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Non-porous polybutadiene-coated silicas as stationary phases in reversed-phase chromatography

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ABSTRACT

Non-porous silica of mean particle diameter 1.7 μm (Monospher) was coated with polybutadiene (PBD) following a published procedure. The silicas were prepared with graduated polymer loads up to 8% (w/w). Examination of the PBD-coated packings by means of electron spectroscopy for chemical analysis, scanning electron microscopy, diffuse reflection infrared fourier transform spectroscopy and differential thermal gravimetry indicated that the optimum polymer load was between 1 and 3% (w/w) with regard to a dense coverage corresponding to an average layer thickness of about 4 nm. No silanophilic interactions could be monitored using 1–3% (w/w) coated silicas under reversed-phase conditions. The small particle diameter combined with the chemically stable polymer coating allowed extremely fast and efficient separations of peptides and polypeptides under gradient elution conditions in less than 1 min.

INTRODUCTION

Major advances have occurred in recent years in the design and development of high-performance liquid chromatographic (HPLC) stationary phases. One focus was on the elucidation of the pore and particle structure of HPLC packings with regard to better mass transfer kinetics, higher column performance and faster analysis. This led to the family of macroporous packings of mean pore diameter of >50 nm and to non-porous packings[1,2]. Both types have been shown to generate outstanding separation capabilities for large biomolecules. The other focus was on novel concepts and improvements of the stationary phase chemistry of HPLC packings.

The objectives were to prepare stationary phases with defined and accessible functional groups, to minimize or eliminate adsorptive effects of the matrix, concur-

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rently improving the chemical stability and lifetime of the packing whilst maintaining a high mechanical stability. The most successful approach is to immobilize defined polymer layers at the surface of the rigid inorganic packing in such a way that a solute-impermeable layer results, which, *a priori*, carries functional ligands or appropriate ligands were introduced by chemical reactions. Numerous polymer-load types of HPLC packings made by different immobilization chemistries[3–8] are available.

Although the immobilization procedures have advanced, few attempts have been made to assess the properties of the immobilized polymer layers in terms of chemical structure, physical structure and morphology, and to demonstrate the way in which variation of these structural parameters affect the chromatographic properties.

This work was aimed at elucidating the structural properties of a well known polymer coating, polybutadiene (PBD), on non-porous microparticulate silicas. The lack of pores and the spherical nature of the particles provide suitable conditions for immobilizing PBD layers of different thicknesses and for studying their layer composition and morphology by surface analytical methods. Chromatographic tests with appropriate polar solutes permit residual silica adsorptivities to be monitored, thus indicating the quality of the coating. The products obtained were most suited for the rapid analysis of peptide mixtures owing to their extremely small particle size of 1.7 μm. Such packings were required for rapid peptide monitoring in process control and allow analyses to be performed in much less than 1 min.

EXPERIMENTAL

Chemicals and materials

Monospher, support (non-porous silica of mean particle diameter 1.7 μm), methanol, acetonitrile, *n*-pentane and trifluoroacetic acid were obtained from E. Merck (Darmstadt, F.R.G.). The peptides for the reversed-phase test and silanol monitoring were obtained from the Department of Biochemistry, University of Alberta, Canada. Polybutadiene and dicumyl peroxide were supplied by Aldrich Chemie (Steinheim, F.R.G.). Proteins were supplied by Boehringer (Mannheim, F.R.G.) and Serva (Heidelberg, F.R.G.). Paracelsine peptides were donated by the Institut für Lebensmitteltechnologie, Universität Hohenheim, F.R.G. ODS and TMS silanization reagents, alkylbenzenes and the polarity test mixture were donated by the Max Planck-Institut für Kohlenforschung (Mülheim, F.R.G.).

Water was deionized with a Milli-Q system (Millipore–Waters, Eschborn, F.R.G.).

Support

To clean the surface of the Monospher support from impurities caused by the manufacturing process and to remove remaining micropores, the material was calcined at 1273 K for 48 h in a shallow bed. After cooling to room temperature, 200 g of calcined Monospher were suspended in 500 ml of nitric acid (15%, v/v) and refluxed for 18 h to rehydroxylate the surface. The material was washed with deionized water until the pH of the washings was neutral. The material was then dried at 440 K under vacuum (0.1 mbar) for 30 h.

Coating of silica with polybutadiene (PBD)

The apparatus consisted of a Rotavapor, a chamfered flask and an argon purge system. Dried Monospher (15 g) was stirred in *n*-pentane (30 ml) until a homogeneous suspension was obtained. Defined amounts of polybutadiene (molecular weight 4500, 45% vinyl, 55% 1,4-*cis*/*trans*) and dicumyl peroxide (DCP) were added according to the desired loading. The batches made were as follows:

PBD batch No.	*PBD (mg)*	*DCP (mg)*
0.2	30.0	1.5
0.5	75.0	3.8
1.0	150.0	7.5
2.0	300.0	15.0
3.0	450.0	22.5
5.0	750.0	37.5
10.0	1500.0	75.5

The solvent in each batch was evaporated and the PBD was immobilized in a chamfered flask at 440 K for 4 h, the mixture being rotated at 30 rpm in a dry argon atmosphere. The loaded silicas were suspended in dioxane–toluene (1:1, v/v) and subjected to ultrasonic treatment. The suspension was then filtered through a porous PTFE membrane of 0.2 μm porosity (Cartridge system; Sartorious, Göttingen, F.R.G.), applying a pressure of 0.4–0.5 MPa. The product was washed with tetrahydroforan, methanol and diethyl ether consecutively to remove the oligomers of polybutadiene. The reaction scheme is shown in Fig. 1.

