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Aligned fiber columns for size-exclusion chromatography

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SUMMARY

Chromatographic columns are being developed that contain, instead of porous particles as in classical packed columns, bundles of aligned porous silica fibers of claimed diameter 18 μm and average pore size 270 Å. From these properties, the material is comparable to conventional silica particles as a stationary phase for high-performance liquid chromatography. However, as fibers can be packed much more densely than spherical particles, the interstitial volume of the packing is significantly lower, while the pore volume can be higher. This combination of geometrical properties is important for size-exclusion chromatography. The performance of a prototype Aligned Fiber Column was tested by measuring the elution times and band broadening of polystyrene molecular weight standards with methylene chloride as the eluent. The results were compared to those found for columns packed with 10- or 3-μm silica particles. In view of possible applications for the separation of biopolymers, the surface of the column was modified by grafting alkyl diol groups in an *in situ* silanization process. The results obtained with several different proteins show a very low residual activity of the surface (only strongly basic proteins are retained), a slight decrease in the pore volume and pore diameters, with little change in the porosity ratio. As this was the first attempt at *in situ* diol bonding of any silica material, some polymerization of the silane occurred, seriously decreasing the efficiency of the column.

INTRODUCTION

In size-exclusion chromatography (SEC) the biggest molecules can move only between the particles of the packing and their elution volume is equal to the interstitial volume (V_0). For small molecules the pore volume (V_p) is also accessible and they are eluted with the mobile phase volume ($V_m = V_0 + V_p$). Hence the first peak of the chromatogram appears after V_0 and the separation takes place within the time corresponding to the elution of one pore volume. For easier comparison, the different contributions to the column volume are expressed as fractions of the empty column volume (porosities ε). Here we use the ratio V_p/V_0 to represent the geometrical characteristics of the column. Some workers prefer to use $(V_p + V_0)/V_0 = 1 + V_p/V_0$. The conversion is easy.

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In practice, for most columns packed with microparticulate silica, the interstitial volume is about 40% of the total volume of the bed and the pore volume accounts approximately for another 40%. This means that the first half of the analysis time is spent waiting for the first peak and that the peak capacity is limited accordingly. Grushka[1] showed that the peak capacity, n, in SEC is given by

$$n = 1 + (\sqrt{N} / 4) \cdot \ln[1 + (V_p / V_0)] \quad (1)$$

The peak capacity is the number of Gaussian peaks that can be placed in the chromatogram, while maintaining a resolution of unity between them; N is the number of theoretical plates of the column. Eqn. 1 shows that, together with the column efficiency or plate number, the ratio of pore volume and interstitial volume will determine the separation power of the column as measured by the peak capacity.

Therefore, a higher pore volume and a lower interstitial volume would be desirable. For an ideal packing of spheres the volume fraction between particles can be calculated to be 26%, assuming the densest hexagonal arrangement. Slight variations in the particle size and shape, however, make it difficult to approach this theoretical limit closely. On the other hand, the pore volume cannot be increased very much without sacrificing the mechanical stability of the particles. The same is true also for packing materials based on organic polymers. The porosity ratio, V_p/V_o, of most commercial phases for SEC is between 1.0 and 1.3. This means that the two segments of the chromatogram, from the time of injection to elution of the first peak, and from there to the elution of the last peak, are about equal. If we use an exceptionally low value of 0.35 for the interstitial porosity ε_0 (ref. 2) and assume that as much as 80% of the particle volume is accessible as pore volume, we find an upper practical limit for the porosity ratio of $V_p/V_0 = 1.5$. The upper theoretical limit would correspond to totally porous particles with $V_p/V_0 = 0.65/0.35 = 1.9$. Therefore, in practice, there is not much hope of improvement beyond this value for packings of particles.

However, when the particles are replaced with parallel porous fibers, we can expect a significantly higher packing density. From analogous geometrical considerations we find that an ideal packing of identical cylindrical fibers would fill almost 91% of the column volume, leaving an interstitial porosity of only 9%. If it is possible to manufacture porous silica fibers with a specific pore volume (ml/g) similar to that of conventional silica particles, the time range available for the actual separation would be increased with respect to the total analysis time. In practice, packings of fibers have an external porosity between 0.15 and 0.18. Again using a porous fraction for the fibers of 80%, but with $\varepsilon_0 = 0.15$, we find a porosity ratio of $V_p/V_0 = 4.5$. If a 10% interstitial porosity could be achieved, the porosity ratio would reach a value of 7.2. Hence the use of aligned fiber columns offers a large potential improvement in SEC performance.

It has been shown that a bundle of (non-porous) fibers can be regarded as a system of parallel capillaries[3,4]. Between three neighboring fibers a flow channel is created, the cross-sectional area and flow resistance of which depend on the radii of the respective fibers. As all channels are fed with the same inlet pressure, slightly different radii will result in different flow velocities (the velocity being proportional to the square of the fiber radius). The signal of the detector is the sum of all the individual chromatograms. Hence fluctuations in the fiber radius will lead to an additional

contribution to the broadening of the chromatographic bands. Together with the dispersion in each of the capillaries, which has been shown by Golay[5] to be large for star-shaped cross-sectional channels, this will result in excessive band broadening. Therefore, it is essential to have access to a source of fibers with an extremely homogeneous distribution of their diameters. Further, careful packing of these fibers is critical. Buckling or misalignment of a few fibers results in the formation of channels with an average velocity considerably higher than the average channel velocity, which may account for a considerable fraction of the total flow-rate. Such packing errors result in important band broadening or even in multiple bands for a single compound.

