ELSEVIER

Journal of Chromatography A, 773 (1997) 73–83

JOURNAL OF CHROMATOGRAPHY A

Preparation and use of ion-exchange chromatographic supports based on perfluoropolymers

Graham E. McCreath*, Ryan O. Owen, Dominic C. Nash, Howard A. Chase

Department of Chemical Engineering, University of Cambridge, Pembroke Street, Cambridge CB2 3RA, UK

Received 4 March 1996; revised 17 January 1997; accepted 7 February 1997

Abstract

A poly(vinyl alcohol) (PVA) coated particulate perfluoropolymer (FEP) support has been functionalised with ion-exchange groups for use in ion-exchange chromatography of proteins. Anion-exchange (DEAE and Q) and cation-exchange (SP) groups were introduced to PVA-FEP which had previously been activated using cyanuric chloride. The equilibrium adsorption capacities of SP-PVA-FEP were 31.8 and 25.2 mg ml^{-1} for lysozyme and IgG respectively while for DEAE-PVA-FEP, the equilibrium adsorption capacities were 14.9 and 9.7 mg ml^{-1} for β-lactoglobulin and HSA respectively. The equilibrium adsorption capacities for Q-PVA-FEP were determined to be 17.2 and 13.5 mg ml^{-1} for β-lactoglobulin and HSA respectively. Experiments carried out to investigate the resolving power of the materials showed that both SP and Q-PVA-FEP were able to resolve proteins with only small differences in their isoelectric points and that this resolution could be maintained at a flow-rate of 1500 cm h^{-1}. SP-PVA-FEP was used to purify lysozyme from egg whites where a 50-fold purification, to homogeneity, was achieved in 98% yield. The anion exchanger, Q-PVA-FEP could be used to purify G6PDH from a clarified homogenate of bakers' yeast 14.3-fold in 81% yield.

Keywords: Ion exchangers; Stationary phases, LC; Perfluoropolymers; Proteins

1. Introduction

Perfluoropolymer (FEP) adsorbents, in both solid and liquid form, have shown promise as versatile media for the separation of a variety of biological products ranging from enzymes to whole microbial cells. The versatility of perfluoropolymers arises from their unique properties which include high density and chemical stability of both solids and liquid emulsions [1,2]. The normally hydrophobic surface properties of perfluorocarbons can be masked using surface active polyhydroxyl polymers such as poly(vinyl alcohol) (PVA). The PVA acts not only as a masking and stabilising agent but also allows for the immobilisation of ligands through primary and secondary hydroxyl groups. Perfluorocarbon affinity emulsions have been used in expanded beds and novel reactors for the purification of enzymes directly from preparations of disrupted cells using triazine dyes as affinity ligands [3,4]. Proteinaceous ligands have also been immobilised onto triazine activated perfluorocarbon emulsions and used for the isolation and enrichment of microbial cells [5,6]. Perfluoropolymer solids have recently shown particular promise in operations whereby proteins can be extracted continuously from crude feedstocks. This application is possible due to the high mechanical strength of FEP solids which enables them to be

*Corresponding author.

0021-9673/97/$17.00
PII S0021-9673(97)00191-X

pumped continuously around process equipment. A continuous 4-stage counter-current contacting device has recently been developed which allows for continuous protein purification by transporting adsorbent sequentially through loading, washing, elution and regeneration stages [7]. In order to increase the range of applications of FEP adsorbents we have investigated the synthesis of adsorbents carrying ion-exchange groups as ion-exchange chromatography remains a widely used and potent technique for the purification of proteins [8,9] and could be used readily in continuous operations. Presently there are no available ion-exchange adsorbents that could be used in devices we have designed for the continuous purification of proteins; this has inspired the synthesis of adsorbents described in this paper.

As most methodologies for the preparation of ion-exchange adsorbents are propriety, there is little information in the literature about their synthesis. However, it is expected that many ion-exchange matrices are prepared in a similar manner to those first outlined by Peterson and Sober in 1956 [10] where anion- and cation-exchange groups were introduced onto a cellulose matrix. Alternatively, rather than the direct attachment of charged groups, the use of polymer grafting to produce charged 'tentacle' type adsorbents has also been described [11]. Copolymerisation of ethylene dimethacrylate and hydroxyethyl methacrylate (HEMA) also enables the preparation of hydrophilic surfaces onto which ion-exchange groups can either be directly introduced or incorporated through the use of various ionic monomers [12–15]. The synthesis of a variety of ion-exchange supports based on PVA-FEP has been described recently [16]. Using techniques of direct attachment of ionogenic groups and graft polymerisation of charged monomers, a range of anion- and cation-exchangers were produced. However, the majority of the PVA-FEP synthesised supports displayed low binding capacity for proteins and poor recovery. In the case of direct attachment, the poor performance was attributed to limited association between the surface attached charged groups and the protein. Grafting charged monomers increased the capacity and recovery of the PVA-FEP supports but involved complex chemistries. The approach that we have adopted is to incorporate charged groups onto PVA-FEP that has previously been activated using cyanuric chloride. This then allows the immobilisation of various charged ligands bearing amino or other nucleophilic groups onto the surface of the matrix via a spacer molecule; this should enable better association of the protein with the charged group. In this paper we provide preliminary results for the generation of ion-exchange chromatographic supports using PVA-FEF.

