 h at 80°C. The

particles were finally washed with water and dried. Thus, a new medium of strong cation ion exchange packings was obtained.

Characterization of Polymeric Particles

The particle size, surface morphology, specific surface area, and pore distribution of the synthesized $P_{GMA/EDMA}$ resins were measured by scanning electron microscopy and mercury intrusion methods, respectively. The pore structure of the resins was also characterized by gel permeation chromatography (GPC). The $P_{GMA/EDMA}$ particles were packed into a stainless steel columns by a slurry method with ethanol. The GPC experiments were carried out in an HPLC instrument with tetrahydrofuran (THF) as eluent and various polystyrenes as molecular weight standards, with detection at 254 nm at room temperature.

Determination of Epoxy Groups

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

Determination of the Activity of Lysozyme

The bioactivity of Lys was determined by following the decrease in absorbance at 450 nm of a 0.25 mg/mL *Micrococcus lysodeikticus* suspension in 0.067 mol/L phosphate buffer, pH 6.2.[12]

RESULTS AND DISCUSSION

Preparation of Monodisperse Porous Beads

Because of good miscibility of both solvents (cyclohexanol and dibutyl phthalate) with the monomers, the mixture of the two solvents was chosen as the porogen diluents for the preparation of $P_{GMA/EDMA}$ resins in this study. As is well known, the porous structure of the resins can be controlled by adjusting the proportion of the porogen in the organic phase. In order to increase the content of the epoxide groups in the polymer which are necessary for the subsequent chemical modification, a high percentage of GMA must be used. The ratios of monomer to porogen (35/65, v/v) and GMA to EDMA (60/40, v/v) were selected for this research. Such proportions not only provide the resins with macroporous and high mechanical properties, which is required for

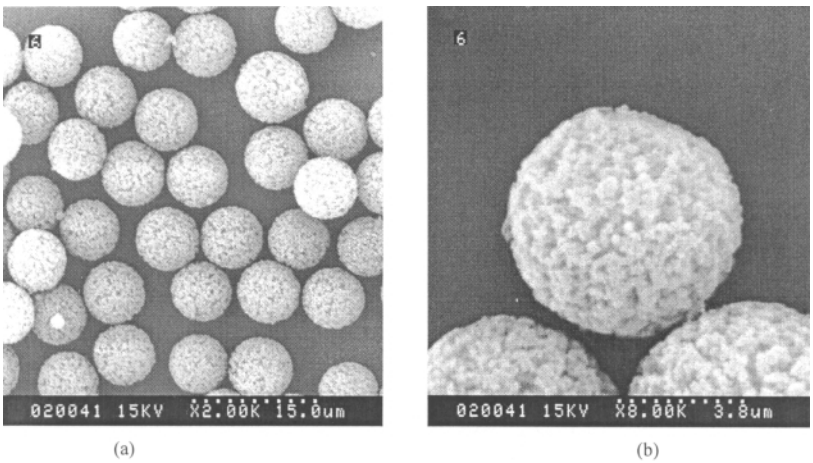
protein separation by HPLC, but also yield a resin with quite a high content of epoxide groups.

The size of the final particles was well controlled by the seed diameter and the amount of organic phase. As organic phase consisting of GMA, EDMA, and diluents is fully absorbed by the seeds in an effective swelling range, the final particle diameter could be calculated according to the following simple equation:[13]

$$\log D = \log d + \frac{1}{3}\log \frac{M+m}{m} \tag{1}$$

where d and D are the diameters of the seeds and the final particles, respectively, M and m are the amounts of organic phase and the seeds, respectively. The value of $(M+m)/m$ is the swelling multiple. The experimental results showed that, when 3.5 μm of seed particles were used and the swelling multiple was controlled to 50, a series of monosized $P_{GMA/EDMA}$ resins with particle diameters in the range of 6–12 μm could be obtained.

Figure 1(a), (b) shows scanning electron micrographs of the prepared particles; they indicate that the prepared beads in this study are uniform in size and have a macroporous structure.



(a) (b)

Figure 1. Scanning electron micrographs of the monosized porous beads (a) and their surface structures (b).

