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Novel affinity separations based on perfluorocarbon emulsions

Use of a perfluorocarbon affinity emulsion for the purification of human serum albumin from blood plasma in a fluidised bed

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ABSTRACT

A perfluorocarbon affinity emulsion has been generated by homogenisation of a saturated perfluorocarbon oil with a polymeric fluorosurfactant based on poly(vinyl alcohol) (relative molecular mass 9000–10 000) previously derivatised with the triazine dye CI Reactive Blue 4. This affinity emulsion has subsequently been cross-linked *in situ* and used in a fluidised bed for the purification of human serum albumin (HSA) from blood plasma. HSA was quantitatively recovered in a semi-continuous fashion from plasma at an average purity of 90 ± 3.3%. The albumin binding capacity of the emulsion has been shown to be 0.59 mg/ml by frontal analysis corresponding to a mol/mol ligand usage of 13.5%. In all regards, when used in a fluidised bed, the emulsions have been shown to behave as a normal chromatographic material. They are stable under operational conditions with no coalescence being observed for periods greater than 1 year. These novel liquid affinity supports present an exciting opportunity to develop a range of unit operations for the continuous purification of proteins.

INTRODUCTION

Affinity chromatography exploits the natural specific recognition between biological molecules [1]. Because of the high specificity offered, affinity chromatography is increasingly being recognised as the method of choice for the purification of high-value proteins [2]. The technique of affinity chromatography is usually carried out by immobilising the affinity ligand onto an inert, hydrophilic solid support and using this support in a column to purify the desired compound. However, certain limitations are imposed by both the support and the usual column mode of operation. The support, usually a macroporous polysaccharide bead such as agarose, is limited in its stability at the high pH ranges needed for efficient sterilisation and depyrogenation. Fixed-bed operations, although still the most popular contacting technique, are somewhat prone to fouling and the entry streams have to be free from any particulates. These problems necessitate the incorporation of a filtration or centrifugation step upstream of the column. This is both expensive in terms of equipment and processing time and ultimately leads to a lower recovery of the protein. In this report we describe the development of an affin-

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ity support based on a liquid perfluorocarbon emulsion and the application of these emulsions in the purification of proteins.

Perfluorocarbons are synthetic molecules comprised solely of carbon and fluorine atoms. They are characterised as being chemically and biologically inert, have a high density and are almost completely insoluble in aqueous and organic solvents. Solid perfluorocarbons have been used as packings for reversed-phase high-performance liquid chromatography [3], as adsorbents for the extraction of organic pollutants [4] and as stationary phases in gas–liquid chromatography [5]. Because of their propensity to dissolve large amounts of respiratory gases [6], perfluorocarbon liquids and emulsions have been used as oxygen transport fluids. Subsequently, they have been used to supply oxygen to microorganisms in culture [7] and to immobilised microbial cells in bioreactors [8]. However, until recently the inertness and hydrophobic character of perfluorocarbon surfaces has precluded their use in affinity chromatography where hydrophilicity and ease of derivatisation are deemed desirable features [9]. Perfluorocarbons are wetted in water only in the presence of fluorosurfactants which adsorb to the surface [10]. The preparation of affinity labelled surfactants and their adsorption to solid and liquid perfluorocarbon surfaces has been described [11]. By the synthesis of a perfluoroalkyl derivative of the dichlorotriazine dye CI Reactive Blue 4, rabbit muscle lactate dehydrogenase was successfully purified on both solid and liquid perfluorocarbons. The perfluorocarbon adsorbents were found to be stable in a range of acids, bases and salt solutions with no ligand leakage being detected. However, ligand leakage was detected in the presence of certain aprotic solvents and albumin solutions indicating that the dye was insufficently anchored. Stronger anchorage was obtained when the neutral polymer poly(vinyl alcohol) (PVA) was derivatised with fluoroalkyl groups and used to coat the perfluorocarbon surface [12]. This produced a very stable adsorbent that successfuly purified rabbit muscle lactate dehydrogenase in 100% yield with an 8-fold purification. This report describes the use of similar technology to generate perfluorocarbon affinity emulsions and the use of such liquid affinity droplets for the purification of human serum albumin (HSA) from human plasma.

