Journal of Chromatography, 476 (1989) 21–35
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1582

NEW POROUS ORGANIC MICROSPHERES FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

RICHARD W. STOUT*, HENRY J. LEIBU, ALBERT T. ROUSAK and RICHARD C. WRIGHT
E. I. du Pont de Nemours & Co., Inc., Medical Products Department, Glasgow Site, Wilmington, DE 19898 (U.S.A.)

SUMMARY

A process for producing spherical porous organic microspheres, based on urea–formaldehyde (UF) polymer, has been developed. These microspheres exhibit exceptional mechanical strength and resiliency and have minimal tendency toward shrinking or swelling in aqueous, organic, or hydroorganic media. The geometric parameters of the microspheres are conveniently adjusted by process variables, which allow precise control of pore and particle dimensions. The surface of the UF microsphere contains organic functional groups suitable for chemical modification so that ligands of choice may be covalently attached for operation in the anion-exchange or hydrophobic interaction modes.

INTRODUCTION

Considerable effort has been expended on the design of stationary phases for high-performance liquid chromatography (HPLC). The first requirement for a suitable support involves consideration of a set of geometric parameters. These parameters include mean particle diameter, mean pore diameter, specific pore volume and other factors. These parameters are optimally held within narrow range specifications in order to allow a packed column with a regular, well-defined geometry to be obtained. A regular, controlled geometry is essential to maintaining high theoretical plate efficiency. The mean diameter of the particles which comprise the bed dictates the system operating pressure during use with flowing mobile phases and also influences the column packing procedure. This sets an upper limit on the number of theoretical plates attainable. The dual requirements of small particles and regular geometry of the support place restrictions on the manufacture and mechanical stability of the support. Not all chromatographic supports fulfill these essential requirements. Silica gel has been utilized to a far greater extent than other substances that could ostensibly serve as alternatives.

Silica gel is unique among numerous substances in its ability to fulfill so many requirements as a chromatographic stationary phase. Its cubic expansion is essentially zero under normal operating temperatures. Several convenient processes are available for the manufacture of porous microspheres with narrow size distribution and well-defined spatial geometry. The silica surface is conveniently modified to suit the various chromatographic requirements by the introduction of covalently attached ligands. The pore size and specific pore volume may be adjusted by several techniques to permit separation of substances of varying molecular dimensions. Silica gel is an admirable stationary phase for the separation of small molecules having a wide range of chemical functional groups.

Silica gel is less useful with water-rich hydroorganic eluents, since the surface of the gel slowly dissolves in water at all pH levels. The dissolution process eventually causes the bonded phase to hydrolyze. The rate at which this occurs becomes rapid above pH 8 but is less of a problem at lower pH levels. Several measures to prevent rapid destruction of the silica surface have been incorporated, but none are completely satisfactory when sterilizing agents, such as dilute alkali solutions, are pumped through the columns. Thus, the question becomes: Can organic substances be conveniently substituted for silica gel as chromatographic supports and is it possible to keep their geometric parameters within narrow limits?

The literature contains numerous descriptions of organic chromatographic supports which can function in most operating modes[1-15], and most of these may be obtained commercially. The available supports may be conveniently divided into two broad categories. The first group[1-9] consists of supports that are vinyl polymers. The second group[10-15] includes substrates derived from agarose, dextrans, polypeptides, or substances other than polymers.

Porous organic microspheres may be produced from vinyl monomers, polymers, and other ingredients by using suspension or emulsion polymerization techniques. The patent literature discloses some aspects of the methods of producing organic microspheres. The pores are introduced by allowing the polymerization to proceed around an inert substance, such as toluene, which is washed away after polymerization is terminated. Polystyrene–divinyl benzene (PS–DVB) is the most common type; it is described in some detail by Helfferich[16].

While not labile in the presence of strong bases in most cases, the organic substrates suffer from other problems. Their most serious disadvantages over their silica counterpart are swelling and insufficient mechanical strength. Organic eluents, commonly employed in the practice of HPLC, will solvate many polymers and thus induce undesirable changes in particle and pore geometries. The swelling effects may be minimized by employing cross-linking agents in varying amounts. A high degree of cross-linking ensures mechanical strength, while a low degree usually produces a particle which has insufficient mechanical strength to withstand the pressure necessary for packing into columns or for sustained operation at the usual pressures. The cross-linking agents may cause additional difficulties by introducing secondary surface interactions, which, in turn, lead to band-broadening or irreversible adsorption. These factors frequently limit the lower boundary of the mean particle size, which dictates the highest number of theoretical plates that can be achieved. Thus, large particle diameters are preferred for separations when such materials are utilized, and lower efficiency and long separation time must be tolerated.

Several porous organic microspheres described in the literature are reported to perform about as well as silica analogues in terms of mechanical parameters. Most of these supports are copolymers of vinyl monomers. Ugelstad[1] has patented processes for the production of monodisperse microspheres. Vlacil *et al.*[2,3] and Kas *et al.*[4]

described porous organic microspheres, based on hydroxyethyl methacrylate copolymers. Hirata *et al.*[5] and Hanai *et al.*[6] worked with similar materials based on polyvinyl alcohol (PVA). Wojaczynska and Kolarz[7] reported on particles made from copolymers of acrylonitrile and DVB. Tanaka *et al.*[8] used particles of 5–7 μm diameter, produced from copolymers of stearyl methacrylate and ethylene dimethacrylate. Dawkins *et al.*[10,11] described microspheres based on polyacrylamide. Most manufacturers disclose little or nothing concerning the exact composition of their chromatographic products produced from vinyl compounds. However, swelling and mechanical strength are routinely discussed. These properties must be tested in detail, since they are the cause of the two most important deficiencies in organic packing materials.

