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Modification of polystyrenic matrices for the purification of proteins Effect of the adsorption of poly(vinyl alcohol) on the characteristics of poly(styrene–divinylbenzene) beads for use in affinity chromatography

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Abstract

A poly(styrene–divinylbenzene) (PSDVB) chromatography matrix, CG1000-sd (TosoHaas), has been modified using poly(vinyl alcohol) (PVA) to create a matrix suitable for the attachment of functional groups for the selective purification of proteins. The characteristics of the modified matrix have been studied using a BET nitrogen adsorption/desorption technique and it has been found that the adsorption of PVA results in the bead micropores being filled whilst the bead macropores are left essentially unaltered. There was no protein adsorption onto the modified matrices. A dye ligand (Procion Blue MX-R) has been covalently attached to the PVA–PSDVB matrix and the lysozyme capacities of the PVA–PSDVB matrix have been determined. The matrix compares well with commercial Blue Sepharose Fast Flow, an affinity matrix based on cross-linked agarose. The dye–PVA–PSDVB matrix is stable when subjected to sanitisation with sodium hydroxide.

Keywords: Stationary phases, LC: Poly(styrene–divinylbenzene) matrix; Affinity adsorbents; Proteins; Poly(vinyl alcohol)

1. Introduction

Affinity chromatography has long been established as a high resolution technique for the purification of proteins. Its use at preparative scale has however been restricted by problems associated with the support matrix. The most popular matrix to date, agarose, is limited by its lack of mechanical rigidity, even in heavily cross-linked varieties, thus restricting its application in large-scale operations. It is imperative that high-performance liquid chromatography matrices should be mechanically strong, as lightly cross-linked semi-rigid polymer networks are easily compressed and can only be operated under low–medium pressure.

Poly(styrene-divinylbenzene) (PSDVB) beads have been used for separations since 1964 when Moore [1] synthesised porous cross-linked polystyrene. Although these rigid PSDVB matrices can be operated under high pressures without collapsing, they cannot be used directly to purify proteins, as the material is difficult to derivatise with affinity ligands and hydrophobic interactions between the matrix and protein often result in irreversible adsorption or loss of yield.

The recent trend towards modification of PSDVB with hydrophilic groups to mask its hydrophobic surface aimed at improving its use in protein chro-

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matography, has produced both covalently bonded carboxyl groups [2] and adsorbed hydrophilic groups [3–5]. Modification of PSDVB by the adsorption of hydrophilic polymers has, however, proved most popular, polyethyleneimine, for example, has been used as an adsorbent coating to create an ion-exchange resin [6].

Extensive research into the adsorption of polyoxyethylene onto PSDVB [7–9] has shown that polyoxyethylene groups, being amphiphilic, will bind to PSDVB in such a way that the hydrophobic regions will bind to the PSDVB whilst the hydrophilic regions are only weakly adsorbed and will loop outwards from the PSDVB surface. Cross-linking increases both the chemical and the mechanical strength of the adsorbed layer. Such coatings are relatively complicated to prepare and have limited applications.

The coating of surfaces with poly(vinyl alcohol) (PVA) is a relatively recent modification procedure. We have previously shown that PVA can be adsorbed by and used to shield perfluorocarbon surfaces [10]. PSDVB matrices have also been coated with PVA to shield their surfaces from hydrophobic interactions [11,12].

This paper investigates the adsorption of PVA onto PSDVB matrices as a method for producing an affinity chromatography matrix. Experiments have been carried out to determine the effect of PVA adsorption on the physical and chemical characteristics of the PSDVB matrices. The effect of PVA molecular mass, degree of hydrolysis and concentration have been investigated using HSA and lysozyme as model proteins to determine the effectiveness of the PVA layer in reducing non-specific adsorption. The adsorption capacity of various PVA coated PSDVB adsorbents have been determined using Procion Blue MX-R as an affinity ligand for lysozyme. The effect of repeated cleaning with 1 *M* NaOH on the dynamic capacity of a fouled bed is also determined.

2. Experimental

2.1. Materials

Poly(vinyl alcohol) (PVA) (M_r 13–23 000, 87–89% hydrolysed, M_r 13–23 000, 97–99% hydrolysed, M_r 31–50 000, 87–89% hydrolysed, M_r 85–146 000, 87–89% hydrolysed, M_r 124–186 000, 87–89% hydrolysed) were purchased from Aldrich Chemical (Gillingham, Kent, UK), as were sodium hydroxide, iodine, potassium iodide, methanol, disodium hydrogen orthophosphate and sodium hydrogen phosphate. Boric acid (99.5% purity), glutaraldehyde and HCl were purchased from Sigma Chemical (Poole, Dorset, UK) and Blue Dextran 2000 was purchased from Pharmacia Biotech (St. Albans, UK).

Chicken egg white lysozyme (M_r 14 000) and human serum albumin (HSA) (M_r 66 000) were obtained from Sigma Chemical (Poole, Dorset, UK).

CG1000-sd beads were kindly donated by TosoHaas (Philadelphia, USA). These beads are composed of polystyrene–divinyl benzene and were characterised as having a particle size range 20–50 μm, a bead surface area of 250 m^2/g and an average pore size of 1000 Å.