When the fibers are porous, however, crossover of solute molecules between neighboring flow channels, by diffusion through a fiber, becomes a possibility. This process will contribute to the relaxation of radial concentration gradients and to a decrease in column dispersion. Local concentration differences will be averaged out in a similar way as in packings of particles. This effect was observed in the preparation of Aligned Fiber Columns (PPG Industries, Pittsburgh, PA, U.S.A.). The efficiency of columns containing porous fibers is higher than that of columns packed with non-porous fibers[6].

EXPERIMENTAL

The size-exclusion properties of an Aligned Fiber Column (AFC) were determined and compared with those of two conventional microparticulate silica materials. The characteristics of these columns were as follows:

PPG Industries AFC: 30 × 2.36 cm, 250 Å, 18 μm;
IMPAQ RG 2010 Si: 25 × 0.46 cm, 198 Å, 9 μm;
Vydac 101 TP B3: 5 × 0.46 cm, 300 Å, 3 μm.

From measurements described below, it was derived that the average cross-sectional areas available to the liquid flow in these columns are 2.4 cm^2 (AFC), 0.15 cm^2 (IMPAQ) and 0.12 cm^2 (Vydac).

The AFC was kindly supplied by PPG Industries. At present no columns of this kind are commercially available. Both the IMPAQ and the Vydac columns were packed in our laboratory from bulk material; IMPAQ, courtesy of PQ (Valley Forge, PA, U.S.A.) and Vydac, courtesy of Separations Group (Hesperia, CA, U.S.A.). The HETP values obtained for these two columns were between 60 and 70 μm for benzene in methylene chloride (1 ml/min), which is significantly higher than what could be expected from these materials if an optimized packing procedure had been used. The high-performance liquid chromatographic (HPLC) system used consisted of a Waters 510 pump (Millipore–Waters, Milford, MA, U.S.A.), a Rheodyne 7125 injection valve (Rheodyne, Cotati, CA, U.S.A.) and a Kratos Spectroflow 757 UV detector (ABI-Kratos, Ramsey, NJ, U.S.A.).

For the determination of the pore-size distribution of these packing materials, a set of thirteen polystyrene standards was used (4–5713; Supelco, Bellefonte, PA, U.S.A.), with molecular weights between 800 and 1 860 000 daltons, and benzene was used as a fully permeating substance. The individual molecular weights are given

as labels in Fig. 2 (in thousands). The polydispersities (M_w/M_n) of the polystyrene standards were given by the manufacturer as $\leqslant 1.06$. The samples were eluted with methylene chloride at various flow-rates (PPG, 10, 5, 2.5, 1 and 0.5 ml/min; IMPAQ, 1, 0.5, 0.1 and 0.05 ml/min; Vydac, 1 ml/min). The evaluation followed the method of Halász and Martin[7]. HETP values were determined by the graphical tangent method, equating the baseline width of the triangles to 4σ.

The *in situ* surface modification of the AFC was based on a standard method[8], which had to be modified because of the lower reactivity of the alkoxysilane compared with chlorosilanes. The silane used was glycidoxypropyltrimethoxysilane, purchased from Silar Labs. (Scotia, NY, U.S.A.).

RESULTS AND DISCUSSION

Porosities

The elution times of benzene and PS 1860 were used to calculate the total mobile phase volume (V_m), the interstitial volume (V_0), and finally their difference, the pore volume (V_p), all expressed as porosities (Table I).

TABLE I

POROSITIES OF AFC AND MICROPARTICULATE SILICAS

Material	ε_0	ε_m	ε_p	$\varepsilon_p/\varepsilon_0$	$\varepsilon_p/(1-\varepsilon_0)$
AFC	0.18	0.54	0.36	2.0	0.44
IMPAQ silica	0.42	0.89	0.47	1.1	0.81
VYDAC silica	0.41	0.71	0.30	0.7	0.51

The interstitial porosities of the two particulate silicas are in the usual range, slightly above 40%, and 1.5 times the theoretical value corresponding to an ideal packing of spheres. As expected, the volume between the fibers of the AFC is significantly lower. The ideal value of 9% is not reached, however, indicating some irregularity of the packing.

The pore volume of IMPAQ silica is at the upper limit for silica particles, whereas that of Vydac silica is low. The ratio between pore volume and interstitial volume of the AFC is twice that for typical silica packings. This can also be seen from the two chromatograms in Fig. 1 which are scaled to equal length to demonstrate the expanded separation period of the AFC. The porosity ratio of the AFC is still low compared with what could be expected (see Introduction).

The last column in Table I lists the porous fraction of each particle or fiber. Typically, values around 70% would be expected. The value for the IMPAQ material is above average, whereas the Vydac particles and the AFC fibers prove to be relatively compact. This result is not surprising for the AFC, in view of the procedure used to prepare the material.

