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Polyvinyl alcohol-coated macroporous polystyrene particles as stationary phases for the chromatography of proteins

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Abstract

A method is described for the hydrophilization of macroporous poly(styrene–divinylbenzene) (PS–DVB) beads by adsorption of polyvinyl alcohol (PVA). PVA adsorbs strongly to PS–DVB surfaces but partially desorbs when exposed to protein solutions. To overcome this problem, the polymeric adsorbed layer was stabilized by crosslinking. We report the effect of polymer adsorption and crosslinking conditions on the amount of adsorbed PVA, on the stability of the polymeric layer and on the hydrophilization efficiency with regard to bovine serum albumin–a strongly hydrophobic protein. The properties of the coated supports were also evaluated by size-exclusion chromatography. PVA coating was shown to greatly reduce hydrophobic interactions. The pore size of modified PS–DVB particles was found to decrease significantly with increasing amounts of adsorbed PVA.

1. Introduction

The large scale production of human and animal proteins by genetic engineering or extraction from natural media, requires efficient separation techniques, e.g. high-performance liquid chromatography. However the development of this technique has been hampered by the lack of suitable packing materials, i.e. sufficiently hydrophilic, chemically and physically stable and with an inert surface to avoid protein denaturation due to strong adsorption. It is thus of significant interest to modify the surface properties of some materials to obtain ideal matrices.

Since the 1970s, most supports have been based on silica, an inorganic matrix which exists in a large range of pore and particle sizes. These supports are very useful but can only be used over a limited pH range [1] as they are readily degraded by alkaline aqueous solutions [2,3]. In addition, adsorption effects are frequently observed [4,5].

To overcome these problems, it has been proposed to use an organic matrix of polystyrene–divinylbenzene (PS–DVB) with high degree of crosslinking, which exhibits excellent mechanical properties and a good chemical stability over a wide pH range [6,7]. Such particles, based on copolymers of styrene and divinyl benzene, exist in a wide range of sizes, from 0.1 μm to more than 100 μm and with pore sizes ranging from 100 to 4000 Å [8] and are used for various applications. However, without modification, they cannot be applied to the chromatography of proteins–except in the reversed-phase mode–because of their hydrophobic properties [1,9,10].

To avoid this drawback and modify the surface

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properties of PS–DVB matrices, they can be coated in the same way as silica supports. Thus, different methods can be used, some of which have already been investigated: (i) adsorption of various charged hydrophilic polymers onto sulfonated polystyrene, leading to ion-exchange materials [11–13]; (ii) adsorption of a hydrophilic monomer on the surface, followed by a polymerization reaction [14]; (iii) adsorption of hydrophobic/hydrophilic polymers, e.g. ethyl(hydroxyethylcellulose) [15] or ethylene oxide–propylene oxide block copolymers [16].

The adsorption of polyvinyl alcohol (PVA) onto polystyrene surfaces is a widely studied phenomenon [17–19] and it is well known that PVA adsorbs strongly to this material but partially desorbs when exposed to protein solutions. Recently, it has also been shown that PVA can be adsorbed onto polytetrafluoroethylene-based matrices, providing a hydrophilic coating on the perfluorocarbon surface [20]. In the present work, macroporous PS–DVB beads have been coated with PVA under various conditions and some properties of the derivatized particles such as hydrophilicity, chemical stability and pore size modification have been investigated.

2. Experimental

2.1. Materials

The wide pore 35-μm diameter styrene-based resin Amberchrom CG162s was a gift from Rohm and Haas (Philadelphia, PA, USA) (average pore size 1000–1400 Å, surface area 200–300 m^2/g).

PVA ($Mr = 85\,000$–$146\,000$, 99 + % hydrolysed) was obtained from Aldrich (St. Quentin Fallavier, France). Bovine serum albumin (BSA) was purchased from Sigma (St. Quentin Fallavier, France). The gel-permeation calibration kit used for protein molecular mass determination (proteins listed in Table 1) was from Pharmacia (Uppsala, Sweden) and pullulan standards were obtained from Sopares (Gentilly, France). Other chemicals were from Aldrich.

