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# Novel affinity separations based on perfluorocarbon emulsions

# Development of a perfluorocarbon emulsion reactor for continuous affinity separations and its application in the purification of human serum albumin from blood plasma

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## ABSTRACT

Perfluorocarbon affinity emulsions are generated by the homogenisation of a perfluorocarbon oil with a polymeric fluorosurfactant previously derivatised with an affinity ligand and subsequently cross-linked *in situ*. This procedure gives rise to a novel liquid affinity adsorbent that can be used for continuous protein purification. Discrete emulsion droplets were found to be unstable when pumped for prolonged periods; however, when flocculated, the emulsion flocculles with diameters of around 125 μm, were very stable and sedimented faster. A four-stage reactor unit (perfluorocarbon emulsion reactor for continuous affinity separations, PERCAS) was designed and constructed to carry out continuous separations, and exploited the unusual properties of the adsorbent, *i.e.* liquid nature and high density. Each of the four stages of PERCAS consisted of a mixing tank, for contacting between emulsion phase and aqueous phase, adjacent to a settling tank for the subsequent separation of emulsion from the aqueous phase. Using PERCAS adsorption, washing, elution and re-equilibration of the emulsion could be carried out continuously with emulsion recycle. Using single-component adsorption of human serum albumin to a perfluorocarbon affinity emulsion derivatised with the triazine dye C.I. Reactive Blue 2, PERCAS was optimised with respect to flow-rates and input concentrations. The work was then extended to the continuous purification of essentially homogeneous human serum albumin from blood plasma.

## INTRODUCTION

Affinity chromatography, based on biological recognition, offers a powerful technique for analytical and process scale bioseparation. However, its usual column mode of operation imposes some limitations on its practicality when moving from analytical to process scale. One of the limitations is the inherent cyclic batch mode of operation (*i.e.* load, wash, elute and re-equilibrate), which means that purified product is only being recovered at one stage of the operation. Furthermore, operational problems are also encountered, e.g. the difficulty of scaling up, column occlusion by particulates neces-

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sitating the incorporation of a filtration or centrifugation step, attainable throughputs etc. Some of these problems have been tackled by changing the contacting stage from a column to some other contactor. Notable examples include continuous affinity-recycle extraction (CARE) [1], in which solid–liquid contacting is carried out in well-mixed reactors (continuous operation is achieved by recirculation of adsorbent between two or more contactors) and expanded bed adsorption which can work in the presence of particulates [2]. By expanding a bed of adsorbent by flowing a solution upwards through the bed, particulates can pass unhindered through spaces generated between the adsorbent particles. Other systems change the physical nature of the adsorbent or use reverse micelles [3], membrane encapsulated ligands [4] and affinity partitioning using two-phase aqueous polymer solutions [5]. All the above processes are amenable to both scale up and continuous operation and have met with some degree of success.

A novel approach to continuous affinity separations is the use of perfluorocarbon affinity emulsions. Perfluorocarbons are a class of synthetic molecules derived from hydrocarbons in which all the hydrogen atoms have been replaced with fluorine atoms and are characterised as being chemically and biologically inert, have low solubility in aqueous and organic solvents and have densities in the region 1.6–2.0 g/ml. In separation science, perfluorocarbons have been used as packings for HPLC [6] and as stationary phases in gas–liquid chromatography [7]. Conceptually, a solid perfluorocarbon would make an ideal chromatographic support because of its stability and mechanical strength, allowing it to be used at high flow-rates and under harsh conditions. However, the inertness and hydrophobic character of perfluorocarbon surfaces has precluded their use in affinity chromatography where hydrophilicity and ease of derivatisation are deemed desirable. Recently, chemistries have been developed that allow for modification of the perfluorocarbon surface with subsequent ease of ligand immobilisation [8]. Perfluorocarbon surfaces are only wetted, in water, in the presence of surfactants that adsorb to their surface. Kobos *et al.* [9] described the technique of perfluoroalkylating enzymes in order to promote their adsorption to fluorocarbon surfaces. This was exploited in the development of a urea electrode by immobilising perfluoroalkylated urease onto the surface of a gas-permeable fluorocarbon membrane of an ammonia sensor. Further work led to the development of immobilisation procedures for a variety of biomolecules ranging from enzymes to affinity ligands on both solid and liquid perfluorocarbon surfaces [10]. More recently, the technique of perfluoroalylation has been used to generate perfluorocarbon based affinity supports based on triazine dyes [11]. Consequently, C.I. Reactive Blue 4 was substituted with perfluoroalkyl groups and used to purify rabbit muscle lactate dehydrogenase on both solid and liquid perfluorocarbons. These affinity supports were found to be quite stable under a range of harsh conditions; however, ligand leakage was detected in certain aprotic solvents and in the presence of albumin solutions. A new range of perfluorocarbon supports were then developed which utilised the coating of the perfluorocarbon surface with a hydrophilic polymer layer [12]: poly(vinyl alcohol) (PVA) was derivatised with perfluoroalkyl groups and was found to adsorb strongly, and essentially irreversibly, to perfluorocarbon surfaces. This approach and subsequent approaches, gave rise to generic chromatographic supports which could be substituted with a range of ligands such as triazine dyes [12], metal chelating groups [13] and Protein A [13].