Another group of chromatographic substrates is based on the use of agarose, dextrans, or other naturally occurring substances[12–14]. The sugar-based supports are widely used for separations of numerous biochemical substances. The chief advantage of agarose lies in the very low non-specific adsorption properties of the complex sugars that form the backbone of the structure. The disadvantages are the lack of mechanical strength and undesirable interactions introduced by the cross-linking agents used to circumvent such problems. Supports based on polypeptides have recently been introduced by Ihara *et al.*[15].

This paper reports on the use of urea–formaldehyde (UF) polymer as an alternative to vinyl-, dextran-, or polypeptide-based supports. The process for producing these microspheres permits convenient selection and control of the most important geometric parameters, such as particle diameter, pore diameter, and specific pore volume. In addition, this polymer exhibits high resiliency and marked resistance toward shrinking and swelling in the presence of organic solvents and is stable up to pH 13.

EXPERIMENTAL

Instruments

Chromatographic experiments were conducted with several instruments, depending on the type of chromatography required. For size-exclusion chromatography (SEC) a Model 8800 HPLC apparatus (E. I. du Pont & Co., Wilmington, DE, U.S.A.), equipped with a fixed photometer, operating at 254 nm, was used. Samples were injected with a Rheodyne Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.). Ion-exchange experiments were performed with a fast protein liquid chromatography (FPLC) system (Pharmacia, Piscataway, NJ, U.S.A.), which included an LCC-500 controller, two P-500 pumps, a mixer, a motor-valve MV-7 injector, a UV-1 single-path monitor with an HR-10 flow-cell and 280-nm filter, and a REC-482 recorder. Analog data were digitized and archived with a Nelson Analytical Model 760 Series interface (Cupertino, CA, U.S.A.), which was interfaced with an HP Series 220 microcomputer (Hewlett-Packard, Fort Collins, CO, U.S.A.). Nelson Analytical software was modified to include calculations for theoretical plates, retention, and skew or peak asymmetry. Archived data were treated with non-linear curve fitting routines by the use of the software package MINSQ (MicroMath Scientific Software, Salt Lake City, UT, U.S.A.).

Chemicals

Tetrahydrofuran (THF) and acetonitrile of HPLC grade were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.) and used as received. Deionized water was produced in a Continental Water System Unit (North Wales, PA, U.S.A.). Tris(hydroxymethyl)aminomethane (Tris), ammonium sulfate, dipotassium hydrogenphosphate, and sodium chloride were obtained from Aldrich (Milwaukee, WI, U.S.A.). The buffer pH levels were adjusted with reagent-grade hydrochloric acid or sodium hydroxide, as appropriate.

Mobile phases and samples

Buffered mobile phase solutions were prepared in single batches and consisted of 2.0 *M* ammonium sulfate or 1.0 *M* sodium chloride, 0.02 *M* dipotassium hydrogen phosphate or 0.05 *M* Tris, and 0.05–0.10% (w/v) sodium azide. The pH of the buffers was adjusted with 1 *M* sodium hydroxide or hydrochloric acid, as appropriate. The protein samples were mixtures prepared from proteins as received by dissolving them gently in the mobile phase and filtering through a 0.45-μm cellulose acetate sterile filter (Nalge Company, Rochester, NY, U.S.A.). Size-exclusion samples were prepared by dissolving polystyrene molecular weight standards in neat THF at concentrations of *ca.* 1 mg/ml.

Carbonic anhydrase, lysozyme, myoglobin, ovalbumin, soybean trypsin inhibitor, and transferrin were obtained from Sigma (St. Louis, MO, U.S.A.). Polystyrene molecular weight standards (PS MW) were obtained from Du Pont.

UF microspheres and columns

UF microspheres were produced by treating coacervated spheres, described elsewhere[17], with ammonium bifluoride in water at ambient temperature. The product was washed with deionized water and the UF surface was chemically modified to introduce chromatographic ligands by proprietary procedures.

UF microspheres were packed into stainless-steel columns of 250 mm × 4.6 or 9.4 mm I.D. by proprietary procedures. Column inlet pressures varied from about 1000 to 10 000 p.s.i. with a variety of different organic solvents. Alternately, UF microspheres were slurry-packed in deionized water into glass columns, 100 mm × 10 mm or 300 mm × 10 mm I.D. at ambient pressure.

Rigidity and resilience experimental protocol

A column of 250 mm × 4.6 mm I.D. was packed with UF microspheres of 3 μm nominal particle diameter and 60 Å pore size. The HPLC pump was directly connected to the inlet of the test column with capillary tubing. The system was thoroughly flushed with THF at a flow-rate of 1.0 ml/min, and the column was purged with this mobile phase for *ca.* 10 column volumes (30 ml). The pump flow-rate was set to 0.25 ml/min. This flow-rate was maintained for 2–3 min or until the pressure reading indicated steady-state operation. This reading was recorded, and the flow-rate was increased in 0.25 ml/min increments with pressure readings being taken at each new step until the inlet pressure approached 350 bar. The flow-rate was then decreased by the same procedure in reverse, and pressure readings were again recorded until the final flow-rate was 0.25 ml/min. These data are shown in Table I.

