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Reversed-phase liquid chromatography of proteins and peptides using multimodal copolymer-encapsulated silica

Yufeng Shen[a], Xiaowen Shao[b], Kim O'Neill[b], Jerald S. Bradshaw[a], Milton L. Lee[a,*]

[a]*Department of Chemistry and Biochemistry, Brigham Young University, Provo, UT 84602-5700, USA*
[b]*Department of Microbiology, Brigham Young University, Provo, UT 84602-5700, USA*

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Abstract

Multimodal copolymer-encapsulated particles for liquid chromatography were prepared by bonding 1-octadecene and unsaturated carboxylic acids on silica particles (5 μm diameter, 300 Å pores) for liquid chromatography of proteins. These multimodal copolymer-encapsulated particles can provide both hydrophobic and hydrogen bonding interactions with polar compounds. The chromatographic performance of these multimodal copolymer-encapsulated particles for peptide and protein separations was evaluated under reversed-phase conditions. Compared with typical C_8-bonded silica, polymer-encapsulated particles were more stable in acidic mobile phases and provided better recoveries, especially for large proteins ($M_r > 0.5 \cdot 10^6$). Totally hydrophobic polymer-encapsulated particles were found to produce broad peaks for proteins, and significant improvements were observed by introducing hydrophilic groups (–COOH) onto the polymer-encapsulated surface to form a multimodal phase. For the reversed-phase liquid chromatography of peptides and proteins, improved selectivity and increased solute retention were found using the multimodal polymer-encapsulated particles. More peaks were resolved for the separation of complex peptide mixtures such as protein digests using the multimodal polymer-encapsulated particles as compared to totally hydrophobic polymer-encapsulated particles.

Keywords: Polymer-encapsulated silica; Stationary phases, LC; Peptides; Proteins

1. Introduction

Reversed-phase liquid chromatography (RPLC) is among the most common methods used for the analysis of proteins [1–5]. Low salt content mobile phases and narrow-bore packed columns make this technique more suitable compared to conventional packed columns for hyphenation with detectors such as mass spectrometry. In order to improve the RPLC separation of proteins, an acidic ion-pairing reagent [e.g., trifluoroacetic acid (TFA) or heptafluorobutyric acid (HFBA)] is often added to the mobile phase [6]. Unfortunately, such additives cause rapidly deterioration of the Si–O bonds [7] which exist in the native silica and in most reversed-phase (RP) bonding reagents.

Several approaches have been developed to extend the RPLC column lifetime when using mobile phases under extreme pH conditions [8–15]. These approaches include bonding bidentate or bulky silanes on the silica particle surface [8], using polymer-encapsulated particles [9–14], and utilizing poly-

*Corresponding author. Tel.: +1-801-378-2135; fax: +1-801-378-5474.
E-mail address: milton_lee@byu.edu (M.L. Lee)

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meric beads [15]. Bonded bidentate or bulky silanes can partially protect the surface from attack by acidic mobile phase additives, however, it is difficult to completely isolate the bonded surface from the mobile phase. Polymeric beads have inherent resistance to attack by reactive solvents. The major concerns with the use of these beads are particle size uniformity and swelling when using organic solvent-containing mobile phases. For polymer-encapsulated particles, the matrix can be either siliceous or non-siliceous (e.g., zirconia), and they can exhibit similar chemical properties to polymeric beads and similar mechanical characteristics to inorganic matrices.

Carbon–carbon (C–C) backbone polymers (e.g., polystyrene or polybutadiene) have been successfully used to prepare polymer-encapsulated RP packings for protein separations [9–14]. The coated C–C polymers are stable in typical acidic mobile phases. One of the major differences between polymer-encapsulated particles and typical bonded RP siliceous phases is that the accessible silanol groups on the particle surface are covered by the polymer coatings. This has been demonstrated by experiments in which no silanophilic interactions were observed for polymer-encapsulated particles [11]. The elimination of accessible silanol groups on the particle surface has a positive effect in RPLC, especially for basic compounds [16,17].

Hydrophobic interactions between the solute and the stationary phase form the basis for RPLC separation of proteins. However, it has been found that a limited number of free silanol groups may be desirable for efficiency and selectivity [18–20], which suggests that some hydrophilicity of the particle surface is important.

Multimodal or mixed-mode columns [3,21–23] and controlled hydrophobic polymer-encapsulated particles [24,25] have been used to optimize the RPLC separations of proteins and peptides. Introducing hydrophilic ionic groups onto a hydrophobic particle surface or using a mixture of reversed-phase and ion-exchange packing materials can affect the selectivity. However, recoveries can be relatively low under RPLC conditions because of the strong charge–charge interactions between the particle surface and proteins. Using carefully synthesized hydrophobic polymers to prepare polymer-encapsulated particles, the selectivity for protein separations was changed. However, decreasing the hydrophobicity of the particle coatings resulted in a decrease in retention for proteins, and a resultant decrease in RPLC dynamic range. Using phosphate functionalized polymer-encapsulated zirconia to prepare multimodal packing materials, poor column efficiencies were obtained [21,22].

In this study, a new method to prepare polymer-encapsulated particles for the separation of proteins was developed. After incorporating hydrophobic alkyl chains on the polymer-encapsulated particle surface, polar carboxyl (–COOH) groups were introduced using unsaturated carboxyl acids. In typical mobile phases (pH~2) used for the RPLC of proteins, the –COOH (pK_a~4) groups remain neutral, and hydrogen bonding interactions exist between these groups and the proteins. Therefore, these particles can provide both hydrophobic and hydrogen bonding interactions. The chromatographic performance of these multimodal copolymer-encapsulated particles was evaluated for peptides and proteins.

2. Experimental

2.1. Materials

Untreated spherical silica and C_8-bonded silica (Nucleosil 300-5 C_8 protein) having 5 μm diameter and 300 Å pores were purchased from Alltech (Deerfield, IL, USA). Octyl-substituted polyhydrosiloxane (PS-C_8, 40–60% substituted) was purchased from United Chemicals (Bristol, PA, USA). 1-Octadecene was purchased from Ethyl (Baton Rouge, LA, USA). 1,13-Tetradecadiene, maleic anhydride and dicumyl peroxide (DCP) were purchased from Aldrich (Milwaukee, WI, USA). Protein and peptide standards were purchased from Sigma (St. Louis, MO, USA). Fused-silica capillary tubing was purchased from Polymicro Technologies (Phoenix, AZ, USA). Other chemicals used were purchased from Sigma or Aldrich.

