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Modification of polystyrenic matrices for the purification of proteins III. Effects of poly(vinyl alcohol) modification on the characteristics of protein adsorption on conventional and perfusion polystyrenic matrices

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Abstract

Poly(styrene–divinylbenzene) (PS–DVB) chromatography matrices, CG1000sd 20–50 μm (TosoHaas), PLRP4000s 15–25 μm, PLRP4000s 50–70 μm (Polymer Laboratories) have been modified by the adsorption and crosslinking of poly(vinyl alcohol) (PVA) to create a matrix suitable for the attachment of dye ligands. The adsorption capacities of lysozyme and HSA on these Procion Yellow HE-3G dyed PVA modified PS–DVB matrices were measured at various flow-rates and the capacities were compared with a Procion Yellow HE-3G dyed OH-activated POROS 20, 20-μm matrix (PerSeptive Biosystems). The adsorption of small proteins was not hindered by the smaller pores of the CG1000sd beads, but as protein size increased, and at high flow-rates, a high mass transfer rate became more dependent on large pore size and small particle diameter. © 1997 Elsevier Science B.V.

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

1. Introduction

Poly(styrene–divinylbenzene) (PS–DVB) matrices have been used in chromatography for many years and these matrices can be operated under high pressures without compression. However, these matrices cannot directly be used to purify proteins, as the surface is difficult to derivatise and strong hydrophobic interactions between the matrix and the protein results in irreversible adsorption and reduced protein yield.

The adsorption and subsequent crosslinking of poly(vinyl alcohol) (PVA) onto PS–DVB surfaces have recently been performed [1–4]. This surface modification technique creates a rigid PS–DVB matrix with a hydrophilic coating with functional groups which allow derivatisation.

The choice of pore size in protein chromatography is critical to process design [5]. Pore size should be optimised for unrestricted access to the immobilised ligand, and this can be achieved by either having pore sizes small enough to totally reject macromolecular penetration (such as non-porous beads) or large enough so that there is no hindrance to diffusion. Pore diameters greater than 4000 Å have been quoted as a desirable size for unrestricted

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diffusion of proteins [6]. However, as the bead pore size increases, the surface area available for protein adsorption decreases. This means that although by using a bead with larger pores the protein mass transfer rate will increase, the maximum amount of protein that can adsorb may decrease.

A number of recent papers have discussed the use of wide-pore 'perfusion' chromatography supports. These have superior adsorption kinetics to traditional supports due to large throughpores (pores greater than 6000 Å) that allow convective mass transfer through the bead [7–9]. These beads are manufactured from crosslinked PS–DVB which needs to be masked in order to shield the hydrophobic properties. PVA adsorbed and crosslinked onto PS–DVB surfaces has been shown to fill the micropores but leave the macropores essentially unaltered [3]. Wide-pore PS–DVB chromatography media could therefore be modified using the PVA coating technique without reducing the size of the larger flowthrough pores needed for the convective mass transfer to occur. The PS–DVB surface of commercial POROS 20 OH-activated beads are masked using an epibromohydrin and glycidol copolymer. If PS–DVB beads with similar wide pores such as PLRP4000s beads [10,11] are modified using the PVA coating technique, then the adsorption characteristics would be expected to show similar perfusion adsorption characteristics to the POROS 20 beads.

2. Experimental

2.1. Materials

PVA (M_r 13 000–23 000, 87–89% hydrolysed) was purchased from Aldrich Chemical (Gillingham, UK), as were ethanol, methanol, disodium hydrogenorthophosphate, sodium hydrogenphosphate and sodium carbonate.

Procion Yellow HE-3G (CI Reactive Yellow 81) was purchased from Sigma (Poole, UK). Blue Dextran 2000 was purchased from Pharmacia Biotech (St. Albans, UK).

Chicken egg white lysozyme (M_r 14 300) and human serum albumin (HSA) (M_r 66 000) were obtained from Sigma.