Occasionally the flow-rate was set to some arbitrary, previously recorded level

TABLE I

RESULTS OF RIGIDITY AND RESILIENCE EXPERIMENTS

Flow-rate, F	*THF*		*Water*		*Acetonitrile*	
(ml/min)	*Pa*[a] *(bar)*	*Pd*[b] *(bar)*	*Pa*[a] *(bar)*	*Pd*[b] *(bar)*	*Pa*[a] *(bar)*	*Pd*[b] *(bar)*
0.25	26	26	53	53	19	20
0.50	53	53	106	106	40	41
0.75	81	81	159	158	61	62
1.00	110	108	209	209	83	83
1.25	138	137	258	255	104	104
1.50	165	164	301	299	125	125
1.75	193	191	344	341	145	145
2.00	220	219			165	165
2.25	247	246			185	185
2.50	274	272			206	205
2.75	293	294			225	225
3.00	323	320			244	244
3.25					264	263
3.50					280	280
3.75					300	300
4.00					318	319
4.25					330	332
4.00					317	317
3.75					299	298
3.50					279	279
3.25					262	261
3.00					241	240
2.75	291	293			223	223
2.50	270	268			204	205
2.25	243	242			184	184
2.00	216	215			164	165
1.75	190	188			145	145
1.50	162	162	294	295	125	125
1.25	136	135	250	249	104	104
1.00	108	108	204	203	84	84
0.75	81	81	155	155	62	62
0.50	53	53	105	104	41	41
0.25	26	26	53	53	20	20

[a] Ascending pressure readings.
[b] Descending pressure readings.

(data not shown) to check for hysteresis effects. The entire procedure was repeated in order to produce additional data for a single mobile phase. The pump was then flushed with the second solvent at a flow-rate of 1.0 ml/min, the column was purged with this mobile phase for *ca.* 10 column volumes, and the entire process was repeated. Finally, a third solvent was used to complete the data set.

THEORY

Synthesis of UF microspheres

Iler patented a process[17] for the production of uniform oxide microspheres. Urea (U) or melamine and formaldehyde (F) are combined with an aqueous silica sol under acidic conditions so that polymerization of the organic constituents produces coacervation of the components into microspheres. The silica sol particles are trapped within the coacervate matrix. The microspheres are dried and heated in air to oxidize the urea–formaldehyde (UF) polymer portion of the matrix, leaving behind porous silica microspheres (PSM). The PSM material is sold commercially as Zorbax™. This paper deals with the organic portion of the coacervate matrix, which is retained while the silica is discarded. This is opposite to the Iler process.

The reaction proceeds according to the following equation

$$n\mathrm{U} + n\mathrm{F} + \mathrm{SiO_2} \rightarrow (\mathrm{UF})_n\text{–}\mathrm{SiO_2}$$

where the term on the right represents the composite organic–inorganic coacervate matrix.

The silica is conveniently removed to leave behind a porous organic microsphere, which contains imprints of the silica sol. The pore size is determined by the diameter of the silica sol analogous to that of Zorbax with several significant differences. While the pore in the silica microsphere is determined by the cavity surrounded by sol particles in a random close-packed arrangement with a coordination number of 5, the situation is different in the porous organic microsphere. The UF matrix is not "solid", in contrast to the silica counterpart, but seems to be a foam. Other investigators have reported on similar microporous substances. Nevejans and Verzele[18] have studied microporosity in detail for the case of PS–DVB copolymers. Intrinsic porosity in the UF material is similar to that of the vinyl analogues and consists of two types: (1) micropores of probably 3–12 Å and (2) some "channels" within this microporous foam. Thus, pores are interconnected by a network of channels. The channel structure facilitates the removal of silica with ammonium bifluoride in a few minutes, and the UF material yields essentially no residue when burned in air. The sol particles are not surrounded by the foam structure in such a way as to limit the effective pore size by some aperture effect (*e.g.*, no 13-Å porous barrier or isolated sol particles). The average pore size is determined by the mean particle diameter of the sol.

Particle and column volumes

The internal volume V_c, of a packed column is given by[19]

$$V_c = V_o + V_s + V_p + V_l \quad (1)$$

where V_o is the interstitial volume outside the microspheres, V_s is the volume of the solid support, V_p is the pore volume, and V_l is the volume of any bonded or coated ligand on the surface of the pores. The volume of spheres when random close-packed into any container is about 0.6 of the volume of the container. Thus, V_o will have a nominal value of 0.4 V_c.

For cases where no ligand is present

$$V_o = 0.4\ V_c \tag{2}$$
$$V_m = V_o + V_p \tag{3}$$
$$V_m = V_c[1-0.6(1-\Phi)] \tag{4}$$

where Φ is the porous fraction and is defined as the ratio of the pore volume to the total volume of the support[20].

$$\Phi = V_p/(V_s + V_p) \tag{5}$$

According to SEC theory, as described by Yau *et al.*[19], the retention volume, V_R, is given by

$$V_R = V_o + V_p(K_D) \tag{6}$$

where K_D is the distribution constant. Eqn. 6 is combined with eqn. 3 to give the following expression in convenient chromatographic volume terms

$$K_D = (V_R - V_o)/(V_m - V_o) \tag{7}$$

Permeability and inlet pressure

The Darcy equation[21,22] predicts the column inlet pressure from eqn. 8

$$U = K_o\, d_p^2\ P/\eta L \tag{8}$$

where U is the linear velocity, K_o is a constant related to the column permeability, d_p is the mean microsphere diameter, P is the pressure drop across the column, η is the viscosity of the mobile phase, and L is the column length.

