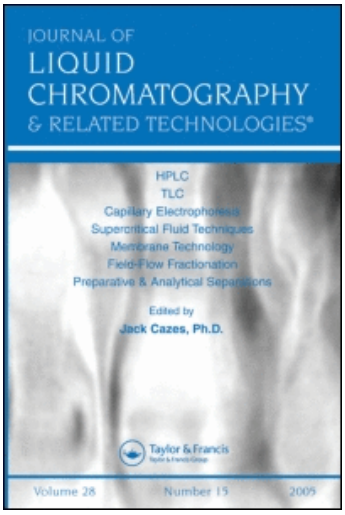

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PREPARATION AND APPLICATION OF MACROPOROUS SILICA-BASED POLYMER-BONDED PACKING FOR HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS

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ABSTRACT

A novel reversed phase chromatography packing to be used for analytical protein separations is described. A macroporous silica support was bonded with diethoxymethylvinyl silane, and then copolymerized with hydroxyethyl methylacrylate and divinylbenzene to produce tailored stationary phase. This packing has high resolution and stability in extent pH range. It was characterized through the separation of two model protein mixtures, the first one containing lysozyme, cytochrome C, β-lactoglobulin, chick egg albumin, ribonuclease and pepsin (porcine mucus), the second mixture containing insulin (two kinds), ribonuclease, lysozyme, serum bovine albumin. Such manipulations of proteins' elution profile are achievable at run times of less than 15 minutes. It is suggested that the macroporous silica-based polymer-bonded packing is a better alternative for the biopolymer separation.

INTRODUCTION

The separation to polypeptides and proteins by reversed phase high performance liquid chromatography (RP-HPLC) has seen a significant increase in recent years.[1,2] The most commonly used solvent systems for RP-HPLC of polypeptides and proteins involve linear increasing gradient modes, starting with water and increasing concentrations of organic solvent (usually methanol, acetonitrile or isopropanol).[3] These solvent systems generally employ low concentrations of perfluorinated organic acids [e. g. trifluoroacetic acid (TFA)] at a concentration 0.05%--0.1% (v:v) in both the water and the organic solvent. Due to the different hydrophobicity of proteins and polypeptides, the packings which possess different hydrophobicity can be used to selectively separate proteins and hold proteins' activity. Lau et al.[4] demonstrated that the primary cause of protein unfolding during RP-HPLC is the hydrophobicity of the stationary phase which disrupts the hydrophobic interactions stabilizing the native conformation.

RP-HPLC separations of proteins have generally been carried out on silica-based matrices containing alkyl (i.e., C_3, C_4, C_8, C_{18}) hydrophobic functional legends. As hydrophobic interactions play a major role in stabilizing the three-dimensional structure of a protein, it is not surprising that the hydrophobicity of such matrix (specially considering the relatively high hydrocarbon loading typical of such stationary phases) could unfold a protein on binding to the column.

In recent years there have been many applications of organic polymer-based supports in HPLC, especially for protein and other biopolymer separations. Demands for increased pH stability are outweighing the advantages of silica-based supports. However, organic polymer-based supports are easy to be shrunk in the organic solvent, and mechanical strength is inferior to silica-based supports.

There have been major advances in recent years in the design and development of stationary phases for RP-HPLC, frequently with a focus on novel concepts and improvement of the stationary phase chemistry of RP-HPLC packings.[5] These packings have both advantages of silica and polymer stationary phase: high mechanical strength and extent pH stability. Two approaches have been used. One approach has been used to immobilize defined polymer layers on the surface of rigid porous inorganic supports as a solute impermeable layer.[5-8] The other approach is that monomer directly polymerized on the surface of inorganic supports.[9]

The approach described in this paper involved that a macroporous silica support was bonded with diethoxymethylvinylsilane, and then it was copolymerized with hydroxyethyl methylacrylate and divinylbenzene to produce tailored stationary phase. This packing has not been reported in other papers. On the macroporous silica-based packing, proteins can be separated more quickly with high recoveries, because proteins and macroporous silica surface have little irreversible effect.

EXPERIMENTAL

Materials

HPLC-grade TFA, Analysis-grade toluene, 1,4-dioxane, diethyl ether, hydrochloric acid, carbon tetrachloride, ethanol, AIBN (azodiisobutyronitrile), deionized water, diethoxymethylvinylsilane (distilled under atm.), hydroethyl methylacrylate, and divinylbenzene (distilled under vacuum). All chemicals are from Tianjin second chemical factory (China). Insulin (MW 5700;2700 Dalton), Cytochrome C (MW 12400 Dalton), Lysozyme (MW 13900 Dalton), Ribonuclease (MW 12600 Dalton), Pepsin (porcine mucus) (MW 34700 Dalton), Chick egg albumin (MW44000 Dalton), β-lactoglobulin (MW 18400 Dalton), Serum bovine albumin (MW 68000 Dalton). The standard samples are from various companies. Casein was prepared from milk in the laboratory. FMDV A type (Foot and Mouth Disease Virus A type), FMDV O type (Foot and Mouth Disease Virus O type), Huang CTCF (Cyticerus Tenuicollis Cystis Fluid), Huang SHCF (Sheep Hydatido Cystis Fluid).