2.2. Chromatographic system

The chromatographic system consisted of an high-performance liquid chromatography pump (Model 590, Waters, Milford, MA, USA), a Rheodyne Model 7125 injection valve (Cotati, CA, USA) equipped with a 20-μl loop, a variable UV-Vis detector (Model L-4200, Merck, Darmstadt, Germany) and a differential refractometer (Model R401, Waters).

2.3. Methods

Coating of PS–DVB beads

Before use, the beads were extensively washed with the organic solvents acetonitrile and methanol and with water. Then 5 ml of aqueous PVA solution, with or without ethanol, were added to 1 ml of beads (52 mg dry weight). The suspen-

Table 1
Proteins used for size exclusion calibration

Protein	M_r[a]	Stokes' radius (Å)[a]	Source[a]
Thyroglobulin	669 000	85	Bovine thyroid
Ferritin	440 000	61	Horse spleen
Catalase	232 000	52.2	Bovine liver
Aldolase	158 000	48.1	Rabbit muscle
Albumin	67 000	35.5	Bovine serum
Ovalbumin	43 000	30.5	Hen egg
Ribonuclease A	13 700	16.4	Bovine pancreas
Phosphotyrosine	261	–	–

[a] Manufacturer data.

sion was stirred at 20°C for 24 h, then centrifuged for 15 min and the supernatant was collected for determination of remaining PVA according to the KI–I_2 method using spectroscopy at 620 nm [21]. The PS–DVB beads were then washed with water and the adsorbed polymer molecules were chemically crosslinked under various conditions. Crosslinking was performed at 20°C with terephtaldehyde (TPA) in 0.1 *M* HCl (40 ml/g PS–DVB), or under alkaline conditions (1*M* NaOH, 80 ml/g PS–DVB) with epichlorhydrin (EpCl) or butanediol-diglycidyl ether (BDGE). Finally, the beads were washed with a 2% sodium dodecyl sulfate solution (SDS) at 40°C for 24 h. The amount of PVA adsorbed on the PS–DVB surface was determined as the difference between the initial amount of polymer added and the PVA remaining in all the supernatants and washing solutions.

Characterization of PS–DVB beads

The size distribution of PS–DVB beads was measured by laser-light scattering, using a Malvern instrument (upper size detection limit: 600 μm). SEM pictures of bead surfaces were obtained with a Jeol JSM T330A apparatus. The amount of PVA adsorbed on the beads was confirmed by FTIR spectroscopy (Bruker IFS 25 spectrophotometer) and the ratio PVA/PS–DVB (g/g) (C_{PVA}/C_{PS}) was calculated from:

$$R = A(\nu_1)/A(\nu_2) \quad (1)$$

where $\nu_1 = 1100\ cm^{-1}$ and $\nu_2 = 795\ cm^{-1}$ and C_{PS} and C_{PVA} are the concentrations (g/g) of PS–DVB and PVA in the KBr disc, respectively.

Since PVA does not absorb at 795 cm^{-1} (C–H out of plane deformation band of aromatics), the absorbance at 795 cm^{-1} is defined as :

$$A(\nu_2) = \epsilon_{PS}^{\nu_2} C_{PS} l \quad (2)$$

where l is the thickness of the KBr disc.

The absorbance at 1100 cm^{-1} is defined as:

$$A(\nu_1) = \epsilon_{PS}^{\nu_1} C_{PS} l + \epsilon_{PVA}^{\nu_1} C_{PVA} l \quad (3)$$

From Eqs. (1), (2) and (3), we obtain $R = K(C_{PVA}/C_{PS}) + K'$ with $K = \epsilon_{PVA}^{\nu_1}/\epsilon_{PS}^{\nu_2}$ and $K' = \epsilon_{PS}^{\nu_1}/\epsilon_{PS}^{\nu_2}$

Measurements were first carried out with samples containing well defined ratios (C_{PVA}/C_{PS}) and the K and K' factors were determined graphically.