Since the linear velocity[21]

$$U = L/t_o \tag{9}$$

where t_o is the column breakthrough time and

$$t_o = V_m/F \tag{10}$$

where F is the flow-rate, by combining eqns. 8–10, we obtain

$$P = \Omega\eta L^2 F/V_m d_p^2 \tag{11}$$

where $\Omega = 1/K_o$.

A packed column has fixed values of d_p, L, and V_m and will experience a pressure drop that is controlled only by changes in F and η, provided the physical properties of the packing material do not change when the mobile phase is changed or the elastic or plastic deformation limits are exceeded. Then the invariant terms may become a constant, and eqn. 12 holds for the following units: P (bar), η (N s/m^2), L (mm), F (ml/min), V_m (ml), and d_p (μm).

$$\varepsilon = 6\ V_m\ d_p{}^2/L^2 \tag{12}$$

or

$$\varepsilon P = \Omega\eta F \tag{13}$$

A plot of εP *vs.* ηF will be a straight line with a slope equal to Ω.

RESULTS AND DISCUSSION

Pore size

Three lots of UF microspheres were produced from three different sol sizes. Nominal sol sizes of 5, 15, and 30 nm were employed to give UF-60 (60 Å), UF-150 (150 Å), and UF-300 (300 Å) porous organic microspheres, respectively. These lots were analyzed by SEC to verify that changing the sol size did directly influence the final pore size. The chromatographic retention volumes for macromolecular polystyrene standards are shown in Table II. Values of the particle porous fraction Φ were calculated from eqn. 5. This gives 0.57, 0.57, and 0.54 for the three packings,

TABLE II

SIZE-EXCLUSION RESULTS FOR UF-60, -150, AND -300

PS MW (daltons)	*UF-60*		*UF-150*		*UF-300*	
	V_R (*cm*^{3})	K_D[a]	V_R (*cm*^{3})	K_D[a]	V_R (*cm*^{3})	K_D[a]
9 000 000	–	–	–	–	5.80	0.000
1 800 000	5.68	0.000	6.97	0.000	6.01	0.019
900 000	6.11	0.065	7.07	0.010	6.04	0.034
233 000	6.39	0.107	7.17	0.027	6.19	0.063
200 000	6.52	0.126	7.21	0.034	–	–
100 000	6.64	0.144	7.26	0.043	–	–
97 200	6.70	0.153	7.32	0.053	6.50	0.113
50 000	6.81	0.170	7.35	0.058	7.00	0.194
35 000	7.03	0.202	7.56	0.094	7.88	0.327
20 000	–	–	7.94	0.158	8.36	0.413
17 500	7.25	0.236	8.29	0.218	8.54	0.442
10 000	7.59	0.287	9.23	0.287	9.00	0.516
9000	7.87	0.329	9.36	0.329	9.27	0.560
4000	8.89	0.482	10.36	0.482	9.79	0.644
2100	9.76	0.613	11.04	0.613	10.12	0.697
800	10.39	0.788	12.29	0.788	10.75	0.798
92[b]	12.34	1.000	12.89	1.000	11.98	1.000

[a] Calculated from eqn. 7.

[b] Toluene.

respectively. When V_0 is calculated from eqn. 2, the values are similar and yield 0.52, 0.57, and 0.48. This indicates that the columns are nearly optimally packed, since the porosity should not be altered during these experiments. The porous fraction Φ may be varied independently (not reported here) by changing process variables. Fig. 1 shows a plot of the logarithm of polystyrene molecular weight standards *versus* K_D, which was calculated from eqn. 7 by using the retention volumes from Table II. Fig. 2 shows a typical SEC chromatogram on UF-60, lot 1, which separates polystyrene molecular weight standards according to accepted theory. The results indicate that the pore size is controlled to a first approximation by the sol mean particle diameter.

Columns were packed by high-pressure slurry techniques in a manner similar to that used for silica packing materials. No obvious deformation was observed that could be attributed to collapse of the packing material under these conditions. No packing collapse was observed in further studies with solvents such as methanol, THF, and similar solvents commonly used for slurry packing. Even when the inlet pressure exceeded 8000 p.s.i., there was no significant rise in column inlet pressure with time, although the initial operating pressures were higher with higher packing pressure. Column steady-state operating pressures remained constant or returned to their

