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Study of the irreversible adsorption of proteins on polybutadiene-coated zirconia

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ABSTRACT

The cause of irreversible adsorption of proteins on polybutadiene-coated zirconia is investigated by comparing the chromatographic properties of polybutadiene-coated zirconia with that of other reversed-phase packing materials such as bonded phase silica, polybutadiene-coated alumina and polybutadiene-coated silica. We find that the polybutadiene-coated zirconia has a micropore size distribution similar to that of the polybutadiene-coated alumina, from which some proteins can be eluted. Thus, the irreversible adsorption of proteins on polybutadiene-coated zirconia is not caused by entrapment of proteins in the micropores of the packing. The high hydrophobicity of the polybutadiene coating and the strong Lewis acid sites on the zirconia surface cause strong interactions between proteins and the stationary phase, the combination of which lead to irreversible adsorption of proteins on polybutadiene-coated zirconia.

INTRODUCTION

The demand for acid and base stable liquid chromatographic packing materials for biomedical separations [1] has motivated the development of zirconia-based HPLC packing materials [2–14]. Uniform micron-sized porous zirconia particles can now be prepared by polymerization induced colloidal aggregation (PICA) [15,16]. Successful separations of proteins on phosphate and fluoride-modified zirconia [4,7,8] by ion-exchange chromatography have shown that this zirconia has considerable potential for use in biomedical separations.

To make a reversed-phase chromatographic support from zirconia, Yu and El Rassi [17] modified the surface of the oxide with monomeric and polymeric octadecyl silanes. They claim that the resulting phase is stable up to pH 12 in phosphate buffer and that elution of proteins can be observed under typical reversed-phase protein elution conditions. A more alkaline-stable reversed-phase, polybutadiene (PBD)-coated zirconia, was developed by Rigney *et al.* [3]. This phase is stable indefinitely in the pH range 1–14 and has been used successfully for the separation of small molecules. However, PBD-coated zirconia is not yet useful for protein separations due to the nearly irreversible adsorption of proteins on its surface [18]. The

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objective of this work is to understand the cause of this irreversible adsorption so that a biocompatible highly alkaline-stable reversed-phase stationary phase can be prepared.

Irreversible adsorption of proteins on reversed-phase stationary phases is generally caused by their entrapment in micropores in the packing materials [19]. Strong hydrophobic interactions between protein molecules and solid surface also cause irreversible adsorption [20]. We consider the following hypothesized mechanisms as possible causes of irreversible adsorption of proteins on PBD-coated zirconia: (1) the zirconia particles have a large volume fraction of micropores that trap proteins, (2) the PBD coating on zirconia is especially hydrophobic toward proteins and (3) strong interactions take place between the carboxyl groups of proteins and the Lewis acid sites [6–12] on the zirconia surface.

To determine whether zirconia has a large fraction of micropores that can trap proteins, the pore size distribution of PBD-coated zirconia was characterized by size-exclusion chromatography (SEC). The use of SEC for pore size characterization is well documented [21–23]. One of the advantages of using SEC to characterize chromatographic packing materials compared to nitrogen sorptometry and mercury porosimetry is that the characterization is done under conditions which closely mimic those in chromatography. To study the hydrophobicity of PBD-coated zirconia, the chromatographic properties of alkylbenzenes and phenylalanine polypeptides were examined. Finally, the surface Lewis acid properties of zirconia and alumina were compared by varying the number of carboxyl groups in several probe peptides.

It has been reported that PBD-coated Biotage alumina and PBD-coated silica allow elution of proteins [24–26]. Therefore, a comparative study on PBD-coated zirconia, PBD-coated Biotage alumina, and PBD-coated wide pore (50 nm) Nucleosil silica should allow us to diagnose the cause of the irreversible adsorption of proteins on PBD-coated zirconia. PBD coatings on zirconia, alumina and silica were prepared identically. In addition, we compared the hydrophobicity of these PBD-coated phases with that of a conventional silica C_{18}-bonded phase.