BSA adsorption

Batch adsorption equilibrium studies

BSA adsorption experiments were carried out in phosphate buffer pH 7 (0.05 *M*). The adsorbed amounts of protein were determined from the supernatant absorbance at 280 nm.

Frontal analysis

Dynamic loading capacities of coated and uncoated beads were determined by frontal analysis, by pumping through the column (50 × 4.6 mm I.D.) a BSA solution (1 mg/ml) at a flow-rate of 0.3 ml/min until the absorbances (at 280 nm) of the output and input were identical.

BSA recovery

Columns (250 × 4.6 mm I.D.) were equilibrated in phosphate buffer (pH 7, 0.05 *M*) and 20-μl BSA samples (2 mg/ml) were injected. BSA recovery was calculated as the ratio between the peak area obtained with columns and that obtained without use of the column. When

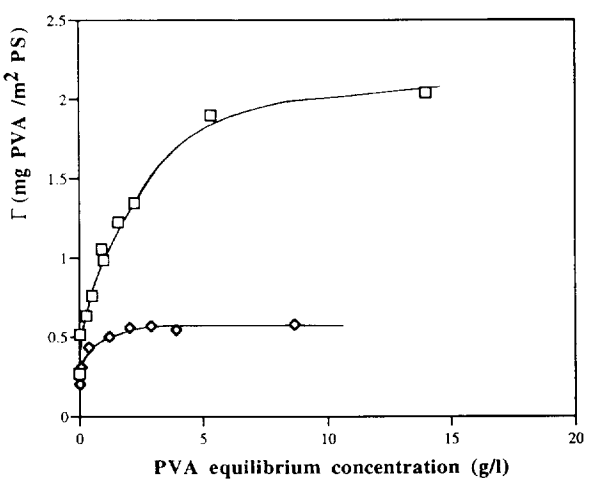

Fig. 1. Adsorption isotherms of PVA on PS–DVB beads in water (□) and in water–ethanol (90:10, v/v) (◇).

the column was disconnected, an empty guard column (50 × 2.1 mm I.D.) was inserted between the loop and the detector in order to obtain some peak spreading. Peak area was integrated with a Spectra-Physics integrator Model SP4290.

Column packing procedure

The coated polystyrene beads were packed by a slurry method, at constant solvent flow (packing velocity 360 cm/h), with a mixture of water and methanol (80:20, v/v).

3. Results and discussion

3.1. PVA adsorption on PS–DVB beads

Fig. 1 shows the adsorption isotherms of PVA in water and in water–ethanol (90:10, v/v). In the presence of 10% alcohol, the amount of PVA adsorbed decreases and the adsorption isotherm is typically of the Langmuir type. The plateau is achieved at a concentration of 2.5 g/l and a Γ value of 0.5 ($\pm$0.05) mg/m^2 was determined. As water is a better solvent for PVA than water–ethanol mixtures, one would expect, according to Hoeve [22], an increase of adsorption per unit area in the presence of alcohol. However, the amounts of adsorbed PVA were found to be higher in water than in water–ethanol. An explanation of this phenomenon is the possible multilayer adsorption of PVA on PS–DVB surfaces, which might be considered given the known aggregation behaviour of PVA in water [23].

3.2. Stabilization of the coated layer

Stabilization of the polymer layer on the beads is a necessary step because PVA partially desorbs when exposed to protein solutions. In order to determine the optimum stabilization conditions, we varied the amount and the nature of the crosslinking reagent (terephtaldehyde, epichlorhydrin or butanedioldiglycidyl ether) and we followed the amount of PVA released during that step. After the reaction, beads were washed with 2% SDS in order to desorb the lossely bound PVA and then with warm water. The amounts of PVA released during the crosslinking and washing steps are shown in Fig. 2. The results indicate that PVA desorption during the crosslinking step increases with the hydrophobicity and with the concentration of the crosslinking reagent, probably by means of competitive interactions with the PS–DVB surface. Thus it should be pointed out that not only the polymer adsorption conditions but also the crosslinking conditions determine the final amount of adsorbed PVA; considering only the adsorption data would result in an overestimation of the true amount of polymer adsorbed. From Fig. 2, it can be concluded that crosslinking with TPA ($7.1 \cdot 10^{-4}$ mol/g of adsorbed PVA) or with EpCl (0.35 mol/g of adsorbed PVA) is a good way to stabilize the PVA layer since only low amounts of the adsorbed polymer were released under these conditions.

