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Performance of wide-pore silica- and polymer-based packing materials in polypeptide separation: effect of pore size and alkyl chain length

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ABSTRACT

The effects of pore size and alkyl chain length of silica- and polymer-based packing materials in the elution of polypeptides with an acetonitrile gradient in the presence of trifluoroacetic acid were studied. Considerable differences were found in the performance of alkylsilylated phases prepared from various wide-pore silica particles assumed to have 30–50-nm pores. The pore size of such silica gels was found to be the critical factor in determining the efficiency for high-molecular-weight polypeptides. Silica C_{18} phases having small pore volumes below 20 nm pore diameter showed comparable performances to C_4 and C_8 phases for polypeptides with molecular weights of up to 80 000, and were more stable. Polymer-based packing materials with adequate pore size provided excellent column efficiencies and recoveries for polypeptides with higher chemical stabilities than silica-based materials.

INTRODUCTION

Silica-based packing materials for reversed-phase liquid chromatography (RPLC) have been extensively studied with respect to silica surface and bonding chemistry in order to produce current high-performance materials. These silica-based packing materials, however, are not totally satisfactory. Relatively low chemical stability in acidic and basic mobile phases and an inadequate performance for high-molecular-weight polypeptides are potential problems with these materials. Although the pore sizes were taken into account when chemically bonded silica gels were applied to the separation of high-molecular-weight solutes [1–10], current wide-pore packing materials have not yet been critically evaluated in this respect. The advantage of wide-pore over small-pore materials in polypeptide separations has been

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well recognized. However, different wide-pore silica gels have rarely been compared with each other.

Polymer gels for RPLC possessing higher chemical stability have been introduced recently. Although their performances with small molecules are generally believed to be lower than those of silica-based materials, they have been employed for the separation of small molecules and polypeptides with adequate performance, especially under severe elution conditions [11].

Silica- and polymer-based packings are frequently compared with each other, and they should complement each other in the separation of small molecules, because silica-based packings are not as chemically stable as polymer gels and polymer gels are generally less efficient, particularly in highly aqueous mobile phases.

One area of RPLC where silica- and polymer-based packings can be in serious competition is the separation of polypeptides under an acetonitrile gradient in the presence of trifluoroacetic acid (TFA). Although a high performance of some polymer gels has been reported [10,12–14], silica-based materials are still mainly used in these applications [10,15]. The most commonly employed packing materials are prepared from wide-pore silica gels having a pore size of *ca.* 30 nm bonded with short alkyl groups such as C_3–C_6 [10,15,16]. These materials however, possess a serious problem with regard to chemical stability [17,18], in spite of their better performance than longer alkyl-chain stationary phases in terms of protein recovery and column efficiency. On the other hand, Szczerba *et al.* [19] reported that a wide-pore C_{18} stationary phase showed a high column efficiency for high-molecular-weight polypeptides.

Users should be better informed about the choice of wide-pore packing materials, whether short-chain alkyl-bonded silica phases are really required, or whether more stable stationary phases can also be used. The effect of chain length should be examined with wide-pore silica gels with sufficiently large pores, as pointed out by Cooke *et al.* [4]. We report here that silica C_{18} and polymer-based packing materials with adequate pore sizes can give high efficiencies for polypeptide separations and higher chemical stabilities than silica C_4 phases. Polymer-based packing materials having C_4 or C_8 alkyl groups provide both high efficiency and high recoveries for high-molecular-weight or hydrophobic polypeptides.

EXPERIMENTAL

Equipment

Two types of liquid chromatograph were used. One consisted of two Model 880 pumps and a Model 875 UV detector (JASCO, Tokyo, Japan) with a Model C-R3A data processor (Shimadzu, Kyoto, Japan), and the other two Model LC-6A pumps, a Model SPD-6A UV detector and a Model C-R3A data processor (all from Shimadzu).

Materials

The silica gels and polymer gels listed in Table I were examined. In addition to the commercially obtained materials, some experimental silica gels, Hypersil 300E, Shiseido 300E and Kromasil 200E, were also examined (according to the manufacturer of Hypersil, the current commercial materials possess the characteristics of Hypersil

300E used in this work). All the silica particles were derivatized to C_4 and C_{18} phases with maximum surface coverage by using alkyldimethylchlorosilanes as reported previously [20]. A C_{18} phase ($C_{18}T$) was also prepared from LiChrospher Si 500 by using octadecyltrichlorosilane instead of monochlorosilane.

Prepacked columns were used for Asahipak (150 mm × 4.6 mm I.D.) and Shodex (150 mm × 6 mm I.D.) materials, and PLRP-S 300, TSK C_{18}-4PW and all the silica-based materials were packed into stainless-steel columns (100 mm × 4.6 mm I.D.) in the laboratory.

Protein standards were obtained from Sigma (St. Louis, MO, U.S.A.). TFA and HPLC-grade solvents, acetonitrile and water were obtained from Nacalai Tesque (Kyoto, Japan).

Characterization of packing materials

Transmission electron microscopy (TEM) was carried out at the Nitto Technical Information Centre. Ultrathin sections of the particles were prepared as reported previously [21].

Nitrogen adsorption measurements were carried out at the Shiseido Research Centre by using Autosorb I (Quantachrome, Syosset, NY, U.S.A.). The pore-size distribution of the polymer gels was also determined by inverse size-exclusion

TABLE I

PORE PARAMETERS OF WIDE-PORE SILICA AND POLYMER GELS

Manufacturer's specifications (with values found experimentally by nitrogen adsorption in parentheses).

Packing material	Surface area (m^2/g)	Pore size (nm)[a]	Pore volume (ml/g)	Particle size (μm)	Manufacturer
LiChrospher Si 500	50(69)	50(40)	0.8(0.90)	10	Merck
Spherisorb 300	190(209)	30(35)	1.5(1.62)	5	Phase Separations
Nucleosil 300	100(109)	30(15,70)	0.8(0.81)	5	Machery, Nagel & Co.
Vydac TP	80(93)	30(26)	0.6(0.69)	10	Separations Group
Hypersil 300(I)	60(58)	30(20)	0.6(0.53)	5	Shandon
Hypersil 300(II)	(47)	(20)	(0.56)	5	
Hypersil 300E	(71)	(35)	(0.73)	5	
Shiseido 300E	(180)	(26)	(1.47)	5	Shiseido
Kromasil 200E	(174)	20(18)	(0.86)	10	Eka Nobel
Kromasil 100	350	10	0.9	10	
Cosmosil 100	330(300)	11(12)	(1.08)	5	Nacalai Tesque
PLRP-S 300	(380)	30(60)	(1.26)	8	Polymer Labs.
TSK C_{18}-4PW	(64)	(50)	(0.64)	10	Tosoh
Asahipak ODP	(108)	(26)	(0.57)	6	Asahi Chem.
Asahipak C8P	(260)	(26)	(0.81)	6	Ind.
Asahipak C4P	(374)	(26)	(0.95)	6	
Shodex D18-613	(56)	–	(0.42)	6	Showa Denko
Shodex D8-613	(98)	–	(0.53)	6	
Shodex D4-613	(100)	–	(0.54)	6	
Shodex DE-613	(354)	(5)	(0.41)	6	

[a] Experimentally obtained values indicate the pore diameter that corresponds to the maximum in the pore volume–pore radius plot.

chromatography using polystyrene standards in tetrahydrofuran as reported by Knox and Scott [22].