EXPERIMENTAL

Materials

PVA (relative molecular mass, M_r 9000–10 000, 80% hydrolysed) was purchased from Aldrich (Gillingham, UK). Perfluorodecalin (Flutec PP6) was obtained from ISC Chemicals (Avonmouth, Bristol, UK). The Coomassie protein assay reagent was purchased from Pierce (Luton, UK). The diagnostic reagent kit for serum albumin determination was purchased from Sigma (Poole, UK) as were the chemicals sodium dihydrogen phosphate, disodium hydrogenphosphate 2-mercaptoethanol and glutaraldehyde (25%, w/v). Pure HSA was also purchased from Sigma, while human plasma was obtained from a known donor at the National Blood Transfusion Centre (Nottingham, UK) and tested negative for HIV III, HBS antigen and syphilis. All other chemicals were purchased from BDH. All solvents used were of analytical grade.

Synthesis of polymeric fluorosurfactant–dye conjugate

PVA (M_r 9000–10 000, 80% hydrolysed, 5 g) was dissolved in distilled water (120 ml) by heating to 70–80°C for 20–30 min. The solution was cooled and triazine dye CI Reactive Blue 4 (2.5 g) added and the solution stirred for 10–15 min at room temperature. The reaction mixture was heated to 70°C with the addition of sodium chloride (2.5 g), and sodium carbonate (2.5 g) and refluxed for 3 h. Sodium hydroxide (3 g) was added and refluxing continued for a further 2 h. Dyed polymeric surfactant was purified by Sephadex G-25 gel filtration (bed volume 120 ml) with the major band being collected. Alternatively, dyed polymeric surfactant could be recovered by precipitation with sodium chloride. The precipitate was washed (× 5) with cold water, dissolved in hot water (60°C, 100 ml) and then dialysed overnight before being lyophilised. Purity of dyed polymeric surfactant could be assessed by thin-layer chromatography (TLC) (ethylmethyl ketone–water–butan-1-ol–acetone, 50:30:30:20, v/v). The extent of dye substitution was calculated by dissolving dyed polymeric surfactant (10 mg) in distilled water (10 ml) and by measuring the molarity of dye, spectrophotometrically at peak absorbance (620 nm for CI Reactive Blue 4), and, by re-iterative calculation estimating the molarity of dye against

weight of conjugate. Molar extinction coefficients of unbound and bound dye were assumed to be equal.

Generation of perfluorocarbon affinity emulsions (liquid affinity supports)

Perfluorodecalin (25 g) was homogenised with a solution of dyed polymeric fluorosurfactant (75 ml, 25 mg/ml) in a cylindrical glass vessel with an aspect ratio of 2.25. Homogenisation was carried out for 1 min using an Ultra-Turrax T-25 homogeniser (half speed). A sample of the resulting emulsion was analysed for droplet size distribution using an Elzone 280 PC particle sizer linked to a Hermes H220 PC for data analysis. The dyed polymeric surfactant adsorbed to the perfluorocarbon droplet was then cross-linked.

Optimisation of cross-linking reaction

The adsorbed layer of polymeric fluorosurfactant-dye conjugate as cross-linked using glutaraldehyde with HCl was the catalyst [13]. An investigation was carried out to determine the optimal conditions and times for cross-linking. A solution of polymeric surfactant (10 mg/ml) (6 ml) was incubated at 40°C in a thermostatted water bath. To this was added HCl (5 *M*) to the desired final concentration and glutaraldehyde (25%, w/v) to the final desired mole ratio (glutaraldehyde:polymeric fluorosurfactant). The reaction could be followed by monitoring changes in turbidity as the reaction proceeded. At time intervals the reaction tube was removed from the water bath and placed into the nephelometer for turbidity measurement.