We have concentrated on the development of liquid perfluorocarbon chromatographic supports using techniques similar to those outlined above. Perfluorocarbon affinity emulsions are generated by homogenisation of a perfluorocarbon oil with a polymeric surfactant previously derivatised with an affinity ligand and subsequently cross-linked *in situ*. Perfluorocarbon emulsions are interesting, in a biochemical engineering sense, because they open up possibilities for continuous affinity separations. We have previously shown [14] that perfluorocarbon emulsions incorporating affinity ligands could be generated which could bind human serum albumin (HSA) and lysozyme. We later showed how an affinity emulsion incorporating the affinity ligand C.I. Reactive Blue 4 could be used in an expanded bed for the semi-continuous purification of HSA from blood plasma [15]. However, these operations were restricted to the use of the emulsions in expanded beds and in order that the emulsions be used for continuous separations, the emulsion phase would

have to be transported. Although the emulsions were stable when used in expanded beds, it was found that when pumped, for prolonged periods of time, the droplets began to coalesce. The stability of the emulsions was found to be dependent on both the molecular mass of the polymeric surfactant used and the degree of substitution of the ligand [16]. In order to make the emulsions more stable a technique of chemically inducing flocculation between discrete emulsion droplets was developed. This was carried out by increasing the molar ratio of the cross-linker used and by maintaining excess polymeric surfactant in solution when cross-linking. A mol/mol ratio of 141:1 (glutaraldehyde:PVA) was found to give a very stable floccule of a predictable size and gave a high recovery of bound protein [17]. Flocculation (20–60 drops/floccule) also increased the sedimentation rate of the emulsion and facilitated collection from the aqueous phase.

Perfluorocarbon affinity emulsions possess unique features not commonly found in conventional affinity supports. Both the high density of the core perfluorocarbon oil (1.92 g/ml), which generates rapid sedimentation times in aqueous solutions, and the inherent transportability of liquid emulsions are features which can be used in continuous affinity separations. In order to perform continuous separations, we have designed and constructed a four-stage mixer settler unit, PERCAS (an acronym for perfluorocarbon emulsion reactor for continuous affinity separations). And in this paper we describe the use of PERCAS for the continuous separation of HSA from blood plasma.

## EXPERIMENTAL

### *Materials*

PVA ($M_r$ 115 000, 100% hydrolysed) was purchased from Aldrich (Gillingham, UK) as was sodium thiocyanate. Perfluorodecalin (Flutec PP6) was obtained from ISC Chemicals (Avonmouth, Bristol, UK). Sodium acetate (anhydrous) was purchased from Fisons (Loughborough, UK). The diagnostic reagent kit for serum albumin determination was purchased from Sigma (Poole, UK) as were the chemicals sodium dihydrogenphosphate, disodium hydrogenphosphate, 2-mercaptoethanol and glutaraldehyde (25%, w/v). Pure human serum albumin 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. C.I. Reactive Blue 2 was purchased from Polysciences. All other chemicals were purchased from BDH (Dagenham, UK). Masterflex peristaltic pumps (Cole-Palmer, UK) were partly used for delivery to the PERCAS unit, the remaining delivery pumps were a Pharmacia P-1 (Pharmacia Biotechnology, Milton Keynes, UK) and a Minipuls II (Gilson, France).

### *Synthesis of polymeric fluorosurfactant–dye conjugate*

The polymeric fluorosurfactant–dye conjugate was synthesised essentially as described previously [15]. Hence, PVA ($M_r$ 115 000, 100% hydrolysed, 10 g) was dissolved in distilled water (200 ml) by heating to 80–90°C for 30–40 min. The solution was cooled and the triazine dye C.I. Reactive Blue 2 (5 g) added and the solution stirred for 30 min at room temperature. The reaction mixture was heated to 70°C with the addition of sodium chloride (5 g) and sodium carbonate (5 g) and refluxed for 3 h. Sodium hydroxide was added (5 g) and refluxing continued for a further 5 h. Dyed polymeric fluorosurfactant was purified and characterised as described previously [15].

### *Generation of perfluorocarbon affinity emulsions*

Perfluorodecalin (95 g) was homogenised with a solution of dyed polymeric fluorosurfactant (200 ml, 25 mg/ml) in a cylindrical glass vessel. Homogenisation was carried out for 2 min using an Ultra-Turrax T-25 homogeniser (Sartorius, Surrey, UK) (full speed). The resulting emulsion was then cross-linked and flocculated.

### *Cross-linking and flocculation*

Perfluorocarbon affinity emulsion (60 ml) was added to a solution of free dyed polymeric fluorosurfactant in distilled water (100 ml, 10 mg/ml, pH 7.0). To this stirred solution was added glutaraldehyde to a final molar ratio of 141:1 (glutaraldehyde:PVA). After 10 min, HCl (5 *M*) was added to give a final concentration of 0.1 *M*. Cross-linking and flocculation was carried out for 1.5 h and terminated by the addition of sodium hydroxide to 0.5 *M*. The emulsion was allowed to settle and the

aqueous phase discarded and replaced with 60 ml distilled water and mixed. This solution (emulsion and water) was transferred to a buchner flask and vigorously degassed for 2 h. The settled emulsion was collected and washed with 20 m*M* sodium phosphate buffer, pH 5.0 until washings were clear of free dyed polymer as determined spectrophotometrically at peak absorbance of the ligand (620 nm). A sample of the emulsions was then analysed for floccule size distributions using a Mastersizer M6.02 (Malvern Instruments).