Apparatus

The HPLC instrument consisted of a Hewlett-Packard (Avondale, PA, USA) HP 1090 Liquid Chromatography, coupled to a HP 9153C UV detection system. HP 9000 Series 300 computer. Support: Porous silica (7±1μm,100nm pore size) support was self-made in the laboratory.

Preparation of Packings

The packing was preparated in following steps:

20g silica and 100mL hydrochloric acid/water (20/80, V/V) were heated under reflux at 100°C for 4 hours, then cooled and filtered, and washed with

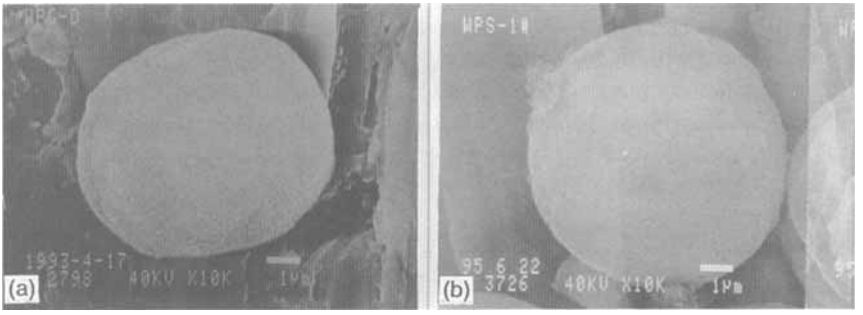

Figure 1. Scanning electron micrograph; (a) Macroporous silica, (b) Macroporous silica-based polymer-bonded phase.

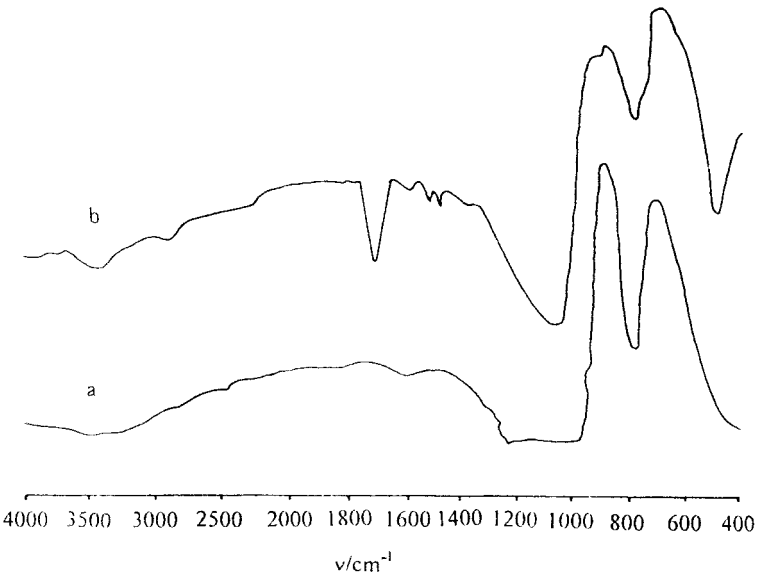

Figure 2. Transmittance fourier transform infrared spectrum; (a) Macroporous silica, (b) Macroporous silica-based polymer-bonded phase.

deionized water till pH7.0, dried under vacuum. 5g macroporous silica, 3mL dried diethoxymethylvinylsilane, and 50mL dried toluene were heated under reflux for 18 hours, cooled and filtered. The remaining solvent was removed by washing with toluene and diethyl ether. 3g vinyl-bonded silica, 0.5g hydroethyl methylacrylate, 0.05g divinylbenzene, 0.03g AIBN, and 50mL toluene were refluxed at 115°C for 18 hours.

Stirring all the time, the solid product was then washed with toluene and diethyl ether. The bonded porous packing was then extracted with methanol for 24 hours.

Column Packing

Silica-based packing was suspended in 1,4-dioxane/carbon tetrachloride (1:2,V:V)[3] and packed by the downward flow method into stainless-steel cartridges (150mm×4.6mmi.d.), using ethanol as the packing solvent.