Silanization of silica

TMS presilanization. Monospher was silanized with trimethylsilyl enol ether

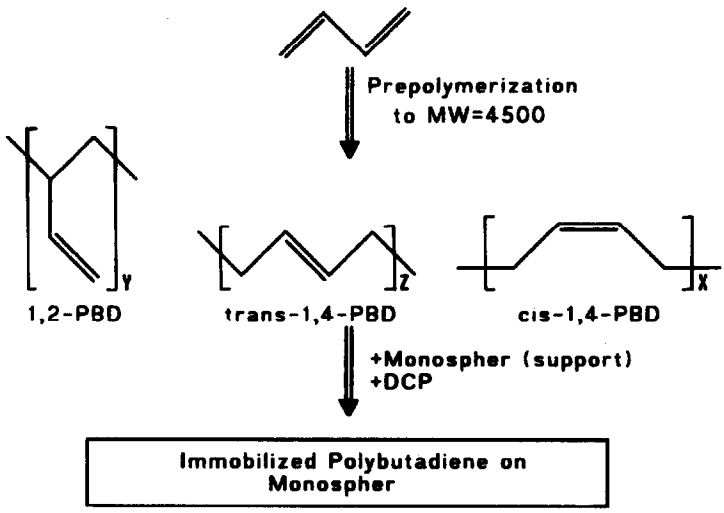

Fig. 1. Reaction scheme of the coating procedure. MW = Molecular weight.

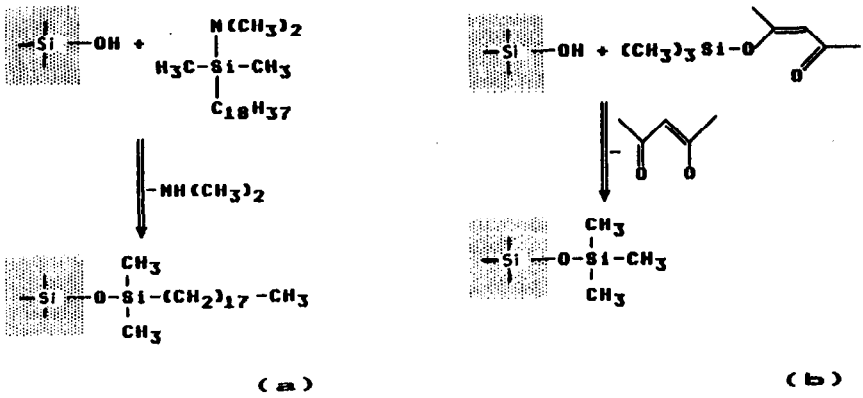

Fig. 2. Reaction scheme for the TMS and ODS modifications.

(TMS) following a procedure described by Schomburg *et al.*[9] (Fig. 2b). Silanized Monospher was also coated by the procedure described above.

ODS silanization. The same procedure was used to immobilize octadecylsilyl (ODS) residues on Monospher. Dimethyl(dimethyloctadecylsilyl)amine was used under the conditions described in ref. 9 (Fig. 2a).

Characterization of the bulk Monospher

Elemental analyses of parental and coated Monospher were obtained on a model 240 B Elemental Analyzer (Perkin-Elmer, Überlingen, F.R.G.). Duplicate analyses for carbon and hydrogen were performed. The reproducibility of the measurements was within ±0.2% (absolute standard deviation).

Thermogravimetric analysis (TGA), differential thermal analysis (DTA) and differential scanning calorimetric (DSC) measurements were conducted with a Du Pont Model 2100 TGA-Analyzer. The temperature was increased to 1120 K at 2 K/min in a dry air atmosphere. The samples were maintained in the analyser until no further weight loss was observed[10].

Diffuse reflection infrared Fourier transform (DRIFT) spectroscopy was performed on a Series 7000 FT-IR spectrometer (Nicolet, Offenbach, F.R.G.).

Electron spectroscopy for chemical analysis (ESCA) was carried out by Dr. Herzog (Hoechst Frankfurt/Main, F.R.G.) on a modified Leybold Hereaus ESCA instrument.

A Chemscan Cambridge, U.K. Series 4 scanning electron microscope was used for morphological investigations.

Chromatographic experiments

Chromatographic experiments were performed with two sets of equipment. With set 1, the screening experiments were performed with a Merck–Hitachi system equipped with a Model 655A-12 pump system, an L-5000 controller, a Model 655 A UV detector and a Rheodyne 7125 injector with a 20 μl-loop (E. Merck). The chromatograms were plotted with a C-R3A integrator (Shimadzu Europa, Duisburg, F.R.G.).

With set 2, rapid peptide separations were performed with a Bischoff (Leon-

berg, F.R.G.) LC unit consisting of an Autochrom 300 benchtop gradient controller system/terminal, two Model A2200 LC pumps and a Rheodyne 7410 injector with a 1 μl-loop. A Shimadzu SPD-6VA UV detector with a 0.6-μl cell and a time constant of < 50 ms and a Shimadzu C-R3A integrator were also used.

All eluents were degassed with a stream of helium leading through the solvent reservoirs.

Stainless steel columns of 33 × 4.6 mm I.D. (Bischoff) were used with compression fittings composed of metal screens and paper filters (Type 827; Schleicher and Schüll, Dassel, F.R.G.). The following materials were packed: (a) polybutadiene-coated Monospher (mean particle diameter 1.7 μm) with loadings of PBD between 0.2 and 8% (w/w); (b) PBD-coated Monospher presilanized with TMS; (c) ODS-bonded Monospher; (d) Monospher coated with a styrene–vinylsiloxane copolymer[11,12]. The columns were slurry packed using toluene–dioxane–cyclohexanol (1:1:1, v/v/v). The slurry, containing 1–2% (w/w) of modified Monospher, was treated ultrasonically for 4 h in a cooled bath. Columns were packed at a constant flow-rate under a constant pressure of 60 MPa.

Several test mixtures were applied in chromatographic experiments:

(a) Alkylbenzenes: ethylbenzene (C_2), propylbenzene (C_3), butylbenzene (C_4), *n*-pentylbenzene (n-C_5), isopentylbenzene (i-C_5) and *n*-hexylbenzene (C_6) were dissolved at a concentration of 1 mg/ml in methanol. The injection volume was 1–2 μl and produced satisfactory chromatographic peak sizes at a detection wavelength of 254 nm. The flow-rate was 0.5 ml/min.