MATERIALS AND METHODS

Reagents

All solvents used were HPLC grade and were obtained from Fisher Scientific (Fairlawn, NJ, USA).

Vydac C_{18} phase and bare Nucleosil 500-5 silica bulk packing materials were obtained from Alltech (Deerfield, IL, USA). Bare alumina packing material was obtained from Biotage (Charlottesville, VA, USA).

Polybutadiene of molecular mass 4500 was obtained from Aldrich (Milwaukee, WI, USA). Polystyrene molecular mass standards (M_r = 300, 1000, 2550, 4000, 7600, 19 600, 30 000, 47 000, 207 750, 490 000, $1 \cdot 10^6$, $2.43 \cdot 10^6$, $6 \cdot 10^6$ and $20 \cdot 10^6$) were obtained from Polysciences (Warrington, PA, USA).

All proteins listed in Table II and peptides [phenylalanine (Phe), di-Phe, tri-Phe, tetra-Phe, penta-Phe and aspartyl-phenylalanine (Asp–Phe)] were obtained from Sigma (St. Louis, MO, USA).

Zirconia colloid (Zr100/20) was obtained from Nyacol Products (Ashland, MA, USA). Urea was obtained from Mallinckrodt (St. Louis, MO, USA) and formaldehyde was obtained from Fisher Scientific.

Preparation of uniform porous zirconia particles as an HPLC packing material

Zirconia particles were prepared by a controlled aggregation method based on the work by Iler and McQueston [27], the detailed procedure is described in an earlier paper [15]. A pH 3 Zr100/20 [100 nm diameter, 20% (w/w), where 90% of the total mass of colloid are 100 nm in diameter] zirconia sol was used for preparing the porous zirconia support. The sol was adjusted to pH 2 by adding concentrated nitric acid. A 75-g amount of urea and 125 ml of formaldehyde were added and mixed with each 1000 ml of zirconia sol. The mixture was allowed to stand for 15 min and the reaction was stopped by diluting the reaction mixture four-fold with water. The particles were collected by settling under gravity, and the urea–formaldehyde polymer was removed by heating at 170°C for 24 h and 375°C for 2 h in air. The particles were then

sintered at 750°C for 6 h and at 900°C for 3 h. The sintered particles were washed in 0.5 *M* hydrochloric acid and then 0.5 *M* sodium hydroxide, and then dried. The particles thus made (Fig. 1) were 8 μm in diameter with 27 m^2/g surface area (BET) and 0.15 ml/g pore volume.

Preparation of PBD coatings

PBD coatings were prepared according to the procedure of Rigney *et al.* [3]. Bare zirconia, Biotage alumina and Nucleosil silica were dried in a vacuum oven at 120°C for 16 h and cooled over phosphorus pentoxide immediately before the coating preparation. Particles were then transferred to a dry round-bottomed flask with a baffle. A solution of 0.1% (w/v) M_r 4500 PBD in hexane was added to the particles. The total amount of PBD added was 0.6 mg PBD per square meter of surface area. While sonicating, a vacuum was applied to the slurry for 5 min to remove the air in the pores. Then, the slurry was gently swirled on a rotary evaporator for 8 h. A 0.05% (w/v) dicumyl peroxide in hexane solution was added to the slurry (2.5 mg of dicumyl peroxide per 100 mg of PBD), and the slurry was sonicated and swirled for an additional hour. The solvent in the slurry was then evaporated over 1 h by applying a mild vacuum at 35°C. The polymer-coated particles were thermally cross-linked in a vacuum oven at 120°C for 1 h and 160°C for 4 h. Finally, the particles were sequentially washed with hexane, dichloromethane, tetrahydrofuran (THF) and hexane.