2.2. Instrumentation

UV–Vis spectrophotometry was carried out using a Shimadzu UV-160A (VA Howe, Oxon, UK). Chromatography was carried out on a Pharmacia FPLC system (Fast Protein Liquid Chromatography) categorised as 2× P-6000 pumps, UV-1, Frac-100, Rec-482 obtained from Pharmacia LKB (Uppsala, Sweden). The column was an HR5/5 with an internal diameter of 0.5 cm. BET analysis was carried out using a micromeritics ASAP 2000 (Norcross, GA, USA).

2.3. Coating of PSDVB beads with PVA

Before coating, the PSDVB beads were washed extensively. This involved washing approximately 10 g of dry CG1000–sd beads in a number 2 sintered funnel and rinsing with 500-ml quantities of acetonitrile, methanol and water. The beads were then dried under vacuum and stored in a dry environment. Prior to coating, the bead samples (0.25 g) were subsequently "wetted" by adding methanol (2 ml) to the dry PSDVB beads in a 7-ml test tube. The tubes were then sealed and shaken to ensure the methanol filled all the beads' pores. The methanol and PSDVB

beads were then washed with water (100 ml) in a number 2 sintered funnel to displace excess methanol from the bead interior. After washing, the PSDVB beads were placed into suitable vessels and PVA solution was added at initial concentrations of 0.1–200 mg/ml and the tubes sealed and incubated on a rotary mixer (Mixer 820, Swelab instrument, Sweden) at room temperature for 1 week. To determine the PVA adsorption kinetics, samples of supernatant were removed on a regular basis and after centrifugation assayed immediately to establish the concentration of PVA.

The PVA solution concentration was measured using the iodine–iodide method described by Garvey [13]. It has been noticed that the coloured end-product of the assay is unstable and therefore the following assay procedure was strictly observed. The following additions were made to a 1-ml polystyrene cuvette in order: boric acid (0.6 *M*, 1 ml), iodine–iodide solution (9 m*M* I_2/41 m*M* KI, 50 ml) and PVA solution (0.1–0.5 mg/ml, 100 ml). The cuvette was inverted twice to mix the contents and then incubated for 1 min at room temperature after which the optical density (OD) at 690 nm was determined. The OD was used to determine PVA concentration by comparing values with a calibration curve. The procedure was followed consistently with a margin of error of less than 10%. The quantity of PVA adsorbed onto the PSDVB beads was calculated from a mass balance on the supernatant concentration and volume.

2.4. Cross-linking of the adsorbed PVA layer

Cross-linking was carried out at room temperature using glutaraldehyde with a ratio of 5.6×10^{-4} mol/g PVA adsorbed (a molar ratio of 10:1 mol/mol for glutaraldehyde to M_r 13–23 000 PVA). The coated PSDVB beads were washed thoroughly with water and returned to a 7-ml test tube. Glutaraldehyde (10 mg/ml) and water were then added to ensure a total liquid volume of 4.9 ml. The beads were left for 30 min to allow the diffusion of glutaraldehyde to the bead interior, then HCl (5 *M*, 0.1 ml) was added to catalyse the cross-linking reaction. The sealed tubes were left for 16 h on a rotary mixer at room temperature.

2.5. Determination of non-specific protein adsorption on PVA–PSDVB matrices

Non-specific protein adsorption was carried out using lysozyme and HSA. The PVA modified and cross-linked PSDVB samples were placed into 20-ml centrifuge tubes and protein solution (lysozyme, 1 mg/ml, 10 ml or 20 ml in 50 m*M* phosphate buffer pH 5.0; HSA, 1 mg/ml, 10 ml or 20 ml in 25 m*M* phosphate buffer pH 5.0) was added. The samples were left for 16 h on a rotating mixer at room temperature, after which the protein concentration in solution was determined by measuring the OD in a quartz cuvette at 280 nm. The OD value was used to calculate protein concentration by employing extinction coefficients of 2.64 ml mg^{-1} cm^{-1} and 0.53 ml mg^{-1} cm^{-1} for lysozyme and HSA respectively [14]. The quantity of protein bound non-specifically was calculated from a mass balance on the supernatant.

2.6. BET analysis of modified matrices

BET analysis was carried out on a Micromeritics ASAP 2000 analysis machine. The modified cross-linked and washed samples (i.e. those that had no contact with protein) were left to dry in crucibles at 60°C for 1 week. A measured sample was then placed into a BET analysis tube and heated to 120°C under a high vacuum for at least 12 h. The sample was reweighed and then loaded onto the automatic BET machine and the nitrogen adsorption/desorption isotherms measured. An equilibrium interval of 5 s was used in determining the isotherm values.

2.7. Dyeing of modified matrices

PVA–PSDVB samples (1 ml) were washed thoroughly with water and placed into 7-ml test tubes to which was added Procion Blue MX-R (200 mg) in water (5 ml). After mixing, NaCl (22% w/v, 1 ml) and sodium carbonate (50 mg) were added. The samples were left on a rotary mixer (Mixer 820, Swelab instrument, Sweden) at 60°C for 16 h. After dyeing, the beads were thoroughly washed with both water and methanol. The quantity of dye that had bound to the PSDVB was determined from the supernatant absorbance at 620 nm, using an extinction coefficient of 10 500 l mol^{-1} [15].