(b) Polarity test: 1 mg/ml of acetophenone, benzophenone, benzyl benzoate and *n*-octylpyridine were dissolved in methanol. The chromatographic conditions were the same as in (a).

(c) Reversed-phase peptides: the following decapeptides (S1–S5), synthesized by C. T. Mant and R. S. Hodges (University of Alberta, Canada), were used in a reversed-phase test[13–15]:

S1: Arg–Gly–Ala–Gly–Gly–Leu–Gly–Leu–Gly–Lys;
S2: Arg–Gly–Gly–Gly–Gly–Leu–Gly–Leu–Gly–Lys;
S3: Arg–Gly–Ala–Gly–Gly–Leu–Gly–Leu–Gly–Lys;
S4: Arg–Gly–Val–Gly–Gly–Leu–Gly–Leu–Gly–Lys;
S5: Arg–Gly–Val–Val–Gly–Leu–Gly–Leu–Gly–Lys;

All peptides contained an N^{α}-acetylated N-terminal and a C-terminal amide, except peptide S1, which was identical with peptide S3 but had a free α-amino group. The separations were carried out in the gradient mode, using acetonitrile–water–0.1% trifluoracetic acid (TFA) as eluent. The flow-rate was 1 ml/min and the detection wavelength was 214 nm.

(d) Monitoring free silanol groups: as described in ref. 16, a method for free silanol monitoring was applied by using a mixture of undecapeptides differing in the number of lysine residues:

1: Gly–Gly–Gly–Leu–Gly–Gly–Ala–Gly–Gly–Leu–Lys;
2: Lys–Tyr–Gly–Leu–Gly–Gly–Ala–Gly–Gly–Leu–Lys;
3: Gly–Gly–Ala–Leu–Lys–Ala–Leu–Lys–Gly–Leu–Lys;
4: Lys–Tyr–Ala–Leu–Lys–Ala–Leu–Lys–Gly–Leu–Lys;

Each peptide contained an N^{α}-acetylated N-terminal and a C-terminal amide. Gradient elution was performed at pH 2, using acetonitrile–water–0.1% TFA as eluent. The chromatographic conditions were the same as in (c).

(e) Paracelsine is a membrane-active polypeptide with antibiotic activity and is secreted by the mould *Trichoderma reesi*. Its composition has a light inhomogeneity, consisting of four peptides (A–D), differing only in two positions:

A: Ac–Aib–Ala–Aib–Ala–Aib–*Ala*–Gln–Aib–*Val*–Aib–Gly–Aib–Aib–Pro–Val–Aib–Aib–Gln–Gln–Pheol

B: Ac–Aib–Ala–Aib–Ala–Aib–*Ala*–Gln–Aib–*Leu*–Aib–Gly–Aib–Aib–Pro–Val–Aib–Aib–Gln–Gln–Pheol

C: AC–Aib–Ala–Aib–Ala–Aib–*Aib*–Gln–Aib–*Val*–Aib–Gly–Aib–Aib–Pro–Val–Aib–Aib–Gln–Gln–Pheol

D: Ac–Aib–Ala–Aib–Ala–Aib–*Aib*–Gln–Aib–*Leu*–Aib–Gly–Aib–Aib–Pro–Val–Aib–Aib–Gln–Gln–Pheol

The peptides were N-terminal acetylated and C-terminated with phenylalaninol. Acetonitrile–water (36:64, v/v) containing 0.1% TFA was used as the mobile phase for the isocratic separation. The peptides were detected at a wavelength of 214 nm. The flow-rate was varied from 1 to 3 ml/min.

(f) The separation of proteins was carried out by gradient elution with acetonitrile–water–0.1% TFA using the following proteins: (1) Ribonuclease A, (2) lysozyme, (3) transferrin, (4) conalbumin, (5) β-lactoglobulin and (6) ovalbumin.

RESULTS AND DISCUSSION

Characterization of polybutadiene-coated silicas

Elemental analysis. In the immobilization procedure the load of PBD on silica was adjusted to discrete values between 0.2 and 10% (w/w). There are two additional means of assessing the load of PBD: elemental analysis of carbon and weight loss during ignition by TGA. The carbon content of the coated silica measured by elemental analysis should correspond to the load adjusted for the immobilization, assuming that no losses of PBD occurred during the procedure. Table I gives the data

TABLE I

POLYMER LOAD OF SILICA DERIVED FROM THE WEIGHT OF IMMOBILIZED PBD, CARBON ELEMENTAL ANALYSIS AND THE CALCULATED PBD CONTENT AND THERMOGRAVIMETRY

d_s = apparent layer thickness of PBD according to elemental analysis.

Sample	*PBD employed (%, w/w)*	*Carbon content (%, w/w)*	*Calculated PBD content*[a] *(%, w/w)*	*Weight loss (%, w/w)*	d_s *(nm)*
PBD 0.2	0.2	0.14	0.16	0.28	0.8
PBD 0.5	0.5	0.35	0.39	–	1.8
PBD 1.0	1.0	0.83	0.93	1.19	4.1
PBD 2.0	2.0	1.59	1.79	2.31	7.9
PBD 3.0	3.0	2.42	2.72	2.87	12.0
PBD 5.0	5.0	3.77	4.25	5.13	18.8
PBD 10.0	10.0	7.38	8.31	9.48	36.9

[a] From carbon elemental analysis.

derived from different methods. It also includes the apparent film thickness, d_s, of PBD-coated silicas calculated with the equation

$$d_s = X_{PBD}\, \rho_{PBD}/a_s$$

where X_{PBD} is the load of PBD (%, w/w) derived from carbon analysis, ρ_{PBD} is the density of PBD (g/ml) and a_s the specific surface area of the silica according to the BET method (a_s = 1.8 m^2/g). The load obtained on the basis of the carbon content is less than the amount of PBD employed in the immobilization procedure even when corrected by a factor of 1.126 to take the content of hydrogen in PBD into account. The weight loss of loaded silicas derived from TGA is seen to be slightly higher than the amount of PBD loaded, which is probably due to the removal of non-carboneous constituents from the silica during ignition. The apparent layer thickness increases proportionally with the load of PBD and ranges from about 1 nm at low loads to 40 nm at high loads.