2. Experimental

2.1. Materials

PVA (M_r 13 000–23 000, 98% hydrolysed) was purchased from Aldrich (Gillingham, UK) as was 3-amino-1-propanesulfonic acid, glycidyltrimethyl ammonium chloride and cyanuric chloride. Bovine serum albumin (BSA), human serum albumin (HSA), β-lactoglobulin, β-lactoglobulin a, cytochrome *c*, lysozyme, egg whites (hen, dried), *Micrococcus Lysodekticus* cells, 2-(diethylamino)ethylamine, glutaraldehyde (25% (w/v), 1,6-diamino hexane and all buffer salts were purchased from Sigma (Poole, UK). Human immunoglobulin G (IgG) was obtained from Pharmacia (Uppsala, Sweden). The non-porous, irregular shaped FEP particles (6–8 $m^2 g^{-1}$) were obtained from Du Pont de Nemours (Wilmington, DE, USA).

2.2. Preparation of PVA-FEP support

This was carried out essentially as described previously [4]. FEP particles (14.5 g) were washed in a solution of acetone (250 ml) for 2 h after which the acetone was aspirated and replaced with fresh acetone (100 ml). The FEP particles were then drained on a sinter (grade 2) and added (still wet) to a stirred solution (200 ml) of PVA (M_r 13 000–23 000, 98% hydrolysed) (20 mg ml^{-1}). The PVA was allowed to adsorb for 5 h after which 100 ml of the supernatant was removed and replaced with 100 ml of distilled water. Glutaraldehyde (10 mg ml^{-1}, 5 ml) was then added and the suspension mixed for 30 min while heating to 40°C. HCl (5 *M*, 20 ml) was added and the adsorbed PVA crosslinked for 2 h. The

material was then washed with hot distilled water (60°C, 500 ml) and distilled water (200 ml).

2.3. Activation of PVA-FEP with cyanuric chloride

PVA-FEP (2.5–5 ml settled volume) was washed with ice cold NaOH (1 *M*, 250 ml) over a sintered disc (30 min) after which ice cold cyanuric chloride (50 m*M*) in acetone (250 ml) was poured slowly over the wet particles; this usually took 20–25 min. After activation the PVA-FEP was washed with 10 ml of ice cold distilled water and used immediately.

2.4. Preparation of DEAE-PVA-FEP using 2-(diethylamino)ethylamine

To 10 ml of 5 *M* NaH_2PO_4 was added 2-(diethylamine)ethylamine (3 ml), this resulted in a final pH of 9.6. Ice cold triazine activated PVA-FEP (2.5 ml) was added and the suspension stirred overnight at 40°C and then for 5 h at 60°C. After immobilisation, the DEAE-PVA-FEP was washed on a sinter (grade 2) with distilled water (200 ml), 0.4 *M* NaOH (200 ml) and finally distilled water (200 ml).

2.5. Preparation of SP-PVA-FEP using 3-amino-1-propanesulphonic acid

To 10 ml of 1 *M* Na_2HPO_4 was added 3-amino-1-propanesulphonic acid (0.5 g) which generated a final pH of 9.1. Ice cold triazine activated PVA-FEP (2.5 ml) was added and the suspension stirred overnight at 40°C and then for 5 h at 60°C. After immobilisation, the SP-PVA-FEP was washed on a sinter (grade 2) with distilled water (200 ml), 0.4 *M* NaOH (200 ml) and finally distilled water (200 ml).

2.6. Preparation of 6-aminohexyl PVA and synthesis of trimethylammonium derivative

1,6-Diaminohexane (4 g, 34 mmol) was dissolved in 25 ml of 1 *M* $NaHCO_3$ to give a solution with a final pH of 12. Activated PVA-FEP (4 ml) was added and the suspension stirred at 40°C for 17 h and then at 60°C for 5 h. After this time the material was washed with distilled water (200 ml) and then with 0.1 *M* sodium carbonate (pH 10) before adding to glycidyltrimethyl ammonium chloride (20 ml). The suspension was then heated to 40°C and mixed for 44 h. After immobilisation, the Q-PVA-FEP was washed on a sinter (grade 2) with distilled water (200 ml), NaOH (0.4 *M*, 200 ml) and finally distilled water (200 ml).

2.7. Chromatographic operation

All packed bed chromatography experiments were carried out using an FPLC system (Pharmacia) which comprised LCC-500 controller, two P500 pumps, UV-1 spectrophotometer, FRAC-100, REC-482. Columns used were HR 5/5 (5 mm I.D.). Flow-rates used during single component adsorption tests (frontal analysis) were 1 ml min^{-1} (300 cm h^{-1}) unless stated otherwise using the following buffer systems. For anion-exchange chromatography (DEAE and Q), 20 m*M* sodium phosphate buffer (pH 8.0) was used whereas for cation-exchange chromatography (SP), 20 m*M* sodium phosphate buffer (pH 6.0) was used.

2.8. Protein determination and enzyme assays

For pure protein solutions, concentrations were determined using the appropriate extinction coefficients measured at 280 nm in a Shimadzu UV-160A spectrophotometer (VA Howe, Oxon, UK); lysozyme 2.64 ml mg^{-1} cm^{-1}, human serum albumin, 0.53 ml mg^{-1} cm^{-1}, bovine serum albumin 0.66 ml mg^{-1} cm^{-1}, β-lactoglobulin 1.08 ml mg^{-1} cm^{-1}, ovalbumin 0.71 ml mg^{-1} cm^{-1} and human immunoglobulin G 1.4 ml mg^{-1} cm^{-1} [17]. Protein determination in crude solutions was carried out using the Pierce Coomassie assay reagent using either BSA or ovalbumin as standards.

Lysozyme was assayed by adding enzyme solution (167 μl) to a 1-ml disposable polystyrene cuvette containing a suspension of *Micrococcus Lysodekticus* (0.25 mg ml^{-1}) in 100 m*M* sodium phosphate buffer (pH 6.24) (833 μl). Enzyme activity was measured spectrophotometrically (20°C) at 450 nm by monitoring the reduction in turbidity caused by lysis of the cells by lysozyme. Results are expressed in units of enzyme activity where 1 unit is defined as being that amount of enzyme causing a decease in absorbance of 0.001 per min.