2.8. Determination of non-specific protein adsorption on dye–PVA–PSDVB matrices

Non-specific protein adsorption onto dye–PVA–PSDVB matrices was tested using frontal analysis techniques as described below. HSA and lysozyme solutions (1 mg/ml) were adsorbed in the presence of the appropriate eluent to ensure no protein binding to the dye ligand (HSA adsorbed in 0.5 *M* NaSCN in 50 m*M* phosphate buffer, pH 8; lysozyme adsorbed in 1 *M* NaCl in 50 m*M* phosphate buffer pH 7.5).

2.9. Frontal analysis

Dynamic loading capacities of dyed PVA–PSDVB matrices were determined using frontal analysis, which entailed pumping a lysozyme solution (1 mg/ml, 50 m*M* phosphate buffer pH 7.5) through the column (bed height 50 mm, internal diameter 5 mm) at a flow-rate of 1 ml/min until the absorbance (at 280 nm) of the output and input streams were identical. The column was then washed (20 ml) with running buffer and eluted using 1 *M* NaCl in 50 m*M* phosphate buffer pH 7.5. When the absorbance of the outlet returned to the base line, the column was washed once again with the adsorption buffer. Dynamic capacities were calculated at 5% breakthrough (the outlet absorbance at 280 nm being 5% that of the inlet). The void volume of the bed was determined using Blue Dextran 2000.

2.10. Equilibrium capacities

Washed and dyed PVA–PSDVB samples were placed into 20-ml centrifuge tubes and lysozyme solution (4.5 mg/ml) in 50 m*M* phosphate, pH 7.5 was added. The equilibrium capacity was calculated from the difference in supernatant absorbances at 280 nm after 16 h adsorption.

2.11. Chemical stability of modified matrix

A Procion Blue MX–R PVA–PSDVB sample was incubated at room temperature in a lysozyme solution for a period of 1 month. Frontal analysis experiments were undertaken both initially and after every 3 NaOH washes (1 *M*, 20 column volumes). The dynamic capacities were measured using the technique described above.

3. Results and discussions

3.1. PVA adsorption onto CG1000-sd beads

Fig. 1 shows the kinetics of PVA (M_r 13–23 000, 87–89% hydrolysed) adsorption onto PS–DVB CG1000-sd beads. It can be seen that PVA adsorption is complete in the period up to 1 h. The higher the stock solution concentration, the sooner equilibrium is achieved and this is due to the greater concentration gradients between the bulk and pore interior solutions. The maximum amount of PVA adsorbed increases from 200 mg/g to 250 mg/g with initial liquid phase PVA concentration over the range 0.5 to 10 mg/ml.

The PVA–PSDVB adsorption relationship is effectively irreversible due to multipoint attachment of the PVA on the PSDVB surface [16]. The amount of polymer adsorbed is dependent on the solution concentration; the higher the concentration of poly-

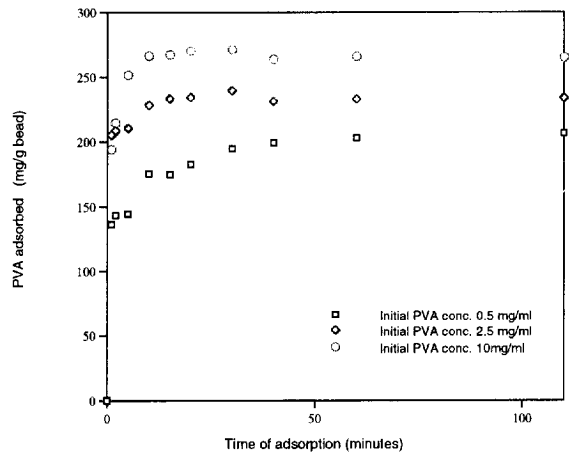

Fig. 1. Adsorption of PVA (M_r 13–23 000, 87–89% hydrolysed) onto PSDVB beads with respect to time. Dried bead samples (0.25 g) were "wetted" with methanol and then washed with water. PVA solution (0.5 mg/ml, 120 ml; 2.5 mg/ml, 30 ml and 10 mg/ml, 10 ml) was added and the tubes sealed and left on a rotary mixer. Samples were taken as indicated and assayed after centrifugation using the iodine–iodide method as described in the Experimental (Section 2). The quantity of PVA adsorbed onto the PSDVB beads was calculated using a mass balance.

mer in solution, the greater the film thickness becomes. It is thought that the PVA adsorbs as a monolayer on PSDVB surfaces; the higher the concentration of PVA in solution the more the PVA will loop outwards from the PS–DVB surface, thus increasing the film thickness [17].

The maximum amounts of PVA adsorbed onto the PSDVB beads for a number of types of PVA are shown in Fig. 2. It can be seen that 97–99% hydrolysed PVA adsorbs to a much lesser degree than 87–89% hydrolysed PVA. PVA adsorbs to the PSDVB surface as a result of hydrophobic forces and there are hydrophobic regions on all PVA molecules. The acetate groups present on the PVA with lower degree of hydrolysis ensure that these PVA molecules are substantially more hydrophobic than PVA molecules with high degrees of hydrolysis. The amount of PVA adsorbed onto the PSDVB beads remains fairly constant with changes in molecular mass (PVA, 87–89% hydrolysed), at around 250 mg/g. It is difficult to predict the degree of adsorption of polymers onto porous materials, as the surface area available for adsorption to occur is dependent on polymer size (the larger the polymer, the less accessible the bead interior), but in general the adsorption of polymers onto non-porous surfaces is dependent on the molecular mass of the polymer; the higher the molecular mass, the thicker the adsorbed layer and so the greater the amount of polymer adsorbed [16].