The metal contents of the silica particles were examined by inductively coupled plasma atomic emission spectrometry (Jobin-Yvon, Longjumeau, France).

The chemical stabilities of the packing materials, Shodex D4-613, LiChrospher C_4 and C_{18} and also $C_{18}T$, were tested by keeping the materials in columns in 0.1% TFA at 60°C. The columns were flushed with 20 ml of tetrahydrofuran at intervals at room temperature, and the retention of alkylbenzenes was measured in 60% methanol at 30°C.

Chromatographic measurements

Two types of linear acetonitrile gradient were used: (I) from 20% acetonitrile (0.1% TFA) to 60% acetonitrile (0.1% TFA) in 20 min, and (II) from 0.1% TFA in water to 60% acetonitrile (0.1% TFA) in 30 min. Type I was used for the evaluation of packing materials with the three polypeptides, cytochrome *c*, lysozyme and bovine serum albumin (BSA), and type II for the elution of a wider range of peptides (molecular weight in parentheses), glycyltyrosine (238), α-endorphin ($1.7 \cdot 10^3$), Leu-enkephalin (556), insulin (6000), cytochrome *c* ($12 \cdot 10^3$), transferrin ($80 \cdot 10^3$), BSA ($66 \cdot 10^3$), β-lactoglobulin ($18 \cdot 10^3$) and γ-globulin ($160 \cdot 10^3$).

Protein recovery was examined with a type I gradient by observing the peak areas of cytochrome *c*, lysozyme, BSA, β-lactoglobulin and ovalbumin in the gradient elution and two subsequent runs without sample injection. The ratio of the peak area in the first gradient run to the total peak areas of the three runs obtained with the C_4 phase was taken as the recovery of proteins. The recoveries on the other phases were calculated by normalizing the peak area by using BSA as a standard.

RESULTS AND DISCUSSION

Silica-based packing materials

Several wide-pore silica gels, shown in Table I, including the commercially obtained materials and the experimental batches (Hypersil-300E, Shiseido 300E and Kromasil 200E) and also small-pore materials (Kromasil 100 and Cosmosil 100) were examined. Average pore sizes determined by nitrogen adsorption agreed well with the specifications of the commercial material, either 30 or 50 nm for wide-pore silica gels, although considerable variations were found in the pore-size distributions. The pore size corresponding to maximum pore volume of each packing material is given in Table I. Nucleosil 300, showing a bimodal pore-size distribution in Fig. 1b, is a blended material as previously observed by TEM [21].

As shown in Fig. 1, LiChrospher Si 500 and Spherisorb 300 possess small pore volumes below 20 nm pore diameter, whereas Hypersil 300 (I and II), Vydac TP and Nucleosil 300 showed the presence of considerable pore volumes in the pore-size range 10–20 nm. The two experimental batches of silica gel, Hypersil 300E and Shiseido 300E, showed relatively sharp pore-size distributions with small pore volumes below 20 nm. The pore sizes of Kromasil 200E, Kromasil 100 and Cosmosil 100 were *ca.* 18, 10 and 12 nm, respectively. A considerable overlap was seen in the pore-size distributions of Kromasil 200E and Hypersil 300.

All the silica gels were derivatized to C_4 and C_{18} phases and tested in the gradient

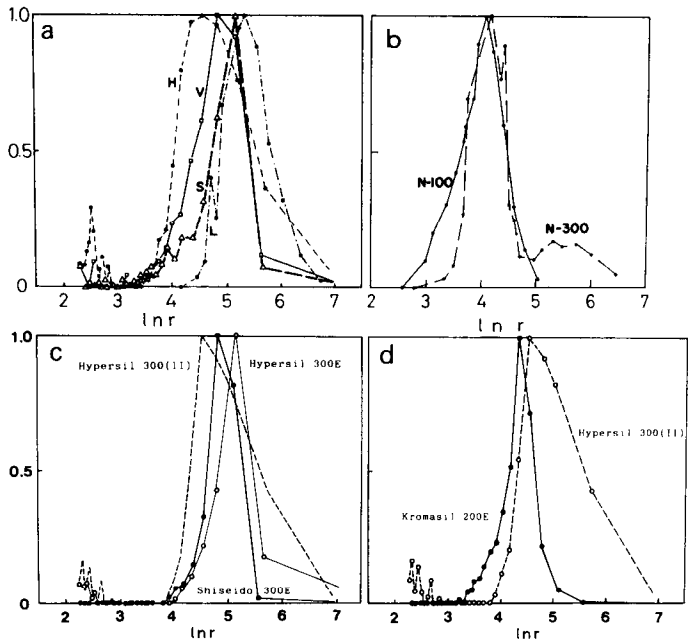


Fig. 1. Pore-size distributions of silica particles determined by nitrogen adsorption. The vertical axis corresponds to the fraction of pore volume; r = pore radius. (a) L = LiChrospher Si 500; S = Spherisorb 300; V = Vydac TP; H = Hypersil 300(I). (b) N-300 = Nucleosil 300; N-100 = Nucleosil 100. (c) Hypersil 300E, Hypersil 300(II) and Shiseido 300E. (d) Kromasil 200E and Hypersil 300(II).

elution of polypeptides. The interaction of the stationary phases with basic substances was minimized by washing the silica particles with acids prior to the bonding reaction with alkyldimethylchlorosilanes to achieve maximum surface coverages. For small molecules, differences in the preparation methods and in the silica particles resulted in a variety of commercial C_{18} phases, providing different selectivities which can be chromatographically characterized by using several sets of solutes [23]. In this study, a variety of packing materials with C_4–C_{18} alkyl groups prepared from various wide-pore silica gels were subjected to examination with polypeptides.

Although silica-based short-chain alkyl-bonded phases have been extensively employed in polypeptide separations [15], the use of C_{18} phases would be preferable if chemical stabilities are to be considered. As shown in Fig. 2, the C_{18} phases of LiChrospher Si 500 and Spherisorb 300 showed excellent performance for all the polypeptides with molecular weight up to 80 000, including BSA and transferrin.