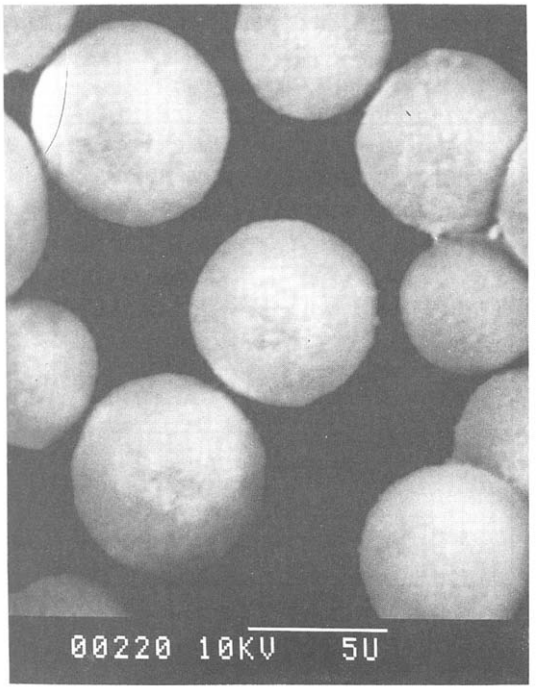

Fig. 1. Scanning electron micrograph of zirconia particles used in this work. The particles were 8 μm in diameter with a surface area of 27 m^2/g, an average pore diameter of 23 nm, and a pore volume of 0.15 ml/g.

Chromatographic experiments

Chromatographic experiments were performed on a Hewlett-Packard HP 1090 liquid chromatograph with detection at a wavelength of 254 nm. The column temperature was 30°C.

PBD-coated supports were suspended in hexane–isopropanol (10:90, v/v), and packed into 50 × 4.6 mm I.D. columns by an upward stirred slurry method. The packing pressure used was 345 bar (5000 p.s.i.). Isopropanol was used as the displacing solvent. Details of the physical characteristics of each packing material and the column dimension are given in Table I.

Comparisons of the retention of small molecules on PBD-coated zirconia, PBD-coated alumina and PBD-coated silica were carried out

TABLE I

CHARACTERISTICS OF PACKING MATERIALS

All columns are 50 × 4.6 mm.

Packing materials	S.A.[a] (m^2/g)	d_{pore}[b] (Å)	*C* (%, w/w)[c]	C (mg/m^2)[d]
PBD/ZrO_2	25	230	1.2	0.45
PBD/Al_2O_3	45	250	1.6	0.46
PBD/SiO_2	28	500	1.7	0.51
Vydac C_{18}	59	300	8.15	1.38

[a] Specific surface area by BET.
[b] Pore diameter of the corresponding bare supports reported by manufactures except for bare zirconia that was measured by BET (average diameter).
[c] Carbon loading per unit mass of packing material.
[d] Carbon loading per square meter of surface area.

in a methanol–water (40:60) mobile phase. The mobile phases used to study the elution of proteins and peptides were: A = acetonitrile with 0.1% trifluoroacetic acid (TFA); B = 0.1% TFA aqueous solution. Gradients from 10 to 90% A in 20 min were employed for protein elution. Isocratic elution schemes were used to study the peptides.

Peptide and protein samples were prepared in mobile phase B at a concentration of 0.5%. A typical injection volume of 10 μl was used.

Pore size characterization by size-exclusion chromatography

The pore size distributions of PBD-coated phases were characterized by SEC based on the work of Knox and co-workers [22,23]. THF was used as the mobile phase at a flow-rate of 0.2 ml/min. Benzene was used as the totally included molecule, and its elution volume V_m is taken as the total pore volume of the column. Polystyrene of molecular mass $20 \cdot 10^6$ was used as the totally excluded molecule, so its elution volume V_0 is taken to be the interstitial pore volume. Polystyrene samples were prepared in THF at a concentration of 0.05%. The elution volume of each polystyrene standard, V_i, was measured four times to obtain an average value. The relative standard deviations of elution volumes for all samples were less than 0.5%. Each column was characterized twice to ensure precision. The distribution coefficient K was calculated as $K = (V_i - V_0)/(V_m - V_0)$, which is the volume fraction of pores accessible to the corresponding molecular mass polystyrene molecule. A plot of K *vs.* the effective size of each polystyrene standard ($r_{eff} = 0.123 M_r^{0.588}$ [21], where M_r is the molecular mass of polystyrene) gives the calibration curve.