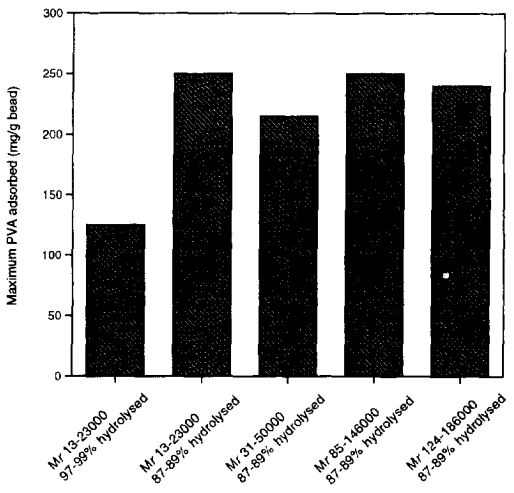

Fig. 2. Maximum PVA adsorption values of PVA (M_r 13–23 000, 97–99% hydrolysed; M_r 13–23 000, 87–89% hydrolysed; M_r 31–50 000, 87–89% hydrolysed; M_r 85–146 000, 87–89% hydrolysed and M_r 124–186 000, 87–89% hydrolysed) onto PSDVB beads. Dried bead samples (0.25 g) were "wetted" with methanol and then washed with water to displace the methanol from the bead interior. PVA solution (0.1–200 mg/ml, 1.2–600 ml) was added and the tubes sealed and left on a rotary mixer at room temperature. Samples were taken after 1 week and assayed using the iodine–iodide method as described in the Experimental (Section 2). The quantity of PVA adsorbed onto the PSDVB beads were calculated using a mass balance.

The amount of PVA that adsorbs onto PSDVB surfaces is quoted to be around 2.5 mg/m^2, for 87–89% hydrolysed PVA [18,19]. Using this estimation of PVA adsorption, it can be calculated that the pore area available for PVA adsorption in the CG1000-sd beads is about 100 m^2/g, and this compares to the total pore area available on the PSDVB beads of 250 m^2/g.

3.2. Non-specific protein adsorption

Experiments to measure non-specific protein adsorption were carried out to determine the effectiveness of the coating in masking possible hydrophobic interactions. Both lysozyme and HSA were used as test proteins.

Fig. 3 shows the effect of PVA (M_r 13–23 000, 87–89% hydrolysed) adsorption onto CG1000-sd beads on the subsequent non-specific adsorption of lysozyme and HSA. Lysozyme is substantially smaller than HSA (M_r lysozyme is approximately 14 500 compared to 66 000 for HSA) and so it is expected that there would be more accessible area available to lysozyme than HSA on these matrices which contain a wide range of pore sizes. Uncoated CG1000-sd beads bound 68 mg/g lysozyme and 40 mg/g HSA and it was only when the amount of adsorbed PVA was greater than 180 mg/g that there was no non-specific adsorption for lysozyme and only beyond 220 mg/g that there was no non-specific adsorption of HSA.

The effect of PVA type on the modification of PSDVB beads can be seen in Table 1. In this set of experiments uncoated beads adsorbed 38 mg/g lysozyme. For PVA M_r 13–23 000, 87–89% hydrolysed and PVA M_r 31–50 000, 87–89% hydrolysed there was no non-specific adsorption of lysozyme. For the M_r 13–23 000, 97–99% hydrolysed PVA, however, there was slight lysozyme adsorption,

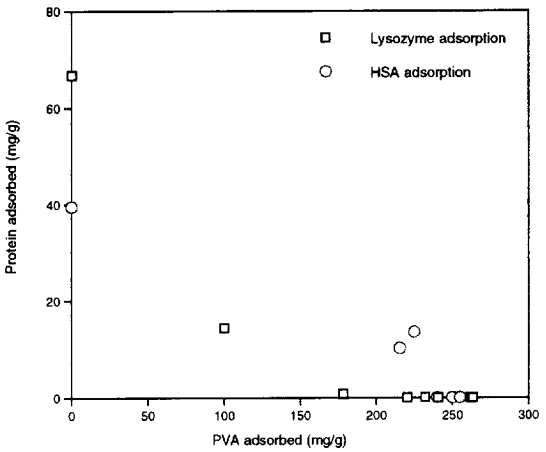

Fig. 3. Effect of PVA (M_r 13–23 000, 87–89% hydrolysed) adsorption on PSDVB beads on non-specific protein adsorption. Non-specific protein adsorption was carried out using lysozyme and HSA. The PVA modified and cross-linked PSDVB samples were placed into 20-ml centrifuge tubes and protein solution (lysozyme, 1 mg/ml, 20 ml in 50 m*M* phosphate buffer pH 5; HSA, 1 mg/ml, 20 ml in 25 m*M* phosphate buffer, pH 5) was added. The samples were left on a rotary mixer for 16 h for adsorption to occur. The protein concentration in solution was determined by measuring the O.D. in a quartz cuvette at 280 nm. The quantity of protein bound was determined from a mass balance on the supernatant.

indicating that despite the lower amount of PVA adsorption that occurs with the 97–99% hydrolysed PVA type, there is still a semi-effective hydrophilic coating. This shielding of the hydrophobic surface at reduced amounts of PVA adsorption could only have occurred if the PVA with the greater degree of hydrolysis had adsorbed much more flatly on the PS–DVB surface.