2.2. Preparation of polymer-encapsulated silica particles and packed capillary columns

The untreated silica particles were first deactivated with 40–60% octyl-substituted polyhydrosiloxane

using a procedure previously described [26]. Using this pretreatment, it is believed that a near monolayer of polymer was formed on the particle surface because the reaction takes place primarily between the silanol groups on the particle surface and hydride groups in the PS. This process produced a similar critical surface tension as the surface tension of the hydrophobic polymers used for encapsulation.

Totally hydrophobic polymer-encapsulated particles were prepared by cross-linking unsaturated hydrocarbon monomers. PS-C_8 treated silica (0.2 g), 1,13-tetradecadiene (or 1-octadecene) (0.06 g), and DCP (0.006 g) were dissolved in CH_2Cl_2 (~5 ml) in a specially designed reaction vessel [24]. The solution was bubbled with an argon gas purge (~40 ml min^{-1}), and the solvent was evaporated at room temperature. Cross-linking of the coated polymer was carried out by increasing the temperature from 40 to 190°C at 4°C min^{-1} and holding at 190°C for 2 h. The product was washed with CH_2Cl_2 (~10 ml), and then dried at room temperature.

Multimodal copolymer-encapsulated particles were prepared by cross-linking unsaturated hydrocarbon and carboxylic acid monomers. Using the same procedure as for the preparation of totally hydrophobic polymer-encapsulated particles, PS deactivated silica particles (0.2 g) were coated with a mixture (0.06 g) of maleic anhydride–1-octadecene (1:1, molar ratio) containing 0.006 g of DCP. The coated layer was cross-linked by heating under the same conditions as for the preparation of totally hydrophobic polymer-encapsulated particles. The anhydride groups on the surface were hydrolyzed to yield –COOH groups in acidified water (1% TFA, v/v).

A CO_2 (SFC grade, Scott Specialty Gases, Plumsteadville, PA, USA) slurry packing method was used to prepare packed fused-silica capillary columns [27]. The columns were conditioned at room temperature and 300 atm in an ultrasonic bath for ~30 min (1 atm=101 325 Pa).

2.3. Packed capillary column LC experiments

Fig. 1 shows the configuration of the packed capillary LC system used in this study. An LC pump (1) (Varian 8500, Walnut Creek, CA, USA) was used to deliver the mobile phase. A slave amplifier reservoir (2) (Ref. [28]) was used to introduce a second solvent to carry out gradient elution. A mixing chamber (3) (~1.5 ml) was stirred with a magnetic stirrer. The packed capillary column was directly connected to a manual liquid injector valve (4) (Valco, 60-nl sample loop). A 30 cm×100 μm I.D. fused-silica capillary was used to connect the column outlet to a UV absorption detector (5) (UV–Vis Model 203, Linear Instruments, Reno, NV, USA), however, the distance between the column outlet and the detection window was ~1 cm. A Model SP 4270 integrator (6) (Spectra-Physics, San Jose, CA, USA) was used to record the chromatograms.

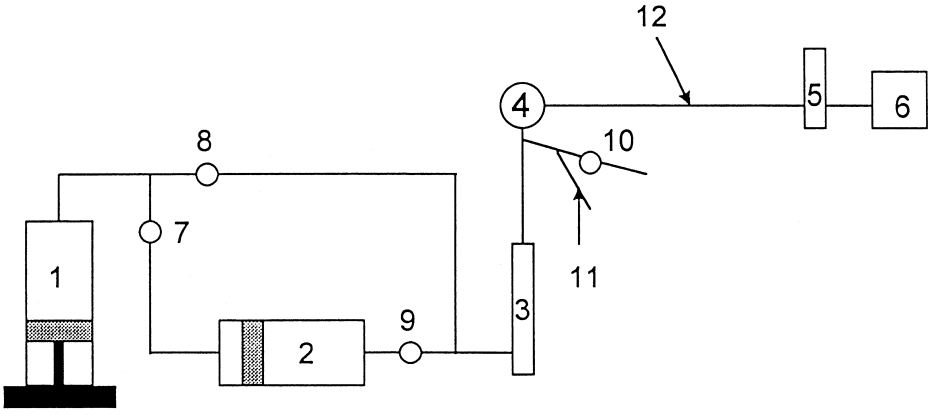

Fig. 1. Schematic diagram of the packed capillary column LC configuration used in this study. Numbered components are identified and described in the text.

When isocratic elution was desired, stop-valves (7), (9) and (10) were closed and pump (1) provided a uniform mobile phase flow. The relative standard deviation (RSD) of retention time and column efficiency for 10 runs using naphthalene as solute was within 3%. The relatively low RSD (~3%) for retention resulted from the use of a syringe pump for packed capillary column LC. When a component gradient was used, the mixing chamber (3) was filled with eluent A from pump (1) and the packed capillary column was fully equilibrated for ~20 min with eluent A. By closing valve (8) and opening valves (7) and (9), a mobile phase gradient was generated by displacing solvent A in chamber (3) with eluent B from reservoir (2). After each run, valves (7) and (9) were closed and valves (8) and (10) were opened to replace eluent B with eluent A (~1 min). When valve (10) was closed, the separation column (12) was re-equilibrated for ~20 min to prepare for the next run. A 22 μm I.D. fused-silica capillary was used as a split line (11) before the injector valve, and the split line length depended on the gradient speed required, as illustrated in Fig. 2. The retention time and peak width RSD values for 10 runs were <5% for naphthalene when using this gradient system.

Model proteins and peptides were dissolved in HPLC grade water at concentrations of 0.05–0.5 mg ml^{-1}. Using an injection sample loop with a volume of 60 nl, the sample amount injected into the packed capillary column was less than 0.03 μg. Tryptic digestion of proteins was carried out according to the method described in Ref. [29].

3. Results and discussion

In order to investigate the influence of the polymer-encapsulated surface on the separation of proteins, various chemical surface structures were created, as illustrated in Fig. 3. Fig. 3A and B represent totally hydrophobic polymer-encapsulated surfaces. By using monomers which have unsaturated double bonds at both ends (e.g., 1,13-tetradecadiene), we attempted to form a flat polymer-encapsulated surface (i.e., polymer backbones lie essentially horizontal on the particle surface), while a surface having densely spaced, extended hydrophobic chains were favored by cross-linking monomers

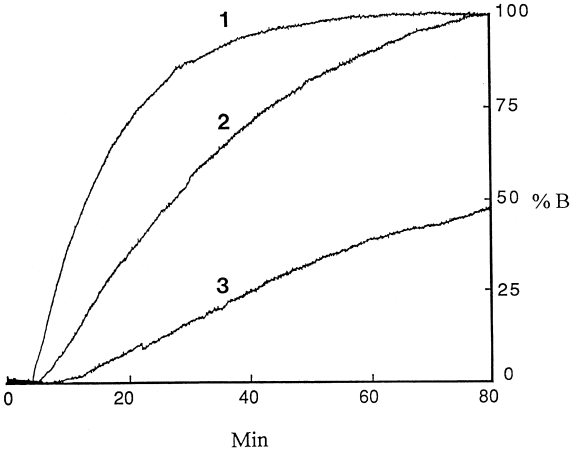

Fig. 2. Mobile phase component gradient profiles obtained with the LC equipment used in this study. Conditions: 35 cm×250 μm I.D. capillary columns containing 1-octadecene–maleic acid co-polymer-encapsulated particles (5 μm diameter, 300 Å pores), 350 atm column inlet pressure, (A) ACN–water (30:70, v/v, 0.2% TFA), (B) ACN–water (60:40, v/v, 0.2% TFA), 20 μl of phenol was added in B. Data plotted for (1) 15 cm, (2) 30 cm, and (3) 60 cm×22 μm I.D. fused-silica capillary split lines at the injector.