RESULTS AND DISCUSSION

Rigney [18], Schafer [28] and Weber [29] found that a number of common proteins, such as insulin and bovine serum albumin, are irreversibly adsorbed to a variety of zirconia based PBD-coated supports. These materials ranged from PBD-coated native zirconia [18], PBD coated on phosphate modified zirconia [28] and PBD coated on chemical vapor deposited carbon covered zirconia [29]. Given the tremendous alkaline stability of PBD-coated zirconia and the importance of alkaline stability for large scale processing of biopharmaceuticals [1], this has been a very disappointing finding. The purpose of the present work has been to study and hopefully find a solution to this problem.

Retention of small non-polar molecules

The comparable PBD loading per unit surface area on zirconia, alumina and silica (Table I) indicates that they all have similar surface coverages with PBD. The linear relationship between log k' and the number of alkyl carbon for the above three phases (Fig. 2) indicates that all three have reversed-phase characteristics. Flow-rate studies show that PBD-coated zirconia and PBD-coated alumina have similar mass transfer properties (Fig. 3). Thus we conclude that the three PBD-coated phases are similar both chemically and chromatographically for the RPLC separation of small, non-polar solutes.

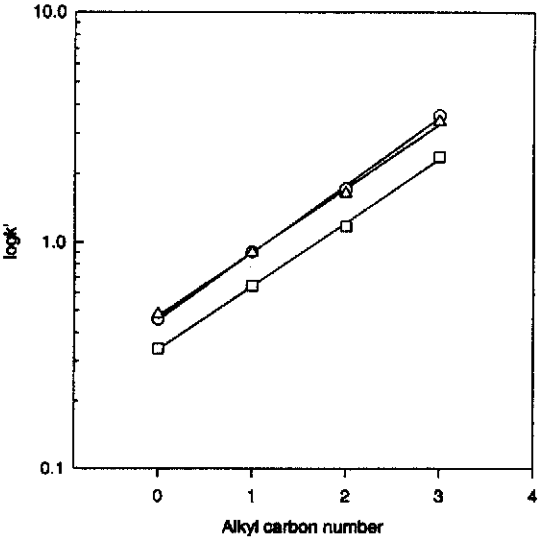

Fig. 2. Retention of benzene, toluene, ethylbenzene and propylbenzene *vs.* side chain alkyl carbon number for PBD-coated zirconia (○), PBD-coated alumina (□) and PBD-coated silica (△). The mobile phase was methanol–water (40:60). The flow-rate was 1 ml/min, 25°C.

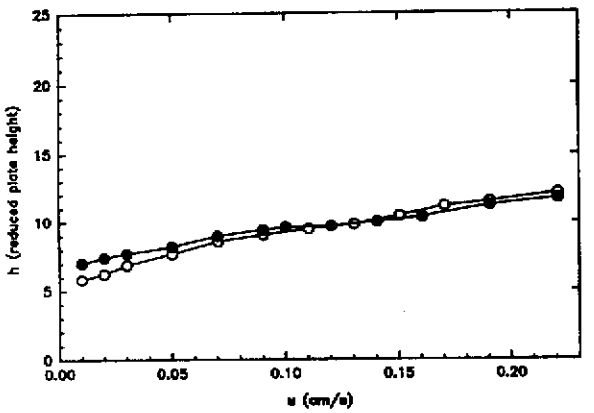

Fig. 3. Comparison of column efficiency of PBD-coated zirconia (●) and PBD-coated alumina (○). Mobile phase used was methanol–water (40:60). Toluene was the probe solute.

Elution of proteins

Although PBD-coated zirconia, alumina and silica have similar properties regarding small molecules, they behave very differently toward proteins. Under the same elution conditions, a number of proteins can be eluted from PBD-coated alumina (Table II) while no elution of any protein was observed on either PBD-coated zirconia or PBD-coated silica. It is well known that C_{18} bonded phase silica is an excellent material for the separation of proteins so it was not included in this study. Ghost protein peaks were observed with PBD-coated alumina during subsequent "dummy" gradient runs in which no protein was injected. This indicates low recovery of protein from PBD-coated alumina. In order to understand the cause of the difference between PBD-coated alumina and PBD-coated zirconia, we compared the pore size distributions and surface chemical properties of these two phases.