The PSDVB beads coated with PVA of molecular masses 85–146 000 (87–89% hydrolysed) and 124–186 000 (87–89% hydrolysed) exhibited non-specific hydrophobic interactions with lysozyme. The amounts of PVA adsorbed onto PSDVB beads were similar to the amounts of PVA of lower molecular masses adsorbed onto the PSDVB beads suggesting that the PVA types of higher molecular masses could not access and thus mask the internal pore area that was accessible to the smaller lysozyme molecule.

For effective hydrophilic coatings of PSDVB matrices, PVA of molecular mass 13–23 000, 87–89% hydrolysed or molecular mass 31–50 000, 87–89% hydrolysed should be used to eliminate any non-specific hydrophobic interactions between the PVA–PSDVB matrix and protein.

3.3. BET analysis

The effect of PVA adsorption on the BET characteristics of modified PS–DVB matrices can be seen in Table 2.

The BET total pore areas of the PSDVB matrices are reduced considerably with modification by adsorption of PVA with differing molecular masses and degrees of hydrolysis. The uncoated PSDVB matrices were found to have a BET pore area of 217 m^2/g, compared with the manufacturer's values of pore area of 250 m^2/g [20], and this drops to about 50 m^2/g with adsorption of 87–89% hydrolysed PVA, regardless of molecular mass. The 97–99% hydrolysed PVA modified PSDVB matrices have slightly higher pore area values than the 87–89% hydrolysed PVA (around 80 m^2/g) and this is probably due to the adsorbed PVA of 97–99% hydrolysis lying flatter on the PSDVB surface.

The BET desorption total pore volumes of the PVA–PSDVB modified matrices decrease with modification, but do not decrease by the same degree as pore areas. The unmodified PSDVB matrix has a total pore volume of 0.69 cm^3/g and this volume drops to around 0.4 cm^3/g for all the modified matrices, irrespective of the adsorbed PVA type.

The mean average pore diameter for the PVA–PSDVB matrices is calculated from a ratio between pore volume and pore area, using the following relationship:

$$\text{average pore diameter} = 4 \times \frac{\text{pore volume}}{\text{pore area}}$$

If, with modification, the matrix total pore volume decreases at a slower rate than pore area, then the average pore diameter increases. The mean average pore diameter for underivatised beads, measured by the BET method is 101 Å and the volume modal average is approximately 550 Å and this contrasts with the manufacturer's quoted modal average pore diameter of approximately 1000 Å. Determination of the average pore size is dependent on the characterising technique. The manufacturers used mercury porosimetry to determine the average pore size and this technique categorises macropores with greater

Table 1
Overall summary of PVA adsorption onto PSDVB matrices, the dyeing of subsequent PVA–PSDVB matrices and the lysozyme adsorption characteristics and recoveries on Procion Blue MX-R PVA–PSDVB beads

Sample number	PVA type adsorbed	PVA initial conc. (mg/ml)	PVA adsorbed (mg/g beads)	Non-specific lysozyme adsorption (mg/g beads)	Immobilised dye (μmol/ml bed)	Dynamic 5% capacity (mg/ml bed)	Saturation capacity (mg/ml bed)	Recovery of bound enzyme (%)	Ratio dyn./sat. capacity (%)
1	M_r 13 000–23 000	8.4	180	0.0	18.0	23.3	30.6	97.2	84
2	87–89% hydrolysed	32.0	216	0.1	16.5	30.6	34.8	99.9	88
3		49.1	240	0.1	18.5	32.6	37.9	100.3	86
4		85.2	240	0.0	17.5	37.2	42.3	98.6	88
5	M_r 13 000–23 000	8.5	125	1.7	10.4	22.5	27.0	88.8	83
6	97–99% hydrolysed	30.1	125	0.6	13.2	24.7	30.6	92.9	81
7		46.3	125	0.9	17.8	31.8	36.5	96.1	87
8	M_r 31 000–50 000	8.3	174	0.2	11.3	30.1	38.1	98.6	79
9	87–89% hydrolysed	33.0	223	0.0	12.1	28.6	33.4	100.6	86
10		43.4	250	0.0	12.5	30.8	35.7	100.0	87
11		78.1	213	0.0	13.0	33.7	38.2	99.7	88
12	M_r 85 000–146 000	8.6	186	5.8	–	–	–	–	
13	87–89% hydrolysed	34.4	255	0.6	–	–	–	–	
14		47.9	281	0.7	–	–	–	–	–
15	M_r 124 000–186 000	8.5	156	18.1	–	–	–	–	
16	87–89% hydrolysed	34.7	236	1.3	–	–	–	–	
17		41.7	240	5.1	–	–	–	–	–
Uncoated	–	–	–	38.0	–	–	–	–	
Blue Sepharose	–	–	–	–	7.3±0.9	21.5	30.7	99.4	70

Operations carried out as described in Section 2.