RESULTS AND DISCUSSION

Characterization of Packing

Figure 1 is the scanning electron micrograph; (a) the distribution of the macroporous in macroporous silica. (b) the macroporous in silica-based polymer-bonded packing. In Figure 1(a), the distribution of the macroporous in macroporous silica can be seen clearly; but in (b), the pore can hardly be seen. Because the packing with a network-structured surface was produced, the macroporosity in silica has been covered partly with polymer-bonded layer.

Figure 2 is the transmittance Fourier transform infrared spectrum. In Figure 2 (a), there is only the absorption peak of silica; but in (b), at 3050cm^{-1}, there is the C-H absorption peak of V_{C-CH}, at 1720cm^{-1}, there is the C=O typical absorption peak of $V_{C=O}$, at 1580cm^{-1}, 1500cm^{-1} and 1480cm^{-1}, there are the typical absorption peaks of benzene skeleton structure, at 1380cm^{-1}, there is the C-H absorption peak of V_{C-C-H}.

The FT-IR spectra shows that the packing has both phenyl and carbonyl. The skeleton symbol of the packing group is shown in (Figure 3). The network-structure is obvious.

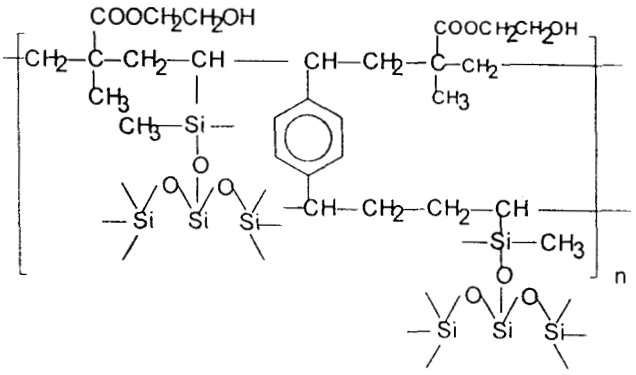

Figure 3. Structure of the packing

Table 1

Repetition of Synthesized Packings

Batch Number	Content of Carbon
1	2.68
2	2.66
3	2.67
4	2.68
5	2.66

Repeatability of Synthesized Packing

The packing was synthesized five times repeatedly by using the method described above. The repeatability of synthesized packings was checked by element analysis. The results are shown in Table 1. The columns were used for protein separation; the same elution profiles were obtained.

Permeability of Column

$K°=\eta Lu/\Delta P$; $K°$: permeability factor; η: viscosity of methanol (0.6CP); L: the length of column (15 cm); u: linear flow rate (0.1 cm/s); ΔP: pressure

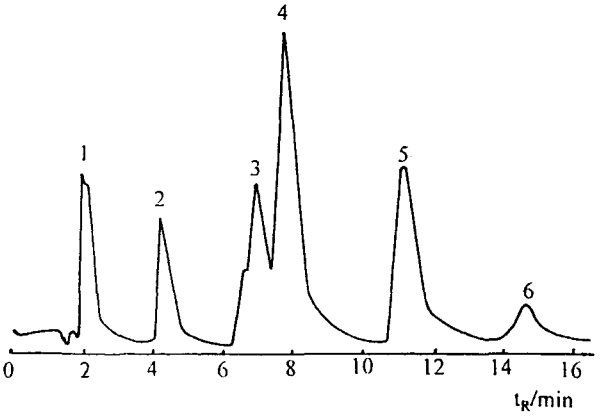

Figure 4. Elution profile of six model proteins; Chromatographic conditions: Column, macroporous silica- based polymer-bonded phase(150mm×4.6mmi.d.); UV-280nm; mobile phase: A:95%acetonitrile+0.1%TFA in water, B:5%acetonitrile+0.1%TFA in water, flow rate: 1mL/min, 20-min gradient from 20%A to 80%A. Peaks: 1. pepsin(porcine mucus), 2. ribonuclease, 3. cytochrome C, 4. lysozyme, 5. β-lactoglobumin, 6. chick egg albumin samples were dissolved in deionized water

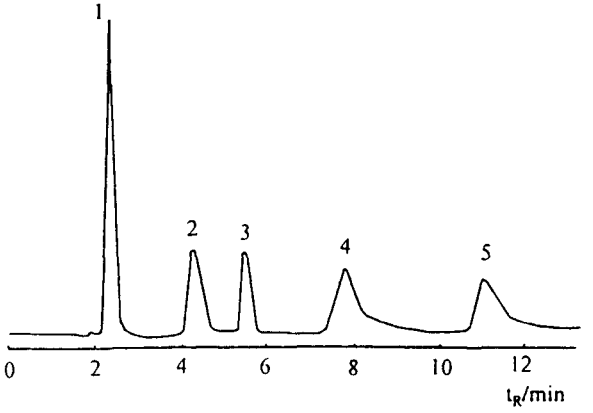

Figure 5. Elution profile of five model proteins; Chromatographic conditions as in Figure 4. Peaks:1.insulin A, 2.ribonuclease, 3.insulin B, 4.lysozyme, 5.serum bovine albumin

Table 2

Resolution of Neighboring Proteins

Sample Pair	Resolution
1/2	3.6
2/3	3.3
3/4	0.9
4/5	3.0
5/6	2.9

Sample pair: 1. pepsin (procine mucus), 2. ribonuclease 3. cytochrome C, 4. lysozyme, 5 β-lactoglobumin, 6. chick egg albumin.