In contrast, the C_{18} phase from 10-nm pore silica gel and those from the three wide-pore silica gels, Nucleosil 300, Vydac TP and Hypersil 300(I), having relatively small pores, showed peak broadening and tailing for BSA, as shown in Fig. 2. The peaks of BSA and transferrin with Nucleosil 300-C_{18} are reasonably sharp, but they are tailed and much broader than the other peaks. The results indicate that the peak broadening and tailing seen with materials containing relatively small pores are caused by the high molecular weight of the polypeptides, not by the high hydrophobicities,

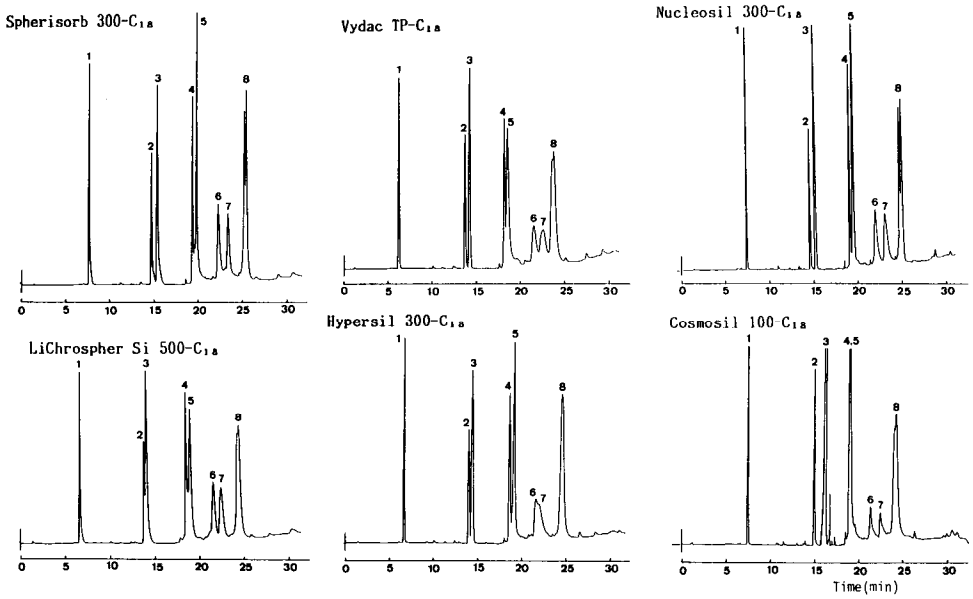


Fig. 2. Elution of peptides [(1) glycyltyrosine; (2) α-endorphin; (3) Leu-enkephalin; (4) insulin; (5) cytochrome *c*; (6) transferrin; (7) BSA; and (8) β-lactoglobulin] on C_{18} phases prepared from wide-pore silica gels. Gradient type II (see Experimental) was used.

because the late-eluting β-lactoglobulin was eluted with high efficiency. We therefore included BSA to test other packing materials in the following study.

Fig. 3 shows the effect of alkyl chain length on LiChrospher Si 500 and Spherisorb 300. In the examination of packing materials with polypeptides, including cytochrome *c*, lysozyme and BSA, 1-naphthylmethanol was also included so that poor column packing with some stationary phases can be taken into account. When the small molecule shows peak tailing, as with Spherisorb C_4, the peak tailing with polypeptides can be discounted.

All the stationary phases from Spherisorb 300 and LiChrospher Si 500, regardless of the alkyl chain length, showed satisfactory performance for the polypeptides. The peak shape on the C_{18} phase was comparable to that on C_4 or C_8 phases. The results indicate that a C_{18} phase instead of C_4 can be selected for polypeptide separation if desired separations can be achieved with acceptable recovery. There seems to be little difference in peak capacity among these phases.

The C_{18} phases from Hypersil 300E and Shiseido 300E also showed excellent performance for the three polypeptides, as shown in Fig. 4. Although BSA was separated into two sharp peaks appearing as a shoulder with Hypersil 300E, the peak tailing on the C_{18} phases prepared from commercial Hypersil 300 (I and II) disappeared. It should be noted that a small pore volume was found below 20 nm with these experimental silica gels of 30 nm pore size, as shown in Fig. 1c. Hypersil 300E showed considerable improvements in the extent of tailing compared with the older type commercial products. The results shown in Figs. 2–4 indicate that the alkyl chain length of the stationary phase is not a critical factor in determining the column efficiency for the polypeptides under the present conditions [1,5,19].

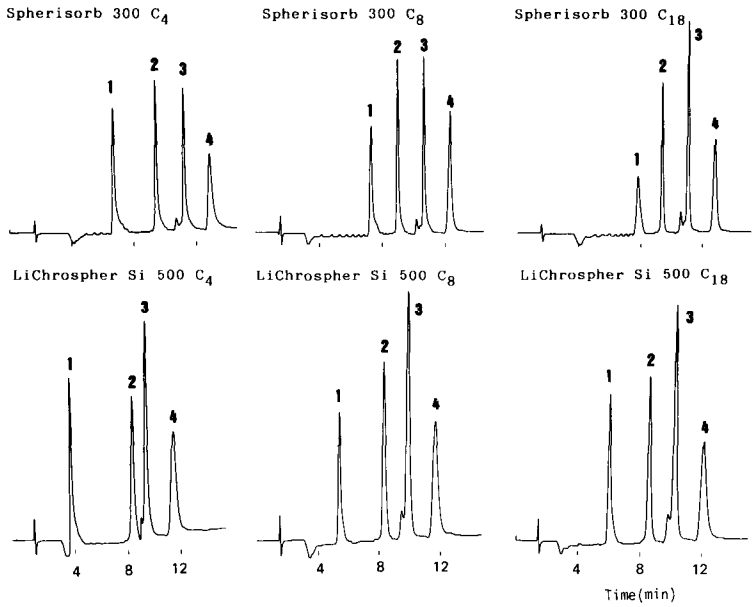


Fig. 3. Effect of alkyl chain length on the elution of (1) 1-naphthylmethanol, (2) cytochrome *c*, (3) lysozyme and (4) BSA using gradient elution type I with alkyl-bonded phase prepared from Spherisorb 300 and LiChrospher Si 500.

However, the C_4 and C_{18} phases prepared from Kromasil 100 and 200E and the commercial batch of Hypersil 300(II) showed instances where one could obtain a better performance with a C_4 bonded phase than a C_{18} phase as suggested generally. As shown in Fig. 5, both C_4 and C_{18} phases from 10-nm pore silica gels showed poor performances for the peptides, especially BSA. With an increase in pore size from 10 to about 20 nm, an improved performance was seen with the C_4 phase, whereas not much improvement was seen with the C_{18} phases for BSA.

Considerable differences between C_4 and C_{18} phases in performance for BSA can be seen for silica particles having pore sizes in the range *ca.* 15–20 nm. Some earlier

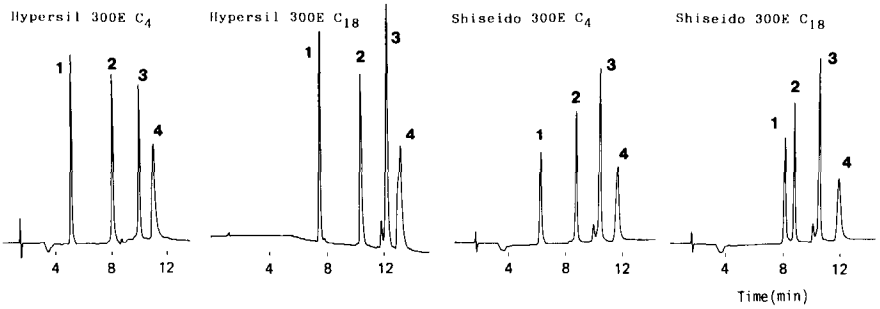


Fig. 4. Performance of C_4 and C_{18} phases prepared from Hypersil 300E and Shiseido 300E experimental wide-pore silica gels. Conditions and peaks as in Fig. 3.