Determination of immobilised ligand concentrations

Immobilised ligand concentration was determined by taking an aliquot of non-cross-linked emulsion (10 ml) and placing it in an oven (200°C, 2 h). After evaporation of the aqueous and perfluorocarbon constituents of the emulsion, water (5 ml) was added to the dried polymeric fluorosurfactant–dye conjugate which was dissolved. The solution was assayed spectrophotometrically at the peak absorbance of the ligand (620 nm). The immobilised ligand concentration could then be expressed as a molarity per ml of settled emulsion.

Protein determination and assay

Protein determination was routinely carried out using the Pierce Coomassie protein assay reagent. Protein concentrations in stock solutions were initially determined by absorbance at 280 nm assuming an extinction coefficient of 0.53 ml mg^{-1} cm^{-1} [14]. Serial dilutions of stock solution (20 μl) were incubated with assay reagent (1 ml) by mixing for 10 min at room temperature. The absorbance was then read at 595 nm against a buffer blank to prepare a standard curve.

Serum albumin content in plasma was determined using the bromocresol green assay. Diluted (1 in 4 with buffer) and filtered (0.45 μm) plasma samples (100 μl) were added to the standard assay reagent (1 ml) and mixed at room temperature for 10 min followed by measuring the absorbance at 628 nm against an appropriate buffer blank.

Bed expansion test for liquid affinity supports

A perfluorocarbon affinity emulsion was produced based on M_r 9000–10 000 PVA (80% hydrolysed) derivatised with CI Reactive Blue 4 (0.95 mol dye/mol PVA) ($6.5 \cdot 10^{-8}$ mol/ml settled emulsion) and cross-linked with glutaraldehyde (10:1 mole ratio). A suspension of emulsion in water was poured into a 2 cm internal diameter column and allowed to settle (settled bed height 4.8 cm, 15.1 ml). The adsorbent bed was fluidised with sodium phosphate buffer (20 m*M*, pH 5.0) at flow-rates up to 0.5 ml/min (0.16 cm/min) with the expanded bed height being recorded at each incremental flow-rate. The bed was also washed by this procedure until washings were clear of free dyed polymer, as determined spectrophotometrically at 620 nm.

Frontal analysis of HSA

Using the same system as above the perfluorocarbon affinity emulsion was fluidised at a volumetric flow-rate of 0.5 ml/min (0.16 cm/min) with 20 m*M* sodium phosphate buffer, pH 5.0 giving an expanded bed height of 9.9 cm (31.1 ml). HSA (2 mg/ml) was injected onto the column at the same flow-rate (0.5 ml/min) until the concentration of HSA at the outlet (C) has reached the concentration of HSA at the inlet (C_0) (*i.e.* $C/C_0 = 1$). The bed was washed with sodium phosphate buffer (20 m*M*, pH 5.0) un-

til $C/C_0 = 0$ and bound HSA eluted with 0.5 *M* sodium thiocyanate in 20 m*M* phosphate buffer, pH 8.0.

Semi-continuous purification of HSA from blood plasma

A perfluorocarbon affinity emulsion incorporating the triazine dye CI Reactive Blue 4 ($6.5 \cdot 10^{-8}$ mol/ml) was poured into a 1 cm internal diameter column (settled bed height 6.37 cm, 5 ml) and fluidised with 20 m*M* sodium phosphate buffer, pH 5.0 (0.25 ml/min, 0.32 cm/min) to give an expanded bed height of 14.0 cm (11 ml). A 300-μl sample of human plasma was injected onto the column at the same flow-rate. The bed was washed with phosphate buffer (20 m*M*, pH 5.0) (20 ml) and HSA eluted with 0.5 *M* sodium thiocyanate in 20 m*M* sodium phosphate buffer, pH 8.0. The bed was reequilibrated with sodium phosphate buffer (20 m*M*, pH 5.0) (20 ml) and the cycle repeated. This procedure was carried out until a total of 6 cycles were achieved. Fractions were collected throughout and were assayed for total protein and HSA.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out essentially as described by Laemmli [15] using a Pharmacia PhastSystem horizontal electrophoresis unit. Gels used were PhastGel Gradient 8-25 (13 mm stacking gel zone, 32 mm gradient gel zone), 2% cross-linking, used in conjunction with PhastGel SDS Buffer Strips (0.55%, w/v, SDS). Protein samples (1 μl) were loaded and the gels stained with PhastGel Blue R (Coomassie R 350). Gels were stored in a preserving solution containing 10% (v/v) glycerol and 10% (v/v) acetic acid in water.