G6PDH was assayed by adding enzyme solution

(33 μl) to a 1-ml disposable polystyrene cuvette containing: 1.27 m*M* glucose-6-phosphate, 0.5 m*M* $NADP^+$ and 6.6 μ*M* $MgCl_2$ in 100 m*M* triethanolamine–HCl (pH 8.0) (967 μl). Enzyme activity was measured spectrophotometrically (20°C) at 340 nm by monitoring the reduction of $NADP^+$ to NADPH. Results are expressed in units of enzyme activity where 1 unit is defined as that amount of enzyme required to convert 1 μmol of substrate to product in 1 min at 20°C.

2.9. Equilibrium adsorption isotherms

Aliquots (0.1–0.2 ml) of settled ion-exchange PVA-FEP (Q, DEAE or SP) equilibrated with relevant buffers were added to a series of test tubes. To each tube was then added protein solution (3 ml, 0.2–6.0 mg ml^{-1}) of either HSA or β-lactoglobulin for DEAE and Q-PVA-FEP and IgG or Lysozyme for SP-PVA-FEP. The tubes were sealed and rotated overnight after which the supernatant was assayed at 280 nm for protein concentration. By mass balance the amount of protein adsorbed onto the ion-exchangers could be determined.

2.10. Purification of lysozyme from hen egg white using SP-PVA-FEP

SP-PVA-FEP was packed into a Pharmacia HR 5/5 column to give a packed bed volume of 1 ml and then irrigated with running buffer (50 m*M* sodium carbonate, pH 9.2) at 1 ml min^{-1} (300 cm h^{-1}) for 15 column volumes. Chicken egg white solution prepared in running buffer and filtered (0.22 μm) was pumped onto the column at the same flow-rate. After 9 ml had been applied, the column was washed with running buffer (5 ml) and bound material eluted with a linear gradient (0–1.0 *M* NaCl, 5 ml). Fractions (1 ml) were collected throughout the experiment and were assayed for total protein and specifically for lysozyme activity.

2.11. Purification of G6PDH from bakers' yeast using Q-PVA-FEP

Bakers' yeast (4.5 g, wet weight) was added to 50 m*M* sodium phosphate (pH 7.0) (4.5 ml) and cooled on ice for 30 min. This suspension was then added to ice-cold ballotini (0.5 mm, 9 ml) and vortex mixed (2×5 min) with an intervening 5 min cooling on ice. The suspension of homogenised cells was removed, centrifuged (8800 *g*, 10 min) and filtered (0.22 μm). To this clarified solution (8 ml) was added 4 ml of buffer (50 m*M* sodium phosphate, pH 7.0) and this was the stock solution used. Q-PVA-FEP was packed into a Pharmacia HR 5/5 column to give a packed bed volume of 1 ml and then irrigated with running buffer (50 m*M* sodium phosphate, pH 7.0) at 1 ml min^{-1} (300 cm h^{-1}) for 15 column volumes. Stock solution (15.2 mg ml^{-1}, 3.45 U ml^{-1}, 0.2 ml) was injected onto the column at 1 ml min^{-1} after which the column was washed with 5 ml of running buffer. Elution of bound protein was carried out using a linear gradient (0–0.5 *M* NaCl, 15 ml) followed by a step change to 1 *M* NaCl. Fractions (1 ml) were collected throughout the experiment and were assayed for total protein (Pierce Comassie reagent) and specifically for G6PDH activity as described above.

3. Results and discussion

3.1. Incorporation of ion-exchange groups onto PVA-FEP

In order to introduce ion-exchange groups onto the PVA coating, the material was first activated with cyanuric chloride at a low temperature to minimise hydrolysis of the reactive groups formed on the PVA. Fig. 1 shows the scheme for this reaction. The PVA-FEP is first incubated with ice cold NaOH to increase nucleophilicity by way of introducing alkoxide groups on the PVA before adding to cyanuric chloride in acetone. The concentration of reactive groups incorporated could be controlled by the initial concentration of cyanuric chloride; with the use of 50 m*M* cyanuric chloride producing a material with 124±22 μmol Cl^- ml^{-1} settled PVA-FEP. Once activated, the material was added to an aqueous solution of the ion-exchanger (DEAE or SP) present in excess at a pH of between 9.0 and 9.5. After incubation, the material was washed with base to hydrolyse any remaining activated groups before washing with water and the desired buffer.

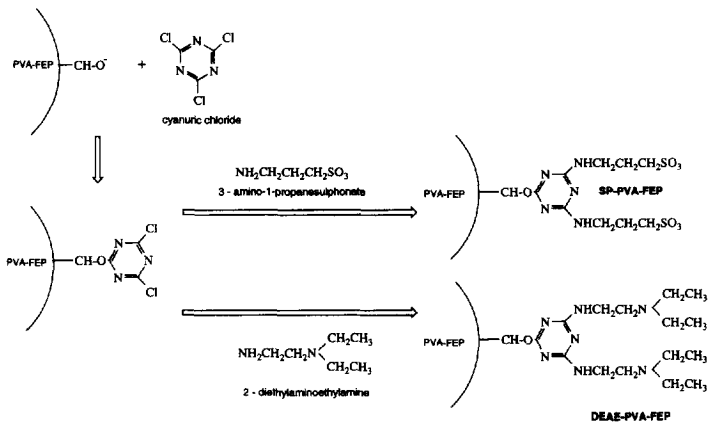

Fig. 1. Synthetic route for the preparation of SP- and DEAE-PVA-FEP as described in Section 2.