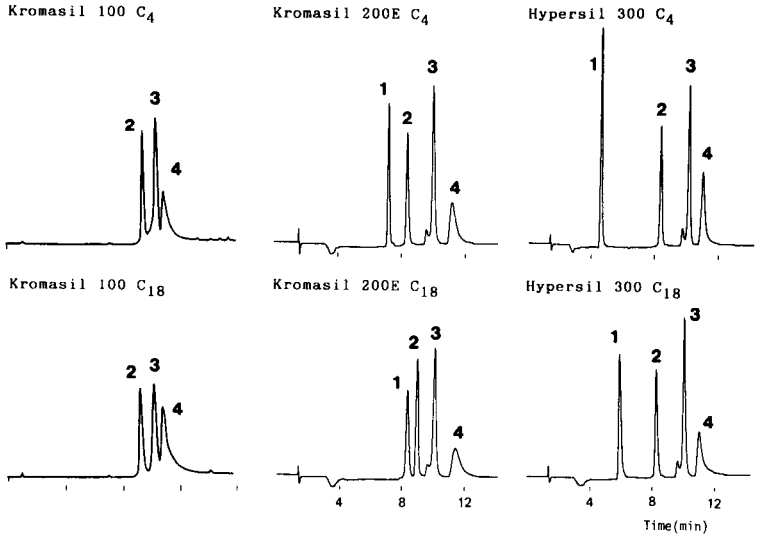


Fig. 5. Performance of C_4 and C_{18} phases prepared from Kromasil 100, Kromasil 200E and Hypersil 300(II). Conditions and peaks as in Fig. 3.

workers might have concluded from such results that the short-chain alkyl stationary phases can give better performances than a C_{18} phase for high-molecular-weight polypeptides. The present results, however, indicate that the effect of alkyl chain length on efficiency is much smaller with packing materials having an adequate pore size or too small a pore size. The effect of alkyl chain length on protein recovery will be discussed later.

In Figs. 2–4, the tailing of the BSA peak was generally accompanied by a narrower band spacing between lysozyme and BSA, indicating the partial steric exclusion for the high-molecular-weight polypeptide from small pores. This indicates

TABLE II

METAL CONTENTS OF WIDE-PORE SILICA GELS

Silica gel	Metal content (ppm)							
	Al	Ca	Fe	Na	Ti	K	Mg	Zr
Hypersil 300[a]	191	11	134	896	58	31	14	122
Hypersil 300E	152	17	46	669	79	15	7	29
Nucleosil 300	22	38	14	405	36	5	27	7
LiChrospher Si 500	1	5	12	315	<1	0	2	0
Spherisorb 300	9	6	8	9	1	0	1	<1
Shiseido 300E	<1	1	<1	7	<1	0	<1	0
Kromasil 100	<1	<1	<1	25	2	<1	<1	<1

[a] A different batch of a commercial product.

the presence of pores that barely permit the permeation of the polypeptide, leading to a more limited performance of C_{18} phases. This effect might be related to the limited surface area available with small-pore materials [6]. The presence of such pores that allow solute permeation but do not allow fast equilibration should be minimized.

The presence of metal impurities was shown to be less important. Spherisorb 300, Shiseido 300E and LiChrospher Si 500 were found to be relatively pure silica particles, while Hypersil 300E contained considerable amounts of metal impurities, as shown in Table II, yet all of these materials showed excellent performance for the polypeptides. The results indicate that the presence of the metal impurity itself is not the source of peak tailing, although there is a possibility that the effect of alkyl chain length is enhanced by the presence of metal impurities.

The TEM photographs provide a common observation for the wide-pore silica gels which gave high efficiencies for high-molecular-weight polypeptides. Spherisorb 300 and Shiseido 300E showed very similar appearances with a thin skeleton and a highly porous structure, as shown in Fig. 6. In contrast, Hypersil 300(I) and Vydac TP showed the presence of a thick skeleton, or large primary spheres in a particle, and a much denser structure with fewer pores.

The size of the primary structures of Hypersil 300E is larger than those in Spherisorb 300 and in Shiseido 300E, but smaller than those in commercially obtained Hypersil 300(I). Fig. 6f shows the internal structures of one of the two types of particles having the smaller pore size found in LiChrospher Si 500 [21]. This material also possesses a highly porous structure with a variety of sizes of primary spheres. The pores in the particles having smaller porosity are assumed to be more winding. Details of the pore structure study of wide-pore silica and polymer gels will be presented elsewhere [24]. The difference in the pore structure is probably related to the method of preparation of these particles.

The present study indicates that the major factor determining the column efficiency of silica-based packing materials for high-molecular-weight polypeptides seems to be the porosity and the pore size, not the average pore size of 30 nm, but the absence of pores below 20 nm. These wide-pore silica gels, possessing a satisfactory pore size distribution and a low metal content, can be regarded as truly high-performance wide-pore packing materials. One aspect to be noted is that the large-pore materials with higher porosity are usually more fragile than those with smaller pores, and care should be taken with column packing.

The present results will be of help in selecting wide-pore silica packing materials depending on the molecular weight of the solutes. Although a C_4 phase might be better for the separation of polypeptides with higher molecular weights or more hydrophobic polypeptides [5,8,10], C_{18} phases prepared from 30-nm pore silica gels can give high efficiencies for polypeptides with molecular weight up to 80 000. The C_{18} phases are much more stable than C_4 phases in TFA solution, as shown below.

Polymer-based packing materials

Various types of polymer-based packing materials have become available recently for RPLC. They include polystyrene gel (PLRP-S 300), esterified poly(vinyl alcohol) gels (Asahipak C4P, C8P and ODP), alkyl ethers of poly(hydroxyalkyl methacrylate) gels (Shodex D4-613, D8-613 and D18-613) and a poly(alkyl methacrylate) gel having short alkyl groups (Shodex DE-613). The polymer gels are stable