Thermal analysis. The TGA data showed that the burning of PBD started at about 200°C and was completed at 500°C. Above 600°C no further weight loss occurred. In differential thermal analysis (DTA) of the PBD silicas three peaks appeared at 120, 300 and 500°C. DTA and differential scanning calorimetric (DSC) experiments on pure PBD as reference material gave nearly the same pattern as observed for PBD-coated silicas. Therefore, apart from the weight loss, no conclusions can be drawn from thermal analysis about the homogeneity of the PBD coating.

Diffuse reflection infrared Fourier transform spectroscopy (DRIFT). DRIFT was carried out on PBD-coated silicas in the range 1600–3200 cm^{-1} (Fig. 3). For the identification and semi-quantitative determination of PBD the C–H absorption band at 2916 cm^{-1} was employed. The intensity of this band was found to be proportional to the load of PBD. Owing to the lack of molar absorption coefficients, the quantification of PBD load from FT-IR measurements was not possible. Whereas the absorbance of the band at 2916 cm^{-1} increased with increase in the PBD load, the absorption band at 1869 cm^{-1} assigned to the silica diminished. Normally additional information would be obtained by monitoring the absorbance at 3750 cm^{-1}, assigned to the stretching vibration of free silanols. However, owing to the low surface area of

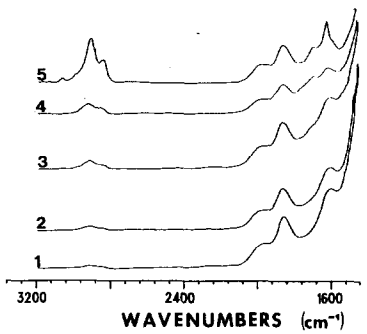

Fig. 3. DRIFT spectra of silicas with increasing PBD load in the range 1600–3200 cm^{-1}. PBD silica samples: 1, 0.5; 2, 1.0; 3, 2.0; 4, 5.0; 5, 10.0 (pure samples, no KBr).

TABLE II

DISTRIBUTION OF CARBON, SILICON AND OXYGEN IN THE LAYER OF PBD-COATED SILICAS

Sample	*Amount of PBD*[a] *(%, w/w)*	$C_{surface}$ *(atom-%)*	$Si_{surface}$ *(atom-%)*	$O_{surface}$ *(atom-%)*
Monospher	–	12.38	30.22	57.40
PBD 0.5	0.39	43.35	21.60	35.05
PBD 1.0	0.93	59.80	15.40	24.80
PBD 3.0	2.72	69.30	10.00	20.70
PBD 5.0	4.25	65.50	9.78	24.72
PBD 10.0	8.31	70.05	7.10	22.85
$RP\text{-}18_{ref.}$	–	26.97	26.88	46.15

[a] From carbon elemental analysis.

non-porous silica, the absolute concentration of free silanols is so low that they could not be detected by DRIFT.

DRIFT serves as a means of identifying the polymer used for coating and allows a crude estimate of the extent of loading, but it cannot be utilized for the assessment of the homogeneity or defects of the coating.

*Electron spectroscopy or chemical analysis (ESCA).*ESCA provides a sensitive

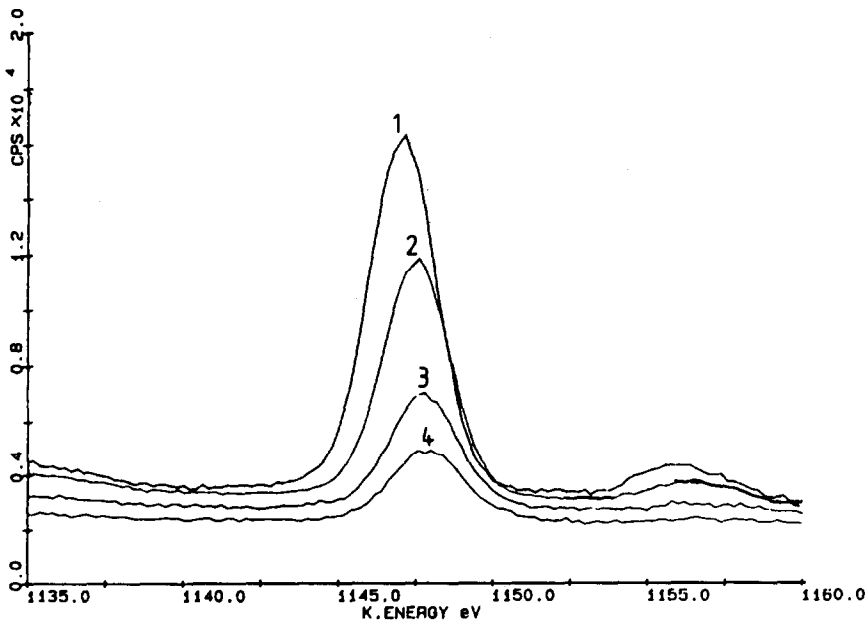

Fig. 4. Intensity of the X-ray photoelectron spectroscopic band at 1147.5 eV due to silicon at various loads: 1 = PBD 0.5; 2 = PBD 1.0; 3 = PBD 3.0; 4 = PBD 10.0.

means of characterizing the chemical composition of the surface layer by following the photoelectron bands of carbon at 966 eV, silicon at 1147.5 eV and oxygen at 718 eV[17,18]. Integration of these bands and correction of the data by equipment-specific factors (carbon 0.2, silicon 0.195, oxygen 0.61), the contents of carbon, silicon and oxygen in atom-% of the layer can be calculated (Table II) According to the penetration depth of photoelectrons, the values reflect the distribution of the surface layer at a film thickness of about 5 nm.