*Protein determination and assay*

Protein determination was carried out routinely using the Pierce Coomassie protein assay reagent. Protein concentrations in stock solutions were initially determined by absorbance at 280 nm using an extinction coefficient of 0.53 ml $mg^{-1}$ $cm^{-1}$ for HSA [18] and 0.66 ml $mg^{-1}$ $cm^{-1}$ for bovine serum albumin [19]. Spectrophotometry was carried out using a Shimadzu UV-160A spectrophotometer (VA Howe, UK). Serial dilutions of stock solutions (20 $\mu$l) 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 standard curves.

Serum albumin content in plasma was determined using the bromocresol green assay [20]. A standard curve was prepared by taking serial dilutions of stock HSA (200 $\mu$l) and adding assay reagent (1 ml). The absorbance was read at 628 nm after 10 min incubation. Plasma was centrifuged (8800 *g*, 5 min) and then filtered (0.45 $\mu$m). Diluted (1 in 50) samples were assayed for total protein. Dilutions (1 in 10) were assayed specifically for HSA.

*Equilibrium adsorption isotherms for HSA*

The equilibrium adsorption isotherm for HSA was determined using the procedures outlined by Chase [21]. An emulsion slurry (1 ml of a 1:1 emulsion:buffer) (20 m*M* sodium phosphate buffer, pH 5.0) was pipetted into each of a series of Eppendorf micro test tubes. The emulsion phase was allowed to settle and the supernatant discarded. To each of these tubes was added HSA (1 ml) in 20 m*M* sodium phosphate buffer, pH 5.0 to give a range of concentrations up to 5 mg/ml. The test tubes were rotary mixed for 2 h at room temperature before the emulsion was settled and the aqueous phase assayed for remaining protein. The amount bound to the emulsion can be calculated from a knowledge of the equilibrium concentration and the starting concentration.

In order to determine the recovery of bound HSA, the supernatant was removed and the emulsion washed (×3) with 20 m*M* sodium phosphate buffer, pH 5.0 (1 ml), HSA was eluted with sodium thiocyanate (0.5 *M*) in 20 m*M* sodium phosphate buffer, pH 8.0 (1 ml) and all fractions were assayed for protein. The recovery of bound HSA could be determined by constructing a simple mass balance.

*Single stage batch adsorption of HSA using PERCAS*

PERCAS (containing 30 ml of settled emulsion) was equilibrated with the appropriate buffers (stages 1, 2 and 4, 20 m*M* sodium acetate, pH 5.0; stage 3, 0.5 *M* sodium thiocyanate in 100 m*M* sodium phosphate, pH 8.0) at the flow-rates shown in Fig. 3a. Once a steady state had been reached (1 h) where the levels of emulsion in the settling chambers were equal, the buffer input into stage 1 was changed to HSA (500 $\mu$g/ml). The experiment was continued for 3 h with aliquots (200 $\mu$l) being taken from the top phase of settling chambers 1 and 3 every 15 min. The flow over from each stage was also collected throughout the experiment. All samples were assayed for HSA and the flow over was also monitored for change in pH.

*Continuous purification of HSA from plasma using PERCAS*

PERCAS was equilibrated as described above using the flow-rates shown in Fig. 3b. Again, once a steady state was achieved the buffer input into stage 1 was changed to plasma (0.75 mg/ml). Samples were again collected every 15 min for 4 h, the flow over was also collected. All samples were assayed for total protein and specifically for HSA. Samples were taken for analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

*SDS-PAGE*

SDS-PAGE was carried out essentially as described by Laemmli [22] using a Pharmacia Phast-System horizontal electrophoresis unit. Gels used

were PhastGel Homogeneous 12.5 (13 mm stacking gel zone, 32 mm separation gel zone), used in conjunction with PhastGel SDS Buffer strips (0.55%, w/v, SDS). To the samples was added SDS to 2.5% (w/v) and 2-mercaptoethanol to 5% (v/v). The samples were than heated to 100°C for 5 min and bromophenol blue added to approximately 0.01% (w/v). Protein samples (4 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 distilled water.

## RESULTS AND DISCUSSION

### *Qualitative aspects of PERCAS design*

Fig. 1 presents a diagramatic representation of PERCAS. The unit was made entirely from Perspex to allow ease of construction and to enable visualisation of the process. Mixing was induced in the four chambers by use of a small rectangular impeller connected via a stainless-steel shaft to a belt and pulley system. The impellers were rotated using a simple variable speed a.c. motor. The settling chamber was designed to ensure that emulsion phase