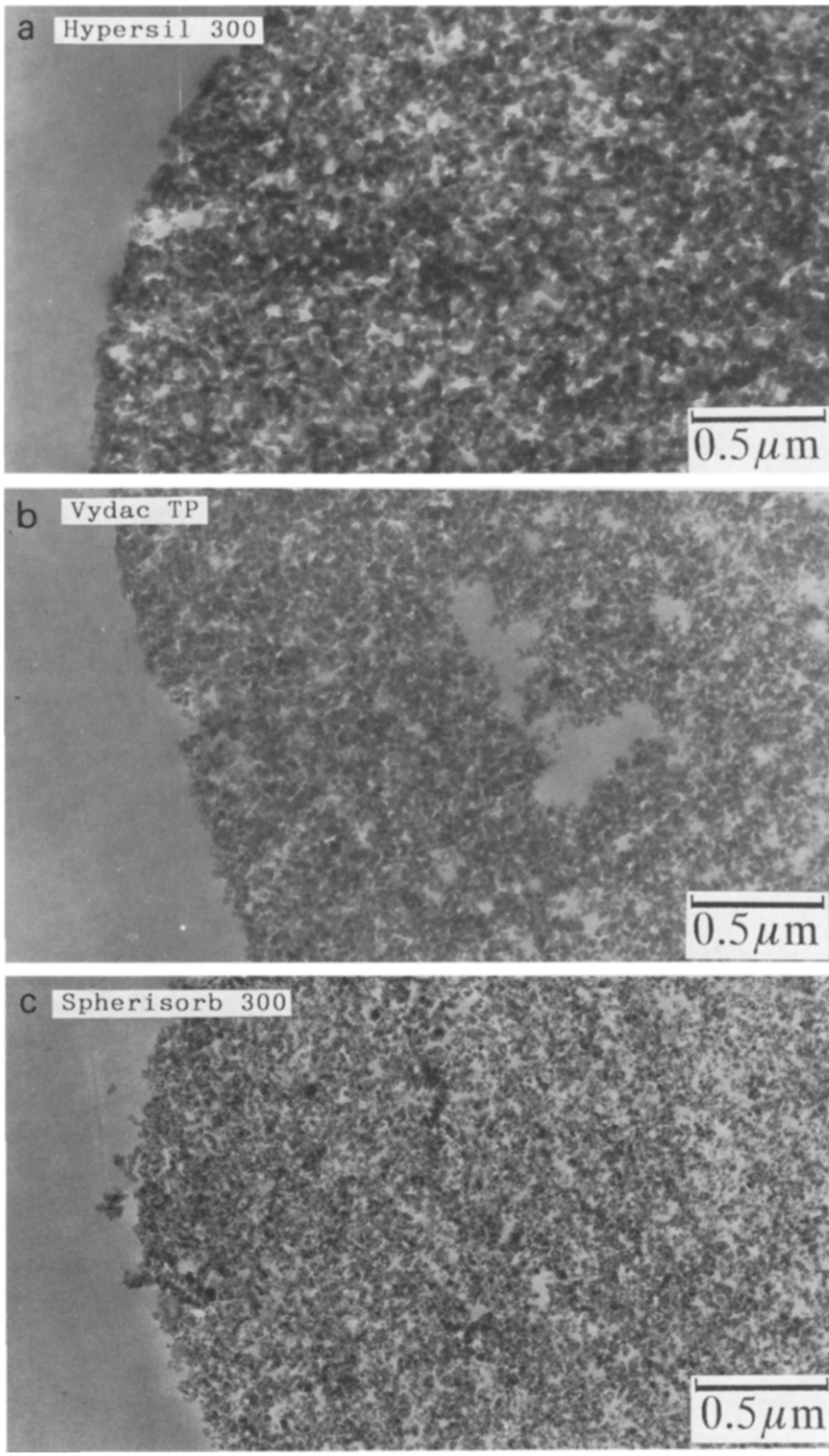


Fig. 6.

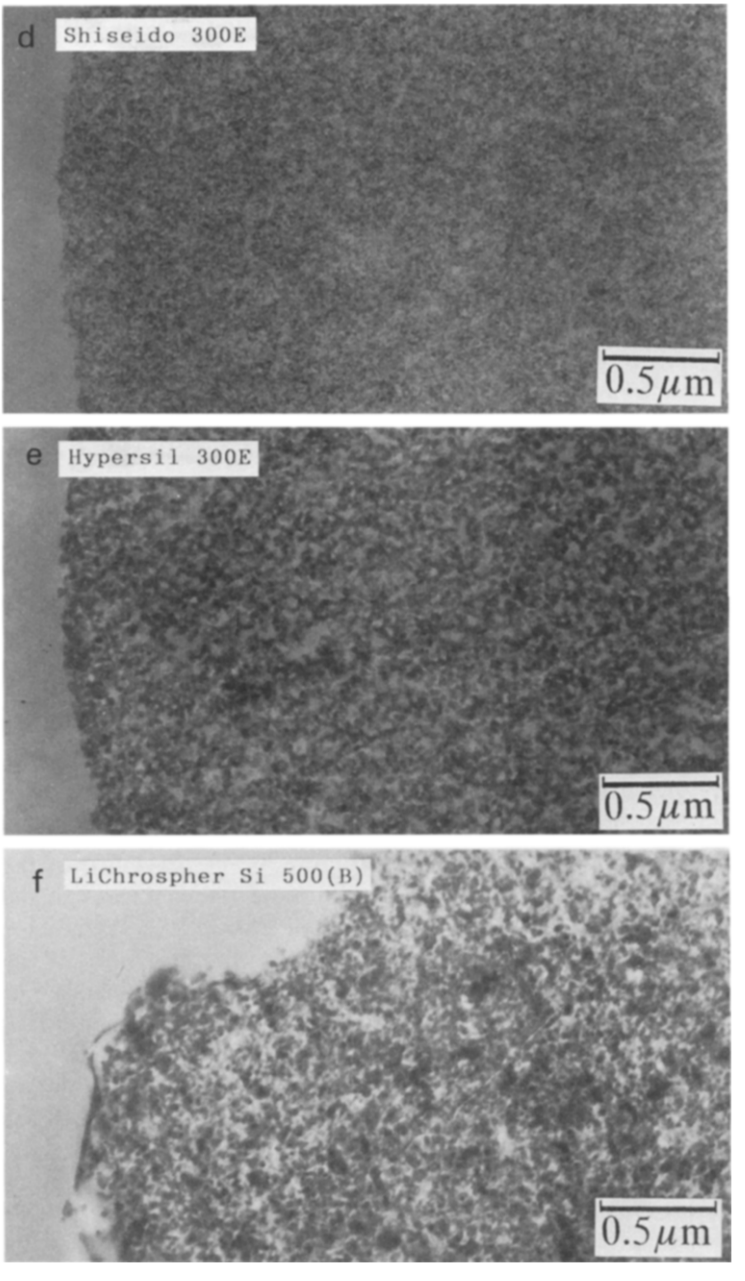


Fig. 6. TEM photographs of wide-pore silica gels.

between pH 2 and 12, and some of them are intended to be used for polypeptide separation [12–14,25,26].