CG1000sd beads were purchased from TosoHaas (Philadelphia, USA), PLRP4000s beads (15–25 and 50–70 μm diameter) were kindly donated by Polymer Laboratories (Church Stretton, UK), and POROS 20 (OH-activated) beads were purchased from PerSeptive Biosystems (Cambridge, MA, USA).

2.2. Instrumentation

UV–Vis spectrophotometry was carried out using a Shimadzu UV-160A (VA Howe, 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 used was an HR5/5 of 5×0.5 cm I.D. (volume 1 ml).

2.3. Coating of PS–DVB beads with PVA

The coating procedure was essentially the same as described in a previous paper [4]. The best PS–DVB shielding through coating with PVA was obtained by adsorbing PVA (M_r 13 000–23 000, 87–89% hydrolysed) and subsequent crosslinking with glutaraldehyde at a crosslinking ratio of 50 mol glutaraldehyde/mol PVA.

2.4. Dyeing of modified PS–DVB matrices

The PVA–PS–DVB modified matrices and the OH-activated POROS 20 beads were both dyed using identical methods. The PVA–PS–DVB samples were washed thoroughly with water and 5-ml samples were placed in a round bottomed flask (50 ml). Water was then added to give total volumes of 20 ml. Procion Yellow HE-3G dye (2000 mg) was added and the dye mixture mixed for 30 min. After mixing, NaCl (22%, w/v, 4 ml) was added to the dye mixture and left for another 30 min to ensure adequate mixing. Sodium carbonate (200 mg) was then added and the samples left for 3 days at 60°C to ensure binding of the dye to the hydroxyl groups on the beads. The dyeing procedure was repeated once more. After the dyeing processes, the beads were thoroughly washed with an excess of water and methanol, and were washed again a number of times over a period of a week. Determination of dye ligand

concentration could not be determined by digestion of the matrices as the PS–DVB was chemically and thermally stable, and so the quantity of dye that had bound to the PS–DVB matrices was determined from a mass balance calculated from the dye added to the beads and from the dye in the wash supernatant. Dye concentrations in solution were determined using the extinction coefficient of 35 600 l mol^{-1} at 400 nm [12]).

2.5. Frontal analysis

Dynamic loading capacities of dyed-modified PS–DVB matrices was determined using frontal analysis. The protein solutions used in these experiments were lysozyme (1 mg/ml, adsorbed 50 m*M* phosphate buffer, pH 7.5, eluted in 1 *M* NaCl in 50 m*M* phosphate buffer, pH 7.5) and HSA (1 mg/ml, adsorbed in 25 m*M* phosphate buffer, pH 5.0, eluted in 0.5 *M* NaSCN in 50 m*M* phosphate buffer, pH 8.0). The frontal analysis was carried out on an HR5/5 column (5×0.5 cm I.D.) at flow-rates ranging from 0.5 to 8 ml/min (superficial velocity from 153 to 2445 cm/h). A strict protocol was adopted to ensure that the flow-rate experiments were reproducible and that there was minimal irreversible protein binding. The flow-rates were conducted in the following order: 1 ml/min (306 cm/h), 1 ml/min, 0.5 ml/min (153 cm/h), 2 ml/min (611 cm/h), 4 ml/min (1222 cm/h), 8 ml/min (2445 cm/h) and 1 ml/min (the dynamic capacities of the first, second and last experiments at a flow-rate of 1 ml/min were compared to ensure that they had not altered over the series of experiments).

The system voidage (column and associated tubing) was determined for each protein on the undyed PVA–PS–DVB beads as there were slight differences in the void volume of the column between the different-sized proteins. The system voidages were measured to be 0.81 ml for lysozyme and 0.67 ml for HSA.

The dynamic capacity was determined at 5% breakthrough (the outlet absorbance at 280 nm being 5% that of the inlet). The 5% dynamic capacity was calculated by determining the quantity of protein applied to the column at the point at which the outlet stream had a protein concentration 5% that of the feed stream, and deducting from this value the amount of protein contained in the system void volume.