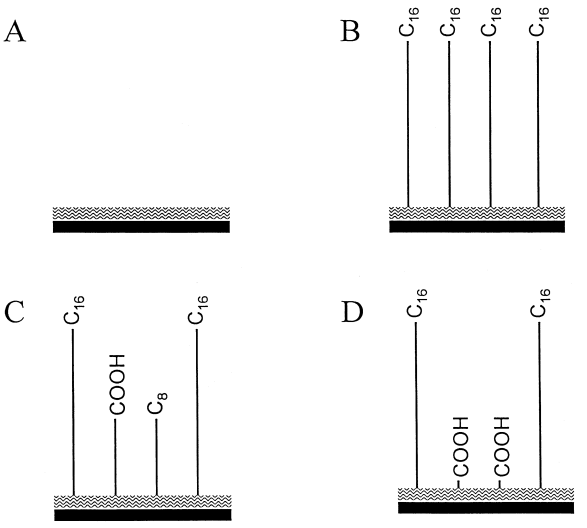

Fig. 3. Polymer-encapsulated particle molecular surface structures. (A) Totally hydrophobic flat polymer-encapsulated surface, (B) totally hydrophobic polymer-encapsulated surface with long extended alkyl chains, (C, D) multimodal polymer-encapsulated surfaces with hydrophilic –COOH groups.

which contain an unsaturated double bond at only one end (e.g., α-olefins). Although some cross-linking may occur at point(s) in the middle of the long chains during the cross-linking reaction, it is expected that extended functional groups largely exist on this polymer-encapsulated particle surface because of the high reactivity of unsaturated double bonds in free radical initiated reactions. Mild cross-linking conditions should yield a more orderly extended structure. Fig. 3C represents a multimodal polymer-encapsulated surface, on which hydrophilic –COOH groups are introduced. The acidic (pK_a~4) –COOH groups were selected because they are chemically more stable on silica than basic groups and are neutral at pH~2. The concentration of –COOH groups and the length of the spacer between the –COOH group and the silica surface can be adjusted by selecting the appropriate unsaturated acid reagent and its concentration during polymerization. In this study, maleic acid was used to prepare this multimodal polymer-encapsulated surface structure.

A pair of small probes, $NaNO_3$ and $NaNO_2$, were used to detect the presence of –COOH (and silanol) groups on the prepared multimodal polymer-encapsulated surfaces. At pH~2, $NaNO_2$ is converted to HNO_2 (pK_a~3.9) which can be retained by silanol or –COOH groups on the particle surface by hydrogen bonding. Experiments showed that these two compounds were not separated on any of the columns using various ratios of acetonitrile (ACN) and water without adding TFA to the mobile phase. The selection of these salts as unretained markers to measure the dead time (t_0) in RPLC [30] should be valid in this situation. However, when adding 0.1% TFA to the mobile phase, a significant separation was observed using a column containing C_8-bonded particles (Fig. 4A). This indicates that the residual silanol groups on the C_8-bonded silica surface interact with HNO_2. In an acidic aqueous mobile phase (pH ~2), only a small percentage of the residual silanol groups on typical bonded RP particles are ionized, and most of them remain neutral.

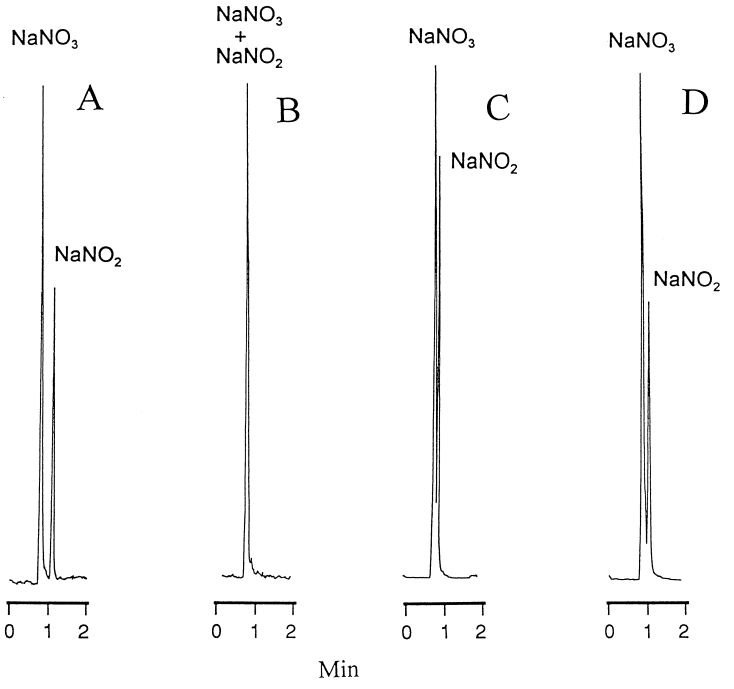

Fig. 4. Chromatograms showing hydrogen bonding interactions for C_8-bonded, totally hydrophobic polymer-encapsulated and multimodal copolymer-encapsulated particles. Conditions: 20 cm×250 μm I.D. capillary columns containing (A) C_8-bonded, (B) poly(1-octadecene)-encapsulated and (C) 1-octadecene–maleic acid copolymer-encapsulated particles (5 μm diameter, 300 Å pores); ACN–water (50:50, v/v, 0.1% TFA) mobile phase; room temperature; UV (215 nm) detection.

This has been confirmed by a recent study of electroosmotic flow (EOF) for typical bonded RP particles in capillary electrochromatography (CEC) [31]. The interactions between nonionic silanol groups and HNO_2 should be hydrogen bonding interactions.