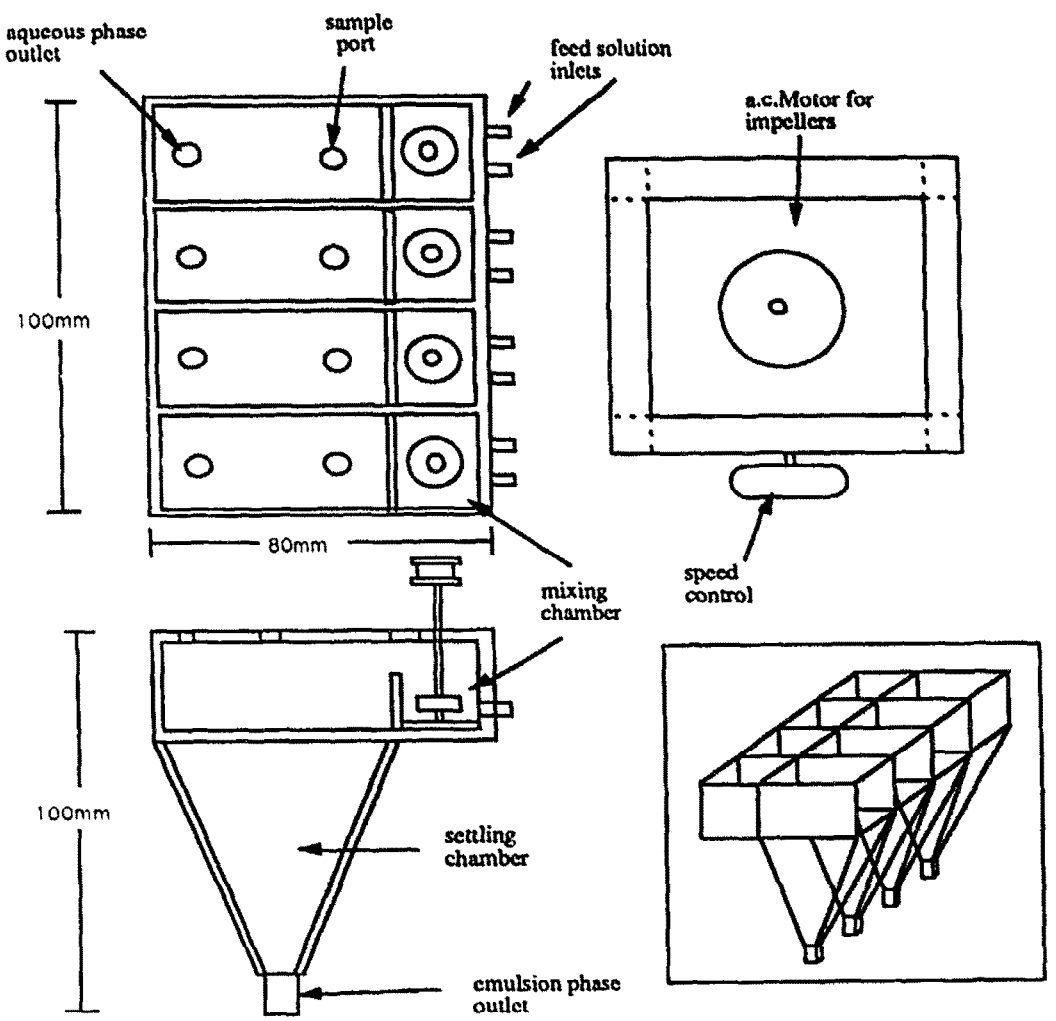



Fig. 1. Plan view and side elevation of the PERCAS unit. Insert box, schematic representation of complete PERCAS unit minus the drive unit and support stand.

contamination of the upper aqueous phase was kept to a minimum. The upper aqueous (or non-perfluorocarbon phase) was actively pumped out of the settling chamber which also includes a sample port for sampling of the aqueous phase. Fig. 2 shows the operational principle behind continuous affinity separations using perfluorocarbon affinity emulsions. Emulsion and aqueous protein phase are pumped into the first mixing chamber where adsorption takes place between the affinity emulsion and the target protein. After a predetermined residence time, which is governed by the flow-rates, the contents begin to spill over a weir into the adjacent settling tank. Here, because of the high density, separation of the emulsion phase from the aqueous phase occurs rapidly. The aqueous phase containing non-adsorbed components is pumped out and the emulsion phase is pumped into the second mixing chamber. A flow of wash buffer into this second chamber removes any contaminants entrapped within the loaded emulsion phase. Again, the contents spill over into a settling tank where phase separation takes place. The washed emulsion is then pumped into the eluting stage where the eluent flow-rate is controlled so as to maximise the concentration of protein eluted into the aqueous product. The emulsion phase is then pumped into the fourth mixing chamber where the emulsion is washed free of entrapped eluent with loading buffer. After settling, the emulsion is recycled back to the first stage. The flow-rate of the emulsion phase throughout the unit is kept constant while the flow-rates of the various aqueous phase constituents can be varied. By using PERCAS it is possible to process large volumes of protein containing solutions by using a relatively small volume of emulsion because the emulsion is continuously recycled.