The polymer gels possess a bimodal pore-size distribution when they are macroporous, as shown in Fig. 7. Although the results of nitrogen adsorption

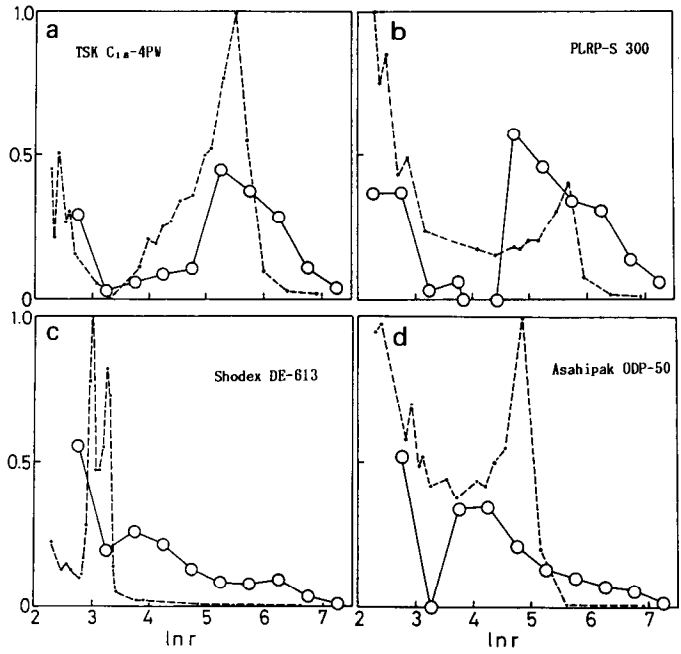
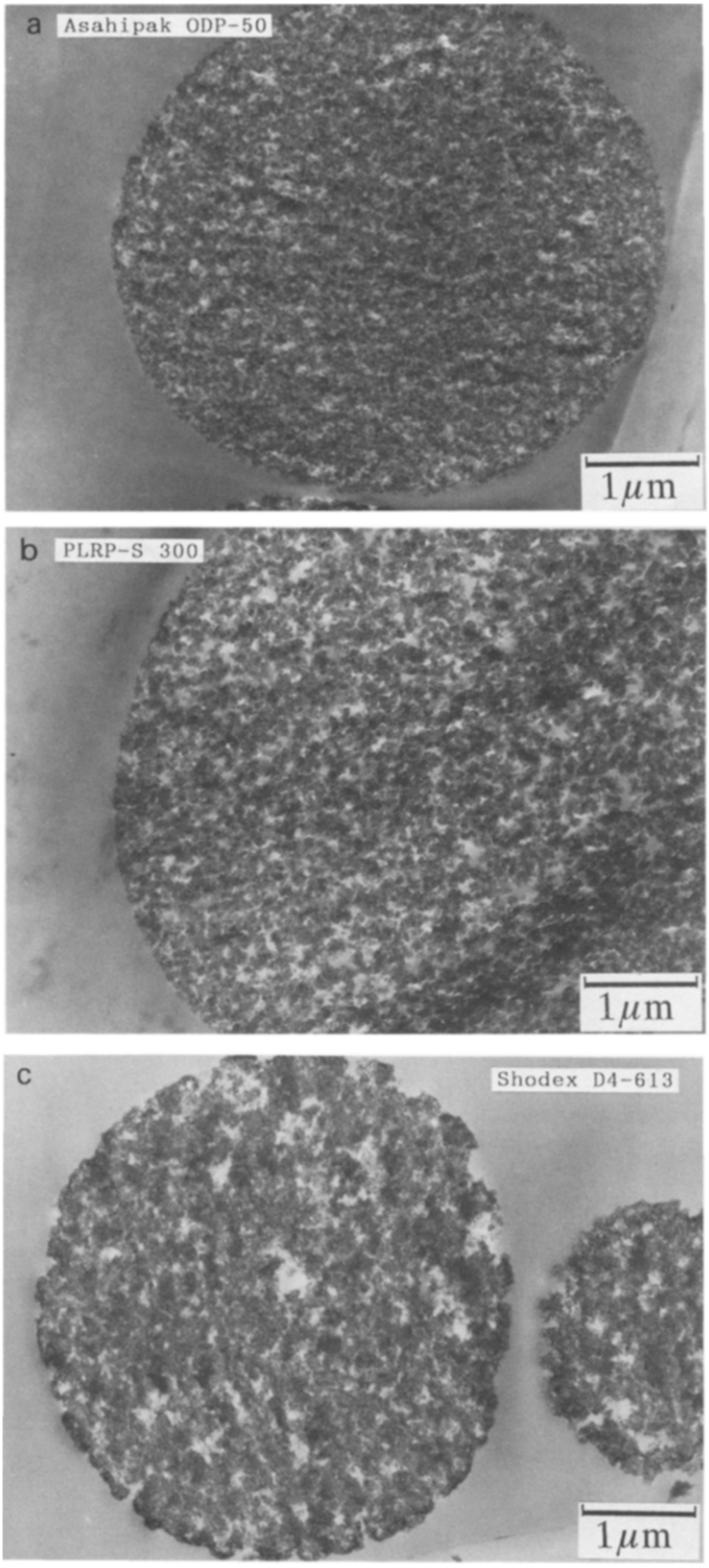


Fig. 7. Pore-size distributions of polymer gels measured by nitrogen adsorption (dashed lines) and size-exclusion chromatography (solid lines). The vertical axis corresponds to the fraction of pore volume, normalized in the case of inverse SEC; r = pore radius.

measurements are thought to be of less significance for polymer gels, the results with PLRP-S 300 were not affected by the use of tetrahydrofuran prior to drying or by the drying temperature between room temperature and 100°C. The micropores of the polymer gels are assumed to be provided by dense network structures, which give selective binding of rigid compact solutes [27,28]. One of the characteristics of the pore-size distribution of these polymer gels is the small pore volume for pore sizes in the range between 5 and 20 nm. This seems to be advantageous for polypeptide separations, considering the results obtained with silica particles.

In Fig. 8, the TEM photographs of TSK C_{18}-4PW, PLRP-S 300 and Asahipak show the presence of macropores throughout the particle. TSK C_{18}-4PW has the appearance of a network structure. Shodex DE-613 and D4-613 from the same manufacturer showed a clear difference in internal structure: whereas D4-613 showed the presence of macropores throughout the particle, DE-613 showed the presence of macropores only at the periphery of the particle. Some steric exclusion effects can be expected for large peptides with DE-613 packing material.

Wide-pore TSK C_{18}-4PW and PLRP-S 300 and also ODP-50 showed an excellent performance for the three polypeptides, as shown in Fig. 9. Shodex DE-613 eluted BSA together with lysozyme, presumably owing to steric exclusion. The retention of a small molecule, 1-naphthylmethanol, indicates how micropores are participating in the retention of small solutes [28]. The greatest contribution of the micropores was seen with Shodex DE-613.