RESULTS AND DISCUSSION

Gel filtration was found to be an effective method for purifying the polymeric fluorosurfactant–dye conjugate with TLC analysis showing one unresolved band (PVA is immobile on silica wetted with this solvent system). Similarly, precipitation of the polymeric fluorosurfactant–dye conjugate followed by exhaustive dialysis lead to one unresolved band on the TLC plate, consequently both methods could be used for purification of the conjugate.

The extent of dye substitution on the PVA (M_r 9000–10 000, 80% hydrolysed) was estimated to be 0.95:1 (dye:PVA, molar ratio). This result is similar to that observed by Stewart *et al.* [12] who postulated that dye substitution occurs via the more reactive

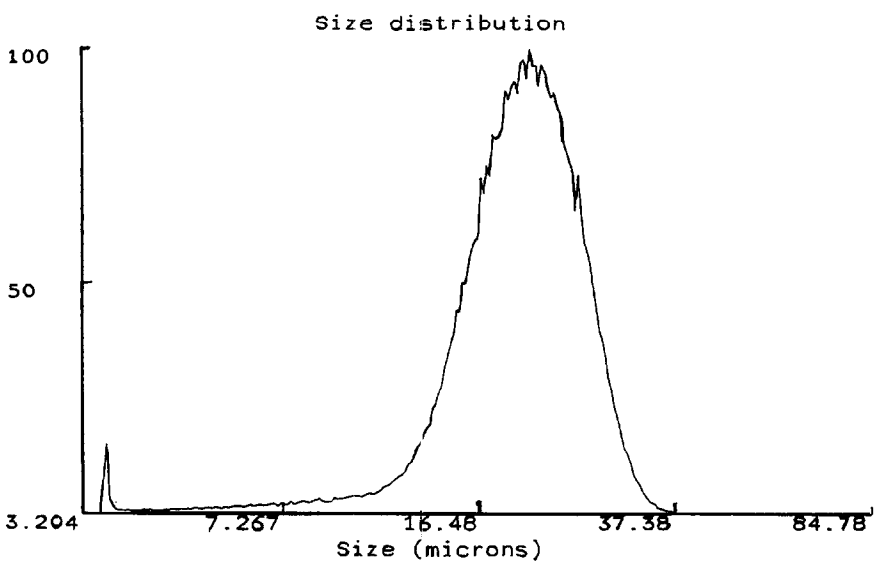

Fig. 1. Size distribution curve for the generation of perfluorocarbon affinity emulsions. Perfluorodecalin (25 g) was homogenised with dyed polymeric fluorosurfactant (75 ml) (25 mg/ml) for 1 min in an Ultra-Turrax T-25 homogeniser. The scale on the y-axis represents the number of droplets.