Fig. 3a and b present, diagramatically, the vari-

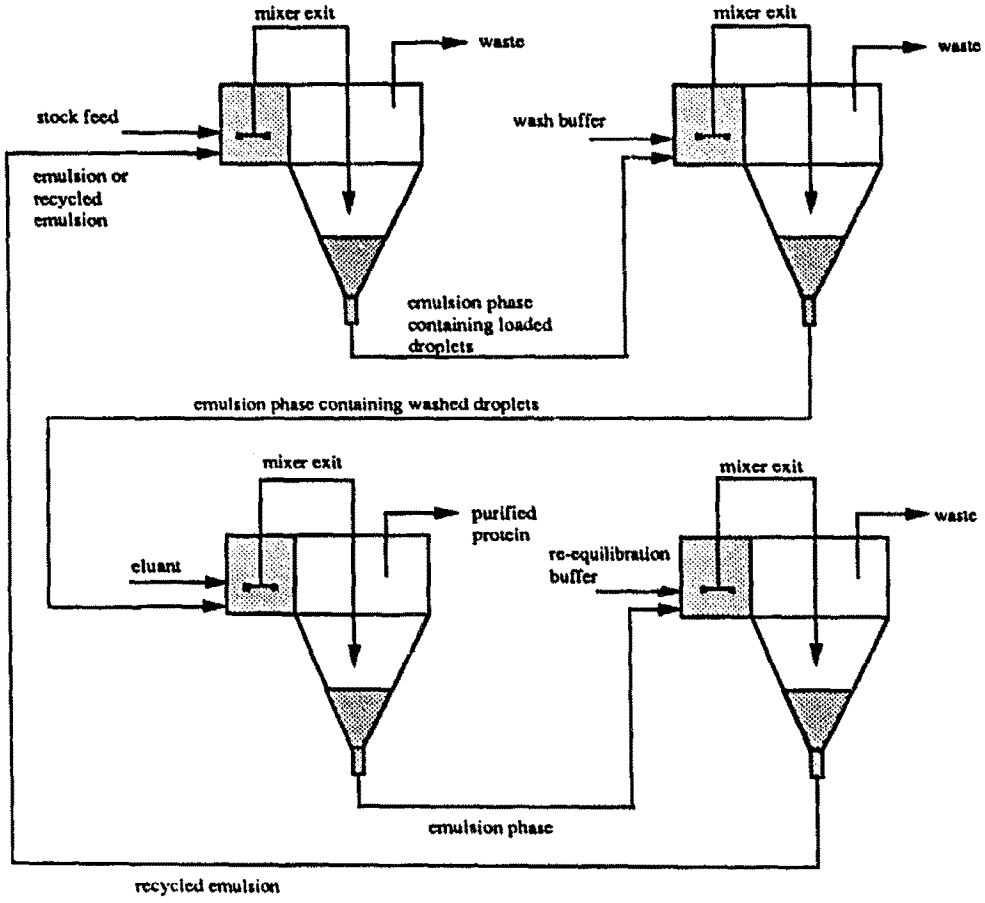
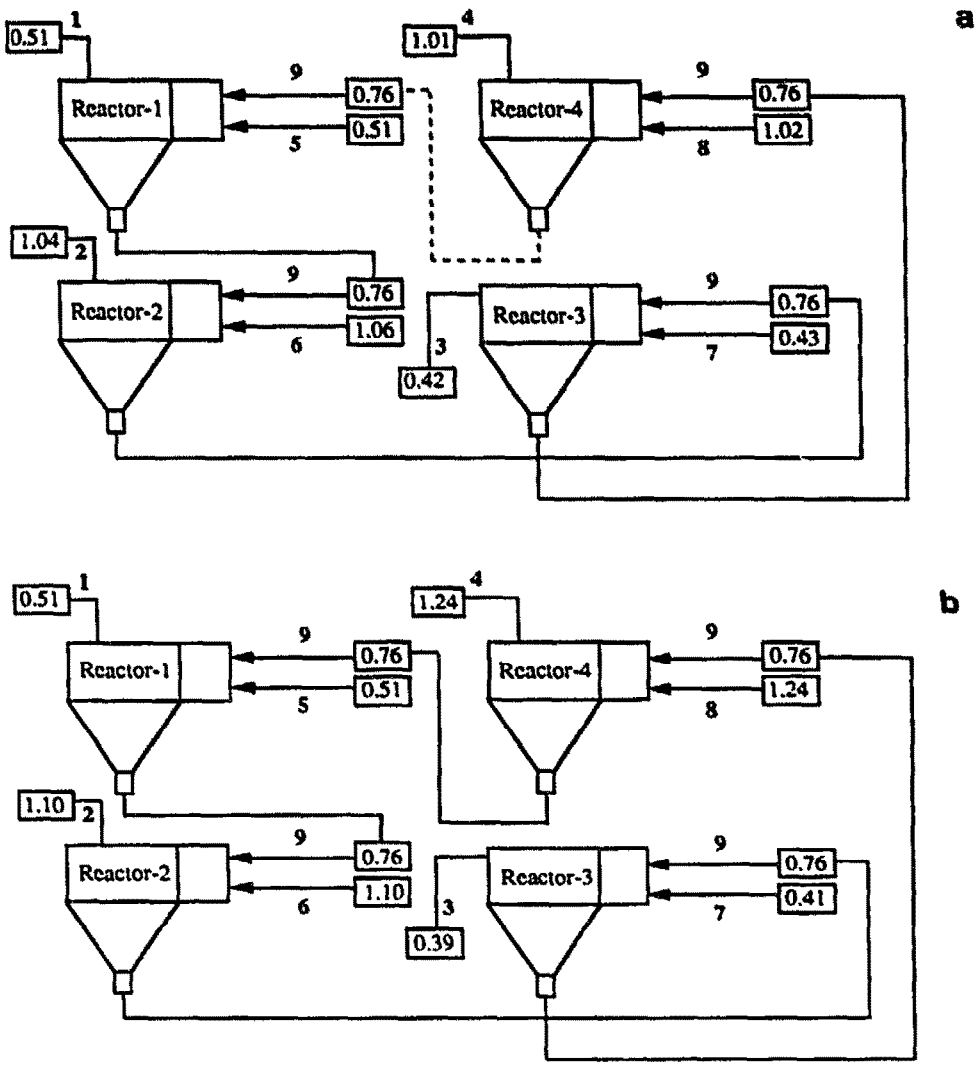



Fig. 2. Operating principle of continuous affinity separations using PERCAS as described in introduction.




| Stream | Description |
|---|---|
| 1 | Flow out of settler 1 |
| 2 | Flow out of settler 2 |
| 3 | Flow out of settler 3 |
| 4 | Flow out of settler 4 |
| 5 | Protein feed solution |
| 6 | Wash buffer |
| 7 | Eluate |
| 8 | Re-equilibration buffer |
| 9 | Emulsion (recycled) |

Fig. 3. Diagramatic representation of operational set-up of PERCAS using flocculated C.I. Reactive Blue 2 perfluorocarbon affinity emulsion showing a description of all flow streams. (a) Flow-rates for the single component adsorption test for HSA; (b) flow-rates used for the continuous purification of HSA from plasma. All flow-rates quoted are in ml min.

ous pump positions and descriptions of feed compositions for the PERCAS unit. Streams 1, 2, 3 and 4 are pumped using a single multichannel pump; which could have been omitted if each settling chamber had a weir. Stream 9 (*i.e.* the emulsion phase stream from each settler) also used a multichannel pump. The remaining streams 5, 6, 7 and 8 all used single-channel pumps, which makes the total inventory of pumps 6. This inventory could be reduced to 2 if a series of weirs replaced the single multichannel pump used for streams 1, 2, 3 and 4 and a multichannel pump replaced the single channel pumps used for streams 5, 6, 7 and 8.