Pore size characterization by SEC

The pore size distributions of PBD-coated zirconia, PBD-coated alumina and PBD-coated silica were determined by SEC. The main objective of these experiments was to assess whether PBD-coated zirconia presents a significantly higher fraction of micropores (<5 nm) than PBD-coated alumina, since such micropores might trap proteins. The studies of Tanaka *et al.* [19] indicate the importance of avoiding micropores in protein chromatography. This can be done by examining SEC calibration curves [21,22].

Fig. 4 shows the SEC calibration curves for the PBD-coated phases. The distribution coefficient K is independent of molecular mass of polystyrene in the range of $1 \cdot 10^6$ to $20 \cdot 10^6$ for all three phases. This indicates that there is no adsorption of polystyrene on any of these phases and that the $20 \cdot 10^6$ molecular mass polystyrene is completely excluded from the internal pores. The calibration curves of the three PBD-coated phases overlap in the region less than 5 nm in diameter. This indicates that PBD-coated zir-

TABLE II

REVERSED-PHASE ELUTION OF PROTEINS FROM PBD-COATED PACKING MATERIALS

Mobile phase A was 0.1% TFA in acetonitrile and mobile phase B was 0.1% TFA in water. Gradients from 10 to 90% A in 20 min at 1 ml/min were used.

Proteins	Molecular mass	Isoelectric point	k' (PBD/Al_2O_3)	k' (PBD/ZrO_2)	k' (PBD/SiO_2)
Insulin	5700	5.7	9.3	n.e.o.[a]	n.e.o.
Lysozyme	14 000	11.0	10.3	n.e.o.	n.e.o.
Cytochrome *c*	11 000	10.2	10.1	n.e.o.	n.e.o.
Ribonuclease *b*	14 700	9.3	8.9	n.e.o.	n.e.o.
Bovine serum albumin	68 000	4.7	13.5	n.e.o.	n.e.o.
β-Lactoglobulin	35 000	5.5	12.9	n.e.o.	n.e.o.
Hemoglobulin	68 000	6.9	13.2	n.e.o.	n.e.o.

[a] n.e.o. = No elution observed.

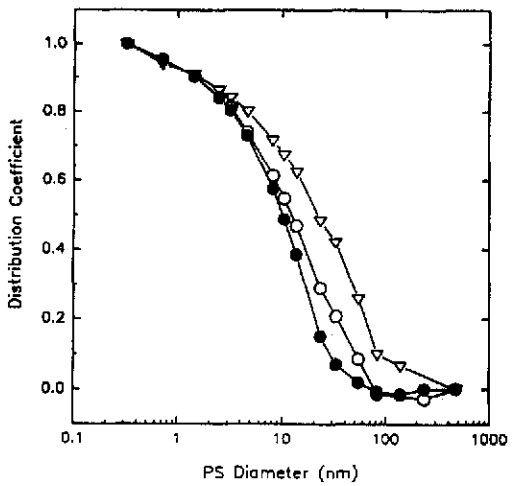

Fig. 4. Comparison of pore size distribution of PBD-coated packings by SEC. ▽ = PBD/SiO_2; ○ = PBD/Al_2O_3; ● = PBD/ZrO_2. THF was used as mobile phase, polystyrene (PS) molecular mass standards were used as the probes. The experiment was done at 30°C.

conia has no more micropores than PBD-coated alumina. Thus we conclude that micro pore entrapment is unlikely to the cause of the irreversible adsorption of proteins on PBD-coated zirconia.

Since wide-pore conventional silane-modified Nucleosil silica is excellent for protein separations [19]. The fact that we could not elute proteins from the PBD-coated silica also indicates that a poor pore size distribution is not the cause of the irreversible adsorption of proteins on PBD-modified zirconia and silica.