The protein feed solution was applied to the column until the absorbance of the outlet stream was approaching that of the inlet stream. The column saturation capacity was calculated from a mass balance by determining how much protein had been applied to the column, and deducting from this value the amount of protein in the system void volume and the amount of protein which had passed through the column unadsorbed.

After the adsorption stage the columns were washed with 20 ml of adsorbent buffer (2 ml/min, superficial velocity 611 cm/h) and then the adsorbed protein was eluted. The bed was then washed with 20 ml of the adsorbing buffer (2 ml/min; superficial velocity, 611 cm/h) before it was used for further protein adsorption experiments.

The solutions applied to the column were collected as three separate fractions: the adsorption, wash and elution stages. Protein concentration in solution was determined by measuring the absorbance in a quartz cuvette at 280 nm. The A_{280} value was used to calculate protein concentration by employing extinction coefficients of 2.64 and 0.53 ml mg^{-1} cm^{-1} for lysozyme and HSA, respectively [13].

3. Results and discussion

3.1. Bead characteristics and protein properties

The bead characteristics are shown in Table 1. The PLRP4000s and POROS 20 beads have pores of a much wider diameter (4000–6000 Å) than the 1000-Å diameter pores of the CG1000sd beads. These larger 'perfusive' pores should act as flowthrough pores, further enhancing the mass transfer kinetics. The PLRP4000s, 15–25 μm particles, and POROS 20 particles are similar to each other and have smaller particle diameters than the PLRP4000s, 50–70-μm beads.

Two proteins were used in the flow-rate experiments investigating the effect of flow-rate on adsorption performance. The properties of these proteins are summarised in Table 2. Lysozyme was the smaller protein tested, hence having the higher diffusion coefficient. HSA has a molecular mass

Table 1
The bead characteristics of the matrices used in the adsorption study. Values are shown are provided by the manufacturers

	Bead (Manufacturer)			
	CG1000sd (ToSoHaas)	PLRP4000s (Polymer Laboratories)	PLRP4000s (Polymer Laboratories)	POROS 20 (PerSeptive Biosystems)
Particle diameter range (μm)	20–50	15–25	50–70	20
Average pore diameter (Å)	1000	4000	4000	6000
Total surface area (m^2 g^{-1})	250	139	139	100
Dry bulk density (g ml^{-1} bed)	0.25	0.25	0.25	0.305

approximately 5 times that of lysozyme and a diffusion coefficient half of the diffusion coefficient of lysozyme. The shapes of the proteins are similar, being globular, with an ellipsoidal factor ranging from 1.2 to 1.3.

3.2. The effect of flow-rate on protein frontal curves and dynamic capacities

The variation of the shapes of the frontal curves for HSA adsorption with flow-rate for each matrix are shown in Fig. 1a–d. The column dimensions were the same in all the adsorption experiments tested: an HR5/5 column of 5×0.5 cm I.D.

The 5% dynamic capacities of HSA and lysozyme adsorption onto Procion Yellow HE-3G dyed modified PS–DVB matrices are shown in Fig. 2 and Fig. 3, respectively. These are summarised together with the maximum binding capacity (Q_m), dissociation constant (K_d) and the equilibrium capacity of the matrices at a protein concentration in solution of 1 mg/ml determined from isotherm measurements in Table 3. Protein was applied to the dyed-modified matrices at 1 mg/ml and at varying flow-rates. The column dimensions were the same in all the adsorption experiments tested: an HR5/5 column of 5×0.5 cm I.D.