*Emulsion preparation*

The degree of ligand substitution on the PVA ($M_r$ 115 000, 100% hydrolysed) was estimated to be 0.98:1 (dye:PVA molar ratio) and is similar to that we have reported previously [14]. Homogenisation (20%, v/v, perfluorodecalin) produced emulsion

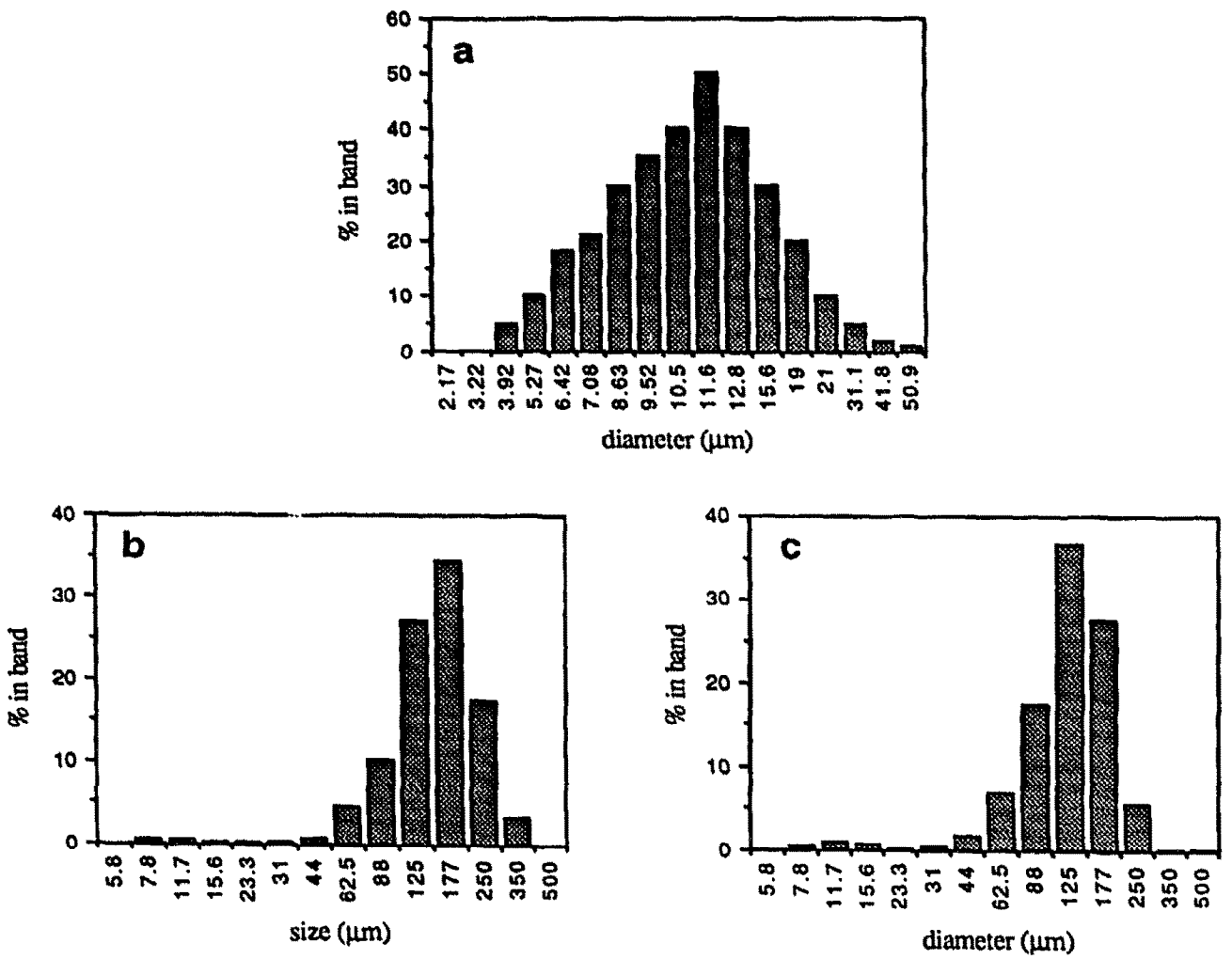



Fig. 4. Size distribution curves for the generation of C.I. Reactive Blue 2 perfluorocarbon affinity emulsion floccules. Perfluorodecalin (95 g) was homogenised with dyed polymeric fluorosurfactant (200 ml, 25 mg ml) for 2 min using an Ultra-Turrax T-25 homogeniser (full speed). Resulting emulsion was cross-linked and flocculated by the addition of settled emulsion (60 ml) to a solution of dyed polymeric fluorosurfactant (10 mg ml) in distilled water (100 ml, pH 7.0). Glutaraldehyde was added to a mol mol ratio of 141:1 (glutaraldehyde:PVA) and HCl (5 *M*) added to a final concentration of 0.1 *M*. Cross-linking and flocculation was carried out for 1.5 h when it was terminated by the addition of NaOH to 0.5 *M*. Flocculated emulsion was then degassed for 2 h. (a) Size distribution of perfluorocarbon affinity emulsion after homogenisation; (b) flocculated perfluorocarbon affinity emulsion before degassing; (c) after degassing.