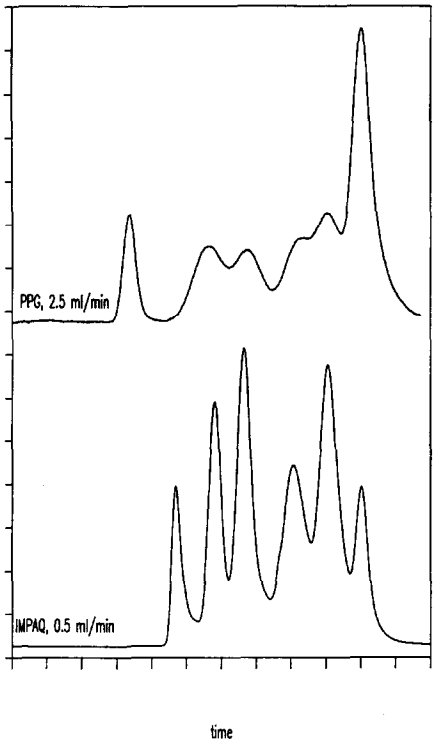

Fig. 1. Size-exclusion separation of a mixture of six polystyrene standards in methylene chloride on two columns (retention scaled to elution of last peak). Molecular weights were 1860, 47.5, 28.4, 9, 2 and 0.078 kilodaltons.

Pore size distributions

For molecular weight calibrations in SEC, the logarithms of the molecular weights are plotted against the elution times of the polystyrene standards (Fig. 2; molecular weights in thousands as labels). The elution times have been replaced by the size-exclusion distribution coefficient, $K_{sec} = (V_e - V_0)/V_p$ (where $K_{sec} = 0$ corresponds to total exclusion, $K_{sec} = 1$ to the benzene peak). For the AFC there is an approximately linear region of the calibration graph from a molecular weight of 100 000 to *ca.* 10 000 daltons, followed by a steeper region down to small molecules. This means that the best molecular weight discrimination (relative, on a logarithmic scale) is found with the molecular weights centered around 30 000 daltons, but also that in the range around 1000 daltons a rough estimate remains possible.

The calibration graphs of the two particulate silica columns, also given in Fig. 2, are more symmetrical and do not possess the sudden cut-off at the exclusion limit. Also, the molecular weight ranges are wider and therefore the selectivity between solutes in the center of the chromatogram will be lower.

For the characterization of size-exclusion packings, it is possible to convert the calibration graphs to pore size distributions following a complex procedure described by Knox and co-workers[9,10]. For our purposes the semi-empirical method of Halász

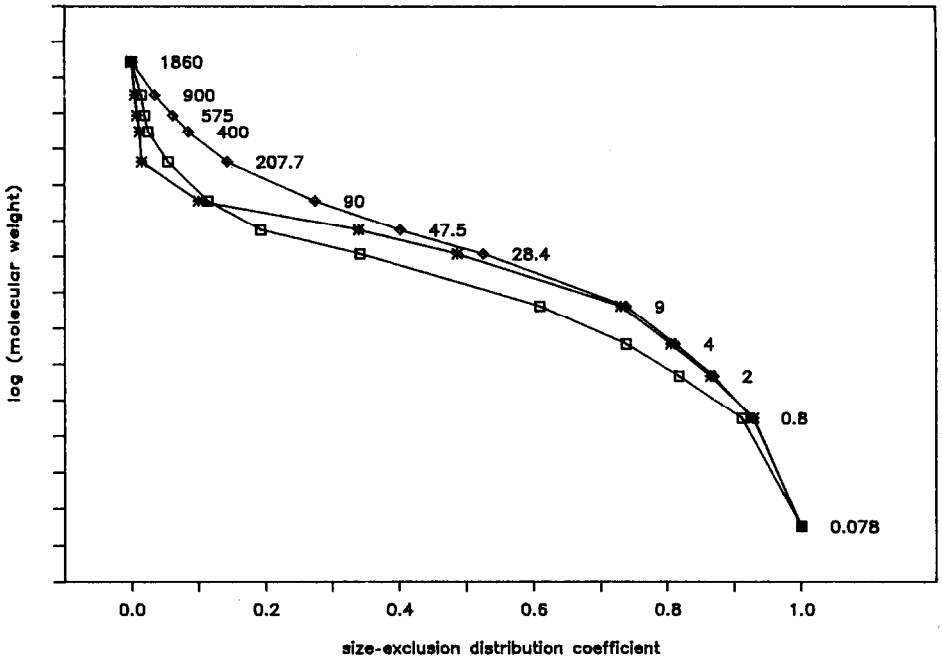

Fig. 2. Size-exclusion calibration graph for polystyrenes in methylene chloride on three different columns (labels: molecular weight in thousands). ◇ = Vydac; □ = IMPAQ; ✳ = PPG.

and Martin[8] was chosen. As it constitutes merely a linear transformation of the calibration graph, the resulting 'pore size distributions' are too broad and the mean pore sizes may not agree very well with those determined with other methods. However, this procedure is useful and practical for the comparison of the three columns.