For totally hydrophobic polymer-encapsulated particles, the hydrogen-bonding interaction is shielded by polymer encapsulation, and no detectable interaction to retain HNO_2 was observed (Fig. 4B). Using the multimodal polymer-encapsulated particles, these probes were again separated (Fig. 4C). This indicates that –COOH groups were successfully introduced onto the polymer-encapsulated particle surface, and they produced hydrogen bonding interactions with HNO_2. The lower resolution of the test probe compounds on the multimodal polymer-encapsulated particles compared with the C_8-bonded particles suggests that the polarity of –COOH is weaker than silanol, which produces weaker hydrogen bonding interactions with HNO_2.

Column efficiency is an important factor for separation, especially when a complex matrix exists. In order to compare the column efficiency for proteins on various packed capillary columns, the uniformity of the packed bed must be evaluated, which can be probed by using solutes having no retention on the stationary phase [32]. In this study a small $NaNO_2$ analyte with retention of <0.2 was used for this purpose. As previously observed [31], a slight improvement in column efficiency was observed with capillary columns containing polymer-encapsulated particles, as illustrated in Fig. 5. Reduced plate height of ~2.5 was obtained, which indicates that a relatively uniform packed bed was produced.

When reducing the ACN concentration in the mobile phase to increase solute retention ($k>4$), very low efficiency (<600 plates m^{-1}) was observed for proteins on all columns. This is probably the reason why the separation of proteins is typically carried out using a mobile phase gradient [19]. The low efficiency is believed to result from slow mass transfer of the proteins in both mobile and stationary phases.

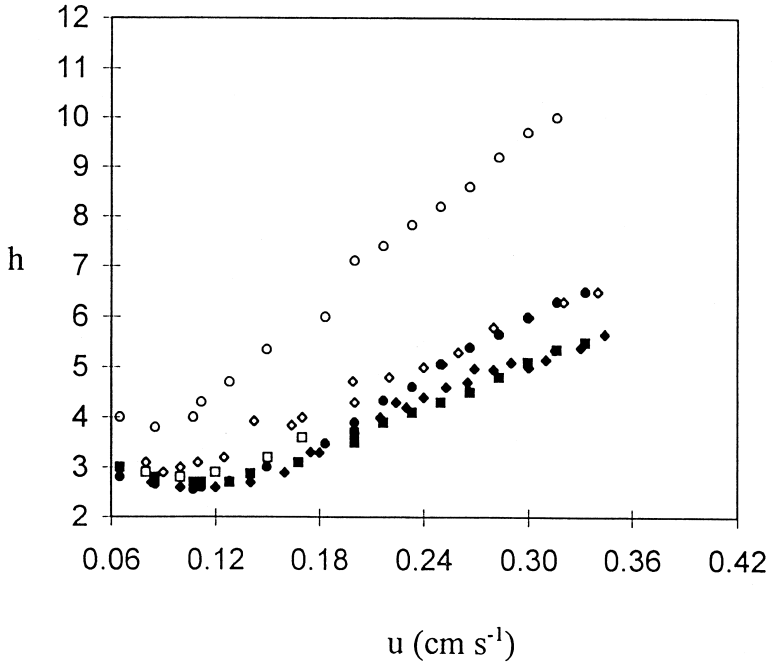

Fig. 5. Plots of h versus u for small molecules on columns containing bonded and polymer-encapsulated particles. Conditions: CH_3NO_2 as test marker (k~0.2 on various columns); other conditions as in Fig. 4. (●) C_8-bonded, (■) poly (1-octadecene)-encapsulated and (♦) 1-octadecene–maleic acid copolymer-encapsulated particles; (○) C_8-bonded, (□) poly(1-octadecene)-encapsulated and (◇) 1-octadecene–maleic acid copolymer-encapsulated particles after five weeks rinsing.

Few reported efficiency data are available in the literature to compare with the results obtained in this study.

By increasing the ACN content in the mobile phase, the efficiency was improved greatly as a result of changing the *C* term of the van Deemter (or Knox) equation. However, it was found that different efficiencies were measured for different proteins on the packed columns, even though the retention factors for the proteins were less than 0.3 (Fig. 6). This suggests that even minor interaction between the test proteins and the particle surface significantly affects the mass transfer in the stationary phase under RPLC conditions. The efficiencies measured for proteins when there is a high ACN concentration in the mobile phase are representative of the separation efficiencies obtained when using component gradient programming as commonly applied in the RPLC of proteins. Additionally, column efficiencies for proteins are relatively low at low mobile phase linear velocities (*u*), and there are no significant losses in reduced plate height (*h*) when increasing *u*. This probably relates to changes in protein structure due to high pressure [33] or hydrophobic interactions.

In these experiments, it was found that the totally hydrophobic polymer-encapsulated (both flat and extended chain structure) particles produced more serious peak broadening than typical C_8-bonded RP particles for most of the test proteins (Table 1). The peak widths of proteins on the totally hydrophobic polymer-encapsulated particles were narrowed when introducing hydrophilic groups (–COOH) on the surface (e.g., for hemoglobin variant 3, ~40% decrease in peak width), and the efficiency approached that obtained using C_8-bonded phases.

For totally hydrophobic polymer-encapsulated particles, the bonded phase structures (e.g., flat or with extended chains) had limited influence on protein retention, and the elution times for model proteins varied between ±7%. However, as high as 10–20% increase in elution time was found for hemoglobin variant 3 (Table 1) when –COOH groups were introduced onto the polymer-encapsulated surface.

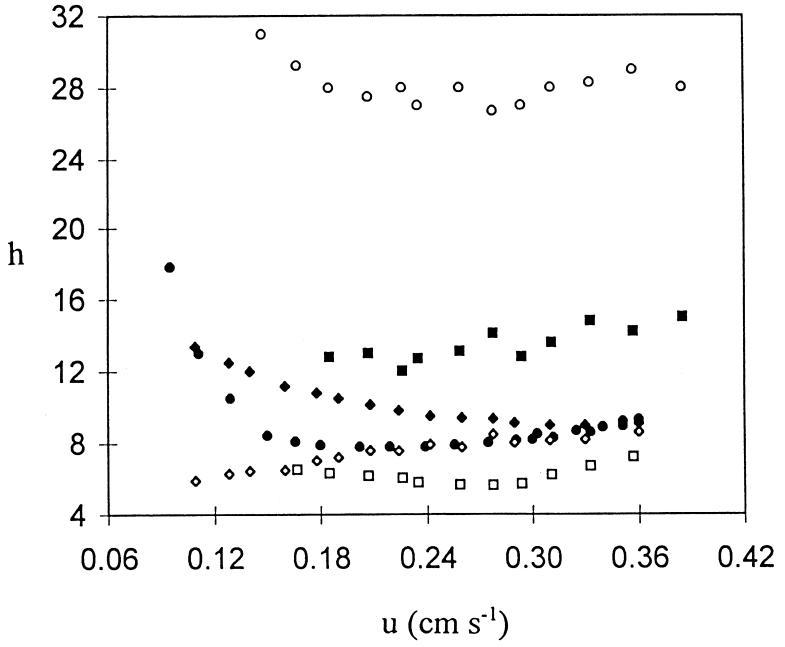

Fig. 6. Plots of *h* versus *u* for proteins on columns containing C_8-bonded, totally hydrophobic polymer-encapsulated, and multimodal copolymer-encapsulated particles. Conditions as in Fig. 4. (●) cytochrome *c* (bovine heart) and (○) myoglobin (horse heart) on C_8-bonded particles, (■) cytochrome *c* (bovine heart) and (□) myoglobin (horse heart) on poly(1-octadecene)-encapsulated particles, (♦) cytochrome *c* (bovine heart) and (◇) myoglobin (horse heart) on 1-octadecene–maleic acid copolymer-encapsulated particles.