Inspection of the uncorrected spectra (Fig. 4) reveals that at high loadings (8% w/w) of PBD, silicon surface atoms can still be monitored. Consequently, this observation indicates that high loadings do not guarantee complete coverage of the native surface of the silica support. A loading of PBD of 8% (w/w) corresponds to an apparent layer thickness, according to elemental analysis, of about 35 nm, which should be completely impermeable to photoelectrons.

The calculated silicon content of the surface layer decreased with increasing load of PBD up to 8% (w/w) at the highest loading. The carbon content was about 43% at a 0.4% load, increasing to 60% at a 0.9% load and approaching values between 66 and 70% at higher loadings.

From the results, it can be concluded that a load of PBD above 3% does not yield a more homogeneous and denser coating than lower loadings. Assuming a dense and homogeneous layer of PBD, the layer thickness, d_s', can be calculated as

$$d_s' = \lambda \ln(100/I_s)$$

where λ is the average path length of inelastic electron diffraction and I_s the intensity of the electrons of the silica matrix in atom-%. Setting λ equal to 5 nm and using the values of I_s calculated from Table II d_s' values of 4.6, 5.9, 5.3, 6.0 and 1.6 nm are obtained for PBD samples 1.0, 3.0, 5.0 and 10.0 and RP-18_{ref}, respectively. For comparison, the layer thickness of an *n*-octadecyl-bonded silica is 1.6 nm with I_s = 73.0%. There is fairly good agreement on comparing the d_s and d_s' values for the PBD 1.0 silica.

Scanning electron microscopy (SEM). SEM provides a direct means of viewing the surface of native and coated silicas and thus allows an estimate of the morphology of the layer. Scanning electron micrographs were taken at magnifications of 30 000, 80 000 and 120 000 and Fig. 5a–d show the images of four coated silicas at the same magnification.

The images of the particle surface indicate a relatively smooth coated surface for the PBD 1.0 and 3.0 silicas. Minor imperfections are seen on the surface of PBD 5.0 silica and the particles of the PBD 10.0 sample show a flake-like surface with island structures. Beneath these clusters the surface seemed to be densely coated. As demonstrated by the ESCA measurements, the coating is not dense enough to suppress penetration by photoelectrons. The density of the coating and the morphology are reflected by the chromatographic measurements following the retention of selected compounds.

Chromatographic characterization

To establish the lipophilic and hydrophilic character of the coated silicas, several test mixtures were applied. The lipophilicity was monitored by using a mixture of

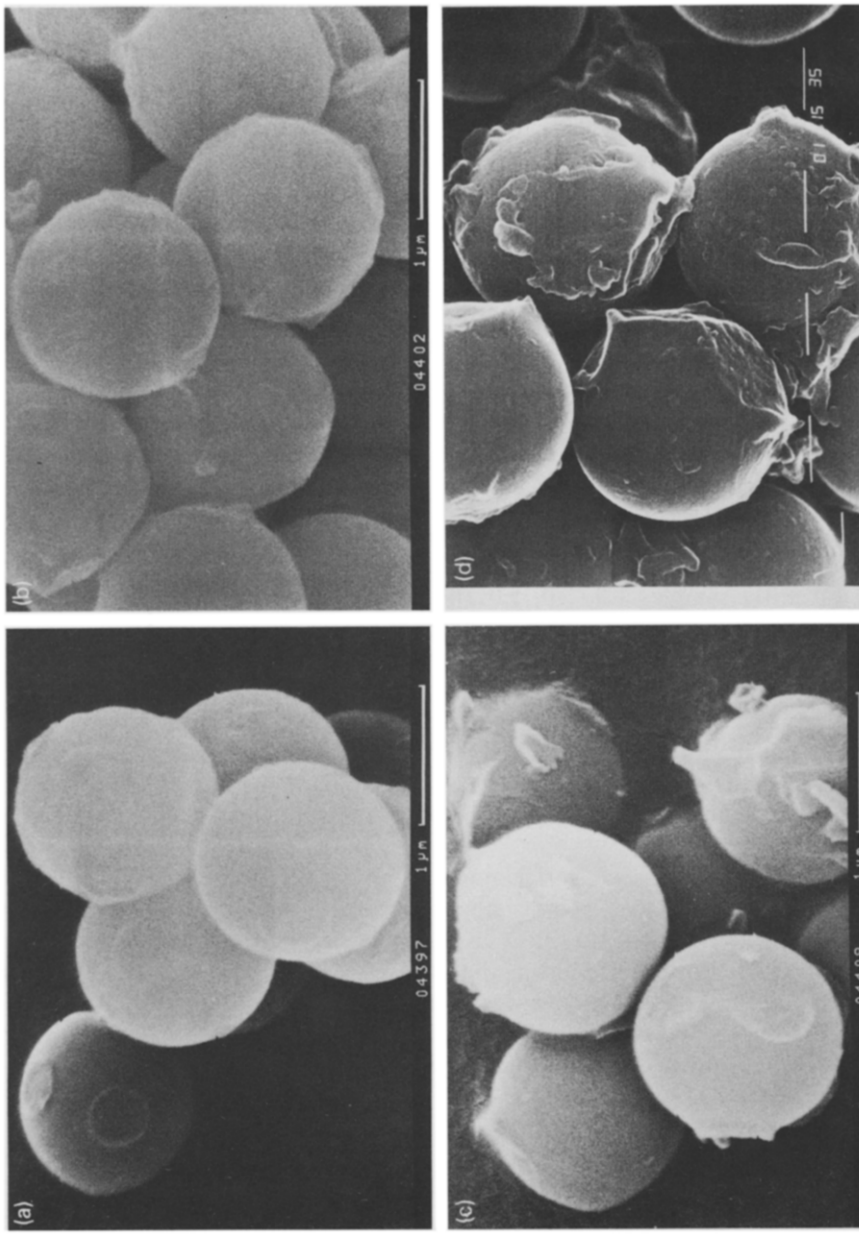

Fig. 5. Scanning electron micrographs of (a) PBD 1.0, (b) PBD 3.0, (c) PBD 5.0 and (d) PBD 10.0.