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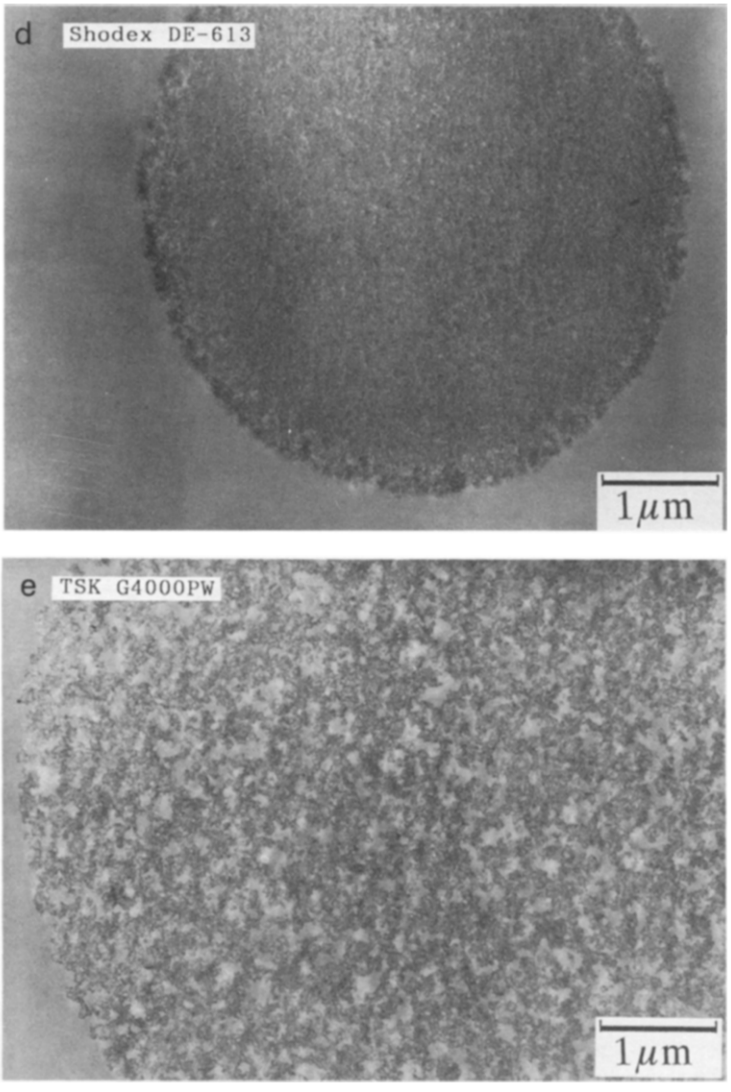

Fig. 8. TEM photographs of polymer gels.

The chromatograms in Fig. 10 show the performance of polymer gels having C_4 to C_{18} alkyl chains. Shodex D4-613 and D8-613 and Asahipak C4P and C8P gave high efficiencies for the peptides. Although tailing was seen for BSA with the C_{18} phases, there is no need to use a C_{18} phase in these cases, because polymer-based C_4 materials are much more stable than silica C_{18} phases. The tailing found with the C_{18} phase could be caused by the smaller pore size, as seen with some silica particles.

Although the packing materials were tested with only the three polypeptides, the elution of a wider range of polypeptides supported the results. Fig. 11 shows that TSK-C_{18}-4PW and PLRP-S 300 can give an even better efficiency than silica-based

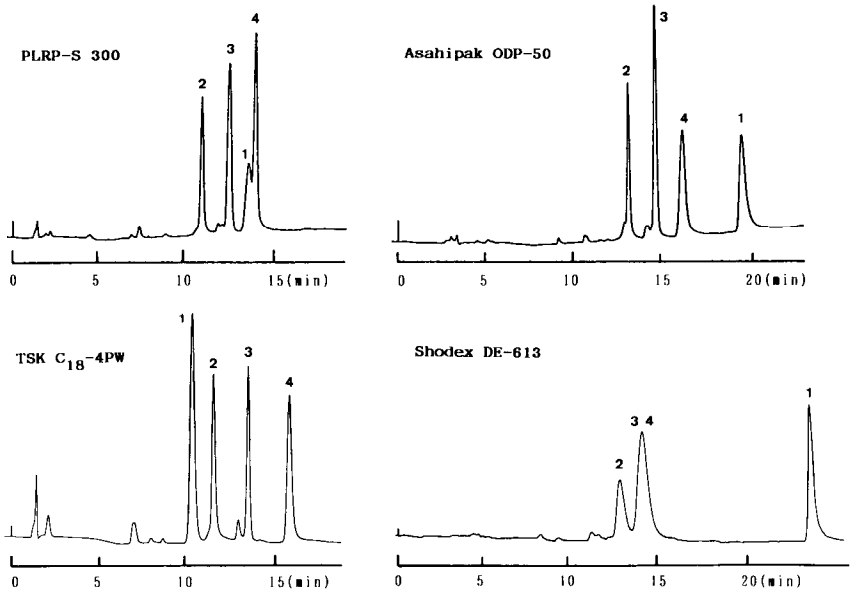

Fig. 9. Elution of polypeptides with polymer gels in gradient type I. Conditions and peaks as in Fig. 3.

materials. Polymer gels having C_4 or C_8 alkyl groups also showed a similar high efficiency. The performance and chemical stability of polymer-based packing materials may make them more attractive than silica-based materials for polypeptide separations, as reported by Burton *et al.* [10] and Welinder [29].

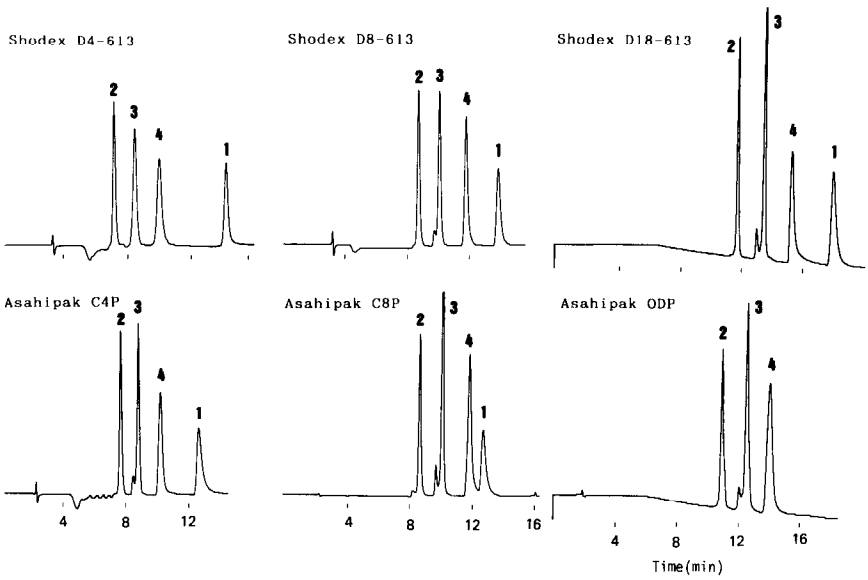

Fig. 10. Performance of polymer gels having C_4, C_8 and C_{18} carbon chains with gradient elution (type I) of polypeptides.