Physical Properties of the Particles

Figure 2 shows the pore size distribution of the synthesized resin in dry state, which was measured by a mercury intrusion method. It is obvious that macroporous (diameter > 30 nm) and super-macroporous (diameter > 100 nm) structures account for most of the total pores within the porous beads. Table 2 lists the properties of the particles. The volumes of porogen diluents used account for about 65% of the total organic phase. This means that the expected porosity and pore volume of the final particles obtained should be approximately 65% and 1.84 mL/g, respectively. The value of the pore volume in Table 2 is very close to the calculated one.

Figure 3 shows that the dependence of the back pressure of the packed column of the synthesized resin upon the flow rate is directly proportional to the flow rate of the mobile phase in the range from 1.0 mL/min to 4.0 mL/min, and the backpressure is only about 3.5 MPa at the flow rate of 4.0 mL/min. This result demonstrates that the beads possess high permeability, which is very favorable to the chromatographic applications.

Chemical Modification of Poly(Glycidyl Methacrylate-co-Ethylenedimethacrylate) Particles

Because of the strong hydrophobicity of the surface of the macroporous $P_{GMA/EDMA}$ resin, it is difficult for it to be employed for the separation of biopolymers. With the chemical modification of the hydrophobic surface by using a hydrophilic reagent, the irreversible adsorption onto the surfaces of the particles and changes in the molecular conformation of protein could be avoided or, at least, diminished.

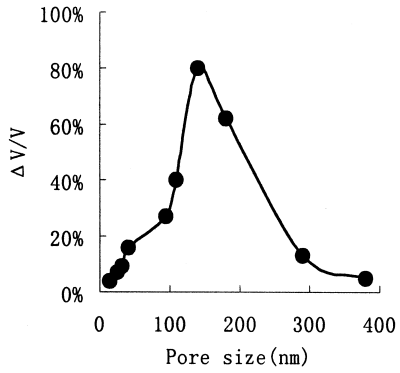


Figure 2. Pore size distribution of a $P_{GMA/EDMA}$ resin.

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Table 2. Properties of porous $P_{GMA/EDMA}$ beads.

Particle size, μm	8.0
Epoxide groups, mmol/g	2.8
Specific pore volume, mL/g[a]	1.74
Median pore diameter of GPC, nm[a]	89.1
Median pore diameter of mercury porosimetry, nm	111.3
Polystyrene exclusion limit, MW[a]	1.8×10^6

[a]According to the GPC.

Many reactions can be used for the chemical modification of the epoxide groups existing on the surface of the $P_{GMA/EDMA}$ resin. Figure 4 shows the reaction path designed for the preparation of SCX packings; it includes the following three steps.

First, the epoxide groups of the $P_{GMA/EDMA}$ particles completely hydrolyzed and were converted to diol particles. Second, with the reaction of the hydroxyl groups of diol particles and epichlorohydrin, catalyzed by boron trifluoride–ethyl complex, particles with higher hydrophilicity were obtained. Third, the particles reacted with sodium hydrogen sulfite to obtain the designed SCX resin.

Elemental analysis revealed the sulfur content to be 1.42%. The IR spectrum of the modified polymer exhibited a large broad adsorption peak at 3433 cm^{-1}, corresponding to hydroxyl. These results prove that the sulfonic acid groups were really bonded to the surface of the prepared polymer.

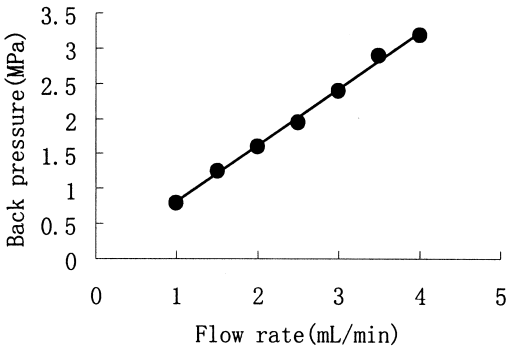

Figure 3. Effect of flow rate on column back-pressure. Column: 100 × 8 mm I.D.; Mobile phase: 0.02 mol/L sodium phosphate buffer (pH 6.5).