3.3. Effect of hydrophilic coating technique on adsorption characteristics

PVA-modified PLRP4000s, 15–25-μm and POROS 20 beads are similar to base PS–DVB particles [10,11], but their highly hydrophobic surfaces are shielded by two different hydrophilic adsorption techniques. It can be seen from Fig. 1a and b that the frontal curves for HSA adsorption are essentially similar, and that PLRP4000s, 15–25-μm and POROS 20 beads do not show diffusion-dominated mass transfer frontal curves. As flow-rate increased for the PLRP4000s, 15–25-μm and POROS 20 matrices, breakthrough occurred at lower applied volumes. However, instead of taking longer for the outlet concentration to approach the feed concentration, as would be expected if diffusion were the dominant mode of mass transfer. The gradients for the curves with PLRP4000s and POROS 20 remained approximately constant. For these two matrices it was found that the outlet concentration approaches the initial concentration

Table 2
The properties of the proteins used in the adsorption experiments on different matrix types

Protein	M_r	p*I*	Ellipsoidal factor	Stokes' radius[a] (Å)	Diffusion coefficient[b] (10^{-7} cm^2 s^{-1})
Lysozyme	14 300	11.35	1.21	17	11.2
HSA	66 000	4.70	1.29	29	6.10

Data supplied by Sigma, or from CRC Handbook of Biochemistry [13].
[a]Determined using method as outlined by Chase [17].
[b]Diffusional coefficients are quoted for water at 25°C.