Comparison of hydrophobicity

The retention of small solutes in reversed-phase chromatography can be approximated as a function of mobile phase composition by the equation:

$$\log k' = \log k'_w - S\varphi \quad (1)$$

where φ is the volume fraction of organic modifier, k'_w is the hypothetical capacity factor of retention in pure water and S is the slope of the relationship. S is directly related to the free energy of transferring a solute from organic modifier phase to pure water. Examination of the differences in S and k'_w for PBD-coated zirconia, PBD-coated alumina and C_{18} silica might allow us to diagnose why proteins did not elute from PBD-coated zirconia. The data shown in Fig. 2 suggest that for small solutes there is not much difference in the hydrophobicity of the three materials. We decided to test the hydrophobicity with a series of homopeptides.

Phe is an amino acid with a phenyl side chain and is therefore very hydrophobic. Homopeptides of Phe composed of one to five Phe units were used as probe solutes to study the hydrophobicity of the C_{18} bonded phase silica, PBD-coated zirconia and PBD-coated alumina. Plots of the retention of these homopeptides *vs.* volume fraction of acetonitrile are shown in Fig. 5. The C_{18} bonded phase is significantly more retentive than either PBD-coated zirconia and alumina. Retention on a PBD-coated alumina is so weak that PHE and di-PHE are virtually unretained and thus are not shown in Fig. 5c.

A very startling observation is that the phenylalanine monomer is more retained than is the dimer at *all* mobile phase compositions (Fig. 5b). At 30% acetonitrile it is even more retained than is the pentapeptide. Further the k' of the monomer is virtually independent of mobile phase composition. These observations are probably due to the overwhelmingly strong interactions between the dissociated carboxyl group on Phe and the Lewis acid sites on the zirconia surface [6–12].

The slopes (S) and intercepts ($\log k'_w$) of the plots shown in Fig. 5 are given in Figs. 6 and 7 as plots *vs.* the number (n) of Phe units. The slope of the $-S$ *vs.* n plots for the PBD-coated phases are significantly larger than that of the C_{18} bonded silica phase. In other words, the increase in retention with increase in hydrophobicity of PHE homopeptides is much faster on PBD-coated phases than that on the Vydac C_{18} bonded silica. Thus, both types of PBD-coated phases are more hydrophobic than the Vydac C_{18} phase, which could lead to the irreversible adsorption of proteins on PBD-coated phases. This difference in hydrophobicity could be due to the association of acetonitrile with PBD and the bonded phase, the details of which will be explored in future studies. Fig. 7 shows that the slopes of $\log k'_w$ *vs.* the number of Phe units do not differ significantly among the three phases. This indicates