Table 2
Effect of adsorbed PVA on the matrix characteristics of PSDVB CG1000-SD beads as determined by the BET nitrogen adsorption/desorption technique

Sample number	PVA type adsorbed	PVA initial conc. (mg/ml)	PVA adsorbed (mg/g beads)	BET pore area (m^2/g)	BET desorption pore volume (cm^3/g)	BET average pore diameter (Å)
1	M_r 13 000–23 000	8.4	180	75	0.49	160
2	87–89% hydrolysed	32.0	216	56	0.34	167
3		49.1	240	55	0.24	173
4		85.2	240	51	0.32	172
5	M_r 13 000–23 000	8.5	125	89	0.57	156
6	97–99% hydrolysed	30.1	125	79	0.51	160
7		46.3	125	77	0.45	154
8	M_r 31 000–50 000	8.3	174	68	0.47	164
9	87–89% hydrolysed	33.0	223	53	0.64	198
10		43.4	250	56	0.44	178
11		78.1	213	49	0.35	199
12	M_r 85 000–146 000	8.6	186	70	0.43	155
13	87–89% hydrolysed	34.4	255	47	0.34	184
14		47.9	281	47	0.39	193
15	M_r 124 000–186 000	8.5	156	79	0.63	156
16	87–89% hydrolysed	34.7	236	46	0.38	198
17		41.7	240	57	0.44	190
Uncoated	–	–	–	217	0.69	101

Operations carried out as described in Section 2.

accuracy than the BET method. The BET method categorises micropores with a greater accuracy than mercury porosimetry and so it would be expected that the determined average pore size of the beads characterised by the BET method will be smaller than if characterised by the mercury porosimetry method. After PVA modification of the PSDVB matrices, the mean average pore diameters measured by the BET method increased from 101 Å to about 180 Å, whilst the volume modal average pore size was unchanged at about 550 Å. The 97–99% hydrolysed PVA–PSDVB matrices had a slightly smaller average pore diameter than the 87–89% hydrolysed PVA types although the difference is small. This is due to the increased pore area of the PS–DVB beads modified with PVA of 97–99% hydrolysis in comparison with the PVA types of 87–89% hydrolysis.

An increase in average pore diameter following matrix modification has been observed by Yang and Regnier [7] who, after coating PSDVB beads with polyoxyethylene, found that whilst the porosity of the beads fell from 0.34 to 0.20, the average pore diameter increased from 600 Å to 625 Å.

Fig. 4 and Fig. 5 show the incremental pore

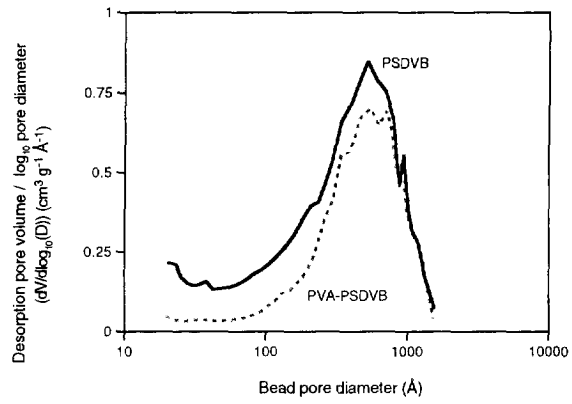

Fig. 4. BET desorption pore volumes of unmodified PSDVB matrices and PVA (M_r 13–23 000, 87–89% hydrolysed) modified PSDVB matrices. BET analysis was carried out on a Micromeritics ASAP 2000 analysis machine. The matrices (unmodified and undyed cross-linked PVA–PSDVB, sample 4) were washed and left to dry in crucibles at 60°C for 1 week. These matrices (0.15–0.3 g) were placed into a BET analysis tube and heated to 120°C under a high vacuum for 12 h. The reweighed sample was then loaded onto the automatic BET reader and the nitrogen adsorption/desorption isotherms measured.

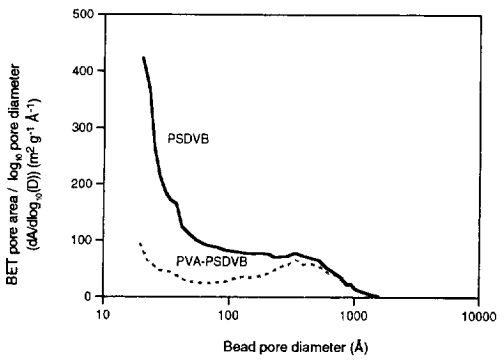

Fig. 5. BET pore areas of unmodified PSDVB matrices and PVA (M_r 13–23 000, 87–89% hydrolysed) modified PSDVB matrices. BET analysis was carried out on a Micromeritics ASAP 2000 analysis machine. The matrices (unmodified and undyed cross-linked PVA–PSDVB, sample 4) were washed and left to dry in crucibles at 60°C for 1 week. These matrices (0.15–0.3 g) were placed into a BET analysis tube and heated to 120°C under a high vacuum for 12 h. The reweighed sample was then loaded onto the automatic BET reader and the nitrogen adsorption/desorption isotherms measured.

volumes and pore areas of the PSDVB and PVA–PSDVB matrices. Fig. 4 shows that pores with a diameter between 200 Å and 1000 Å represent most of the pore volume in the matrices and the greatest incremental pore volume occurs at a pore diameter of about 550 Å. The PSDVB matrix has substantial pore volume in the pores of diameter less than 200 Å whereas following modification, this volume is reduced substantially. The incremental pore volumes are less in the PVA–PSDVB matrix than the PSDVB matrix with pores up to diameters of 800 Å. At 800 Å the incremental pore volumes are equal in both matrix types indicating that, following modification, the bead micropores and mesopores are filled with PVA, whilst the bead macropores remain relatively unchanged.