From the retention times, the fraction of the pore volume that is accessible to molecules of a given molecular weight is determined. If the size of the molecules can be directly correlated with the diameter of the pores (which has been shown to be an oversimplification[9]), then we find the contribution of pores of a given size to the total pore volume. Often this experimental pore size distribution can be idealized as a log-normal curve, which is characterized by its mean value (at an accessibility of 50%) and its standard deviation.

Table II contains the mean Halász diameters and the widths of the pore size distributions (as standard deviations of the logarithms of the pore diameters).

The Halász diameters are smaller than the nominal values, which were determined by other methods, but vary in the same order. The standard deviations correspond to the range of molecular weights that can be separated on the columns. It is obvious that the AFC has a very narrow pore size distribution and therefore the separation between neighboring peaks in this range should be better than with the other two materials.

Other examples for conventional narrow pore size silicas have been given by Kirkland[11]. The slopes of the calibration graphs given for his PSM silica correspond to molecular weight ranges spanning a factor of 90–450. The corresponding values for

TABLE II

MEAN HALÁSZ DIAMETERS AND WIDTHS OF PORE SIZE DISTRIBUTIONS

Material	*Halász diameter (Å)*	σ
AFC (250 Å)	170	0.40
IMPAQ silica (200 Å)	150	0.48
Vydac silica (300 Å)	240	0.54

the IMPAQ and Vydac column are 90 and 120, respectively, whereas for the AFC a value of about 40 is found.

Efficiencies, peak capacities

Due to the low diffusion coefficients of polymer molecules, it is impractical to operate SEC columns packed with conventional SEC stationary phases at the optimum eluent velocity[12,13]. In addition, the band width of polymer peaks depends strongly on the flow-rate (Fig. 3). The HETP increases much more steeply with in-

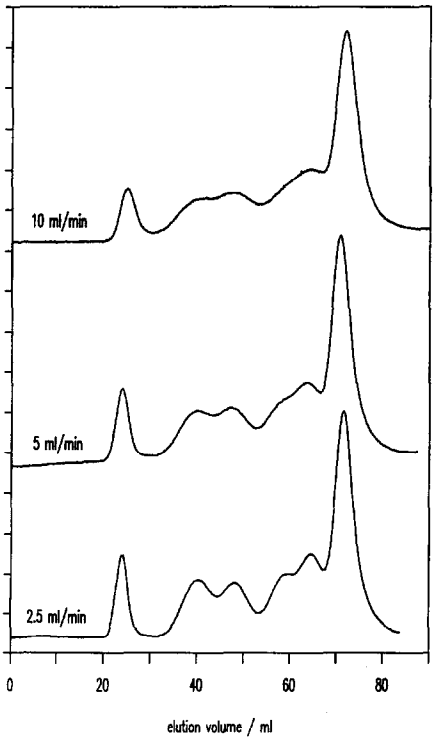

Fig. 3. Improvement in resolution by decreasing the flow-rate on the Aligned Fiber Column.

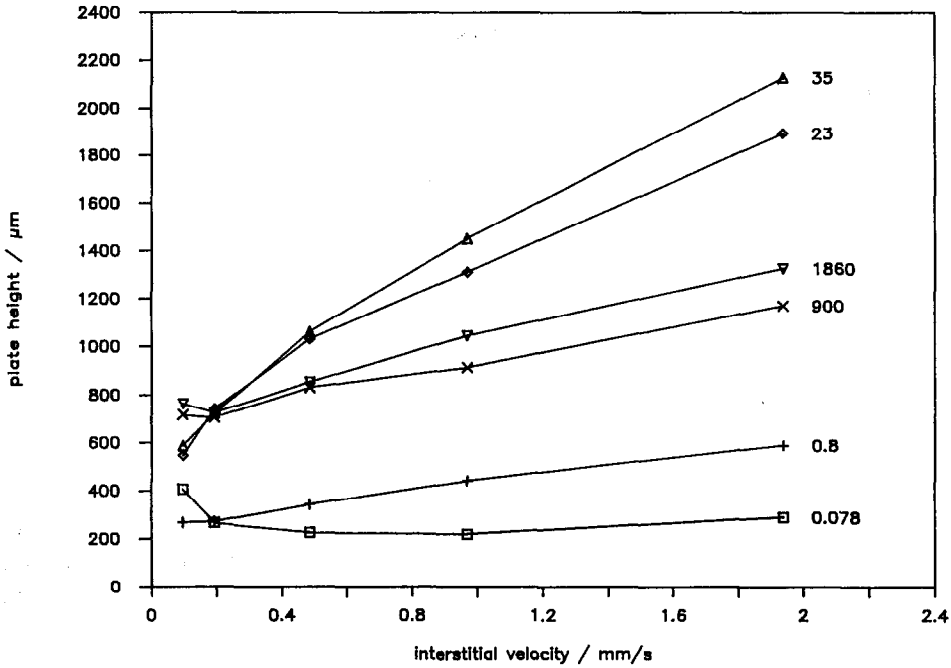

Fig. 4. HETP *vs.* interstitial velocity curves for some polystyrene standards (labels: molecular weight in thousands).

creasing flow-rate than it does for small molecules, *i.e.*, the *C*-term of the Van Deemter equation is larger for polymers. This can be seen in Fig. 4, which shows the velocity dependence of the HETP for a series of polystyrene standards. The optimum velocity is observed only for benzene (mol.wt. = 0.078 kilodaltons). For the other standards the flow-rate would have to be decreased below 0.5 ml/min and the analysis time increased beyond 3 h in order to observe the minimum value of the HETP. The lowest HETP value found for benzene is slightly above 200 µm, corresponding to about 1400 plates. This low efficiency, which is disappointing even for an 18-µm support column, indicates that the packing procedure for the fibers has not yet been optimized.