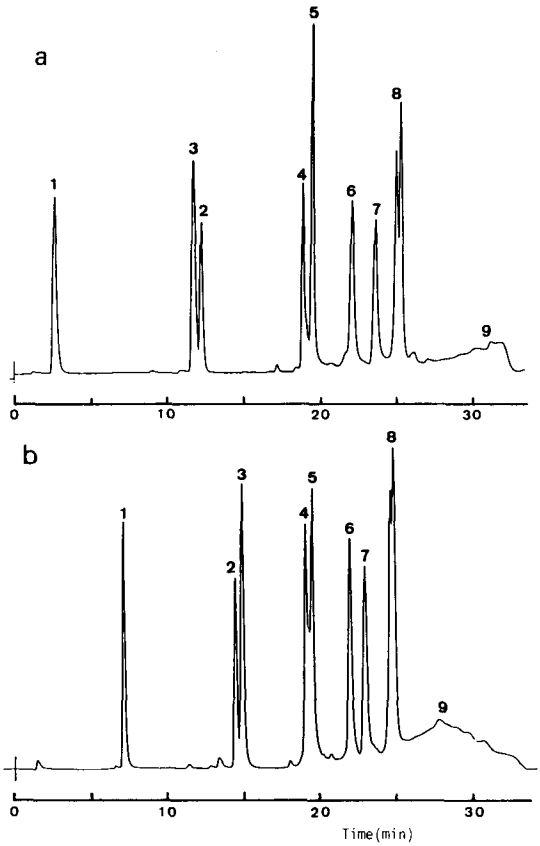

Fig. 11. Elution of polypeptides from polymer-based packing materials with gradient elution (type II). (a) TSK C_{18}-4PW; (b) PLRP-S 300. Peak 9: γ-globulin. Other conditions as in Fig. 2.

Protein recovery

By using the same gradient conditions (type I), consistent peak areas were obtained for cytochrome *c*, lysozyme, BSA and β-lactoglobulin without any indication of carryover (elution of a polypeptide in subsequent gradient runs without sample injection) with all the wide-pore packing materials. A small carryover was seen for BSA with Kromasil 200E-C_{18} and Cosmosil 100 C_{18}, and considerable carryover for ovalbumin with all the packing materials.

As shown in Table III, the recovery of ovalbumin with the first gradient was about 80–90% with C_4 phases. The total peak areas in the three gradient runs on C_{18} phases (one run with sample injection followed by two runs without sample) were 30–50% of those on the C_4 phase, although 60–80% of the total peak areas were obtained with the first gradient. The better recovery of polypeptides, especially ovalbumin, and the better resolution for high-molecular-weight polypeptides were the major reasons for the recommendation of short-chain alkyl-bonded silica phases [2,3,4,16]. The results obtained here are consistent with the reported results. However, the present results also showed that wide-pore materials, including polymer gels and

TABLE III

RECOVERY OF POLYPEPTIDES

Stationary phase	Polypeptide recovery (%)	
	BSA[a]	Ovalbumin[b]
LiChrospher Si 500		
C_4	100	87
C_8	100	88 (92)[c]
C_{18}	100	48 (76)[c]
Spherisorb 300		
C_4	100	87
C_8	100	70 (85)[c]
C_{18}	100	35 (76)[c]
Hypersil 300(II)		
C_4	100	88
C_{18}	100	28 (71)[c]
Kromasil 200E		
C_4	99.6	93
C_{18}	99.4	78 (90)[c]
Cosmosil 100-C_{18}[d]	97	– (76)[c]
Asahipak		
C4P	100	90
C8P	100	92 (84)[c]
Shodex		
D4-613	100	95
D8-613	100	84 (94)[c]

[a] Peak area of BSA in the first gradient was taken as 100% when no carryover was observed and used to normalize the recovery of ovalbumin.

[b] The recovery of ovalbumin on the first gradient run is shown. The total recovery of ovalbumin in three successive gradient runs on C_4 phase was taken as 100%, and used to calculate the recovery of ovalbumin on the other packing materials from the same support.

[c] The ratio of the recovery in the first gradient run to the total recovery in the three runs.

[d] Cytochrome *c* (*ca.* 2%), lysozyme (*ca.* 0.3%) and β-lactoglobulin.

silica C_{18} phases, can give high efficiencies and high recoveries of polypeptides including BSA and β-lactoglobulin, eluting later than BSA. Essentially no carryover was observed with any of the polypeptides except ovalbumin with all the stationary phases based on wide-pore materials, whereas a small carryover was seen for BSA on small-pore materials.

The better polypeptide recovery with short-chain alkyl-bonded silica phases would not justify their use except for polypeptide separations that would be accompanied by poor resolution or very low recoveries with longer chain alkyl-bonded phases. Even in these instances, polymer-based packing materials can provide comparable efficiencies and recoveries with much higher chemical stability. Optimization of the mobile phase and gradient profile may lead to even better recoveries of hydrophobic polypeptides on the more stable polymer gels and on silica C_{18} stationary phases [30]. Further studies of the effect of the structure of silica- and polymer-based packing materials on the recovery of a wide range of proteins are in progress.

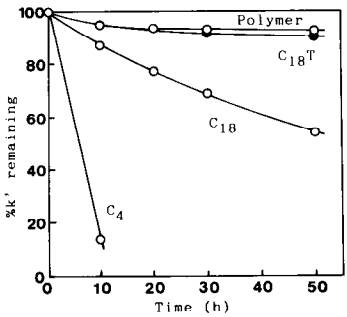

Fig. 12. Stability of packing materials under acidic conditions. The *k'* values for alkylbenzenes (toluene for LiChrospher C_{18}, $C_{18}T$ and Shodex D4-613 and amylbenzene for LiChrospher C_4) were measured in 60% methanol following tetrahydrofuran flushing at intervals while keeping the packing materials in 0.1% TFA solution at 60°C. The initial retention was taken as 100% for each packing material.

Stability of packing materials in TFA solution

Fig. 12 shows the decrease in the retention of alkylbenzenes in 60% methanol observed after keeping the packing materials in 0.1% aqueous TFA solution at 60°C. Whereas polymer-based Shodex D4-613 and LiChrospher Si 500-$C_{18}T$ bonded with trichlorosilane showed relatively high chemical stabilities, the silica-based phases bonded with monochlorosilane, especially the C_4 phase, showed low stabilities. The results obtained under the severe degradation conditions showed fairly good agreement with those reported by Kirkland *et al.* [18] and by Sagliano *et al.* [17]. Asahipak C4P materials have also been shown to be stable under similar conditions [14]. Polypeptides were eluted with considerably broader peaks with extensively decomposed packing materials.

The present results suggest the use of polymer-based packing materials or C_{18} packing materials prepared by reacting trifunctional silanes with wide-pore silica gels having adequate pore size, with regard to both column efficiency and chemical stability, if acceptable recoveries can be obtained.

CONCLUSION

The effect of the alkyl chain length of packing materials was shown to be related to the pore size of wide-pore silica gels when examined with high-molecular-weight polypeptides.

A wide-pore silica gel should contain few pores smaller than 20 nm in order to be a good packing material, then the C_{18} phase can be as efficient as the short-chain alkyl-bonded phases. Such a stationary phase would give a good column efficiency and recoveries for many polypeptides with molecular weights up to 80 000 with much higher stability than C_4 phases. The efficiency and recovery with polymer-based phases are comparable to those with optimized silica-based materials. Polymer-based packing materials having a higher chemical stability may be more efficient than silica-based packing materials in these applications.

The results also showed that the pore structure is of prime importance in wide-pore packing materials, and TEM may help in their characterization and optimization.

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