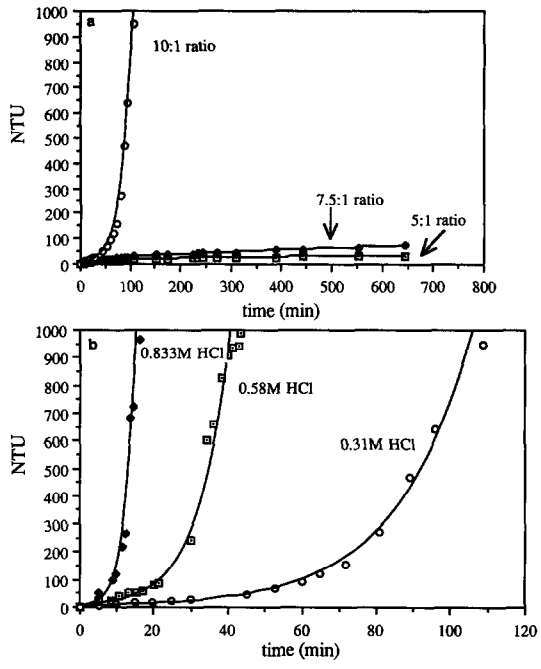

Fig. 2. (a) Effect of glutaraldehyde:polymeric fluorosurfactant ratio on the rate of cross-linking. A solution of surfactant (10 mg/ml) was incubated at 40°C. To this was added HCl to a final concentration of 0.31 *M* and glutaraldehyde (25%, w/v) to the desired mole ratio. Turbidity measurement were taken at time intervals and are expressed as Nephelomeric Turbidity Units (NTU). (b) Effect of HCl catalyst concentration on the rate of cross-linking. A solution of polymeric fluorosurfactant was incubated at 40°C. To this was added glutaraldehyde (25%, w/v) to give a final mole ratio (glutaraldehyde:polymeric fluorosurfactant) of 10:1. HCl (5 *M*) was added to give the final desired concentration. Turbidity measurements were taken at time intervals and are expressed as Nephelometric Turbidity Units (NTU).

primary hydroxyl groups. We have found that on average between 1 and 2 dye molecules are substituted per molecule of PVA depending on the reaction time.

Fig. 1 shows that on homogenisation (15%, v/v, perfluorodecalin) droplets of stabilised oil were produced with a size distribution of between 10 and 37 μm (diameter) and with a mean diameter of 20 $\pm$ 5 μm. By varying the homogenisation speed and time, some measure of control could be obtained over the range of sizes of emulsion droplets produced. However, the larger sizes of droplets (>40 μm) were prone to coalescence when agitated and were deemed unsuitable for further investigation. With a mean diameter of 20 μm, the surface area produced was 0.3 m^2/ml settled emulsion. The immobilised ligand concentration was determined to be 6.5 · 10^{-8} mol/ml settled emulsion. The immobilised ligand concentration was usually in the range 4.5 · 10^{-8} to 9.4 · 10^{-8} mol/ml settled emulsion depending on the degree of substitution and the surface area of the emulsion.

Fig. 2a presents the data for cross-linking a solution of polymeric fluorosurfactant (10 mg/ml) (6 ml) with varying mole ratios of glutaraldehyde to polymeric fluorosurfactant. The results show that at a constant HCl concentration of 0.31 *M* and at 40°C, crosslinking was most rapid at the highest mole ratio used (10:1). Reactions carried out at mole ratios of glutaraldehyde:polymeric fluorosurfactant of 7.5:1 and 5.0:1 showed very limited cross-linking even after a period of 10 h. Fig. 2b presents the data for the cross-linking reaction carried out at a constant mole ratio (glutaraldehyde:polymeric fluorosurfactant) of 10:1 and at a constant temperature of 40°C while varying the concentration of HCl catalyst. The reaction was observed to be most rapid at the highest concentration of HCl used (0.833 *M*) which showed precipitation of the polymeric fluorosurfactant network after only 12 min. Based on these observations all subsequent reactions to cross-link the adsorbed polymeric fluorosurfactant–dye conjugate on the perfluorocarbon surface were carried out at a mole ratio of 10:1 (glutaraldehyde:polymeric fluorosurfactant–dye conjugate) at 0.31 *M* HCl and at 40°C for 1 h. This was found to give a stable cross-linked droplet with-