Fig. 5 shows a plot of the plate heights for all standards at all flow-rates, as a function of their position in the chromatogram, as given by K_{sec}. From benzene to polystyrene 90 000, the HETP increases owing to decreasing diffusion coefficients. Then the HETP drops suddenly, because the pores are no longer accessible and the higher molecular weight polymers experience only limited band broadening in the interstitial volume.

For the IMPAQ column, the plot of HETP *versus* the position of the band in the chromatogram is similar, the only difference being that the maximum plate height is shifted towards lower molecular weights. However, the values of the HETP measured are smaller by at least a factor of 3. For benzene, a minimum value of 70 µm is found.

The peak capacity can also be used as a measure of the column performance. It

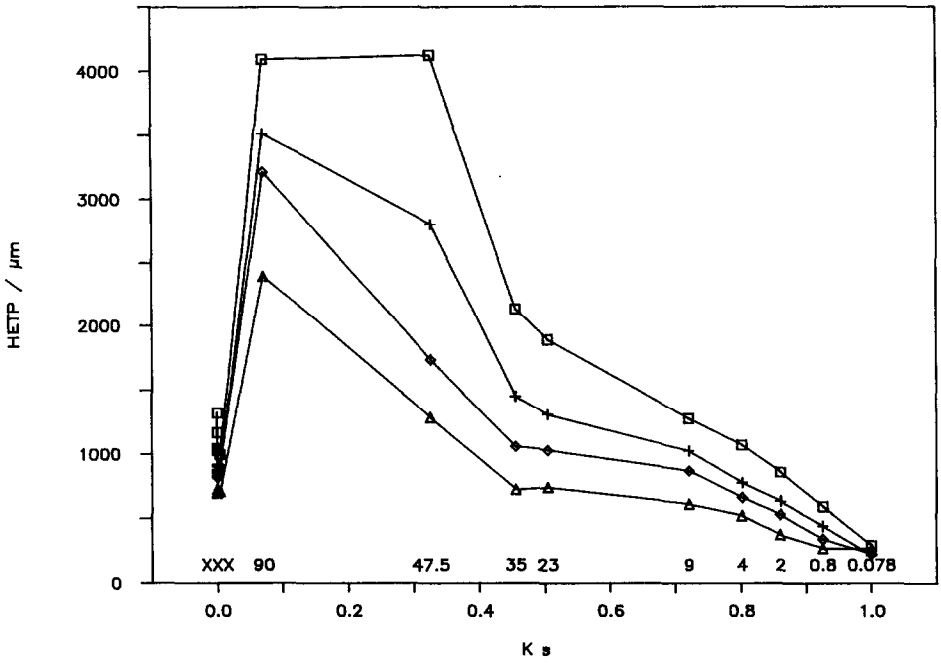

Fig. 5. HETP *vs.* K_{sec} of polystyrene standards af different flow-rates; □ = 10; + = 5; ◇ = 2.5; △ = 1 ml/min.

is the number of peaks that would fit into the chromatogram if all of them were separated by a resolution of 4σ. It can be calculated by dividing the duration of the chromatogram by the baseline width of one peak. To take into account the dependence of the peak width, w_b, on the retention, the peak capacity for the SEC columns studied was calculated from a summation over all standards (Δt = elution time interval between two successive peaks):

$$PC = \frac{(t_m - t_0)^2}{\Sigma(w_b \, \Delta t)} \tag{3}$$

The result is reported in Fig. 6 for both the AFC and the IMPAQ columns as a function of flow-rate. The peak capacity of the AFC is a steep function of the flow-rate. At extremely low velocities and with analysis times of several hours it approaches the values found with the IMPAQ column.

Hence it is possible in SEC to compensate for the poor mass transfer of the column by an exceptionally low flow-rate. More important, it is also possible to compensate for the poor efficiency by an exceptionally large porosity ratio. Higher efficiencies can be expected in the future as a result of continuing research and further optimization efforts[6].

DIOL bonded phase

If the Aligned Fiber Column material is to be used for the size-exclusion chromatography of proteins, the silica surface has to be modified by reaction with a silane