Table 3

Resolution of Neighboring Proteins

Sample Pair	Resolution
1/2	4.1
2/3	2.4
3/4	3.3
4/5	3.5

Sample pair: 1. insulin A, 2. ribonuclease, 3. insulin B 4. lysozyme, 5. serum bovine albumin.

(6 atm.); $K°=1.48\times10^{-9}cm^2$. $\Phi=dp^2/K°$; Φ: resistant factor; dp: the size of packing (6×10^{-4}cm) ; $\Phi=243$. This is lower than common spherical packings (about 500). It suggests that the column has high column permeability. This column has been continuously used more than one year (about 140,000 mL mobile phase), and its efficiency holds almost the same as the initial.

Application of Packings

The reversed phase packing described above was applied to the separation of model proteins and several actual samples.

Figure 6. Elution profile of Casein; Chromatographic conditions: Column, macroporous silica- based polymer-bonded phase(150mm×4.6mmi.d.), UV-254nm; mobile phase: 30%acetontrile+0.1%TFA in water; flow rate:1mL/min,isocratic eluent. Casein was dissolved in mobile phase.

On macroporous silica-based polymer-bonded phase, six model proteins were separated within 15 minutes; results are shown in Figure 4. Elution profile of five proteins was achievable at run time of less than 12 minutes; results are shown in Figure 5. Resolutions of neighboring proteins are shown in Table 2 and 3 respectively. The proteins were separated through an RP-HPLC mechanism. The interactions of macroporous silica-based polymer-bonded phase and proteins are carried out by two different hydrophobic interaction groups. One is the alkyl group of hydroxyethyl methacrylate, the other is the phenyl group of divinylbenzene. The effect of two hydrophobic interaction groups on protein separation is different from the effect of a single alkyl chain hydrophobic interaction group.

Silica-based matrices containing alkyl group (i.e., C_4, C_8, C_{18}) can be stable only in the pH range 2-7.5.[10] However, HEMA (copolymers of ethylene dimethacrylate and hydroxyethyl methacrylate) can be stable in the pH range 2-12.[11] The packing prepared in our laboratory can be used in the pH range 2-10. In addition, its mechanical strength is superior to organic polymer-based packing.

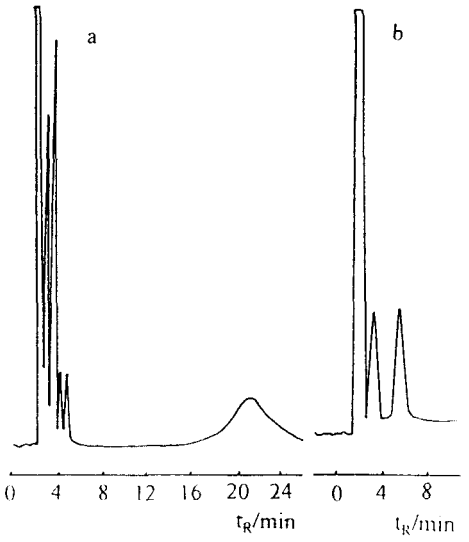

Figure 7. Elution profile of actual samples; (a)Huang SHCF,(b)FMDV O type. Chromatographic conditions can be seen in Figure 6.

Several actual samples were also separated on this packing. Cascin(purified by precipitating at PI) was separated on this packing (Figure 6). The result suggested that its purity was over 95%. Huang CTCF, Huang SHCF, FMDV A type, FMDV O type have also been separated. The elution profile of Huang SHCF and FMDV O type is shown in Figure 7.

CONCLUSIONS

The potential of a novel reversed phase chromatography on macroporous silica-based polymer-bonded phase for protein separations has been evaluated. This packing has two different hydrophobic interaction groups. The effect of two hydrophobic interaction groups on protein separation is different from the effect of a single alkyl chain hydrophobic interaction group. The packing prepared in our laboratory can reduce the probability of silica touched with mobile phase, so this packing can be used in wide pH range. Using this packing, proteins are separated more quickly with higher resolution than other silica-based bonded phase. It suggests that the potential of this packing for biopolymer separations is very promising.

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