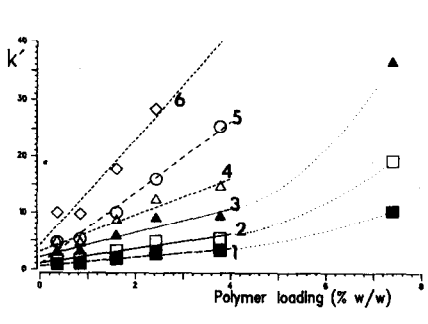

Fig. 6. Dependency of the capacity factor, k' (alkylbenzenes), as a function of the PBD loading on Monospher: 1 = ethylbenzene 2 = *n*-propylbenzene; 3 = *n*-butylbenzene; 4 = isopentylbenzene; 5 = *n*-pentylbenzene; 6 = *n*-hexylbenzene.

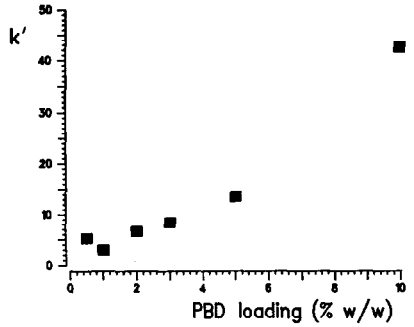

Fig. 7. Plot of the capacity factor, k', of *n*-octylpyridine *versus* the PBD loading on Monospher.

n-alkylbenzenes. A polarity test aiming at measuring the residual adsorptivity of the silica was carried out using acetophenone, benzophenone, benzyl benzoate and *n*-octylpyridine.

To assess the separation capabilities of the PBD-coated silicas for peptide separation, two standard peptide mixtures were chromatographed with an acidic mobile phase under gradient elution conditions with acetonitrile as the organic solvent. An isocratic separation was carried out with paracelsine peptides using a binary acidic mobile phase (water–acetonitrile). Finally, a protein mixture was separated under the same conditions as in the standard peptide test.

Test with n-alkylbenzenes. The capacity factors, k', of a homologous series of *n*-alkylbenzenes (ethyl to n-hexyl) were measured on columns packed with PBD-coated silicas with a constant mobile phase composition. For comparison the same measurements were performed on an *n*-octadecyl-bonded non-porous silica. Fig. 6 depicts the dependence of k' on the PBD loading of the stationary phase. A linear relationship is obtained for each solute up to a 4% PBD loading. Above 4% PBD each curve shows an upward swing to higher retention. This additional increase in the retention of *n*-alkylbenzenes on the PBD 10.0 silica is probably due to the fact that an additional partitioning of solute takes place with the bulky clusters of PBD attached to the underlying PBD coating.

The retention behaviour of the *n*-octadecyl-bonded silica is similar to those of the PBD 0.5 and 1.0 silicas.

Polarity test. Basic compounds such as amines are sensitive markers for monitoring the residual adsorptivity of reversed-phase materials. In this instance *n*-octylpyridine was employed. The plot of k' of *n*-octylpyridine *versus* the PBD loading of the silicas showed that the retention declines from PBD 0.5 silica to a minimum at PBD 1.0 silica and then increases proportionally at higher loadings (Fig. 7). In the first part of the curve *n*-octylpyridine is mainly retained by silanophilic interactions with the stationary phase, which reaches a minimum at the PBD 1.0 silica. On the right-hand side of the curve the lipophilic interactions dominate because the silica support is nearly completely covered by the PBD.

Further evidence of the absence of residual adsorptivity of the silica is provided

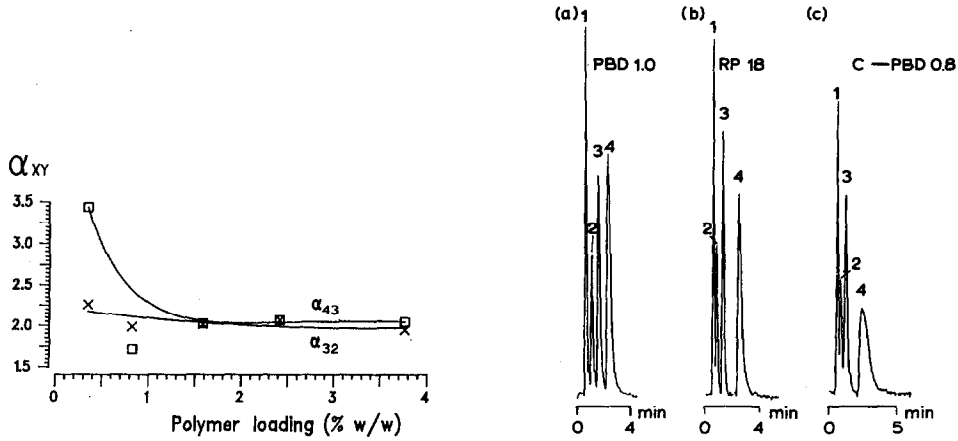

Fig. 8. Plot of the selectivity coefficients, α, of two pairs of solutes *versus* the PBD loading on Monospher.