Fig. 5 shows the incremental change in pore area with change in $\log_{10}$ pore diameter. In the PSDVB matrix, the majority of the total pore area results from pores with diameter less than 50 Å, a pore area not present in the PVA–PSDVB matrices. The total pore area of PSDVB matrices is 217 m^2/g and the pores with a diameter less than 50 Å contribute 100 m^2/g. In the PVA–PSDVB matrix, the pores of diameter less than 50 Å contribute only 15 m^2/g out of a total pore area of 51 m^2/g. As with pore volume, the pore areas of the PVA–PSDVB matrix are less than the PSDVB matrix up to a pore diameter of 800 Å indicating that the PVA is filling the micropores present in the bead and not altering the macropores. This suggestion is contrary to the view of Leonard [11], who hypothesised that PVA molecules fill the large pores and that smaller pores are formed in a network of cross-linked PVA if the adsorbed layer is not dense enough.

3.4. Non-specific protein adsorption on dyed PVA–PSDVB matrices

Table 3 shows the amount of HSA and lysozyme bound onto unmodified PSDVB matrices and dye–PVA–PSDVB matrices in the presence of eluent (0.5 *M* NaSCN in 50 m*M* phosphate buffer, pH 8). The effect of dyeing the modified hydrophilic coating was investigated, as the dyeing process and dye attachment may destroy the effectiveness of the hydrophilic coating. Protein was adsorbed in eluent as under these conditions protein will not bind to the ligand, but can still bind to the PSDVB surface. It is expected that any non-specific adsorption will be due

Table 3
Effect of dyeing on the integrity of the hydrophilic coating of PVA–PSDVB matrices

Bead	Protein	Protein in (mg)	Protein out (mg)	Protein adsorbed (mg)	5% dynamic capacity (mg/ml)
PSDVB	HSA	32.6	3.2	29.4	27.4
	Lysozyme	46.2	7.6	38.6	29.9
Dye–PVA–PSDVB	HSA	2.4	2.2	0.2	0.0
	Lysozyme	6.0	6.0	0.0	0.0

HSA and lysozyme protein adsorption was adsorbed onto PSDVB and dye–PVA–PSDVB matrices in eluent to ensure no binding to the dye ligand and adsorption only onto the matrix. Operations carried out as described in Section 2.

to hydrophobic forces. It can be seen that in uncoated PSDVB matrices there was substantial protein adsorption, with HSA binding at about 30 mg/ml and lysozyme binding at about 40 mg/ml. The dye–PVA–PSDVB matrices did not bind any protein at all, indicating that there was no interactions between the PSDVB surface and proteins in solution. The dyeing process and dye attachment therefore does not interfere with the integrity of the PVA hydrophilic shielding of the PSDVB surface.

3.5. Dynamic capacities of Procion Blue MX-R PVA–PSDVB matrices

Table 1 shows the PSDVB samples modified with PVA and Procion Blue MX–R. Of the 17 samples, only sample numbers 1 to 11 were dyed, as sample numbers 12 to 17 displayed high degrees of non-specific protein adsorption. Similar quantities of dye ligand were bound on all modified matrices.

Fig. 6 shows the values of the 5% breakthrough dynamic capacities and the saturation capacity after 16 h of adsorption. The dynamic capacities are all in the region of 30–40 mg/ml bed. The ratio of 5% dynamic capacity to saturation capacity is fairly constant, being approximately 85% of saturation capacity, indicating that the majority of the lysozyme adsorption sites on the bead are occupied before breakthrough to 5% of the inlet concentration occurs. The recovery of bound lysozyme was high, with recoveries approaching 100% in the matrices modified with the 87–89% hydrolysed PVA. The recovery for sample 5 (modified with 87–89% hydrolysed PVA) was lower at 89% and this was due to lysozyme binding to the PSDVB surface (sample 5 showed non-specific protein adsorption) and not to the dye ligand. The eluent was not able to recover the lysozyme from the PSDVB surface. It can be seen that the adsorption capacities increase with an increase in the amount of PVA adsorbed and that beads modified with PVA types *M*r 13–23 000, 87–89% hydrolysed and M_r 31–50 000, 87–89% hydrolysed have marginally higher binding capacities than those modified with M_r 13–23 000 PVA with greater hydrolysis. All the matrices prepared exhibit higher dynamic and saturation capacities than the Blue Sepharose Fast Flow, an affinity matrix based on cross-linked agarose. Dye leakage was found to be