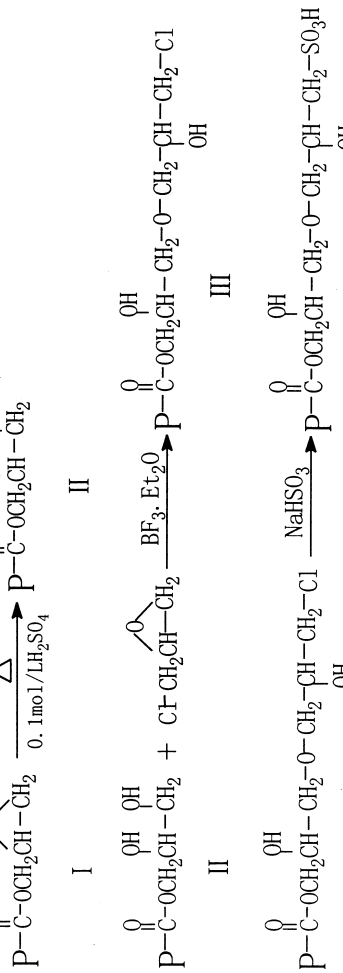

Figure 4. The chemical modification scheme for preparation of the SCX packing.

Separation of Biopolymer by Strong Cation Exchange

Figure 5 shows, as an example, the chromatogram of the separation of six proteins on the SCX column. The better separation and sharpness of the peak shapes, and fast separation, indicate that the synthesized SCX resin in this study is much better than the one prepared from PS-DVB reported in the literature.[14,15]

The effect of hydrophobicity on biopolymer retention 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 times of Myo, RNase-A, α-Chy-A, Lac, and Lys decreased less than 5%. This fact indicates that the hydrophobic interaction between protein and stationary phase is very low, and electrostatic interaction dominates the retention behavior of the proteins. The hydrophilicity of the resin is proved to increase greatly after the chemical modification. Therefore, the three dimensional structure of the separated protein molecules in the circumstance should not be changed. This point was also proved by the high bioactive recovery, e.g., 102 ± 5% for Lys. With three continuous individual measurements obtained from the SCX column, the average mass recoveries of five proteins are listed in Table 3. It is seen that all mass recoveries are greater

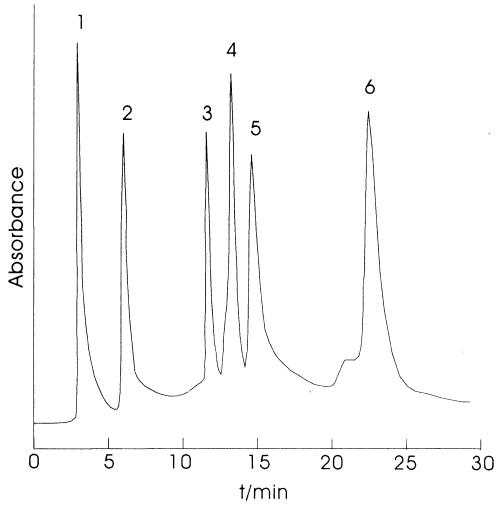

Figure 5. Chromatogram of proteins by SCX mode on the prepared column (100 × 8.0 mm I.D.). Linear gradient elution from 100% solution of 10 mmol/L of phosphate, pH 6.5, to 100% solution of 1.0 mol/L NaCl at a flow rate of 1.0 mol/L NaCl for 30 min with a delay for 5 min; 0.08 A.U.F.S.; detection wavelength 280 nm. Proteins: 1, solvent + α-Amy; 2, Myo; 3, RNase-A; 4, α-Chy-A; 5, Lac; 6, Lys.

Table 3. The average mass recovery of five proteins by using the synthesized SCX column in this study.

Protein	Recovery (%)
Myo	89 ± 2.5
RNase-A	92 ± 2.3
α-Chy-A	91 ± 2.1
Lac	94 ± 1.8
Lys	97 ± 1.6

than 85%. The relative standard deviations of recoveries of five proteins in three parallel tests are all less than ±3%. This result shows that protein can obtain high mass recovery by using the SCX column synthesized in this study.