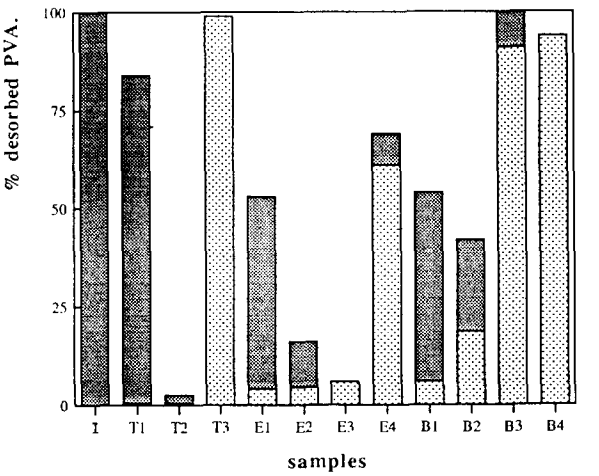

Fig. 2. Relative PVA desorption from coated PS–DVB beads (sample I, 0.5 mg PVA/m^2 PS) during crosslinking (dotted area) and SDS treatment (shaded area). T1, T2 and T3 were crosslinked with TPA (respectively $7 \cdot 10^{-5}$, $7 \cdot 10^{-4}$ and $7 \cdot 10^{-3}$ mol TPA/g adsorbed PVA). E1–E4 and B1–B4 were crosslinked with EpCl or BDGE, respectively ($7 \cdot 10^{-3}$, $7 \cdot 10^{-2}$, 0.35 and 1.4 mol reagent/g adsorbed PVA).

Finally, the stability of the coated layers was assessed under both acidic and basic conditions and no apparent leakage of PVA was observed after 24 h at ambient temperature either in 1 *M*

Table 2
PVA content of the various coated PS–DVB beads

Sample[a]	PVA (mg/m^2 PS) Supernatant[b]	FTIR[c]
A	0.49	0.67
B	0.45	0.46
C	0.77	0.79
D	1.12	1.25

[a] Sample A was coated with PVA in water–ethanol (90:10, v/v). The other samples were prepared in water.
[b] PVA determination according to the supernatant method.
[c] PVA determination by FTIR spectroscopy.

HCl or 1 *M* NaOH (detection limit 5 μg released PVA/m^2).

3.3. Characterization of PS–DVB beads

Four samples (A–D) of beads differing in coating percentage were prepared as described, using TPA as the crosslinking reagent. The PVA content of each sample (Table 2) was determined by the supernatant method and by FTIR spectroscopy. Slightly higher amounts of PVA were found by the second method. Fig. 3 shows the FTIR spectra of native and modified beads.

The size distribution of coated and uncoated samples was analysed by laser-light scattering and SEM. The size of the beads was not significantly modified after coating, except for the beads in sample D which contained the highest amount of PVA. In this latter case, aggregates were formed (15%, w/w), possibly due to the self-association properties of PVA; surprisingly, these aggregates were very large, with a size higher than 100 μm, as illustrated in Figs. 4 and 5. An explanation may be that at high PVA concentrations, i.e. exceeding those required to fully saturate the particle surface, the polymer produces a network entrapping the PS–DVB particles. Nevertheless, the nature and mechanism of formation of such aggregates require clarification.