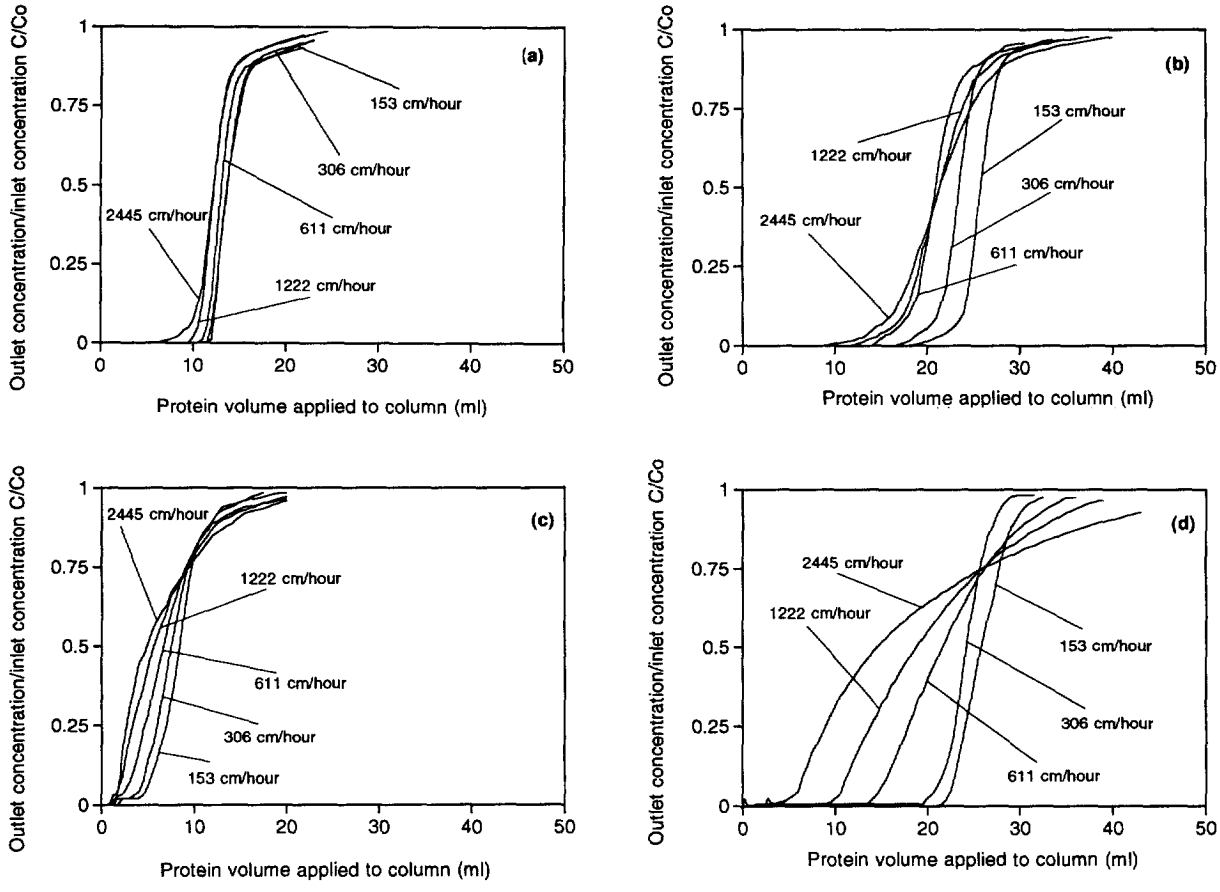

Fig. 1. The effect of flow-rate on the frontal curves of HSA adsorption on Procion Yellow HE-3G dyed modified PS–DVB particles. (a) PVA–PS–DVB PLRP4000s 15–25-μm beads; (b) OH-activated POROS 20 beads; (c) PVA–PLRP4000s 50–70-μm beads; (d) PVA–CG1000sd beads. The PVA modification technique was carried out as described previously [4]. The dyeing procedure was carried out as described in Section 2. HSA (1 mg/ml in 25 m*M* phosphate buffer, pH 5.0) was applied to an HR5/5 column (5×0.5 cm I.D.) at various flow-rates (corresponding to superficial velocities of 153, 306, 611, 1222 and 2445 cm/h). The absorbance (at 280 nm) of the outlet stream was measured and the ratio of this absorbance to feed absorbance is plotted against applied protein volume.

asymptotically. The total amount of HSA adsorbed at saturation was almost the same at all flow-rates.

Fig. 2 and Fig. 3 show that for the modified PS–DVB matrices, the 5% dynamic capacities for lysozyme and HSA on the dyed PVA–PLRP4000s, 15–25-μm and dyed OH-activated POROS 20 show characteristics typical of perfusive flow. This is characterised by an initial drop in capacity with a slight increase in velocity because, at low flow-rates, convective enhancement is negligible and diffusion is the dominant mode of intra-particle mass transport. At high adsorption velocities, convective intra-particle mass transfer dominates and there is little further drop in dynamic capacities with an increase in superficial velocity [14].

The drop in 5% dynamic capacity on the PLRP4000s 15–25-μm particles is smaller than the drop in dynamic capacity for the POROS 20 beads. At a 2445-cm/h superficial velocity the dynamic capacities of the POROS 20 particles was half that of the saturation capacities, whereas the dynamic capacities of the PLRP4000s 15–25-μm particles was