droplets with an average diameter of 12.5 μm and a size range of 4–40 μm (Fig. 4a). The surface area (a parameter determined by the Malvern Particle Sizer) was 0.6 $m^2$/ml settled emulsion. Cross-linking of the emulsion was instigated by the addition of glutaraldehyde with HCl as a catalyst [23]. As excess glutaraldehyde and PVA were used, cross-linking occurs between adsorbed PVA molecules on the surface of the droplet and between free PVA in solution, between adsorbed PVA molecules and also between PVA molecules on separate droplets. This inter- and intra-cross-linking effectively builds up a floccule of discrete emulsion droplets whose growth (for a given concentration of PVA and cross-linker) is dependent on the time of cross-linking. Following cross-linking, the emulsion floccules are vigourously degassed. We believe that degassing leads to a condensation of the PVA on the emulsion floccule and an overall tightening up of the floccule. This is evidenced by a change in the floccule size distribution. Fig. 4b shows the size distribution of a flocculated emulsion before degassing while Fig. 4c shows the same emulsion once degassing has been completed. The mean floccule diameter drops from 177 μm to 125 μm on degassing. A microscopic examination of the supernatant after degassing reveals no evidence either of the floccule breaking up, or of the emulsion breaking down. We believe that on degassing, the outermost PVA layer is pulled to the surface bringing with it bound droplets, if present. Once on the surface, the PVA may react with remaining free aldehyde groups. Therefore, in using these procedures dense emulsion floccules are produced which show superior stability yet still retain the inherent transportability of the discrete emulsion.

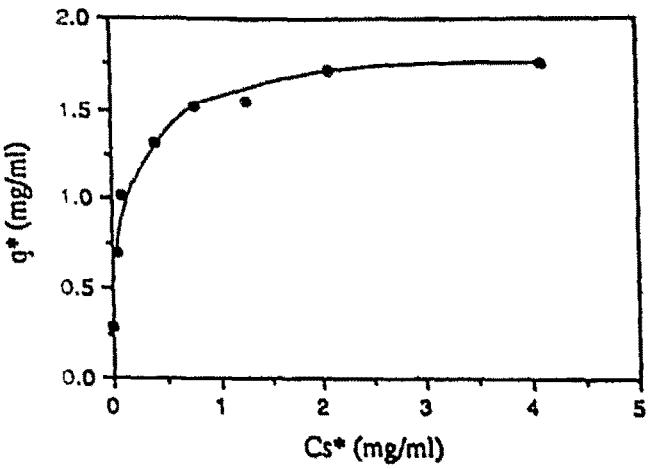



Fig. 5. Equilibrium adsorption isotherm for HSA on flocculated C.I. Reactive Blue 2 perfluorocarbon affinity emulsion. Emulsion (1:1 in 20 m*M* sodium acetate buffer, pH 5.0) was added to a series of micro test tubes, allowed to settle and the aqueous phase removed and replaced with HSA (in 20 m*M* sodium acetate buffer, pH 5.0) in a concentration range up to 5 mg ml (1 ml). Incubation was carried out by rotary tumbling for 2 h at room temperature, the emulsion was allowed to settle and aqueous phase assayed for the disappearance of HSA.

### *HSA adsorption*

The equilibrium adsorption isotherm for HSA (Fig. 5) was found to fit a Langmuir isotherm of the form:

$$q^* = \frac{q_m C_s^*}{(K_d + C_s^*)}$$

which describes the equilibrium capacity of the emulsion ($q^*$) as a function of the equilibrium concentration of protein in the soluble phase ($C_s^*$) [21]

TABLE I

RECOVERY OF BOUND SERUM ALBUMIN

Samples of emulsion with adsorbed HSA were washed (× 3) with 20 m*M* sodium acetate buffer, pH 5.0, bound HSA was eluted by the addition of 0.5 *M* sodium thiocyanate in 100 m*M* sodium phosphate buffer, pH 8.0 (1 ml). Washings and eluttions were collected and assayed as described under Experimental.

| Experiment number | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| HSA added (mg) | 0.19 | 0.38 | 0.57 | 0.95 | 1.43 | 1.90 | 2.85 | 4.76 |
| HSA recovered (mg) | 0.17 | 0.37 | 0.58 | 0.84 | 1.40 | 1.85 | 2.86 | 4.63 |
| Recovery (%) | 89 | 98 | 101 | 88 | 98 | 97 | 100 | 97 |

where $K_d$ is the dissociation constant. The maximum capacity of the flocculated emulsion ($q_m$) for HSA was found to be 1.81 mg/ml settled emulsion with ($K_d$) = 0.12 mg/ml. The $q_m$ of the flocculated emulsion (1.81 mg/ml) is higher than that quoted for a previous discrete emulsion incorporating the same affinity ligand (0.37 mg/ml) [14]. This is to be expected since the flocculated affinity emulsion contains not only emulsion droplets but also bridging dyed PVA molecules which would result in an increase in the surface area compared to a non-porous particle with the same diameter. ($K_d$) is approximately the same as described previously (0.12 mg/ml as opposed to 0.10 mg/ml [14]). It is desirable to have the $K_d$ as low as possible as this will determine, in part, the residence time required in the adsorption stage of the PERCAS unit.