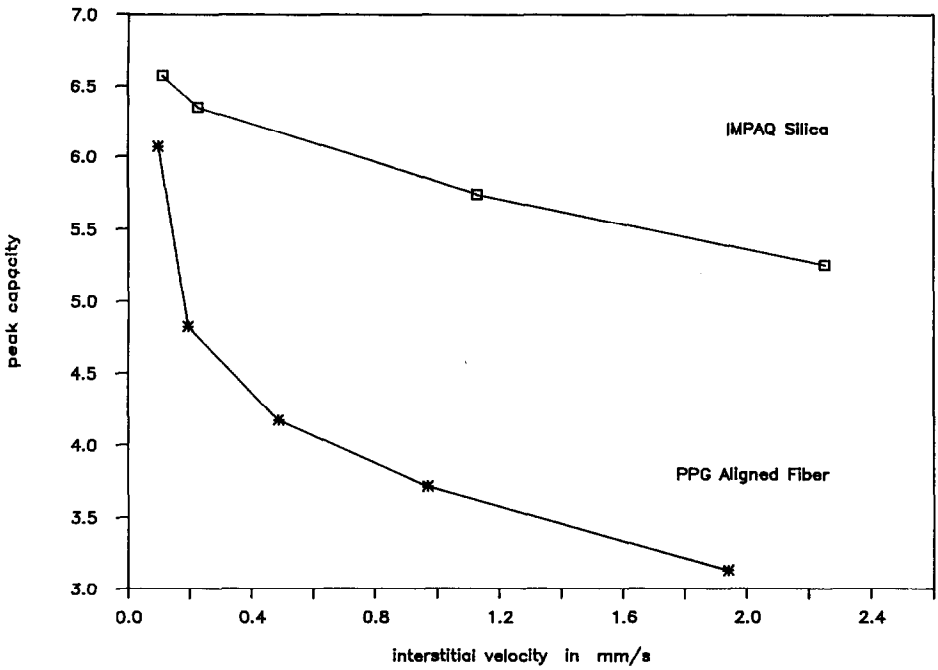

Fig. 6. Peak capacity for polystyrene standards *vs.* interstitial velocity.

carrying a hydrophilic group. The most widely used and most readily available of such silanes is glycidoxypropyltrimethoxysilane, which, after bonding, is hydrolyzed to give the so-called diol phase. With our one column we could not optimize the bonding procedure but had to accept the results produced in our first attempt.

For surface modification, an *in situ* bonding procedure was used[7]. Since this alkoxysilane is far less reactive than chlorosilanes, significant changes in the procedure were necessary. A 300-ml volume of a 10% solution of the diol silane in toluene was recirculated through the column for 500 min at room temperature, then the solution was left in the column overnight and washed out the next morning with methanol. After several hours of equilibration with a phosphate buffer, hydrolysis of the epoxy functional groups to the diol form was assumed to be complete. Initial tests showed that proteins could be eluted within the exclusion volume when phosphate buffer (0.2 mol/l, pH 6.8) was used as the mobile phase.

As preliminary experiments had suggested that some polymerization of the silane had occurred, we tried to use an additional heat treatment to rearrange the polysiloxane molecules adsorbed on the surface and form a more homogeneous layer. The column was dried by passing a stream of nitrogen through it, then the inlet and outlet were sealed with stainless-steel plugs and the column was 'baked' overnight in an oven at 200°C. After washing out all the material that had become dislodged, the pore size distribution of the 'baked diol' column was determined (see below). Repeating the baking procedure a second time did not produce any further significant change.

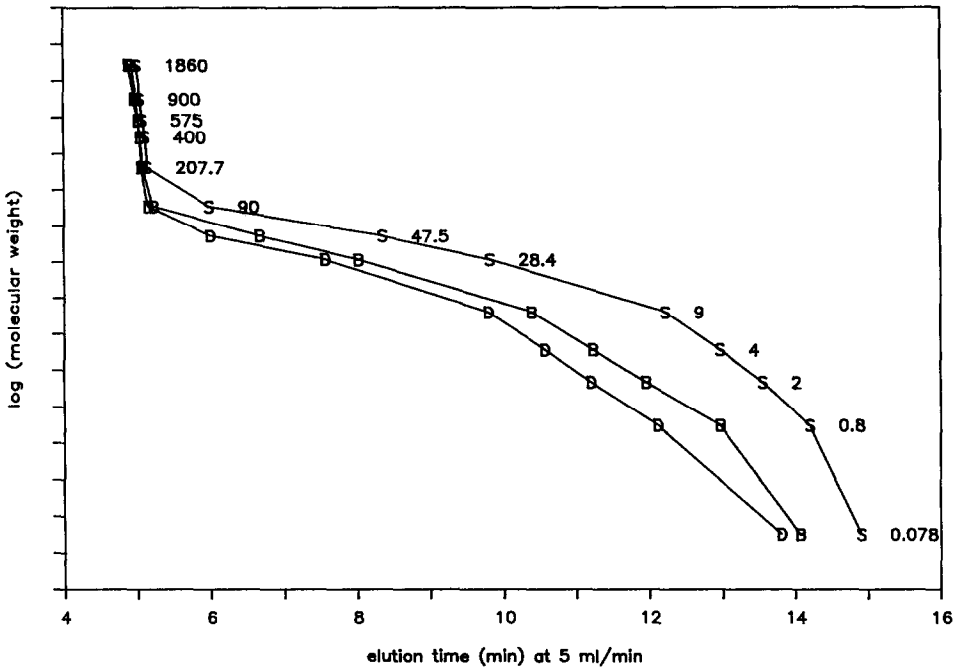

Fig. 7. Size-exclusion calibration graphs for the Aligned Fiber Column before and after silanization to the diol phase. S = Silica; B = baked diol; D = diol.