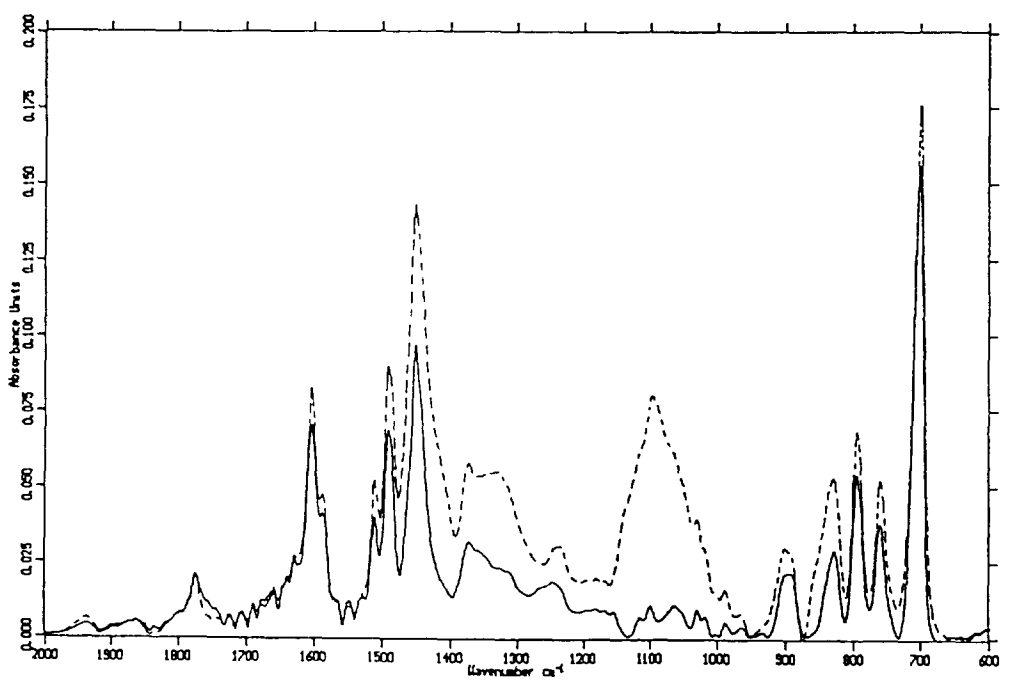

Fig. 3. FTIR spectra of PS (—) and PVA–PS beads (- - -).

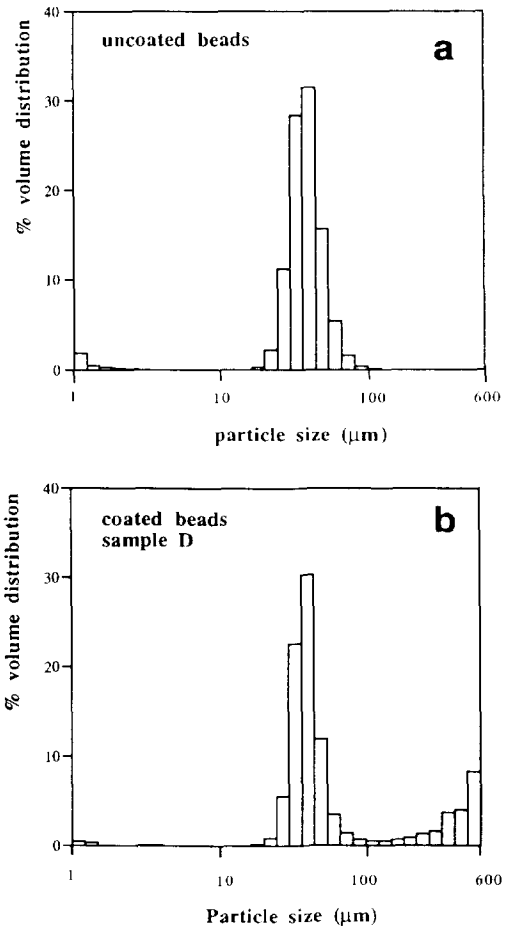

Fig. 4. Laser-light scattering determination of particle size distribution of (a) uncoated, and (b) coated beads.