For preparation of the material bearing quaternary amine groups, activation with cyanuric chloride was carried out as described above and then 1,6-diaminohexane was used to introduce a nucleophilic group onto the PVA (Fig. 2), this also has the effect of introducing a long spacer arm between the ion-exchange group and the matrix. It is probable that any diamine could be used at this stage in the reaction; 1,6-diaminohexane was used because of immediate availability. This was carried out with the diamine present in a 50-fold molar excess to maximise substitution and to minimise crosslinking of reactive groups. This nucleophilic derivative could then be reacted with glycidyltrimethyl ammonium chloride to produce the quaternary amine derivative.

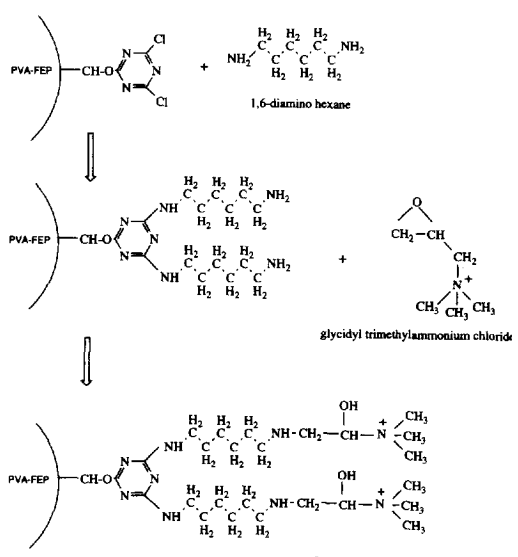

Fig. 2. Synthetic route for the preparation of Q-PVA-FEP as described in Section 2.

The exchange capacity of the DEAE ion-exchanger, determined using Cl^- as a counter ion [16], was found to be 95±15.3 μmol Cl^- ml^{-1} settled bed which corresponds to a coupling efficiency of 77% with regard to initial Cl^- content on the activated material. For SP-PVA-FEP the concentration of immobilised ion-exchange groups, determined using H^+ as a counter ion [16], was found to be 107±11.4 μmol H^+ ml^{-1} settled bed which corresponds to a coupling efficiency of 86%. The exchange capacity of Q-PVA-FEP was again determined using Cl^- as a counter ion and was found to be 84±8.8 μmol Cl^- ml^{-1} settled bed which corresponded to a coupling efficiency of 67%. It is therefore reasonable to assume that in the ion-exchangers formed, the majority were present as bis-substituted derivatives with respect to the charged group with the remaining groups being lost as a result of hydrolysis.

3.2. Adsorption-capacities of perfluoropolymer ion-exchangers

Experiments were carried out to determine the protein binding capacities of all the ion-exchangers produced using frontal analysis and by determining equilibrium adsorption characteristics. An example of a typical breakthrough curve is shown in Fig. 3 for β-lactoglobulin on Q-PVA-FEP. The parameters

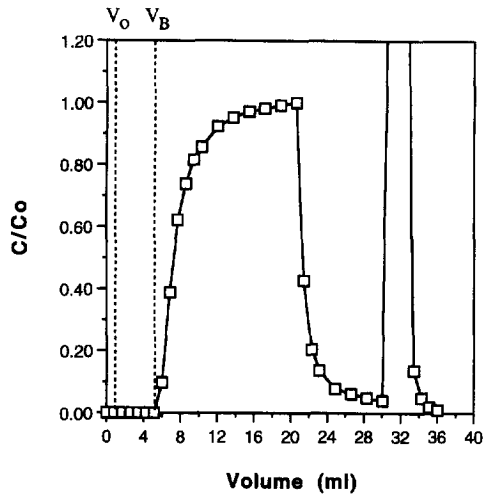

Fig. 3. Frontal analysis of β-lactoglobulin on Q-PVA-FEP. A column (1 ml) of Q-PVA-FEP was irrigated with running buffer (20 m*M* sodium phosphate, pH 8.0) for 10 column volumes before applying β-lactoglobulin (2.0 mg ml^{-1}) in running buffer. The application of β-lactoglobulin was continued until its concentration at the outlet of the column (C) was equal to its inlet concentration (C_0). The column was then washed with running buffer and eluted with 1 *M* NaCl in running buffer. All operations were carried out at 1 ml min^{-1} (300 cm h^{-1}).

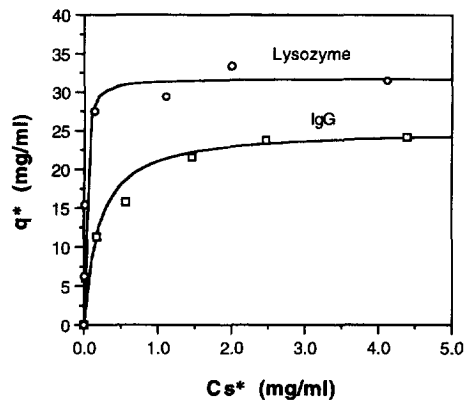

Fig. 4. Equilibrium adsorption isotherms of lysozyme and IgG on SP-PVA-FEP. Aliquots (0.15 ml, settled volume) of ion-exchanger were added to a series of test tubes followed by the addition of either lysozyme or IgG (3 ml) in the concentration range 0.2–6.0 mg ml^{-1}. The tubes were sealed and then rotated overnight after which the supernatant of each tube was assayed at 280 nm. Lines represent best fits to Langmuir isotherms.

obtained from breakthrough experiments were the dynamic capacity of the adsorbent which is defined as the capacity reached when the protein starts to appear in the outlet (V_B). Thus, dynamic capacity in mg ml^{-1} is equal to;

$$\frac{(V_B - V_0)C_0}{V_C}$$

where V_0 is the void volume of the system, V_C is the column bed volume and C_0 is the inlet concentration of protein. The total capacity of the column is defined as the capacity reached when $C/C_0 = 1$ and is calculated by collecting outlet fractions and from a knowledge of the amount of protein added.