Table 1
Comparison of peak widths at half height ($w_{1/2}$) and elution times (t_R) of proteins on various columns[a]

Column	$w_{1/2}$ (min)				t_R (min)				
	Ribo	Insu	Lyso	Hemo 3	DT[b]	Ribo	Insu	Lyso	Hemo 3
1	0.32	0.28	0.32	0.56	3.3	4.8	9.6	19.6	36.2
2	0.35	0.32	0.38	0.66	3.2	4.6	8.4	18.8	35.2
3	0.32	0.40	0.44	0.88	3.2	4.0	7.8	19.2	36.9
4	0.42	0.28	0.36	0.51	3.2	5.8	11.1	21.4	40.0

[a] Conditions: 35 cm×250 μm I.D. columns containing (1) C_8-bonded, (2) poly(1,13-tetradecadiene)-encapsulated, (3) poly(1-octadecene)-encapsulated and (4) 1-octadecene–maleic acid copolymer-encapsulated particles (5 μm diameter, 300 Å pores); split line: 30 cm×22 μm I.D. FS capillary tubing; mobile phase: (A) ACN–water (30:70, v/v, 0.2% TFA) and (B) ACN–water (60:40, v/v, 0.2% TFA); UV (215 nm).

[b] DT is the dead time and was measured by the baseline disturbance injection signal.

This suggests that the –COOH groups on these particle surfaces were involved in retaining the model proteins. The greater retention favors a wider retention window for the RPLC separation of proteins.

Compared with a typical C_8-bonded reversed-phase (Nucleosil C_8), one of the most significant advantages of the polymer-encapsulated particles is the high recovery of proteins. Fig. 7 shows separations of model proteins on columns containing Nucleosil C_8, totally hydrophobic polymer-encapsulated and multimodal copolymer-encapsulated particles. For all tested model proteins, all polymer-encapsulated packed capillary columns yielded

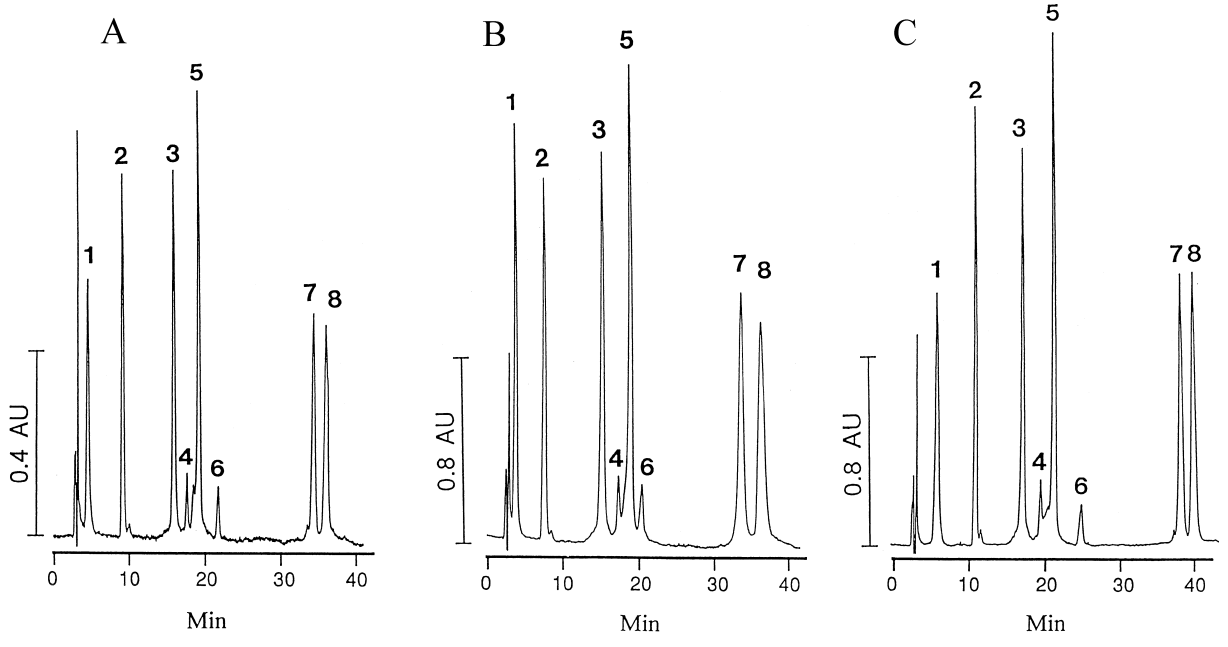

Fig. 7. Chromatograms of model proteins on C_8-bonded, totally hydrophobic polymer-encapsulated and multimodal copolymer-encapsulated particles. Conditions: 35 cm×250 μm I.D. capillary columns containing (A) C_8-bonded, (B) poly(1-octadecene)-encapsulated, (C) 1-octadecene–maleic acid copolymer-encapsulated particles (5 μm diameter, 300 Å pores); 30 cm×22 μm I.D. fused-silica capillary split line; (A) ACN–water (30:70, v/v, 0.2% TFA) and (B) ACN–water (60:40, v/v, 0.2% TFA); room temperature; UV (215 nm) detection. Peaks: 1=ribonuclease A (bovine pancreas), 2=insulin (bovine pancreas) 3=cytochrome *c* (bovine heart), 4=impurity from lysozyme (chicken egg), 5=lysozyme (chicken egg), 6, 7 and 8=hemoglobin A_2 variants (human).

~100% larger peak areas compared to the C_8-bonded phase. Either active sites (Si–OH or $Si–O^-$ groups), or small pores, or a combination of these are suspected to be responsible for the low recovery of the model proteins on the C_8-bonded phase. These results were confirmed by using two or three columns containing each particle type. The introduction of –COOH did not affect the recovery of the proteins.