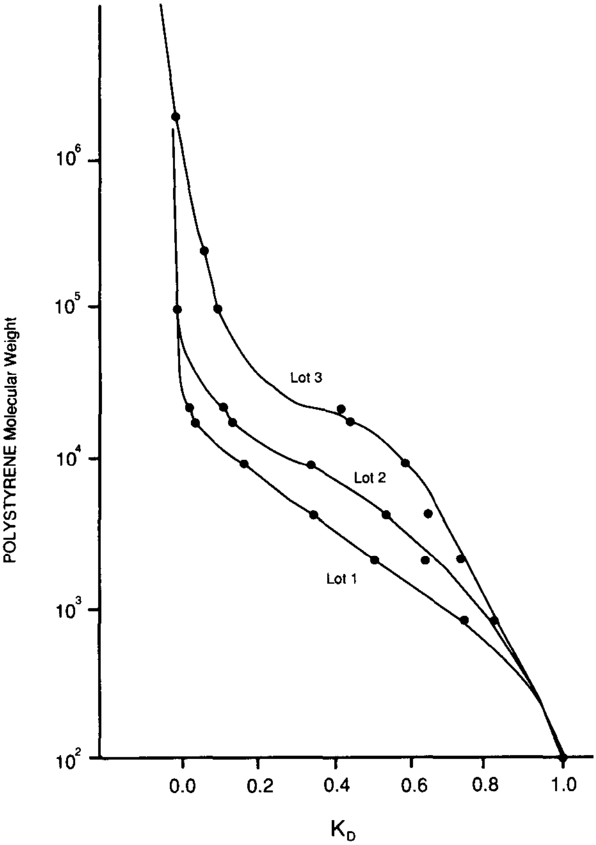

Fig. 1. Plot of log molecular weight *vs.* K_D for polystyrene standards. Lot 1, 60 Å; lot 2, 150 Å; lot 3, 300 Å.

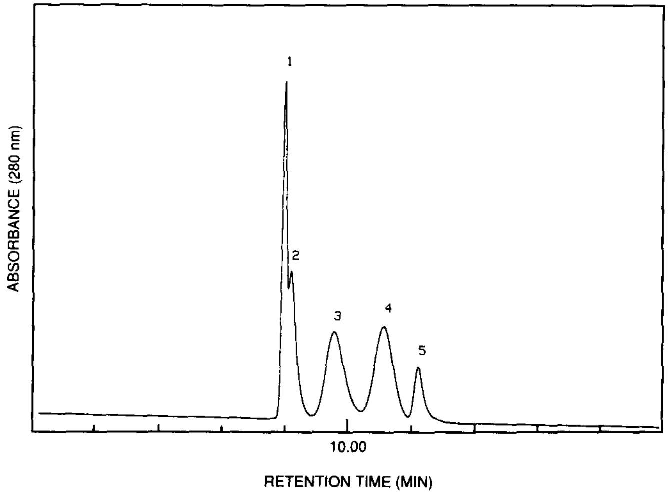

Fig. 2. Typical size-exclusion chromatogram of UF-60. Conditions: column, 250 × 9.4 mm I.D.; particle diameter, 3 μm; mobile phase, THF; flow-rate, 1.0 ml/min; detector, 254 nm, 0.2 a.u.f.s.; inlet pressure, 12 bar. Peak identities: 1 = polystyrene 97 200; 2 = 17 500; 3 = 4000; 4 = 800; 5 = 92 (toluene).

original levels during various evaluation episodes, even though several different mobile phases of neat or hydroorganic composition were employed.

More systematic experiments were designed to investigate the swelling phenomenon of these UF microspheres, since there was no indication in the literature that they had previously been utilized as a chromatographic column material, nor was there any indication of how UF would behave in an environment such as a packed column under pressure.

Swelling of porous supports

Swelling of porous organic particles has been reported by several researchers. In fact, controlled swelling is the basis for the synthesis of some patented processes for the production of monodisperse microspheres[1]. Hirata *et al.*[5] studied hydrophilic gels, derived from copolymers of vinyl alcohol (VA). They used the theoretical plate efficiency as a parameter for estimating the magnitude of swelling effects of organic particles in packed columns. The VA gels maintained a rather consistent efficiency when methanol, ethanol, or dimethylformamide (DMF) were used as the mobile phase but significant decreases in efficiency were observed when acetonitrile, THF, or chloroform were used. When several VA gels were subjected to these tests, these materials were generally observed to shrink in organic solvents. Wojaczynska and Kolarz[7] performed similar experiments on porous particles derived from copolymers of acrylonitrile (AN) and DVB. Maa and Horváth[23] studied non-porous, highly cross-linked microspheres of 3 μm particle diameter and assessed their mechanical stability by performing flow and pressure experiments. Their results indicated that the nominal value of the interstitial volume fraction V_0/V_c (usually 0.4) decreased from 0.38 to 0.24, depending on which solvents were selected for mobile phases. This change was calculated to correspond to a 7.8% increase in particle diameter, and this corresponds to a 25% increase in the volume of the particles in the packed column. The specific permeability was found to be greatest with water.

Thus, even "solid" (described as non-porous in this case) or highly cross-linked vinyl copolymers appear to swell to some extent in the presence of organic solvents. Most researchers will agree that swelling of organic particles in packed columns is highly complicated where linear or predominantly linear vinyl polymers and copolymers are concerned.

The situation is similar in the case of agarose, except that the mobile-phase systems are predominantly aqueous and nearly always buffered. Hjertén[13,14] described highly cross-linked agarose beads with particle diameters between 3 and 10 μm. These materials were deformed during column packing, and a microscopic examination showed that the original spherical shape was restored on the microscope slide after a short period of time. In addition, the cross-linking reagents interfered with the chromatographic process unless suitable precautions to mask their effects were taken. These materials are not equivalent to "linear polymers" in the sense of vinyl copolymers. The chief problem with these packing materials is insufficient rigidity and resilience.