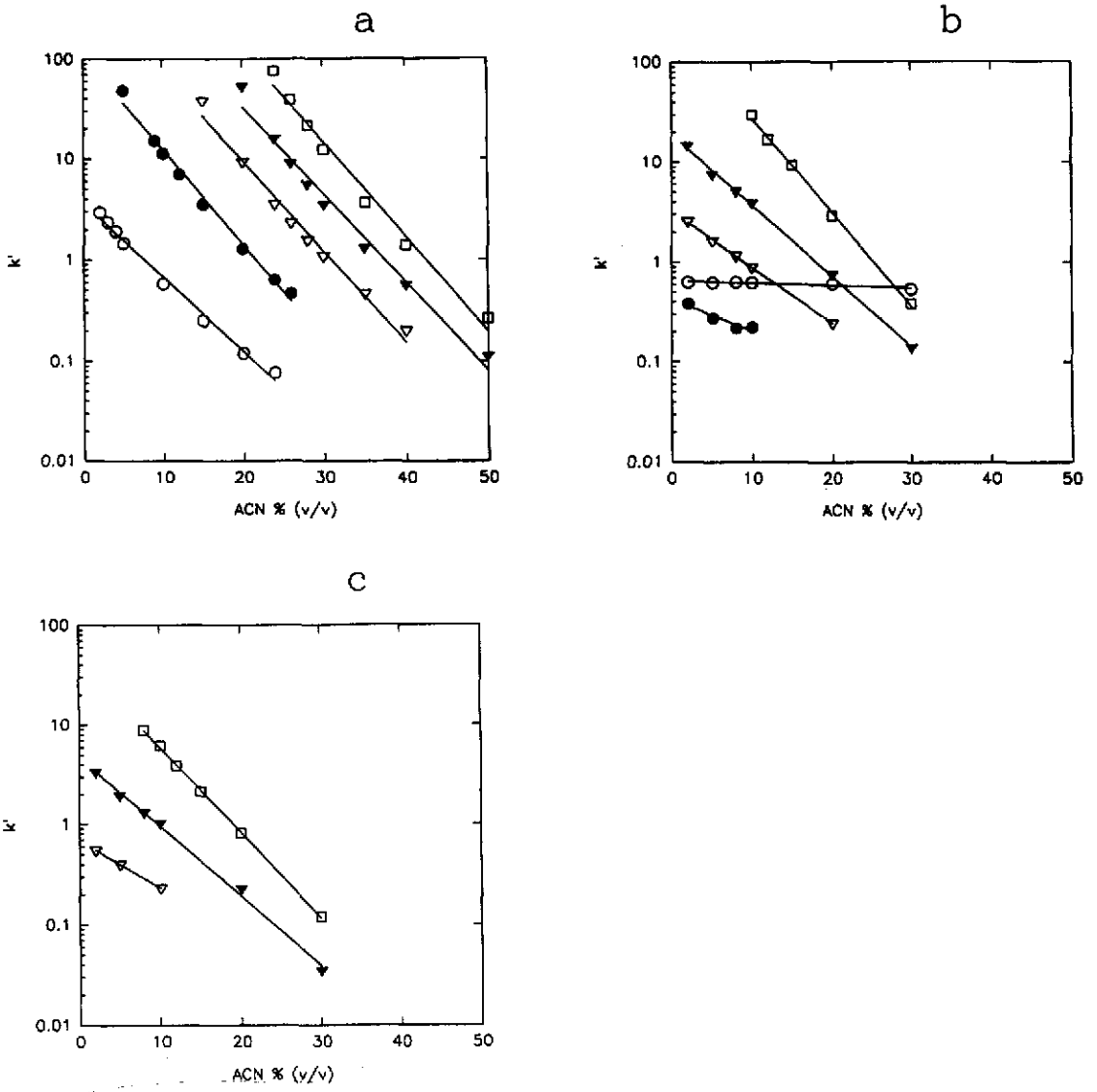

Fig. 5. Retention of Phe polypeptide *vs.* the volume fraction of acetonitrile (ACN) in the mobile phase (acetonitrile–water–0.1% TFA). (a) C_{18} silica; (b) PBD-coated zirconia; (c) PBD-coated alumina. ○ = Phe; ● = di-Phe; ▽ = tri-Phe; ▼ = tetra-Phe; □ = penta-Phe. Experiments were done at 30°C.

that the free energy decrease upon transfer of a Phe unit from pure water to the stationary phase is about the same for all three stationary phases.

The apparently higher hydrophobicity of the PBD-coated phases based on the *S* value could be the cause of the irreversible adsorption of proteins on the PBD-coated zirconia and low recovery of proteins from PBD-coated alumina. However, we feel that we must not rule out the possibility that strong Lewis acid–base interaction sites are ultimately responsible for the irreversible adsorption of proteins on PBD-coated zirconia.