A typical equilibrium adsorption isotherm is shown in Fig. 4 for the adsorption of lysozyme and IgG onto SP-PVA-FEP. The curve could be described using the Langmuir isotherm where:

$$q^* = \frac{q_{max} C_s^*}{K_d + C_s^*}$$

where the superscript * denotes equilibrium conditions. For each system, the isotherm was found to be of this type and therefore the equilibrium parameters q_{max} (maximum adsorption capacity) and K_d (dissociation constant) could be calculated by least squares linear regression. These result are presented in Table 1 with the results from the frontal analysis experiments. The dynamic capacity of the ion exchangers ranged from 8.1–23.6 mg ml^{-1} depending on the system and with each adsorbent the highest capacity was exhibited towards the smallest protein. The convoluted and pitted surface of the perfluoropolymer, and the adsorption of the PVA layer may increase the surface area available for binding and impart on the adsorbent a degree of porosity [4]. The overall capacities for the adsorbents are low in comparison with those achieved with conventional ion-exchange adsorbents based on, for example, Sepharose Fast Flow (50–120 mg ml^{-1}) [18], and are also somewhat lower than other agarose based material designed for use in expanded bed adsorption systems (36–63 mg ml^{-1}) [19]. The lower capacity is a reflection of the essentially nonporous nature of the adsorbents. It is interesting that although ionic capacity of the PVA-FEP is similar to soft gel matrices, the adsorption efficiency i.e. mg protein per μmol ionic group is very much lower. We attribute this to small ionic groups having greater access to

Table 1
Dynamic and equilibrium adsorption capacities of proteins on perfluoropolymer ion-exchangers

	Frontal analysis		Adsorption isotherms	
	Dynamic capacity (mg ml^{-1})	Total capacity (mg ml^{-1})	Equilibrium capacity (mg ml^{-1})	K_d (mg ml^{-1})
SP-PVA-FEP				
Lysozyme	23.6	27.6	31.8	0.032
IgG	15.5	21.8	25.2	0.198
DEAE-PVA-FEP				
β-Lactoglobulin	11.8	13.1	14.9	0.054
HSA	7.2	8.1	9.7	0.189
Q-PVA-FEP				
β-Lactoglobulin	12.2	14.8	17.2	0.089
HSA	8.1	11.2	13.5	0.222

Conditions for dynamic capacity estimation were as follows. All columns used were HR 5/5 with packed beds heights of 5 cm. Flow-rates were 1 ml min^{-1} (300 cm h^{-1}) with the proteins present at the following initial concentrations. For SP-PVA-FEP initial concentrations were, lysozyme 1.85 mg ml^{-1}, IgG 2.12 mg ml^{-1}; for DEAE-PVA-FEP initial concentrations were, β-lactoglobulin 1.82 mg ml^{-1}, HSA 1.98 mg ml^{-1} and for Q-PVA-FEP initial concentrations were β-lactoglobulin 2.0 mg ml^{-1} and HSA 2.1 mg ml^{-1}. In each experiment, protein was applied to the column until the outlet concentration (C) was >95% of the inlet concentration (C_0) i.e. $C/C_0>0.95$

available surface area which results from layering of the PVA. This area would may not be available to larger protein molecules. However, as we are interested in the use of these materials in continuous separation devices, the capacity is not as important a characteristic as mechanical stability or selectivity. The recovery of bound protein could also be calculated and is shown in Table 2 for each of the ion-exchangers used. It can be seen from this table that yields of eluted protein were relatively high for each ion-exchanger demonstrating that there was little non-specific binding. Any non-specific binding observed was the result of the chemistries used to introduce ionogenic groups to the PVA rather than either the PVA or the FEP i.e. when coated with PVA the hydrophobic surface of the FEP is effectively masked and displays no non-specific binding. Non-specific binding could probably be reduced by a thorough optimisation of each step used in synthesis.

Table 2
Recovery of eluted protein from perfluoropolymer ion exchangers during frontal analysis

	Recovery (%)	S.D. (%)	n
SP-PVA-FEP			
Lysozyme	93.7	2.9	4
IgG	86.1	5.3	4
DEAE-PVA-FEP			
β-Lactoglobulin	95.3	4.4	8
HSA	92.4	4.5	8
Q-PVA-FEP			
β-Lactoglobulin	92.2	7.6	5
HSA	95.1	1.7	5

Operations carried out as described in Section 2.

3.3. Resolving power of ion-exchange perfluoropolymers

In order to test the resolving power of the ion-exchangers produced, experiments were performed to resolve proteins with small differences in their isoelectric points. In Fig. 5, results are presented for the resolution of cytochrome *c* (p*I* 10.3) and lysozyme (p*I* 11) on SP-PVA-FEP using an optimised gradient. In the top graph, the experiment was carried out at a flow-rate of 1 ml min^{-1} (300 cm h^{-1}) and it can be seen that baseline separation of the proteins is achieved. When the experiment was repeated at a flow-rate of 5 ml min^{-1} (1500 cm h^{-1}), it can be seen that there is only a small loss in resolution of the proteins which demonstrates that the material is able to operate efficiently at high flow-rates. Similar experiments were carried out using Q-PVA-FEP, where the separation of ovalbumin (p*I* 4.7) and β-lactoglobulin a (p*I* 5.1) was carried out (Fig. 6). In the top graph, the separation