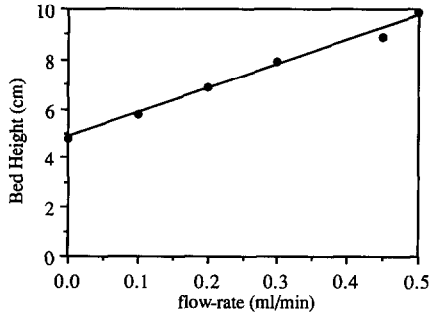

Fig. 3. Bed expansion test for perfluorocarbon affinity emulsion. A slurry of perfluorocarbon affinity emulsion in water was poured into a 2 cm internal diameter column and fluidised at flow-rates up to 0.5 ml/min (0.16 cm/min). The expanded bed height was recorded at each incremental flow-rate.

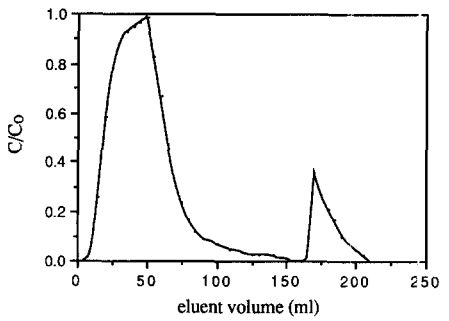

Fig. 4. Determination of dynamic binding capacity for serum albumin by frontal analysis on CI Reactive Blue 4 perfluorocarbon affinity emulsion. Settled bed height 4.8 cm (15.1 ml) fluidised at 0.5 ml/min giving an expanded bed height of 9.9 cm (31.1 ml). Immobilised dye concentration $6.5 \cdot 10^{-8}$ mol/ml settled emulsion; running buffer 20 m*M* sodium phosphate, pH 5.0; eluting buffer 0.5 *M* sodium thiocyanate in 20 m*M* sodium phosphate buffer, pH 8.0; loading 2 mg/ml pure serum albumin, fraction V, Sigma, 9.0 mg bound, 9.0 mg eluted. Protein determined by absorbance of fractions at 280 nm (diluted if required).

out inclusion of free aldehyde groups which may lead to the non-specific adsorption of proteins. The cross-linking reaction could be terminated by the addition of sodium hydroxide to 0.5 *M*.

Fig. 3 shows how a bed of affinity droplets expands as it is fluidised with an upward flow of buffer. The graph shows that the bed expansion is linear with increasing volumetric flow-rate. At the maximum flow-rate used (0.5 ml/min), the bed expanded to 9.9 cm which was 2.1 times the settled bed height. Because of the liquid nature of the adsorbent column operations are restricted to the use of fluidised beds. Use of a fixed bed would compress the droplets into polyhedra with eventual rupture of the interfacial film and emulsion breakage.

The chromatographic behaviour of the perfluorocarbon affinity emulsion was examined through frontal analysis. Fig. 4 shows the chromatogram obtained for determining the dynamic loading capacity for HSA on a fluidised bed containing an emulsion incorporating the ligand CI Reactive Blue 4 ($6.5 \cdot 10^{-8}$ mol/ml settled emulsion); 9.0 mg of HSA bound to the fluidised adsorbent. This was quantitatively eluted with 0.5 *M* sodium thiocyanate (in 20 m*M* sodium phosphate buffer, pH 8.0) in 100% yield giving a dynamic binding capacity of 0.59 mg/ml bound protein/ml settled emulsion. This corresponds to a ligand usage of 13.5% on a molar basis, *i.e.* 0.135 mol albumin bound/mol CI Reactive Blue 4.