Fig. 8 shows a further comparison of RPLC separations of another set of model proteins on columns containing C_8-bonded, totally hydrophobic polymer-encapsulated and multimodal copolymer-encapsulated particles. Columns containing polymer-encapsulated particles provided better recoveries for these model proteins, especially for trypsin (peak 2), than the column packed with C_8-bonded particles. The column packed with multimodal copolymer-encapsulated particles yielded better resolution of β-lactoglobins A and B than the column containing totally hydrophobic polymer-encapsulated particles.

The ability to chromatograph large proteins was investigated using multimodal polymer-encapsulated particles. Fig. 9 shows the separation of γ-globulin (M_r $0.16 \cdot 10^6$), β-galactosidase (M_r $0.5 \cdot 10^6$) and thyroglobin (M_r $0.67 \cdot 10^6$) using capillary columns packed with C_8-bonded and 1-octadecene–maleic acid copolymer-encapsulated particles. These are among the largest proteins separated by RPLC. Adding isopropanol (IPA) (10–15%, v/v) to the

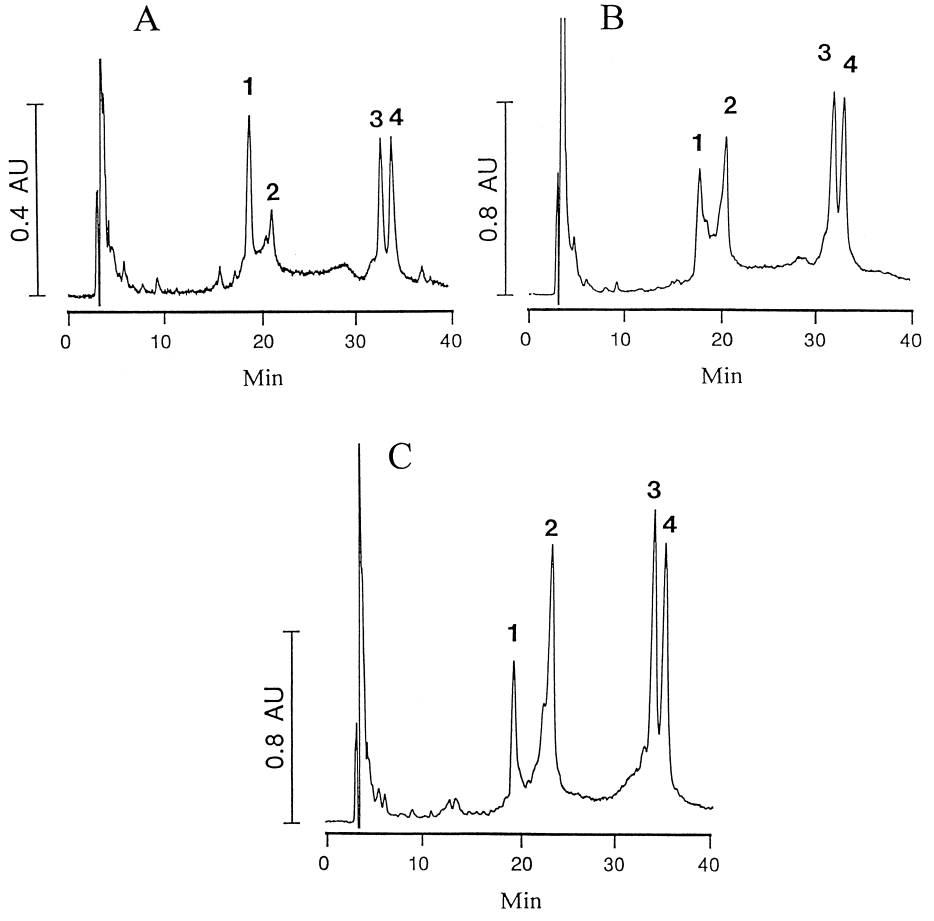

Fig. 8. Chromatograms of model proteins on columns packed with C_8-bonded, totally hydrophobic polymer-encapsulated and multimodal copolymer-encapsulated particles. Conditions for A–C as in Fig. 6A–C, respectively. Peaks: 1=trypsinogen (bovine pancreas), 2=trypsin (porcine pancreas), 3=β-lactoglobulin B (bovine milk) and 4=β-lactoglobulin A (bovine milk).

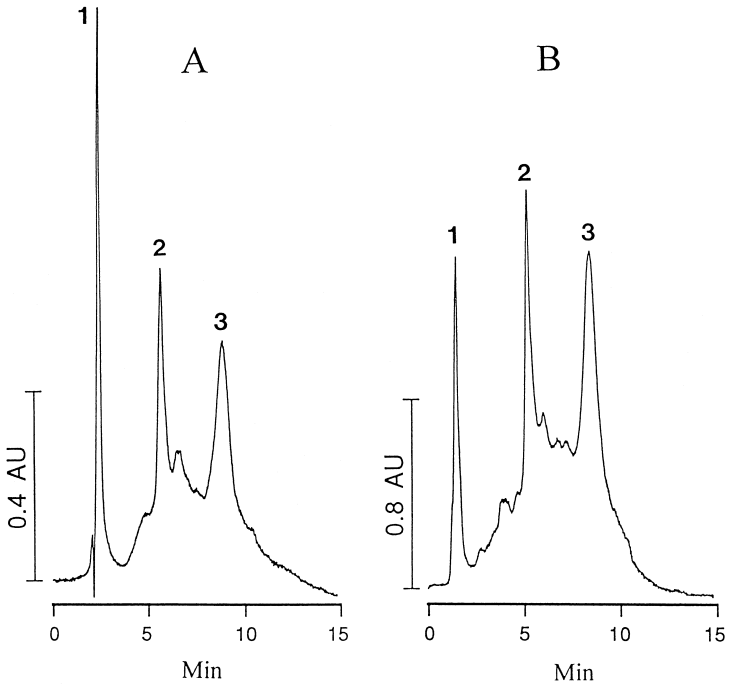

Fig. 9. Chromatograms of large proteins. Conditions: 20 cm×250 μm I.D. capillary columns containing (A) C_8-bonded and (B) 1-octadecene–maleic copolymer-encapsulated particles (5 μm diameter, 300 Å pores); split line: 15 cm×22 μm I.D. fused-silica capillary, (A) ACN–IPA–water (25:15:60, v/v, 0.2% TFA) and (B) ACN–IPA–water (65:15:20, v/v, 0.1% TFA); other conditions as in Fig. 7. Peaks: 1=γ-globulin (human, M_r $0.16 \cdot 10^6$), 2=β-galactosidase (*Aspergillus oryzae*, M_r $0.51 \cdot 10^6$), and 3=thyroglobin (bovine, M_r $0.67 \cdot 10^6$).

mobile phase improved the peak shapes of β-galactosidase and thyroglobin. A 2.3:1 ratio of peak areas of thyroglobin to γ-globulin was obtained using the column containing multimodal copolymer-encapsulated particles compared to a 1.5:1 ratio obtained using the C_8-bonded particles. Totally hydrophobic poly(1,3-tetradecene)-encapsulated particles provided similar results as the multimodal copolymer-encapsulated particles. The difference in recoveries measured for large proteins was more significant than for the smaller proteins. It is not clear whether or not proteins under reversed-phase conditions, where they are partially denatured, have unrestricted access to the 300 Å pores.