Interaction of Lewis acid sites on stationary phases with solutes

It is well known that the surface of zirconia has Lewis acid sites that strongly interact with carboxyl groups [6–12]. Rigney [18] found the PBD coating does not completely block the

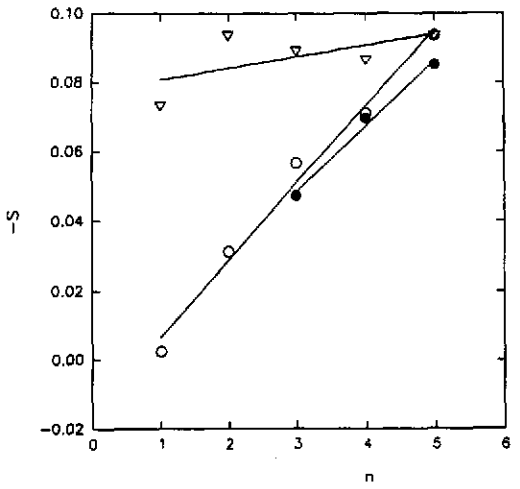

Fig. 6. The slopes of plots in Fig. 5 *vs.* the Phe unit number *n*. ▽ = C_{18} SiO_2; ○ = PBD/ZrO_2; ● = PBD/Al_2O_3.

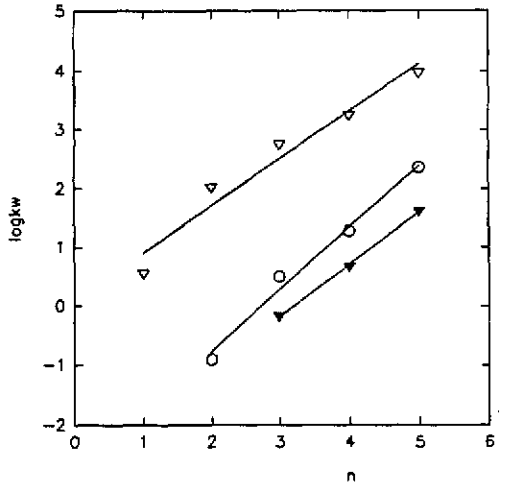

Fig. 7. The intercepts of plots in Fig. 5 are plotted *vs.* the Phe unit number *n*. ▽ = C_{18} SiO_2; ○ = PBD/ZrO_2; ▼ = PBD/Al_2O_3.

access of phosphate and small carboxylates to the Lewis acid sites on the surface of zirconia. The exposed Lewis acid sites might well interact with the carboxyl groups on proteins, causing irreversible adsorption of proteins.

Compared to zirconia, Biotage alumina has much weaker Lewis acid sites [9]. This is supported by the fact that Asp–Phe elutes from PBD-coated alumina but not from PBD-coated zirconia (Table III). In contrast to Asp–Phe (Fig. 5b), we see that Phe homopeptides up to penta-Phe elute from the PBD-coated zirconia. Thus, the additional carboxyl group on aspartic acid relative to any of the homopeptides must be responsible for the irreversible adsorption of Asp–Phe on PBD-coated zirconia. The addition of citric acid to the mobile phase caused the elution of Asp–Phe from PBD-coated zirconia and greatly improved the plate number (N) for Asp–Phe on PBD-coated alumina. Since proteins have many carboxyl groups, it is reasonable to expect that they might follow the trend described here.

CONCLUSIONS

PBD-coated zirconia has a higher hydrophobicity than does a conventional C_{18} bonded phase. On the other hand, the Lewis acid sites on zirconia surface exhibit very strong affinity toward carboxyl groups. Both may contribute to the irreversible adsorption of proteins on PBD-coated zirconia. It is clear that micropore entrapment can be ruled out. Very recent experimental work carried out after the *10th International Symposium on Preparative Chromatography* held

TABLE III

EFFECT OF CITRIC ACID ON THE ELUTION OF Asp–Phe

Stationary phase	No addition of citric acid[a]		50 m*M* citric acid added[b]	
	Recovery (%)	Plate number	Recovery (%)	Plate number
PBD/ZrO_2	0	–	100	45
PBD/Al_2O_3	100	45	100	145

[a] Mobile phase was 20% acetonitrile, 79.9% water and 0.1% TFA at 1 ml/min.
[b] 50 m*M* of citric acid was added in the mobile phase described in footnote *a*.

in June 1993 indicates that many proteins can be eluted from PBD-coated zirconia in TFA–acetonitrile–water system if both phosphoric acid and a high concentration of salt are added to the eluent. This work is in progress and details will be reported in a subsequent communication.

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