Table I presents the data for the semi-continuous purification of serum albumin by chromatography of human plasma on a fluidised bed of the perfluorocarbon affinity emulsion droplets ($6.5 \cdot 10^{-8}$ mol/ml). Over the 6 cycles carried out there was no observable variation in either the purity of HSA obtained or the purification factor. Table II presents the data for pooled and averaged fractions. Albumin was quantitatively recovered at 91% purity and 87% yield and with a purification factor of 1.44. Fig. 5 shows the SDS-PAGE analysis of the puri-

TABLE I

SEMI-CONTINUOUS PURIFICATION OF SERUM ALBUMIN FROM HUMAN PLASMA USING CI REACTIVE BLUE 4 PERFLUOROCARBON AFFINITY EMULSION

Albumin was purified from dilute plasma (1:4) in a semi-continuous fashion using a fluidised bed of CI Reactive Blue 4 perfluorocarbon affinity emulsion. Column volume, 5 ml settled, 11 ml when fluidised at 0.25 ml/min; immobilised dye concentration $6.5 \cdot 10^{-8}$ mol/ml; running buffer 20 m*M* sodium phosphate, pH 5.0; eluting buffer 0.5 *M* sodium thiocyanate in 20 m*M* sodium phosphate, pH 8.0; applied sample, 6×300 µl. Protein measured by the Pierce Coomassie assay; albumin detected by the bromocresol green assay.

	Purification (cycle)number					
	1	2	3	4	5	6
Total protein collected (mg)	3.42	3.27	3.70	3.20	3.35	3.31
Recovery (%)	95	91	103	89	93	92
Protein present in elution (mg)	2.22	2.13	2.41	2.08	2.17	2.15
HSA present in elution (mg)	2.02	1.89	2.28	1.77	1.99	1.95
HSA purity (%)	91	89	95	85	92	91
Purification factor	1.40	1.37	1.46	1.31	1.41	1.40

TABLE II

PURIFICATION TABLE FOR POOLED AND AVERAGED FRACTIONS FOR THE SEMI-CONTINUOUS PURIFICATION OF SERUM ALBUMIN FROM HUMAN PLASMA USING CI REACTIVE BLUE 4 PERFLUOROCARBON AFFINITY EMULSION

Assays carried out as described in the text.

Stage	Total protein (mg)	Albumin (mg)	Albumin content (%)	Yield (%)	Purification (fold)
Crude sample	3.6	2.27	63	100	1
Average fractions	2.19	1.98	91	87	1.44

fication and clearly demonstrates that the eluent fraction (lane A) is of comparable purity to that of a commercially available pure HSA sample (lane C).

CONCLUSION

In this report we have described the development of a novel affinity support based on a stabilised perfluorocarbon emulsion droplet. The emulsions have been shown to behave as normal chromatographic materials when operated in a fluidised bed and have capacities similar to conventional affinity supports of equivalent surface area. Furthermore, ligand immobilisation can be carried out by using well established techniques. In this report the triazine dye CI Reactive Blue 4 was immobilised; we are now extending this work to the immobilisation of proteinaceous ligands by techniques similar to those developed for solid perfluoropolymer supports [16].

Because of their non-porous nature adsorption and desorption kinetics are rapid allowing for short contacting times between adsorbent and target molecule. By exploiting the fast kinetics, together with their inherent transportability (as a result of their liquid nature) and high density, perfluorocarbon affinity emulsions can be used for the continuous purification of biomolecules if suitable contacting reactors can be designed.

Perfluorocarbon affinity emulsions present a novel alternative to conventional affinity supports for the purfication of biomolecules. However their use in biotechnology is not restricted to bioseparations since their use in bioreactors and in continuous immunoassays and flow-injection analysis may be envisaged.