In certain cases, the multimodal polymer-encapsulated particles can provide improved selectivity. Fig. 10 shows an isocratic elution of four test peptides. Using totally hydrophobic polymer-encapsulated particles as packing material, leucine enkephalin co-eluted with angiotensin III. However, they were separated very well on the column containing multimodal polymer-encapsulated particles. Table 2 lists the column efficiencies and retention factors for 10 test peptides obtained using columns containing totally hydrophobic and multimodal polymer-encapsulated particles. These column efficiencies ($<30\,000$ plates m^{-1}) were much lower than that measured using ACN as test solute ($>70\,000$ plates m^{-1}), as demonstrated in Fig. 5. This suggests again that resistance to mass transfer, even for medium size peptides (M_r~1000), leads to a compromise in column efficiency.

From Table 2, it can be seen that greater retention factors are obtained using columns containing multimodal polymer-encapsulated particles for all test peptides. For some peptides (e.g., [Sar^1,Ala^8]-angiotensin II), ~100% increase in retention factor was observed. Since the same ratios of polymer mass to particle mass were used for preparing the total hydrophobic and multimodal polymer-encapsulated

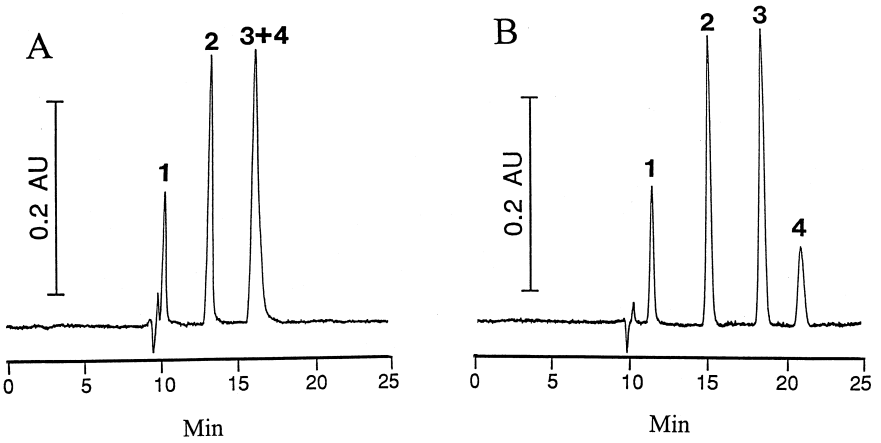

Fig. 10. Chromatograms showing selective separations of peptide standards. Conditions: 68 cm×250 μm I.D. capillary columns containing (A) poly(1-octadecene)-encapsulated and (B) 1-octadecene–maleic acid copolymer-encapsulated particles (5 μm diameter, 300 Å pores); ACN–water (25:75, v/v, 0.2% TFA); other conditions as in Fig. 2. Peaks: 1=[Sar[1],Ala[8]]-angiotensin II, 2=methionine enkephalin, 3=leucine enkephalin and 4=angiotensin III.

particles, the observed increase in retention should be due to the presence of –COOH groups on the multimodal polymer-encapsulated particle surface. Fig. 11 shows separations of a hemoglobin A_2 tryptic digest using columns containing totally hydrophobic and multimodal polymer-encapsulated particles. More than 10 additional peaks were resolved using the column containing the multimodal polymer-encapsulated particles. Using a longer column to obtain higher efficiency, the separation of this complex sample was improved. The limitation in the use of long columns is determined by the maximum pressure limit for the pump or valve injector, both of which are limited ~400 atm. The mobile phase linear velocity was only ~0.1 cm s^{-1} when using 1 m long capillary columns packed with 5 μm particles under 400 atm column inlet pressure.

Even though the –COOH groups on the bonded surface interacted with polar solutes, no increase in the retention factor was observed, i.e., there was not

Table 2
Efficiency and retention data for peptides on columns containing totally hydrophobic and multimodal polymer-encapsulated particles[a]

Peptide	*N* (plates)		*k*	
	Column 1	Column 2	Column 1	Column 2
[Sar[1],Thr[6]]-Angiotensin II	18 000	18 000	0.047	0.12
[Sar[1],Ala[8]]-Angiotensin II	15 000	14 000	0.071	0.16
Angiotensin I/II (1–7)	18 000	16 000	0.11	0.20
Methionine enkephalin	14 000	15 000	0.36	0.51
[Sar[1],Ile[8]]-Angiotensin II	12 000	13 000	0.33	0.60
[Asn[1],Val[5]]-Angiotensin II	14 000	15 000	0.34	0.61
Leucine enkephalin	12 000	16 000	0.58	0.84
Angiotensin III	13 000	14 000	0.59	1.09
Angiotensin II	11 000	12 000	0.66	1.05
Angiotensin I	7 000	10 000	1.68	2.72

[a] Conditions: 68 cm×250 μm I.D. columns containing (1) poly(1-octadecene)-encapsulated and (2) 1-octadecene–maleic acid copolymer-encapsulated particles (5 μm diameter, 300 Å pores); mobile phase: ACN–water (25:75, v/v, 0.2% TFA); UV (215 nm). The dead time was measured from the baseline disturbance injection signal.