Rigidity and resilience of UF microspheres

UF porous microspheres probably belong to a different classification scheme in that they are undoubtedly highly cross-linked, not linear, and not very well solvated by commonly used organic solvents. The exact nature of the UF matrix is unknown and somewhat controversial[24].

Marvel *et al.*[25] suggested that their structure consists of rings formed by the reaction of urea with amino acid amide. In our experiments of surface modifications we found nothing to support this assumption. Even the mole ratio of urea and formaldehyde in UF polymers is in question, since the methods to determine this parameter are laborious and imprecise[26,27].

Experiments were conducted on 3-μm diameter, 60-Å pore-size UF microspheres, since the small particle diameter would offer the highest resistance to flow, a concomitantly high pressure drop, and a high propensity toward plastic or elastic deformation. A column of 250 mm × 4.6 mm I.D. was packed and used at several different flow-rates with different mobile phases. The inlet pressure was recorded under fixed conditions of mobile phase and operating pressure, and then the flow-rate was increased and the new pressure level was recorded after the system was sufficiently equilibrated. Table I shows the results of this experiment with three different solvents, and a plot of the pressure *versus* flow-rate is shown in Fig. 3. The results indicate that the packed column is resistant to geometric changes up to 300 bar with three different solvents commonly used in HPLC. The open circles indicate ascending pressure readings, while the solid triangles indicate descending pressure readings. In addition, random flow-rate selections were made during the experiments (not shown) to verify the absence of any hysteresis effects. Note that the linearity is good, as Horváth[23] and others have previously shown, and the intercept of the line is zero within the experimental error. When slight deviations from linearity are observed, such as points where levels exceed 280 bar, the deviation is seen to be negative with respect to pressure. This negative deviation is probably caused by heat generated by the system. Heat works on the packed column to lower the viscosity of the mobile phase. If the

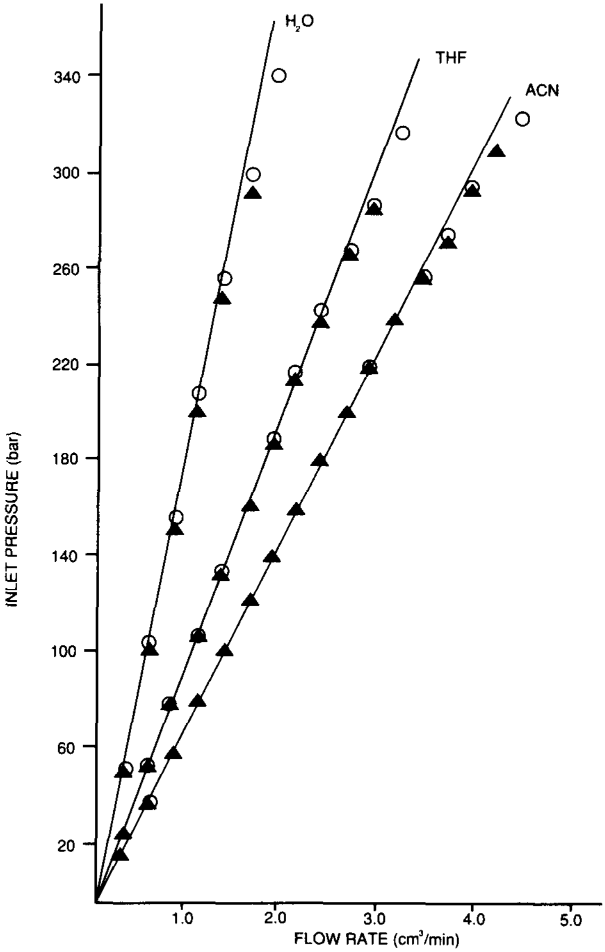

Fig. 3. Plot of inlet pressure *vs.* flow-rate for 3-μm UF-60. For conditions see Experimental section. (○) Ascending pressure readings; (▲) descending pressure readings. ACN = Acetonitrile.

deviation had been observed to occur in the opposite direction and hysteresis effects had been noted, the UF particles would be shrinking, swelling, or changing in shape in some irreversible manner. The chromatographic theory states[22] that there should be some flow resistance parameter, Ω, with a nominal value of 500 for spherical particles. The theory further predicts that the magnitude of this value is invariant with stable packed columns, as is the case for silica, provided the pressure limit of the particles is not exceeded. For this experiment, the constant ε from eqn. 14 has a value of $2.557 \cdot 10^{-3}$, when P is in units of bar, L in mm, V_m in ml and d_p in μm,

$$\varepsilon = 6V_m d_p^2/L^2 \tag{14}$$

and the viscosities of water, acetonitrile, and THF were taken as 0.993, 0.370, and 0.550 N s/m², respectively[28]. According to the Theory section from eqn. 13

$$\varepsilon P = \Omega \eta F$$

where Ω should remain invariant, with a constant value of about 500, while the mobile phase composition is altered. Fig. 4 shows a plot of εP *vs.* ηF with a slope of 535. This experiment clearly shows that UF microspheres exhibit exceptional resistance to swelling and particle deformation in organic solvents. Maintenance of the Ω value of about 500 in various solvents at high pressures shows that, if elastic deformation or other phenomena are occurring, the effects are reversible and a stable packed column is maintained.