Effect of Column Loading on the Resolution of Ribonuclease A and Lactoperoxidase

Column loading and its effect on the separation of biopolymers is an important parameter needed to evaluate the goodness of a column. It is especially significant for its applications on a preparative scale. High resolution is necessary for the goodness of the separation of small molecular solutes under isocratic elution. The separation of biopolymers is usually by gradient elution and elucidated by peak capacity.[16] However, peak loading is difficult to be employed to elucidate the changes in the effect of column loading on the resolution of biopolymers. In this study, we especially used the usual concept of resolution to elucidate the effect on the goodness of the separation of RNase-A and lactoperoxidase under the influence of a linear gradient. Figure 6 shows the effect of the injected amout of RNAse-A and Lac on the resolution of both by a linear gradient elution. It could be seen that the resolution decreased as the injected amount increased. This is a normal phenomenon in HPLC. The resolution of RNase-A and Lac at the injected amount of 1.8 mg for each decreased nearly to one-half that observed using 0.05 mg of each.

Separation and Purification of Combinant Human Interferon-γ by Strong Cation Exchange

The diluted rhIFN-γ solution was obtained by diluting the crude rhIFN-γ extract of 7.0 mol/L guanidine hydrochloride (Gu·HCl).[17] The diluted rhIFN-γ suspension was allowed to stand overnight and then separated from

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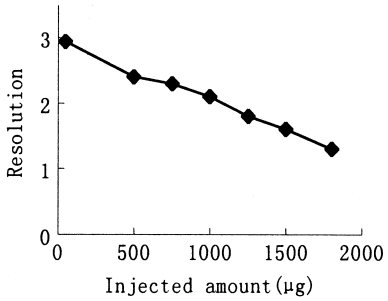

Figure 6. Effect of column loading on the resolution of RNase-A and Lac, except that the solution of 20 mmol/L of phosphate, pH 6.5, was replaced with 10 mmol/L phasphate; other chromatographic conditions are the same as those shown in Fig. 5.

precipitate by centrifugation at 14,000 rpm for 15 min at 4°C. The supernatant only contains about 36% rhIFN-γ. The diluted rhIFN-γ solution was directly injected into the SCX column. Figure 7 shows the chromatogram of the separation and purification of the diluted rhIFN-γ extract solution. Sodium dodecyl sulfonate–PAGE analysis shows one main band of purified

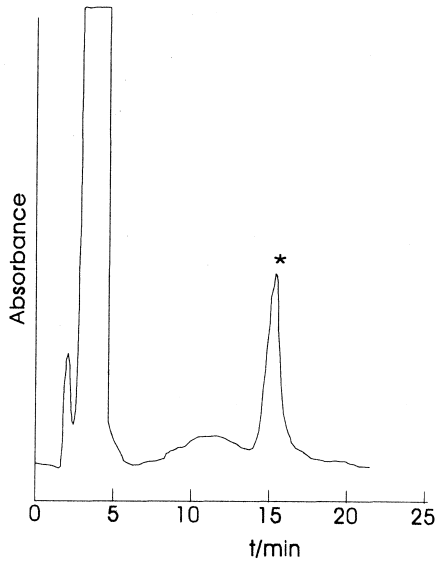

Figure 7. Chromatogram of the diluted rhIFN-γ extract by SCX mode on the prepared column. All chromatographic conditions are the same as those shown in Fig. 6.

rhIFN-γ extract and the purity of the purified rhIFN-γ is more than 93.0% with only one step during 25 min.

CONCLUSION

The monodisperse macroporous poly(glycidyl methacrylate-co-ethylene-dimethacrylate) resins were synthesized by a single-step swelling and polymerization method. The physical properties of the resin were measured and discussed in detail. The results show that the particles have uniform particle size, strong particle rigidity, and the desired macroporosity. Based on this medium, one kind of strong cation exchange resin was synthesized by a new chemically modified method.

The SCX packings exhibit an excellent separations for typical proteins. The column efficiency achieved on the polymer-based SCX column is comparable to that of silica-based packing materials.[18] The SCX column was also used for the separation and purification of the diluted rhIFN-γ extract solution with satisfactory results.

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