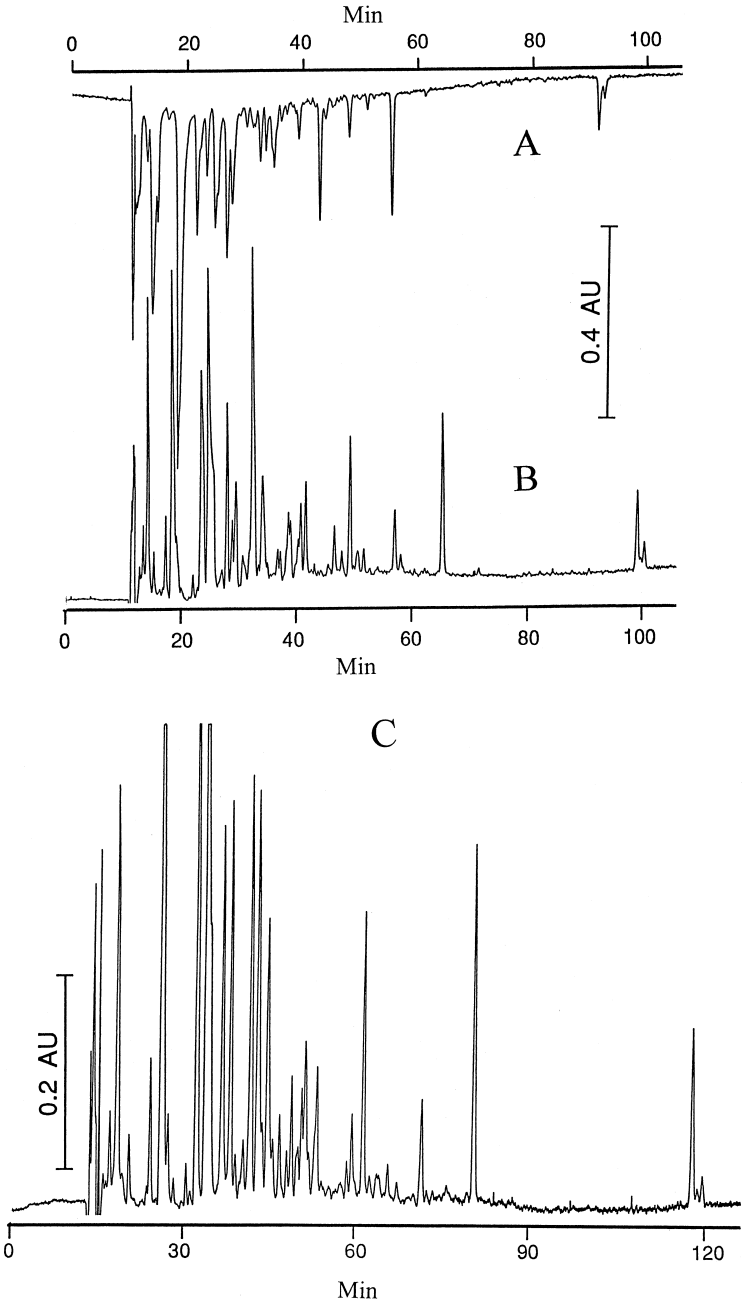

Fig. 11. RPLC of hemoglobin A_2 tryptic digest. Conditions: 80 cm×250 μm I.D. capillary columns containing (A) poly(1-octadecene)-encapsulated and (B) 1-octadecene–maleic acid copolymer-encapsulated particles (5 μm diameter, 300 Å pores); 80 cm×22 μm I.D. fused-silica capillary split line; (A) water (0.2% TFA) and (B) ACN–water (60:40, v/v, 0.2% TFA); (C) 105 cm×250 μm I.D. capillary columns containing 1-octadecene–maleic acid copolymer-encapsulated particles (5 μm diameter, 300 Å pores); 100 cm×22 μm I.D. fused-silica capillary split line; other conditions as in Fig. 2.

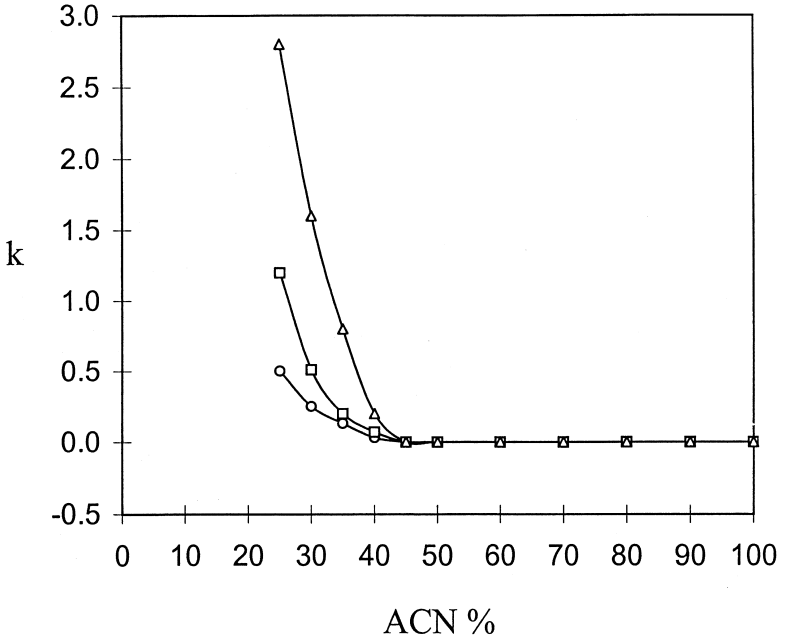

Fig. 12. Relationship between retention factor (k) and acetonitrile content in water (%, v/v, 0.2% TFA) on multimodal polymer-encapsulated particles. Conditions: 68 cm×250 μm I.D. capillary columns containing 1-octadecene–maleic acid polymer-encapsulated particles (5 μm diameter, 300 Å pores), 400 atm column inlet pressure, UV (215 nm). (△) Methionine enkephalin, (□) angiotensin III and (○) angiotensin I.

a ''U'' shape curve when increasing the concentration of ACN from 20% to 100% (v/v) for proteins and peptides. Fig. 12 shows the experimental relationship between retention and ACN concentration using peptides as test solutes. This further confirms that –COOH groups produced a weaker interaction with proteins and peptides than silanol groups in acidic mobile phases containing high organic solvent content.

Polymer-encapsulated particles are stable under typical RPLC conditions. The polymer coating on the particle surface isolates the surface from contact with reactive mobile phases. For the evaluation of column stability, column efficiency was found to be a more sensitive measurement than changes in solute retention [32]. In Fig. 5, van Deemter curves are compared for columns before and after rinsing. After the columns were rinsed with a mixture of ACN–water (70:30, v/v, 0.3% TFA) for five weeks, >50% loss in column efficiency was found for the column containing C_8-bonded silica particles, while <10% loss was observed for columns containing either totally hydrophobic polymer-encapsulated or multimodal copolymer-encapsulated particles. The introduction of –COOH groups onto the surface did not seem to lessen the stability of the particle surface coatings.

4. Conclusions

Coating and immobilizing polymers around silica particles is an effective approach to produce stable packing materials for RPLC of proteins and peptides. High solute recoveries can be achieved during the analysis of proteins, even for large proteins ($M_r > 0.5 \cdot 10^6$). Compared with a typical bonded reversed-phase, broad peaks were observed using totally hydrophobic polymer-encapsulated particles. This disadvantage can be improved by introducing hydrophilic –COOH groups onto the surface to yield a multimodal copolymer-encapsulated surface. Compared with totally hydrophobic polymer-encapsulated particles, the multimodal copolymer-encapsulated

particles provided greater retention and improved selectivity for a variety of proteins and peptides under RPLC conditions.

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