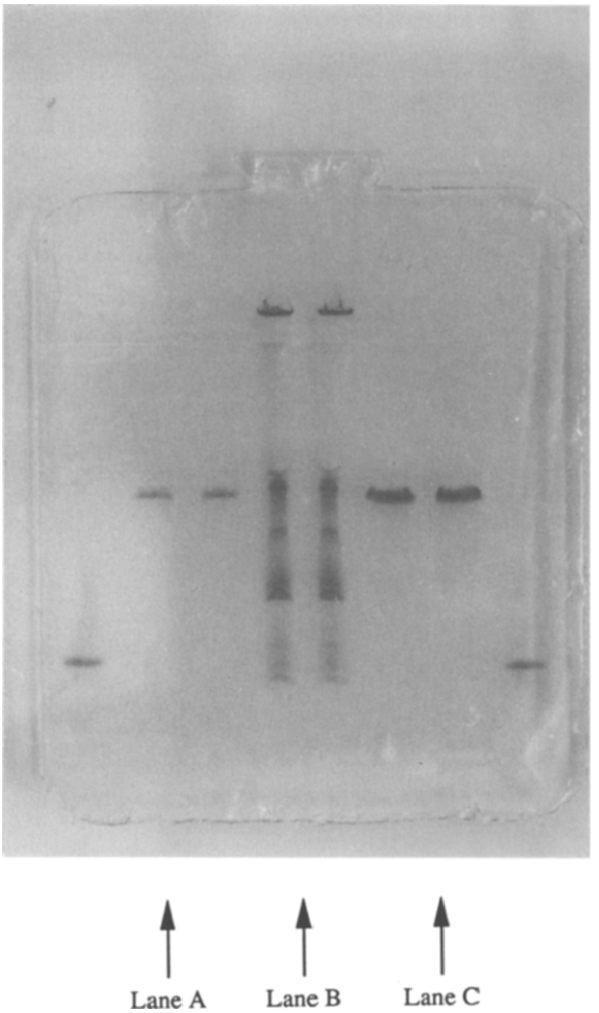

Fig. 5. SDS-PAGE analysis of purification of serum albumin from human plasma. Lanes A = purified fraction, CI Reactive Blue 4 perfluorocarbon affinity emulsion; B = crude human plasma, C = pure human serum albumin, fraction V, Sigma.

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REFERENCES

1 C. R. Lowe and P. D. G. Dean, *Affinity Chromatography*, Wiley, London, 1974.
2 C. R. Lowe, S. J. Burton, N. Burton, D. J. Stewart, D. R. Purvis, I. Pitfield and S. Eapen, *J. Mol. Recognit.*, 3 (1990) 117.
3 R. C. Williams, J. F. Vastner-Russel, L. Glajich and K. Golebiowski, *J. Chromatogr.*, 372 (1986) 63.
4 C. M. Josefson, J. B. Johnston and R. Trubey, *Anal. Chem.*, 56 (1984) 764.
5 J. J. Kirkland, *Anal. Chem.*, 35 (1963) 2003.
6 A. T. King, B. J. Mulligan and K. C. Lowe, *Biotechnology*, 7 (1989) 1037.
7 I. S. Chibata, S. T. Yamada, M. N. Wada, N. Y. Izuo and T. Y. Yamaguchi, *US Pat.*, 3 850 753 (1974).
8 B. Matiasson and P. Adlercreutz, *Ann. N. Y. Acad. Sci.*, 413 (1983) 547.
9 F. V. Groman and M. Wilchek, *Trends Biotechnol.*, 5 (1987) 220.
10 H. E. Bee, R. H. Ottewill, D. G. Rance and R. A. Richardson, *Adsorption from Solution*, Academic Press, London, 1983, p. 155.
11 D. J. Stewart, P. Hughes and C. R. Lowe, *J. Biotechnol.*, 11 (1989) 13.
12 D. J. Stewart, D. R. Purvis and C. R. Lowe, *J. Chromatogr.*, 510 (1990) 177.
13 A. Higuchi and T. Iijima, *Polymer*, 26 (1985) 1207.
14 T. Jr. Peters, *The Plasma Proteins*, Academic Press, New York, 1975, p. 133.
15 U. K. Laemmli, *Nature (London)*, 227 (1970) 680.
16 I. Pitfield, D. R. Purvis, D. J. Stewart and C. R. Lowe, in preparation.