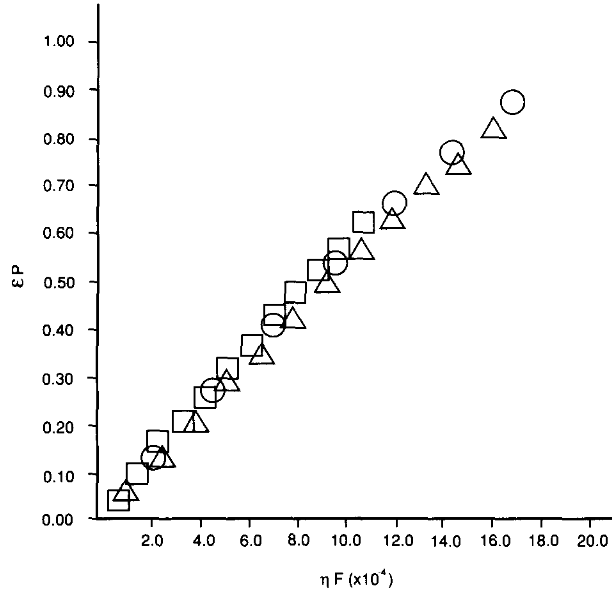

Fig. 4. Normalized plot of inlet pressure *vs.* flow-rate for 3-μm UF-60. For conditions see Experimental section. (○) Water; (□) acetonitrile; (△) THF.

Bonded phases

The surface of the UF matrix is probably complex in terms of chemical functional groups. There are doubtlessly numerous varieties of substituted urea and other similar substructures in this polymer[24]. Nevertheless, the surface may be chemically modified to introduce ligands suitable for use in the interactive modes of HPLC. For example, ionogenic ligands can be covalently attached to the surface so that ion-exchange (electrostatic interaction) chromatography can be performed. A strong anion-exchange packing (SAX) was prepared on a UF matrix by a proprietary process and studied experimentally to determine its suitability for the fractionation of some common proteins. This particular packing material had 300-Å pores and a nominal particle diameter of 10 μm. Fig. 5 shows the separation of several proteins under classical ion-exchange conditions.

Hydrophobic interaction chromatography (HIC) is frequently preferred as a separation technique, since the conditions are mild, and denaturation of precious substances is minimized. The UF surface was chemically modified to produce a weakly hydrophobic environment, such that proteins and other macromolecular substances

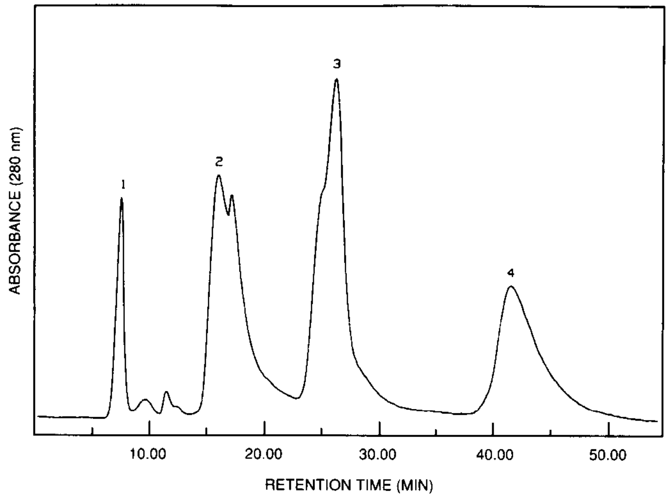

Fig. 5. Typical chromatogram of proteins on UF-300 SAX. Conditions: column, 300 × 10 mm I.D.; particle diameter, 9.5 μm; mobile phase system: (A) 0.01 *M* Tris (pH 8.0)–0.05% (w/v) sodium azide, (B) 0.01 *M* Tris–1.0 *M* sodium chloride (pH 8.0)–0.05% (w/v) sodium azide; gradient: 0% to 100% B linear in 50 min; flow-rate, 2.0 ml/min; detector, 280 nm, 0.1 a.u.f.s.; inlet pressure, 20 bar. Peak identities: 1 = myoglobin (cetine), 2 = carbonic anhydrase, 3 = ovalbumin (hen egg) and 4 = soybean trypsin inhibitor.

can be sorbed under mobile-phase conditions of high salt concentration and desorbed at lower concentrations. Fig. 6 shows a separation of myoglobin, carbonic anhydrase, and lysozyme. The linear gradient runs from a 2.0 *M* salt concentration, held constant