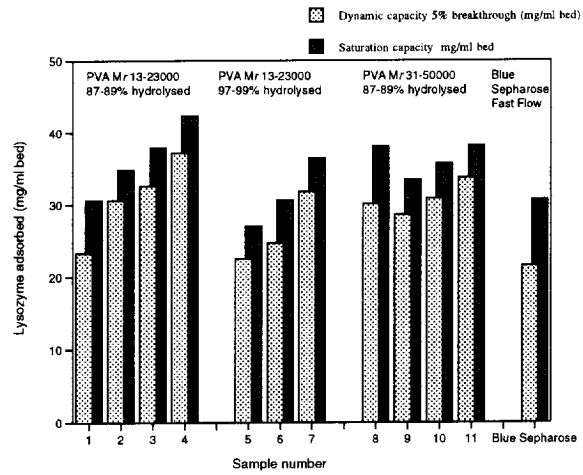

Fig. 6. Comparison of dynamic capacities (5% breakthrough) and saturation capacities of lysozyme on Procion Blue MX-R PVA–PSDVB matrices (1-ml column volume, length 5 cm, diameter 0.5 cm). The capacities were determined using frontal analysis by pumping a lysozyme solution (1 mg/ml, 50 m*M* phosphate buffer, pH 7.5) through the column at a flow-rate of 1 ml/min (300 cm/h) until the absorbances (at 280 nm) of the output and input streams were identical. Dynamic capacities were calculated at 5% breakthrough (the outlet stream absorbance at 280 nm was 5% that of the input). The column was then washed (20 ml) with running buffer and then eluted using 1 *M* NaCl in 50 m*M* phosphate buffer pH 7.5. Saturation capacities were determined by placing the samples into 20-ml centrifuge tubes and adding lysozyme solution (20 ml, 4.5 mg/ml in 50 m*M* phosphate buffer, pH 7.5). The saturation capacity was calculated from the difference in supernatant absorbances at 280 nm after an adsorption time of 16 h.

below detection limits (1 μmol/l), i.e. the level of dye in all solutions that were passed through the bed was below 1 μmol/l.

3.6. Stability of modified matrices to chemical cleaning

In order to determine the chemical stability of the matrices during cleaning, a Procion Blue MX-R (16.5 mm/ml) PVA–PSDVB matrix was deliberately fouled by incubating it with lysozyme at room temperature for one month. It was expected that during this time the protein would become denatured and microbial activity would have occurred ensuring sufficient bed fouling. After this period, the fouled bed (sample 2) was repeatedly applied with a sodium

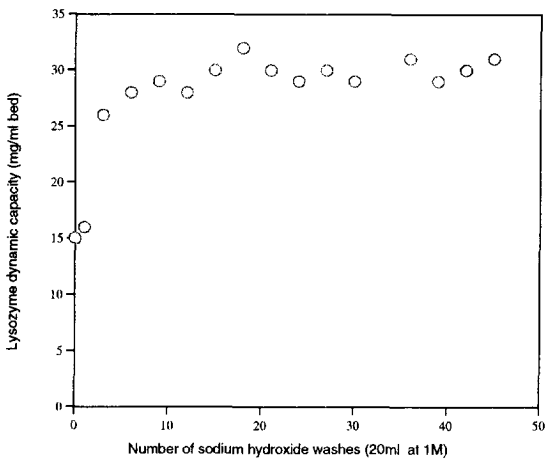

Fig. 7. Effect of NaOH cleaning on the dynamic capacity (5%) of lysozyme adsorption on a fouled Procion Blue MX-R PVA-PSDVB matrix. The matrix (sample 2, 16.5 μm/ml immobilised dye conc.) was incubated in a lysozyme solution for a period of a month at room temperature. NaOH washes (1 *M*, 20 ml) were passed through the column and the dynamic capacities were determined as described in experimental.

hydroxide wash (1 *M*, 20 column volumes). The dynamic capacity of the uncontaminated bed was 30 mg/ml. After fouling, its capacity had dropped to 15 mg/ml. After three washes the dynamic capacity had substantially increased. After ten NaOH washes the capacity had reached the level of the clean bed. The dynamic capacity did not drop with further repeated washes, indicating that the modified coating is chemically stable to 1 *M* NaOH and can therefore be cleaned using common cleaning techniques.

4. Conclusions

A hydrophilic packing material has been created by the modification of PSDVB matrices by the adsorption of PVA. The molecular mass, hydrolysis and solution concentration of the PVA are all important determinants in the subsequent characteristics of the adsorbent beads. The adsorption layer masks the hydrophobic surface and ensures minimal non-specific protein adsorption. PVA of lower degrees of hydrolysis adsorbs to a greater extent than PVA with the higher degree of hydrolysis. The PVA of lower molecular mass and lower hydrolysis shields the hydrophobic surface more effectively than that with higher molecular mass and hydrolysis. To create a practical affinity chromatography matrix, PVA of molecular masses 13–23 000, 87–89% hydrolysis or 31–50 000, 87–89% hydrolysis should therefore be used for coating.

The adsorbed coating provides a chemically stable matrix that allows easy derivitization with dye ligands. The dynamic capacities are high (greater than commercial Blue Sepharose Fast Flow) and under the described conditions of operation, the dynamic capacity is up to 85% of the saturation capacity, an indication that during operation the lysozyme reaches most of the binding areas in the particle interior.

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