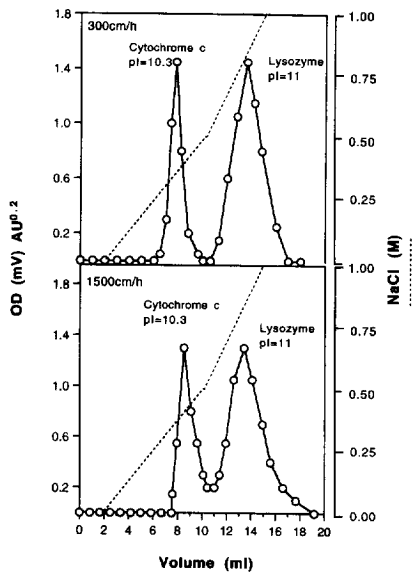

Fig. 5. Resolution of cytochrome *c* and lysozyme on SP-PVA-FEP. A sample (0.5 ml) containing lysozyme (0.5 mg ml^{-1}) and cytochrome *c* (0.4 mg ml^{-1}) was injected onto a column of SP-PVA-FEP at 1 ml min^{-1} (300 cm h^{-1}). The column was washed with 20 m*M* phosphate (pH 6.0) (2 ml) before being eluted with a 3 part gradient, 1st (8 ml) 0–0.5 *M* NaCl in 20 m*M* phosphate (pH 6.0) followed by 2nd (5 ml) 0.5 *M*–1.0 *M* NaCl in 20 m*M* phosphate (pH 6.0) followed by 3rd (3 ml) 1.0 *M* NaCl in 20 m*M* phosphate (pH 6.0). The experiment was then repeated using a flow-rate of 5 ml min^{-1} (1500 cm h^{-1}).

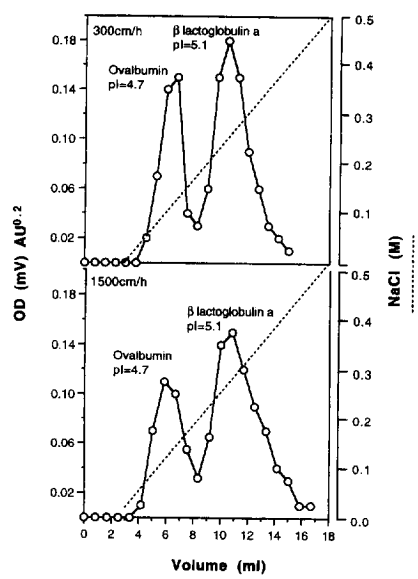

Fig. 6. Resolution of ovalbumin and β-lactoglobulin a on Q-PVA-FEP. A sample (0.1 ml) containing ovalbumin (8 mg ml^{-1}) and β-lactoglobulin (5 mg ml^{-1}) was injected onto a column of Q-PVA-FEP at 1 ml min^{-1} (300 cm h^{-1}). The column was washed with 20 m*M* phosphate (pH 8.0) (3 ml) before being eluted with a gradient, (15 ml) 0–0.5 *M* NaCl in 20 m*M* phosphate (pH 8.0). The experiment was then repeated using a flow rate of 5 ml min^{-1} (1500 cm h^{-1}).

was carried out at 1 ml min^{-1} (300 cm h^{-1}) and it can be seen from this figure that the proteins were resolved quite efficiently. Increasing the flow-rate to 5 ml min^{-1} (1500 cm h^{-1}) did not appear to change the resolution significantly.

3.4. Purification of lysozyme from hen egg white

The purification of lysozyme from hen egg whites was chosen as an example of an cation-exchange purification with which to assess SP-PVA-FEP in a packed bed. Purifications of lysozyme from egg whites using cation-exchange resins have been described previously [20]. But rather than using a low adsorption pH we chose an adsorption pH of 9.2 to increase the selectivity of the ion-exchanger as few proteins in egg white would have isoelectric points above 9.2 where the SP group would still have a negative charge. The chromatogram for this separation is shown in Fig. 7 where it can be seen that there was no breakthrough of lysozyme from the column. Elution of adsorbed protein was carried out using a gradient to 1 *M* NaCl where the protein was eluted in a small volume. The results in Table 3 show that a 50-fold purification of the enzyme was achieved in a yield of 98%, the value of the specific activity 79 926 U mg^{-1} is higher than that obtained with a commercially available pure sample of lysozyme (Sigma, 71 286 U mg^{-1}). In Fig. 8, an SDS-PAGE shows the high purity of the eluted fraction (Lane C) compared with the applied sample (Lane A).

3.5. Purification of G6PDH from homogenised bakers' yeast

G6PDH was purified from a clarified solution of homogenised bakers' yeast using Q-PVA-FEP as a typical example of anion-exchange chromatography. Fig. 9 shows the chromatogram from this purification. G6PDH could be eluted at a salt concentration

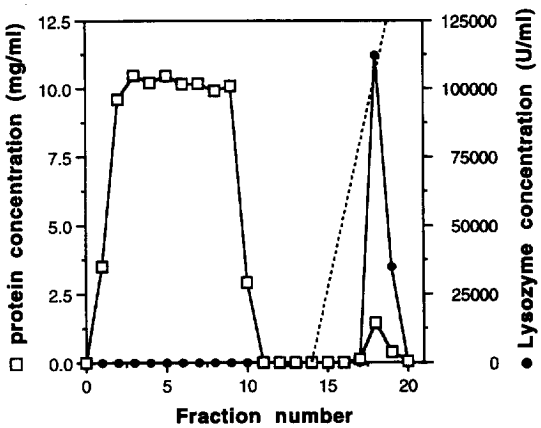

Fig. 7. Purification of lysozyme from hen egg whites using SP-PVA-FEP. A solution (9 ml) of egg whites (10.46 mg ml^{-1}) prepared in 50 m*M* sodium carbonate buffer (pH 9.2) was filtered (0.22 μm) and pumped onto a column (1 ml) of SP-PVA-FEP at 1 ml min^{-1}. The column was then washed with 15 ml of 50 m*M* sodium carbonate buffer (pH 9.2) after which protein was eluted using a linear gradient 0–1.0 *M* NaCl in 50 m*M* sodium carbonate buffer (pH 9.2) (5 ml). Fractions (1 ml) were collected from the column and were assayed for total protein content (Pierce Coomassie assay) and specifically for lysozyme activity as described in Section 2.