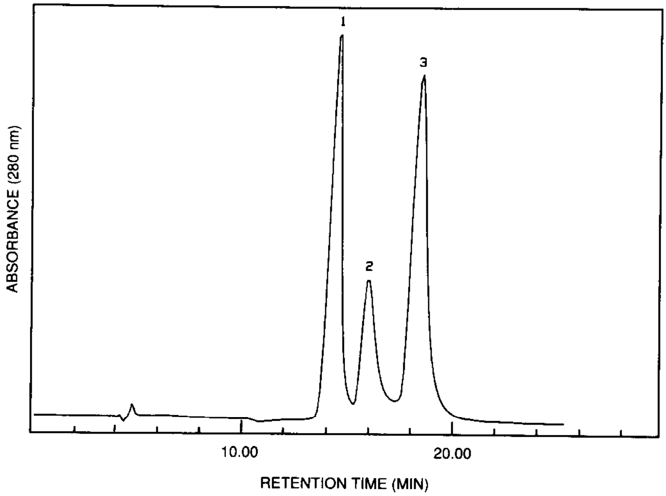

Fig. 6. Typical chromatogram of proteins on UF-300 HIC. Conditions: column, 300 × 10 mm I.D.; particle diameter, 9.5 μm; mobile phase system: (A) deionized water (pH 7.00)–0.05% (w/v) sodium azide, (B) 2.0 *M* ammonium sulfate–0.02 *M* potassium phosphate (pH 7.00)–0.05% (w/v) sodium azide; gradient, 100% to 0% B linear in 10 min, hold at 0% B for 15 min; flow-rate, 3.7 ml/min; detector, 280 nm, 0.1 a.u.f.s.; inlet pressure, 45 bar (at 100% B). Peak identities: 1 = myoglobin (equine), 2 = transferrin and 3 = lysozyme (hen egg).

for 5 min to ensure the sample was loaded, to 0.0 *M* in 15 min. The eluted substances were detected at 280 nm. Nominal 10-μm diameter particles with 300-Å pores were used for this separation.

CONCLUSIONS

The results of this work indicate that UF polymer may be quite suitable for use as a chromatographic support for HPLC. This material is easily formed into spherical microparticles by a well-established process, and the pore size is conveniently controlled by merely selecting silica sols of different sizes. The microspheres resist shrinking or swelling when contacted with common HPLC mobile phases. Furthermore, the UF microspheres exhibit no elastic or plastic deformation when subjected to inlet operating pressures ranging from ambient to levels in excess of 300 bar. The surface can be chemically modified by several procedures to provide for separations of proteins.

REFERENCES

1 J. Ugelstad, *U.S. Pat.*, 4,530,956 (1985); 4,186,120 (1980); 4,113,687 (1978).
2 F. Vláčil and I. Vinš, *J. Chromatogr.*, 391 (1987) 133.
3 F. Vláčil, I. Vinš and J. Čoupek, *J. Chromatogr.*, 391 (1987) 119.
4 J. Káš, M. Marek, J. Beronová and F. Švec, *J. Chromatogr.*, 376 (1986) 269.
5 N. Hirata, M. Kasai, Y. Yanagihara and K. Noguchi, *J. Chromatogr.*, 396 (1987) 115.
6 T. Hanai, Y. Arai, M. Kirukawa, K. Noguchi and Y. Yanagihara, *J. Chromatogr.*, 349 (1985) 323.
7 M. Wojaczyńska and B. Kolarz, *J. Chromatogr.*, 358 (1986) 129.
8 Y. Tanaka, H. Sato, K. Miyazaki and Y. Yamada, *J. Chromatogr.*, 407 (1987) 197.
9 Y. Kato, T. Kitamura and T. Hashimoto, *J. Chromatogr.*, 333 (1985) 93.
10 J. V. Dawkins, N. P. Gabbott, M. Montenegro, L. L. Lloyd and F. P. Warner, *J. Chromatogr.*, 371 (1986) 283.
11 J. V. Dawkins, L. L. Lloyd and F. P. Warner, *J. Chromatogr.*, 352 (1986) 157.
12 T. Anderson and L. Hagel, *Anal. Biochem.*, 141 (1984) 461.
13 S. Hjertén, B.-L. Wu and J.-L. Liao, *J. Chromatogr.*, 396 (1987) 101.
14 S. Hjertén, Z.-Q. Liu and D. Yang, *J. Chromatogr.*, 296 (1984) 115.
15 H. Ihara, T. Yoshinaga and C. Hirayama, *J. Chromatogr.*, 362 (1986) 197.
16 F. Helfferich, *Ion Exchange*, McGraw-Hill, New York, 1962.
17 R. K. Iler, *U.S. Pat.*, 4,010,242 (1977).
18 F. Nevejans and M. Verzele, *J. Chromatogr.*, 406 (1987) 325.
19 W. Yau, J. Kirkland and D. Bly, *Modern Size-Exclusion Chromatography*, Wiley, New York, 1979.
20 R. Stout, G. Cox and T. Odiorne, *Chromatographia*, 24 (1987) 602.
21 P. A. Bristow, *Liquid Chromatography in Practice*, hetp, Cheshire, 1976.
22 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Chromatography*, Wiley, New York, 2nd ed., 1979.
23 Y.-F. Maa and Cs. Horváth, *J. Chromatogr.*, 445 (1988) 71.
24 W. R. Sorenson and T. W. Campbell, *Preparative Methods of Polymer Chemistry*, Wiley, New York, 2nd ed., 1968.
25 C. S. Marvel, J. R. Elliott, F. E. Boettner and H. Yuska, *J. Am. Chem. Soc.*, 68 (1946) 1681.
26 P. P. Grad and R. J. Dunn, *Anal. Chem.*, 25 (1953) 1211–1213.
27 J. C. Morath and J. T. Woods, *Anal. Chem.*, 30 (1958) 1437–1440.
28 R. C. Weast (Editor), *Handbook of Chemistry and Physics*, The Chemical Rubber Co., Cleveland, OH, 50th ed., 1969.