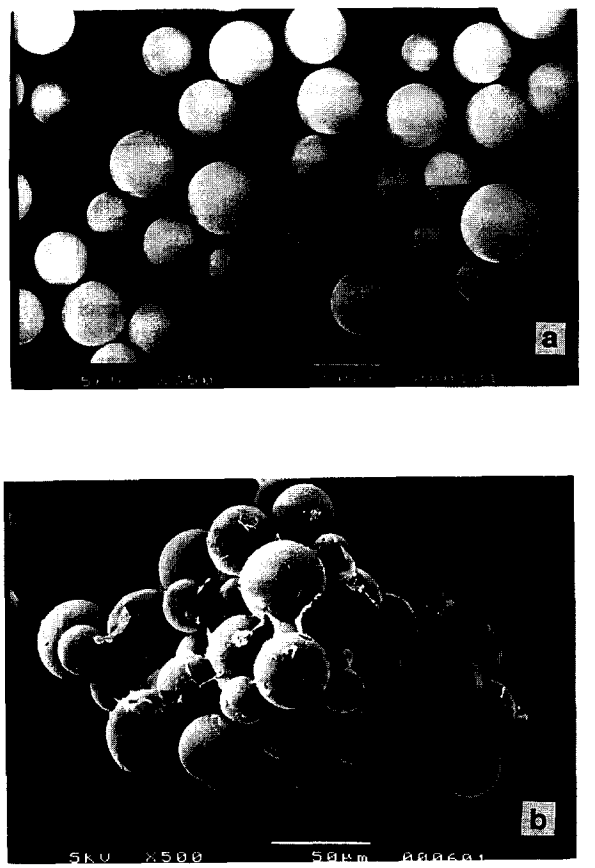

Fig. 5. SEM micrographs of native PS–DVB beads (a) and of aggregated particles in sample D (b).

In order to discard aggregates, sample D was filtered on a 100-μm mesh sieve before its chromatographic evaluation.

3.4. BSA adsorption studies

BSA was used as a test protein for study of the hydrophilization efficiency of PS–DVB surfaces. BSA adsorption isotherms were determined at pH 7, as shown in Fig. 6. Dynamic loading capacities for BSA of coated beads were also compared (Table 3). BSA adsorption was found to decrease significantly with increasing amounts of adsorbed PVA. Under batch conditions, the lowest residual BSA adsorption was obtained with support D which contained the highest amount of adsorbed PVA. Dynamic analysis data show that irreversible adsorption of BSA on supports containing more than 0.8 mg PVA/m^2 (i.e. supports C and D) was negligible. Furthermore, BSA recovery on supports C and D was investigated using the method described in Experimental. The results show a good recovery since 94% and 98% of BSA were eluted from the columns.

3.5. Chromatographic evaluation

Fig. 7 shows the calibration curves for a series of pullulan standards on native PS–DVB beads and on coated supports B and D. It should be noted that the exclusion volume (V_0) of uncoated

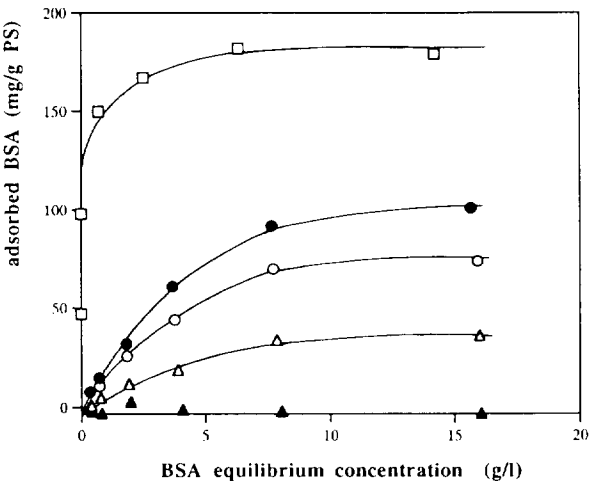

Fig. 6. Adsorption isotherms of BSA on PVA-coated PS (samples A = ●, B = ○, C = △, D = ▲) and on uncoated PS (□) determined in phosphate buffer pH 7, 0.05 *M*.