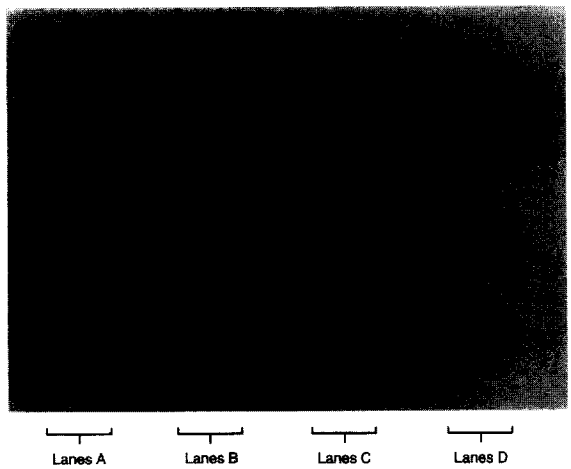

Fig. 8. SDS-PAGE of lysozyme purification from hen egg whites using SP-PVA-FEP. SDS-PAGE was carried out using a Pharmacia PhastSystem with Gradient 8–25 gels and SDS buffer strips. Lanes A = applied hen egg white solution, Lanes B = flow through, Lanes C = eluted fraction (1 *M* NaCl) and Lanes D = commercial lysozyme (Sigma).

of 0.13–0.26 *M* NaCl in a volume of 4 ml. The results in Table 4 show that a purification factor of 14.3 was obtained with an 81% recovery of enzyme activity.

4. Conclusions

FEP adsorbents have shown great promise in downstream processing especially for use in expanded beds and continuous reactors. In this paper we have described ways to prepare ion-exchange adsorbents using FEP materials to increase their scope of application in bioseparations. There are several ways to introduce ionogenic groups onto polyhydroxyl matrices, most of which involve direct attachment or involve grafting or copolymerising charged monomers. We decided, for simplicity, to use a direct approach; however, as most of the hydroxyl groups on PVA are present as secondary hydroxyls, which are relatively unreactive, we introduced an electrophilic spacer (cyanuric chloride) on the PVA layer. This could then be reacted with nucleophilic ligands to generate the desired support. Both DEAE and SP groups are available in primary amine derivatives and could therefore be used. In the case of the quaternary amine it was difficult to locate

Table 3
Purification of lysozyme from crude hen egg white using SP-PVA-FEP

Stage	Volume (ml)	Protein (mg)	Lysozyme (U)	Activity (U mg^{-1})	Yield (%)	Purification (fold)
Load	9	94.14	150 134	1594	(100)	(1)
Elute	2	1.84	147 065	79 926	98	50

SP-PVA-FEP was packed into an HR 5/5 column to give a packed bed volume of approximately 1 ml. The column was equilibrated with 50 m*M* sodium carbonate buffer (pH 9.2) at a flow-rate of 1 ml min^{-1} (300 cm h^{-1}) for 15 bed volumes after which crude hen egg white (10.46 mg ml^{-1}, 9 ml) was pumped onto the column at the same flow-rate. After washing, (5 ml) the lysozyme was eluted (1 ml min^{-1}, 300 cm h^{-1}) using a linear gradient 0–1.0 *M* NaCl in 50 m*M* sodium carbonate (pH 9.2) (5 ml). Fractions (1 ml) were collected throughout the experiment and were assayed for total protein and specifically for lysozyme activity as detailed in Section 2.

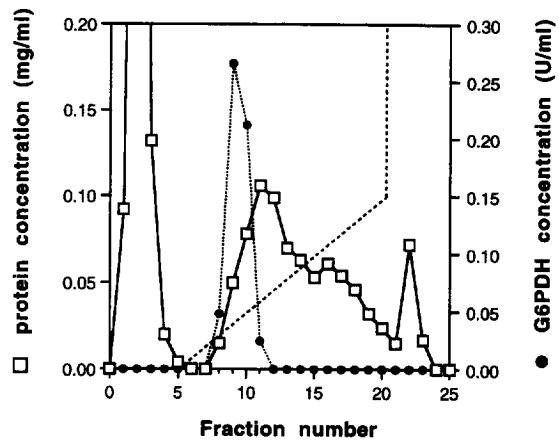

Fig. 9. Purification of G6PDH from bakers' yeast using Q-PVA-FEP. A solution (0.2 ml) of clarified bakers' yeast homogenate (15.2 mg ml^{-1}, 3.45 U ml^{-1}) prepared in 50 m*M* sodium phosphate buffer (pH 7.0) was injected onto a column (1 ml) of Q-PVA-FEP at 1 ml min^{-1}. The column was then washed with 5 ml of 50 m*M* sodium phosphate buffer (pH 7.0) after which protein was eluted using a linear gradient 0–0.5 *M* NaCl in 50 m*M* sodium phosphate buffer (pH 7.0) (15 ml) followed by a step change to 1.0 *M* NaCl in 50 m*M* sodium phosphate buffer (pH 7.0). Fractions (1 ml) were collected from the column and were assayed for total protein content (Pierce Coomassie assay) and specifically for G6PDH activity as described in Section 2.

a suitable nucleophilic compound. Glycidyl trimethyl ammonium chloride has been used previously to generate Q-type ion-exchange adsorbents based on PVA [21] but unless extremely harsh conditions are used we observed the degree of substitution, based on the base-catalysed Williamson synthesis with secondary hydroxyl groups, to be very low. In order to increase the degree of incorporation we used an intermediate step whereby triazine activated PVA perfluoropolymer was incubated with a diamino spacer. This had the effect of increasing the reactivity of the material towards the epoxy group of glycidyl trimethyl ammonium chloride.