Both the freshly synthesized diol and the baked diol columns were characterized with polystyrene standards in methylene chloride at 5 ml/min (Fig. 7). The results are summarized in Table III.

After bonding, the pore volume was found to have decreased by more than 10%, whereas the interstitial porosity had hardly changed. This means that most of the silane was bound in the pores and did not affect the channels between fibers. As expected, the pore diameters also decreased, lowering the exclusion limit to a molecular weight below 90 000 daltons and the average Halász diameter to 106 Å (127 Å after heat treatment).

After silanization, the efficiency of the column had deteriorated markedly, with the formation of double peaks for small molecules such as benzene (Fig. 8). This

TABLE III

POROSITIES OF ORIGINAL AND TREATED AFC

AFC	ε_m	ε_0	ε_p	$\varepsilon_p/\varepsilon_0$
Silica	0.54	0.18	0.36	2.0
Diol	0.50	0.18	0.32	1.8
Baked diol	0.52	0.18	0.34	1.9

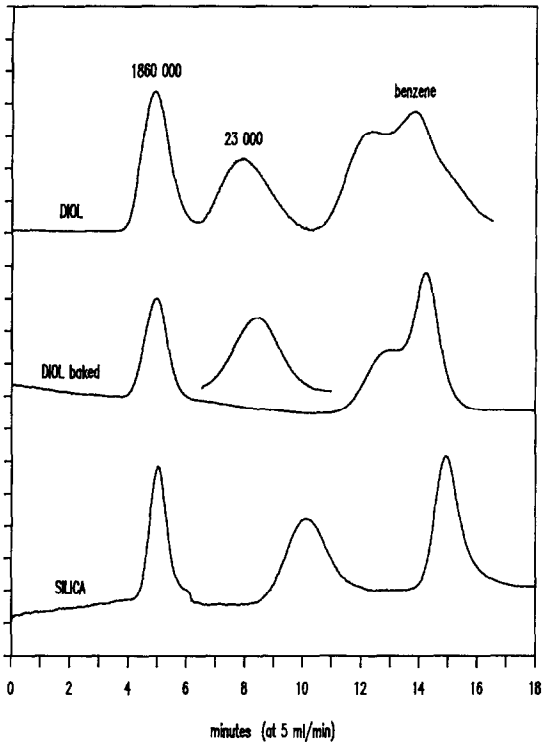

Fig. 8. Comparison of peak positions and band broadening on the diol columns.

indicates that changes in the pore structure took place that hinder diffusion. During the heat treatment, about half on the initially bound silane was removed, as indicated by the change in pore volume. At the same time the efficiency also increased. For the higher molecular weight compounds, the HETP values approached those found before silanization. Small molecules, however, were still eluted as double peaks.

The following figures show the chromatographic behavior of proteins on the diol column. A phosphate buffer at pH 6.8 with a phosphate concentration of 0.2 mol/l was chosen as the mobile phase. The ionic strength is needed to suppress ion-exchange interactions of the basic proteins with the residual silanols found even on a well deactivated modified silica surface.

The size calibration graph (Fig. 9) begins at a molecular weight of about 170 000 daltons and ends with the dead time ($K_{sec} = 1$) at 13.8 min (5 ml/min). As proteins are more tightly coiled than linear polymers, it is expected that the same retention time would be observed for proteins of higher molecular weight than the polystyrene samples. Unlike the random coils of polystyrene standards, protein molecules in their native state differ in structure, shape and compactness. For that reason, a perfectly smooth calibration graph is not to be expected under these conditions. The three proteins eluting close to or after the void volume are strongly basic ($pI > 9$) and hence are slightly retained by cation exchange.