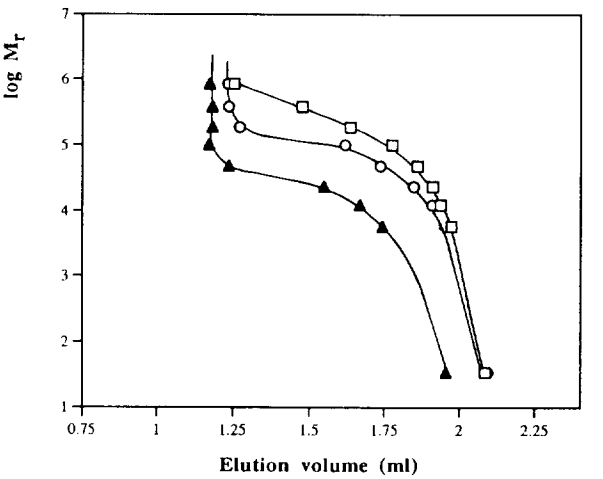

Fig. 7. SEC calibration graph for pullulans on 150 × 4.6 mm I.D. columns (PS = □, B = ○, D = ▲). Eluent: water–methanol (80:20, v/v) at 0.3 ml/min flow-rate; refractometric detection. The total volume of the columns was determined by injection of ethanol.

resin could not be reached since no pullulan standard has a large enough size. After coating, all the retention volumes (V_e) of the pullulans and the limit exclusion molecular mass were decreased. In SEC, the plot of elution volume versus molecular size can be related to the pore size distribution of the adsorbents. As can be seen from Fig. 7, the porous character of the PS–DVB beads was largely altered after PVA coating: the higher the amount of PVA adsorbed, the smaller the pore size. However, it should be pointed out that the porous volume of the columns ($V_p = V_t - V_0$) was not changed significantly. An explanation for this phenomenon may be that PVA molecules fill the large pores, totally or not, and that smaller pores are formed in a network of crosslinked PVA if the adsorbed layer is not dense enough.

Fig. 8 shows the calibration curve for proteins on coated resin D. The mean pore size was estimated as the size of the protein eluting at

Table 3
BSA adsorption data (frontal analysis)

Sample	Adsorbed BSA (mg/ml support)
Uncoated PS	30 (27[a])
A	2.8
B	3.6
C	0.1
D	–

[a] Manufacturer data.
Conditions as described in Experimental.

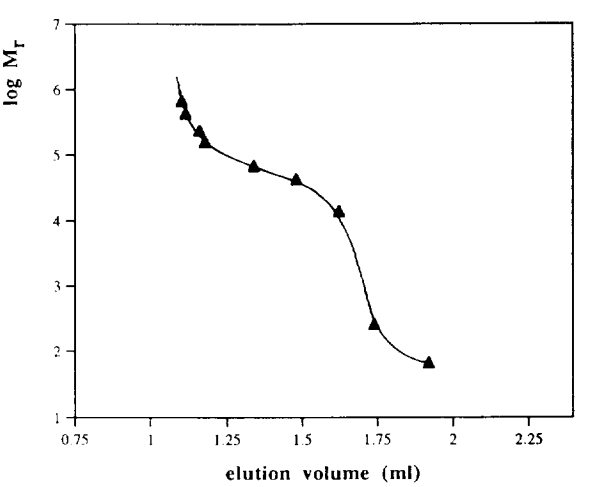

Fig. 8. SEC calibration graph for proteins on coated PS–DVB resin D. Column: 150 × 4.6 mm I.D.; eluent: phosphate buffer pH 7, 0.05 *M*; flow-rate 0.3 ml/min; UV detection at 280 nm. The total volume of the column was determined by injection of NaN_3.

$V_e = V_0 + 0.5V_p$. A value of 60 Å was obtained, which is very small compared to that of the starting material.

4. Conclusions

These preliminary results show that hydrophilization of macroporous PS–DVB beads is possible by adsorbing PVA and by chemically crosslinking the coated film. The porous character of the modified matrices is largely changed but it should be possible to improve the pore size and pore size distribution through optimization of the adsorbed layer thickness and density. Such a procedure could provide hydrophilic HPLC supports not only for gel permeation but also for ion-exchange or affinity chromatography since the PVA layer may be easily derivatized by classical methods.

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