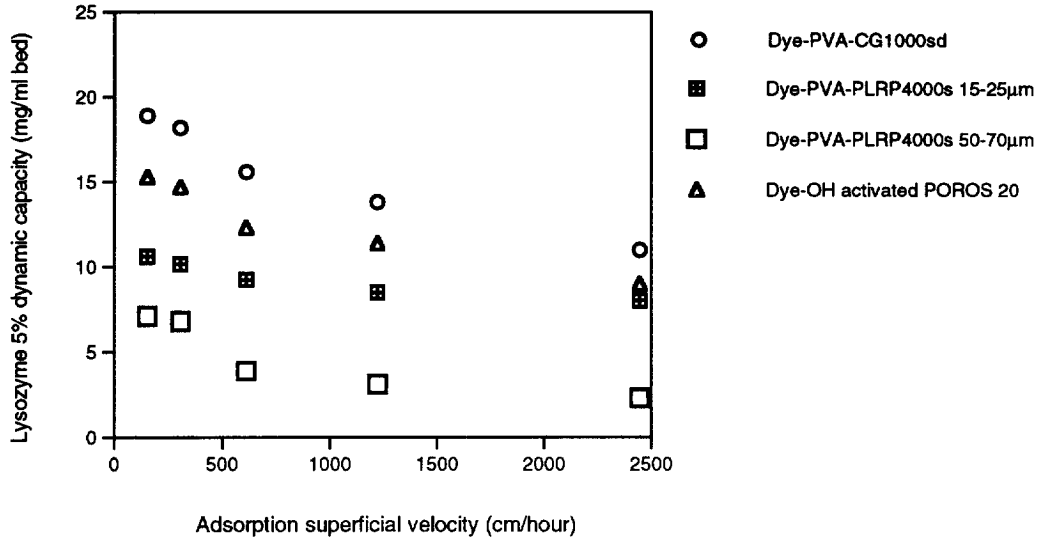

Fig. 2. The effect of flow-rate on the 5% dynamic capacities of lysozyme adsorption on Procion Yellow HE-3G dyed PVA–PS–DVB matrices (CG1000sd and PLRP4000s) and Procion Yellow HE-3G dyed OH-activated POROS 20 matrices. Lysozyme (1 mg/ml in 50 m*M* phosphate buffer, pH 7.5) was adsorbed onto an HR5/5 column (5×0.5 cm I.D.) at superficial velocities of 153, 306, 611, 1222 and 2445 cm/h at room temperature. The absorbance of the outlet stream was measured at 280 nm and the dynamic capacity was calculated from the frontal curve from the point where outlet absorbance was 5% that of the feed.

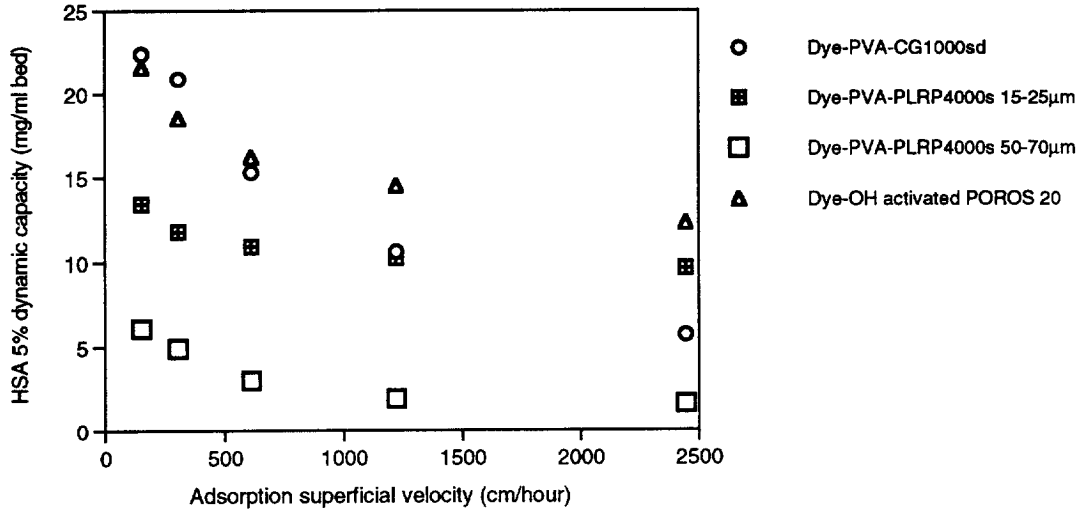

Fig. 3. The effect of flow-rate on the 5% dynamic capacities of HSA adsorption on Procion Yellow HE-3G dyed PVA–PS–DVB matrices (CG1000sd and PLRP4000s) and Procion Yellow HE-3G dyed OH-activated POROS 20 matrices. HSA (1 mg/ml in 25 m*M* phosphate buffer, pH 5.0) was adsorbed onto an HR5/5 column (5×0.5 cm I.D.) at superficial velocities of 153, 306, 611, 1222 and 2445 cm/h at room temperature. The absorbance of the outlet stream was measured at 280 nm and the dynamic capacity was calculated from the frontal curve from the point where outlet absorbance was 5% that of the feed.