Our procedures for introducing ionogenic groups onto PVA-FEP resulted in materials with exchange capacities ranging from 87–107 μmol ml^{-1} and show that our method is quite effective. Capacities of the ion-exchangers were in the range 9.7–32 mg ml^{-1} which although low in comparison to soft gel materials are comparable with capacities of dye–ligand perfluoropolymer supports [1] and were higher than those exhibited by other PVA-FEP ion-exchangers (1.68–26 mg ml^{-1}) [16] where polymer grafting techniques had to be used to achieve reasonable capacities and recoveries of protein. The high mechanical strength of the perfluoropolymer compensates for its low capacity and allows it to be used in continuous reactors where most other chromatographic supports would disintegrate rapidly [7]. To be useful in continuous separation devices which deploy pumping as a means of transporting adsorbent from various contacting stages, an ideal adsorbent would display a high resistance to attrition. In addition, our counter current contactors require that the adsorbent material be sufficiently dense as to allow the use of expanded bed adsorption as a means of contacting. At present, perfluoropolymer particles are probably the only materials available with these characteristics. The ion-exchangers synthesised were able to resolve proteins with only small differences in their p*I* values demonstrating that there were few or no mixed interactions in the ion-exchangers synthesized. In terms of purification, both SP- and Q-PVA FEP were able to purify enzymes with purification factors of between 14 and 50 fold, which

Table 4
Purification of G6PDH from bakers yeast using Q-PVA-FEP

Stage	Volume (ml)	Protein (mg)	G6PDH (U)	Activity (U mg^{-1})	Yield (%)	Purification (fold)
Load	0.2	3.04	0.69	0.23	(100)	(1)
Elute	4	0.17	0.56	3.3	81	14.3

Q-PVA-FEP was packed into an HR 5/5 column to give a packed bed volume of approximately 1 ml. The column was equilibrated with 50 m*M* sodium phosphate buffer (pH 7.0) at a flow-rate of 1 ml min^{-1} (300 cm h^{-1}) for 15 bed volumes after which clarified (8800 *g*, 5 min; filtered 0.22 μm) bakers' yeast homogenate (15.2 mg ml^{-1}, 3.45 U ml^{-1}, 0.2 ml) was pumped onto the column at the same flow-rate. After washing, (5 ml) the adsorbed protein was eluted (1 ml min^{-1}, 300 cm h^{-1}) using a linear gradient 0–0.5 *M* NaCl in 50 m*M* sodium phosphate (pH 7.0) (15 ml) followed by a step change to 1.0 *M* NaCl. Fractions (1 ml) were collected throughout the experiment and were assayed for total protein and specifically for G6PDH activity as detailed in Section 2.

are common figures for ion-exchange chromatography.

In this paper, preliminary results have been provided which demonstrate that PVA-FEP supports can be synthesised with ion-exchange groups covalently attached. A more detailed investigation is currently underway to try and reduce non-specific binding and to more fully characterise the adsorption characteristics of these promising adsorbents.

References

[1] D.J. Stewart, D.R. Purvis, C.R. Lowe, J. Chromatogr. 510 (1990) 177.
[2] J.W. Eveleigh, presented at the 7th International Conference on Affinity Chromatography, Oberammergau, Germany, August 17–21, 1987.
[3] G.E. McCreath, H.A. Chase, C.R. Lowe, J. Chromatogr. A 659 (1994) 275.
[4] G.E. McCreath, H.A. Chase, R.O. Owen, C.R. Lowe, Biotechnol. Bioeng. 48 (1995) 341.
[5] G.E. McCreath, H.A. Chase, Biotechnol. Prog. 12 (1996) 77.
[6] G.E. McCreath, H.A. Chase, J. Mol. Recognit. 9 (1996) 575–584.
[7] R.O. Owen, G.E. McCreath, H.A. Chase, J. Mol. Recognit. 9 (1996) 607–616.
[8] E. Boschetti, J. Chromatogr. 658 (1994) 207.
[9] P.R. Levison, S.E. Badger, R.M.H. Jones, D.W. Toome, M. Streater, N.D. Pathirana, S. Wheeler, J. Chromatogr. 702 (1995) 59.
[10] E.A. Peterson, H.A. Sober, J. Amer. Chem. Soc. 78 (1956) 751.
[11] W. Muller, J. Chromatogr. 510 (1990) 133.
[12] O. Mikes, P. Strop, J. Zbrozek, J. Coupek, J. Chromatogr. 180 (1979) 17.
[13] O. Mikes, P. Strop, Z. Hostomska, M. Smrz, J. Coupek, A. Frydrychova, M. Bares, J. Chromatogr. 261 (1983) 363.
[14] O. Mikes, P. Strop, Z. Hostomska, M. Smrz, S. Slovakova, J. Coupek, J. Chromatogr. 301 (1984) 93.
[15] J. Coupek, I. Vins, J. Chromatogr. A. 654 (1994) 391.
[16] I.D. Pitfield, PhD Thesis, University of Cambridge, 1992.
[17] D.M. Kirschenbaum, Handbook of Biochemistry and Molecular Biology, Vol. 2, CRC Press, Cleveland, OH, 3rd edition, 1976.
[18] Pharmacia Information Booklet, Pharmacia Biotech, Uppsala, Sweden.
[19] R. Hjorth, S. Kampe, M. Carlsson, Bioseparation 5 (1995) 217.
[20] E. Li-Chan, S. Nakai, J. Sim, D.B. Bragg, K.V. Lo, J. Food Sci. 51 (1986) 1032.
[21] J. Yu, Z. El Rassi, J. High. Res. Chromatogr. 17 (1994) 705.