In Fig. 10 some of the chromatograms obtained are reproduced. As the viscos-

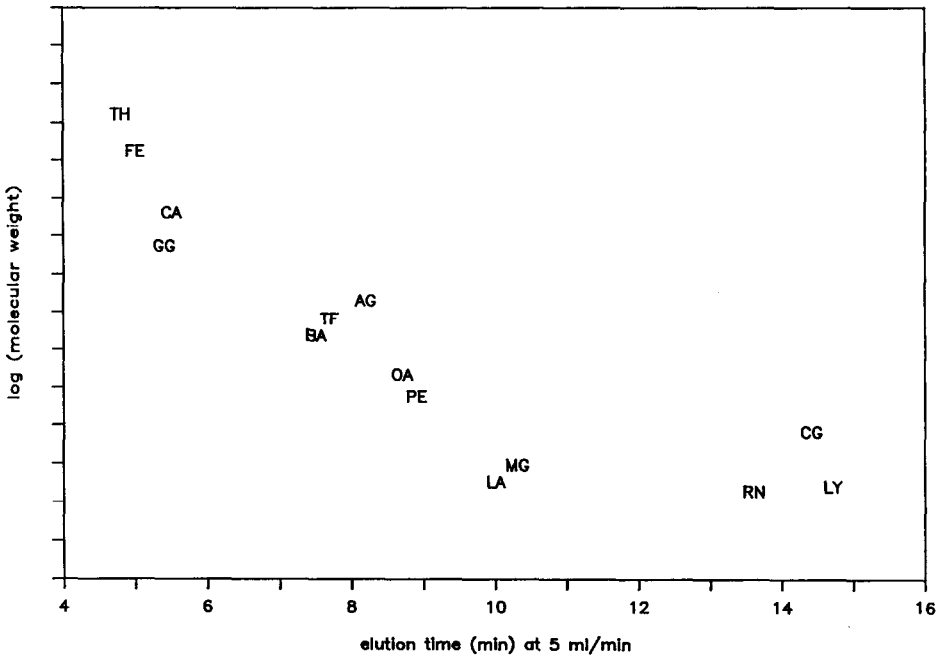

Fig. 9. Size-exclusion calibration graph for proteins on the diol Aligned Fiber Column. Mobile phase: phosphate buffer, pH 6.8, 0.2 mol/l. Proteins and approximate molecular weights (in thousands): TH = thyroglobulin (porcine), 660; FE = Ferritin (equine), 450; CA = Catalase (bovine), 240; GG = γ-globulins (bovine), 170; AG = Amyloglucosidase (*Aspergillus niger*), 97; TF = transferrin (bovine), 80; BA = serum albumin (bovine), 67; OA = ovalbumin (chicken), 45; PE = Pepsin (porcine), 36; CG = chymotrypsinogen A (bovine), 25; MG = myoglobin (equine), 17.8; LA = lactalbumin (bovine), 14.4; LY = lysozyme (chicken), 14.4; RN = ribonuclease A (bovine), 13.7.

ity of aqueous buffers is much higher than that of methylene chloride, the diffusion coefficients of proteins are correspondingly lower, and the bands are extremely broad and do not offer much potential for an actual separation. In addition, the late-eluting proteins show double peaks. A decrease in flow-rate from 5 to 0.1 ml/min results in a significant sharpening of the bands, but the separation time also increases from 15 min to 12 h, becoming prohibitively long.

Hence the diol bonding with our unoptimized method was only a partial success. The coverage is sufficiently dense for the unretained elution of most proteins, but polymerization of the silane led to excessive band broadening. Together with a higher initial efficiency of the packing and a higher pore volume, a better silanization procedure could lead to stationary phases superior to conventional size-exclusion materials.

CONCLUSIONS

Good resolution between polymer peaks in size-exclusion chromatography is favored by three factors. The length of the actual chromatogram, and with it the peak capacity, increases with the pore volume of the stationary phase. A narrow pore size

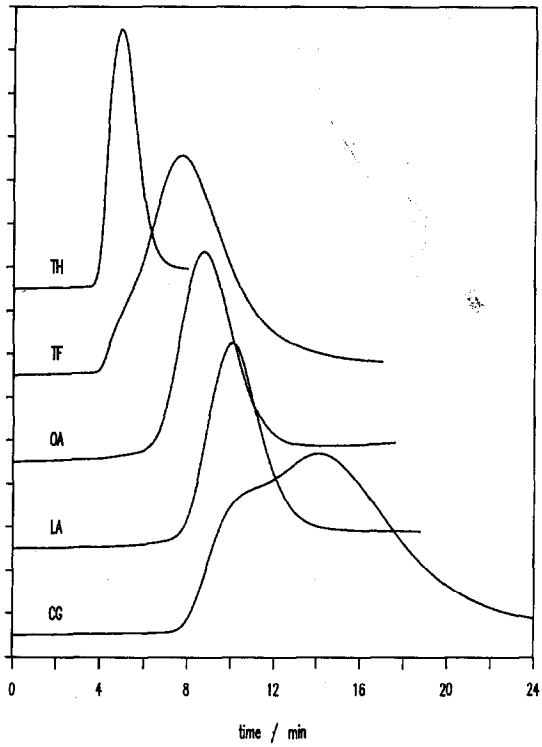

Fig. 10. Elution profiles of some proteins on the diol column. Conditions, protein abbreviations and molecular weights as in Fig. 9.

distribution causes a narrow molecular weight range to be spread over the whole length of the chromatogram. Band broadening must be kept as low as possible.

The porosity ratio of the Aligned Fiber Column excels over those of conventional silica packings. For the purification of monodisperse polymers such as proteins, the narrow pore size distribution should also be an advantage. If the material is to be used for the determination of molecular weight profiles of inhomogeneous synthetic polymers, however, a wider pore size distribution might be needed. Comparison with other silica packings indicates that the specific pore volume of this material could still be increased. According to the manufacturer, fibers with a higher porous fraction have been prepared recently, raising the ratio of pore volume to interstitial volume above 3 (ref. 6). Also, wider pore size distributions could be prepared on demand.

The HETP value for the Aligned Fiber Column tested, on the other hand, did not compare favorably with that of standard silica packings. An improved packing procedure and the future introduction of smaller fiber diameters, however, may lead to a useful alternative to conventional size-exclusion materials.

It was shown that the surface of the column can be effectively deactivated by *in situ* reaction with glycidoxypropylsilane. In the course of the reaction, however, some polymerization of the silane seems to have occurred, leading to excessive band broadening and the formation of double peaks. By partially removing the siloxane it was

possible to restore some of the previous efficiency. The structure of the packing did not seem to suffer from the silanization reaction. The deactivation procedure was not optimized. With a different modification procedure, better results can be expected.

ACKNOWLEDGEMENTS

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