Table 3
The adsorption of various proteins onto Procion Yellow HE-3G dyed PVA-PSDVB matrices and Procion Yellow HE-3G dyed OH-activated POROS 20 beads: all determinations were carried out as described in Section 2

	Immobilised dye concentration (μmol/ml)	Protein bound	Maximum capacity, Q_m (mg/ml)	Dissociation constant, K_d (mg/ml)	Equilibrium capacity at 1 mg/ml (mg/ml)	5% dynamic capacity			
						306 cm/h		2445 cm/h	
						mg/ml	5% dynamic capacity/ equilibrium capacity (%)	mg/ml	5% dynamic capacity/ equilibrium capacity (%)
CG1000sd	14.7	Lysozyme	20.0	0.013	19.7	8.3	92.9	11.0	55.8
		HSA	27.0	0.022	26.4	21.0	79.5	5.7	21.6
PLRP4000s 15–25 μm	8.0	Lysozyme	11.2	0.014	11.0	10.4	94.5	8.0	72.7
		HSA	14.7	0.013	14.5	12.0	82.8	9.6	66.2
PLRP4000s 50–70 μm	10.8	Lysozyme	12.1	0.016	12.0	6.8	56.2	2.3	19.0
		HSA	11.6	0.038	11.2	4.5	38.8	1.6	13.8
POROS 20	11.9	Lysozyme	18.2	0.019	17.9	15.0	83.8	9.0	50.3
		HSA	24.4	0.023	23.8	19.0	79.8	12.3	51.7

about 68% of saturation capacity. However, the epibromohydrin and glycidol surface coating, as used to modify the OH-activated POROS 20 media seems to provide a more suitable surface for the immobilisation of dye ligand, and therefore a subsequently higher binding capacity than the PVA-coated PLRP4000s PS–DVB media.

3.4. Effect of particle size on adsorption characteristics

The HSA frontal curves for the dyed PVA-modified PLRP4000s beads, 15–25- and 50–70-μm, are shown in Fig. 1a and c. It can be seen that, whereas the smaller 15–25-μm particles show perfusive qualities, the larger PLRP4000s 50–70-μm particles show adsorption characteristics typical of diffusive mass transfer. In these larger beads the 5% dynamic capacities of both HSA and lysozyme drop with an increase in superficial velocity, and this is due to the mass transfer of the proteins into these larger particle beads being dominated by diffusive effects due to the lower column pressure drop associated with larger particle sizes. The decline in convection-dominated mass transfer with an increase in particle size is documented. Van Deemter analysis carried out by McCoy et al. [14] for reversed-phase adsorption of lysozyme onto POROS 20 and POROS 50 particles have shown that the split ratio, i.e. the proportion of convective flow that goes through the bead rather than round the bead is smaller for POROS 50 beads than with the POROS 20 particles. Weaver and Carta [15] found no indication of convective mass transport in POROS 50 beads up to a superficial velocity of 5000 cm/h.

3.5. Effect of pore size on adsorption characteristics

CG1000sd beads have a diameter of 20–50 μm and an average pore size of about 1000 Å. The surface area of these beads available for subsequent ligand immobilisation is greater than the surface area in the larger pore perfusive supports, and so the maximum capacity, Q_m, for both proteins was higher in these beads than any other. The frontal curves obtained for the adsorption of HSA on CG1000sd beads are shown in Fig. 1d. This figure shows frontal curves typical of diffusional-dependent mass transfer.