Fig. 9. Chromatograms for the polarity test (1 = acetophenone, 2 = benzophenone, 3 = benzyl benzoate, 4 = *n*-octylpyridine) for the following columns: (a) PBD 1.0 silica, (b) RP-18 (*n*-octadecyl-bonded, not end-capped) silica and (c) presilanized PBD silica (C1-PBD 0.8). Chromatographic conditions: mobile phase, acetonitrile–water (45:55, v/v); flow-rate, 0.5 ml/min; detection wavelength, 254 nm.

by Fig. 8, where the selectivity coefficients, α, of two pairs of solutes, $\alpha_{43} = k'$(*n*-octylpyridine)/k' (benzyl benzoate) and $\alpha_{32} = k'$ (benzyl benzoate)/k' (benzophenone), is plotted against the PBD load of the silica. α_{43} decreases from 3.5 to about 2 from the PBD 0.5 to the PBD 2.0 silica and then remains constant.

Fig. 9 compares the chromatograms, obtained under constant conditions, for

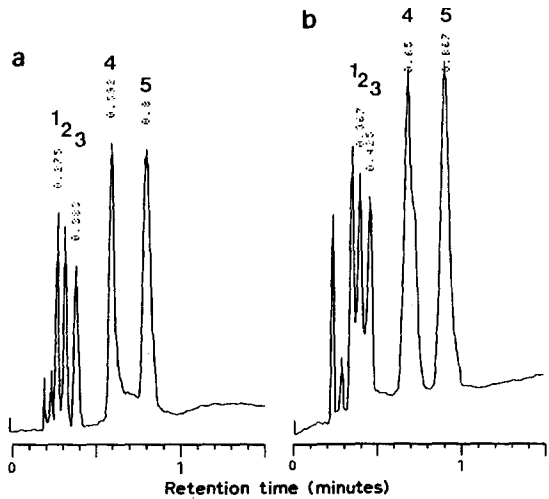

Fig. 10. Chromatograms of reversed-phase peptide standards on (a) PBD 1.0 silica and (b) PBD 3.0 silica. Chromatographic conditions: gradient from 100% A to 75% A–25% B in 1.5 min, where A = 0.1% aqueous TFA and B = acetonitrile–water (75:25, v/v) containing 0.1% TFA; flow-rate, 1.0 ml/min; detection wavelength, 214 nm (Peak numbers correspond to peptides S1–S5, p. 273).

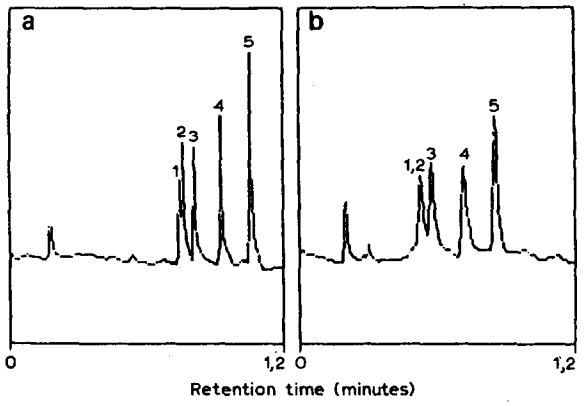

Fig. 11. Chromatograms of reversed-phase peptide standards on (a) *n*-octadecyl-bonded silica and (b) polystyrene–vinylsiloxane-coated silica. For chromatographic conditions, see Fig. 10 (Peak numbers correspond to peptides S1–S5, p. 273).

the test mixture applied to three columns: the PBD 1.0 silica, the *n*-octadecyl-bonded silica and the presilanized PBD 0.8 silica. The PBD 1.0 silica shows a baseline separation and the last eluted peak (*n*-octylpyridine) is fairly symmetrical. The resolution with the other two stationary phase is much worse.

Tests with peptide standard mixtures. The first set of five standard decapeptides enabled us to monitor the hydrophobic character of the stationary phase. Four stationary phases were selected for comparison: PBD 1.0 and PBD 3.0 silica, the *n*-octadecyl-bonded silica and the polystyrene–vinylsiloxane-coated silica. The last material was prepared following the procedure of Kurganov and co-workers[11,12]. The mobile phase was an acidic acetonitrile–water gradient. The chromatograms obtained with the PBD 1.0 and PBD 3.0 silicas are shown in Fig. 10. The first three peptides

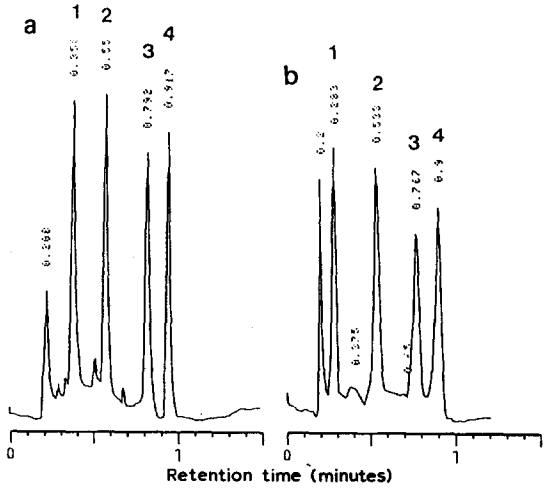

Fig. 12. Chromatograms of the "silanol monitoring" peptide test: (a) PBD 1.0 silica and (b) PBD 3.0 silica. For chromatographic conditions, see Fig. 10 (Peak numbers correspond to peptides 1–4, p. 273).

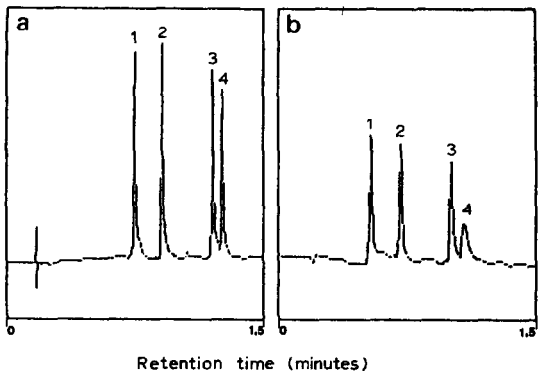

Fig. 13. Chromatograms of the "silanol monitoring" peptide test: (a) *n*-octadecyl-bonded silica and (b) polystyrene–vinylsiloxane-coated silica. For chromatographic conditions, see Fig. 10 (Peak numbers correspond to peptides 1–4, p. 273).

elute closely together as a triplet, followed by peptides 4 and 5 as a doublet. The resolution with the *n*-octadecyl-bonded silica is not as good as that with the two PBD-coated silicas. With the polystyrene–vinylsiloxane-coated silica the triplet is not resolved and the eluted peaks are broader than those in the other columns (Fig. 11).