The smaller-pore CG1000sd beads have a greater lysozyme dynamic binding capacity at all flow-rates than the beads with the larger pores, namely the PLRP4000s and POROS 20 beads. It can be seen in Fig. 2 and Table 3 that the fall in lysozyme dynamic capacity of the CG1000sd beads with increased flow-rate is proportionally about the same as for the POROS 20 beads, and less than the PLRP4000s 50–70-μm beads. Lysozyme has a large diffusion coefficient, and so it is likely that the diffusional mass transfer of lysozyme into the CG1000sd beads will already be high, and under these experimental flow-rates the benefits of the convective-dominated mass transfer associated with perfusion beads will not appreciably enhance their dynamic capacities.

However it can be seen from Fig. 3 and Table 3 that, whereas CG1000sd beads have the highest capacity for HSA at low flow-rates, it was found that at increased superficial velocities the dynamic capacities of the CG1000sd beads dropped substantially. Indeed, the 5% dynamic capacity of CG1000sd dropped below that of the dyed POROS 20 beads when superficial velocity increased beyond 500 cm/h, and the dynamic capacity of the CG1000sd beads dropped below that of the PLRP4000s 15–25-μm beads with a superficial velocity higher than 1200 cm/h. HSA is a larger protein than lysozyme and consequently has a lower diffusion coefficient. The mass transfer of this protein into the bead interior will therefore be slow under diffusional-dominating regimes, and the introduction of convective mass transfer at high flow-rates will ensure that the dynamic capacities of the perfusive beads do not drop as much as the diffusional-dependent CG1000sd and PLRP4000s 50–70-μm particles.

The results obtained are similar to those obtained by other researchers, although it must be appreciated that quoted values of dynamic capacity depend not only on the linear velocity of liquid flow through the bed, but also on the height of the packed bed employed in the determination. Work on bovine serum albumin (BSA) adsorption on uncoated PS–DVB CG1000sd beads [16] (6.2×1.0 cm diameter, 4.9 ml volume) has shown that with an increase in superficial velocity from 150 to 800 cm/h, the 1% dynamic capacity fell from 32 to 26 mg/ml, a drop

of 19%. This compares with a drop of 29% with an increase in superficial velocity from 150 to 610 cm/h on the Procion Yellow HE-3G—PVA–PS–DVB CG1000sd beads.

4. Conclusions

A PVA coating procedure [12,13] can be used to shield the surface of PS–DVB matrices including wide-pore 'perfusion' matrices. The PVA coating procedure created a chromatography matrix suitable for the purification of proteins, however the quantity of ligand that could be immobilised on a PVA-modified PS–DVB matrix was lower than on a similar base matrix modified with an epibromohydrin and glycidol copolymer.

The base PS–DVB support matrix affected the characteristics of the subsequent PVA-shielded and dyed matrix significantly. It was found that small-particle wide-pore 'perfusive' PS–DVB matrices (PLRP4000s 15–25 μm) retained their perfusion mass transfer characteristics after subsequent PVA modification. However, dyed, PVA-modified large-particle wide-pore matrices (PLRP4000s 50–70 μm) showed little indication of convective mass transfer. Dyed, PVA-modified small-pore PS–DVB matrices (CG1000sd) created a chromatography matrix with higher maximum protein adsorption values than the larger pored PS–DVB matrices and the dynamic capacity of lysozyme with this matrix, under the flows tested, was higher than the wide-pore beads. HSA has a lower diffusion mass transfer than lysozyme and the effect of the dominating convective flow associated with the wide-pore particles ensured that the drop in HSA dynamic capacity was lower on small-particle wide-pore matrices than on the small-pore CG1000sd particles.

The identification of the most desirable coated PS–DVB matrix will therefore depend on the nature of the protein and flow-rate to be used in subsequent applications. The higher surface area available in the CG1000sd beads will ensure an overall higher capacity as well as a higher dynamic capacity with small proteins and at low flow-rates than small-particle perfusion matrices. The adsorption of larger proteins or adsorption at high flow-rates will, however, mean that adsorption kinetics will be inhibited by the small pore size of CG1000sd beads, and so beads with through-pores would provide a more desirable matrix.

Acknowledgments

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