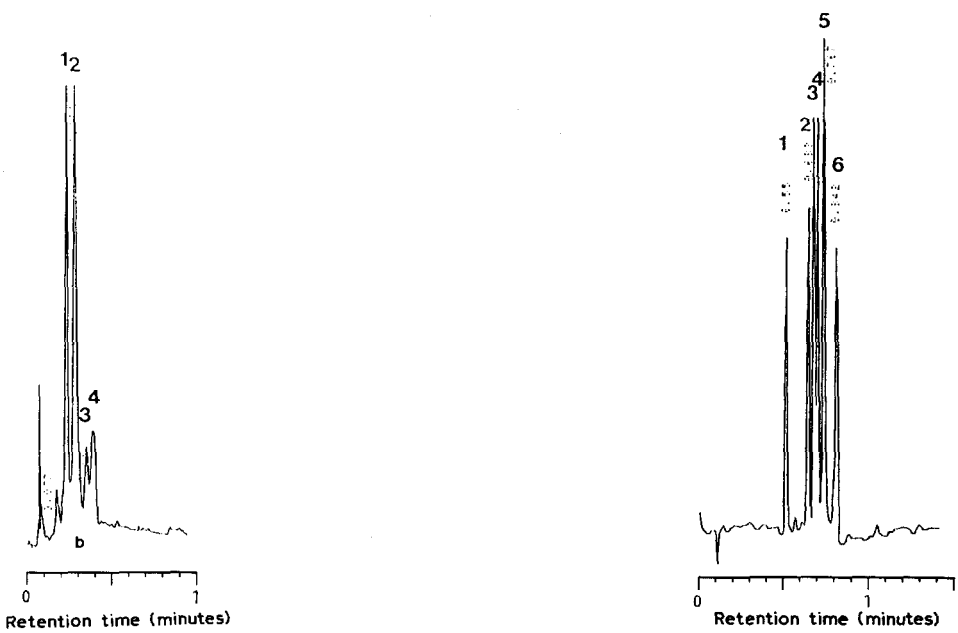

Fig. 14. Isocratic separations of paracelsine peptides on a PBD 1.0 silica column. Chromatographic conditions: Mobile phase, acetonitrile–water (36:64, v/v) containing 0.1% TFA; flow-rate, 3.0 ml/min; detection wavelength, 214 nm (Peak numbers correspond to Paracelsine peptides A–D, p. 274).

Fig. 15. Separation of proteins on a PBD 1.0 silica column. Chromatographic conditions: gradient from 100% A to 100% B in 1.0 min, where A = 0.1% aqueous TFA and B = acetonitrile–water (75:25, v/v) containing 0.1% TFA; flow-rate, 2.0 ml/min; detection wavelength, 214 nm (Peak numbers correspond to proteins, p. 274).

The second set of standard peptides serves for silanol monitoring. The basic character of the peptides increases in the sequence 1 < 2 < 3 <4 owing to the increasing content of lysine. All four peptides are baseline separated on the PBD 1.0 and PBD 3.0 silica columns (Fig. 12) with almost no tailing. The *n*-octadecyl-bonded silica exhibits slightly tailed peaks for the basic peptides. Pronounced peak tailing, particularly of the last-eluting peptide, was observed with the polystyrene–vinylsiloxane-coated silica (Fig. 13).

Paracelsine peptides. This peptide mixture consists of four peptides which differ slightly in two positions. A baseseparation of the four peptides was achieved by Lork *et al.*[19] using a ternary mobile phase and isocratic conditions. Fig. 14 shows the chromatogram of the mixture on a PBD 1.0 silica column with water–acetonitrile (64:36, v/v) as the mobile phase under isocratic conditions, obtained in less than 1 min.

Protein test mixture. A test mixture containing six proteins (1, ribonuclease A; 2, lysozyme; 3, transferrin; 4, conalbumin; 5, β-lactoglobulin; 6, ovalbumin) was chromatographed under the same conditions as applied to the peptide standards. Short gradient times of about 1 min and high flow-rates above 1 ml/min allowed very efficient, high-resolution separations, as already demonstrated by Unger and coworkers[20–23] on *n*-alkyl-bonded non-porous silica. An example is shown in Fig. 15.

CONCLUSIONS

The combination of various physico-chemical methods applied to the characterization of PBD-coated non-porous silicas provided a detailed insight into the chemical structure of the polymer layer and its morphology. For the non-porous silicas investigated, loadings of PBD between 1 and 3% (w/w) provide the most dense and homogeneous coatings.

Chromatographic measurements with various test mixtures indicate a similar lipophilic character of the PBD silica with *ca.* 1% (w/w) of PBD and the *n*-octadecyl-bonded derivative. The polymer clusters formed on the coated PBD 10.0 silica give rise to a substantially higher retention for lipophilic solutes, as expected when extrapolating from lower PBD loadings. This additional retention is probably due to partitioning of the solute between the mobile and bulk polymer phases in addition to the solute–surface interaction with the thin underlying PBD layer.

A minimum load of PBD of about 1–3% (w/w) is required to eliminate the residual adsorptivity of the silica support, as shown by the retention of *n*-octylpyridine in isocratic and basic peptides in gradient elution experiments.

The PBD silicas studied are particularly suitable for rapid chromatographic separations of peptides and proteins under gradient elution conditions in less than 1 min.

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