The possibility of irreversible non-specific adsorption of HSA onto the emulsion floccule was tested by washing and elution of HSA from the emulsion following the isotherm determination. These results are depicted in Table I and show that the average recovery of HSA was 96%. The absence of irreversible adsorption demonstrates that the PVA was effectively wetting the surface of the perfluorocarbon and that there was limited exposure of free aldehyde groups. Consequently, the cross-linked emulsion did not require capping with ethanolamine.

*Continuous adsorption and elution of HSA*

PERCAS, with the flocculated emulsion, was tested for its ability to bind and elute HSA on a continuous basis. In stage 1 contact between the HSA and the affinity emulsion takes place. After settling, the loaded emulsion is transported to stage 2. Any HSA that has not been adsorbed to the emulsion remains in the aqueous phase and is pumped out of the settling chamber (referred to as the flow through). Thus, a concentration profile for non-adsorbed HSA appears as shown in Fig. 6. This is analogous to a breakthrough curve as obtained in column chromatography. The elution of bound HSA from the affinity emulsion takes place in stage 3. Again the concentration of eluted HSA builds up in the settling chamber as seen in Fig. 6 until a steady state is reached. The concentration of HSA used was 500 μg/ml. The emulsion flow-rate (0.76 ml min) and the HSA solution flow-rate (0.51 ml/min) gave an average residence time in the mixer unit of 8 min. An analysis of the results is presented in Table II. By multiplying the volumetric flow-rate by the concentration the data can be used to calculate the yield in each stage of the reactor. The input

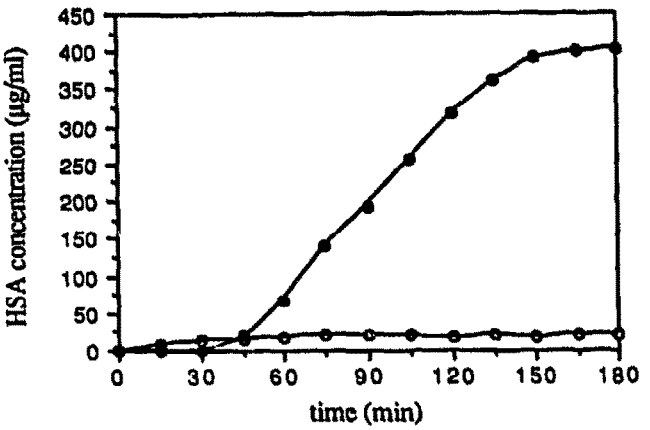



Fig. 6. Single-component adsorption test of HSA on flocculated C.I. Reactive Blue 2 perfluorocarbon affinity emulsion. The PERCAS unit was equilibrated at the flow-rates and buffer compositions shown in Fig. 3. HSA (500 μg/ml in 20 m*M* sodium acetate buffer, pH 5.0) was pumped into the PERCAS unit at 0.508 ml/min. HSA was recovered from stage 3 after elution of the emulsion with 0.5 *M* sodium thiocyanate in 100 m*M* sodium phosphate buffer, pH 8.0 at a flow-rate of 0.42 ml min. Assays performed as described under Experimental. ○ = Flow through; ● = eluted.

TABLE II

DETERMINATION OF HSA YIELD FROM CONTINUOUS ADSORPTION AND ELUTION OF HSA

HSA (500 μg ml) was pumped into the PERCAS unit at a volumetric flow-rate of 0.51 ml min (0.255 mg min). Bound HSA was eluted in stage 3 with 0.5 *M* sodium thiocyanate in 100 m*M* sodium phosphate buffer, pH 8.0. HSA detected by Pierce Coomasie reagent kit as described under Experimental. The stream numbers refer to Fig. 3.

| Stream | Flow-rate (ml min) | Protein concentration (mg ml) | Protein flow-rate (mg min) |
|---|---|---|---|
| (HSA) 5 — in | 0.508 | 0.50 | 0.254 |
| 1 — out | 0.51 | 0.025 | 0.013 |
| 2 — out | 1.04 | 0.008 | 0.008 |
| 3 — out | 0.42 | 0.40 | 0.168 |
| 4 — out | 1.01 | 0.020 | 0.020 |

of HSA into PERCAS was 0.255 mg/min, the sum of the outputs of HSA from each stage equalled 0.21 mg/min which gave the process an overall yield of 82%. Fig. 6 clearly demonstrates that PERCAS is capable of binding and eluting HSA on a continuous basis. The versatility of the system enables residence times, wash rates and product concentration to be controlled by changing the appropriate flow-rates.

*Continuous purification of HSA from plasma*

Fig. 7 presents the results for the continuous purification of HSA from human plasma using a flocculated perfluorocarbon affinity emulsion and PERCAS over a 4-h period. The graph is plotted as total protein concentration against time for the out flow from stages 1 and 3. The experiment was run for 3 h to reach a steady state and then for a further 1 h. Aliquots from the steady state region were collected and used to prepare the data in Table III which shows PERCAS performance in steady state conditions. The input flow-rate of plasma was 0.51 ml/min and the eluted HSA flow-rate was 0.39 ml/min. In both wash streams (stages 2 and 4) the flow-rates were increased from those previously used to 1.10 and 1.24 ml/min, respectively. This was to ensure that contamination of the eluted stream was kept to a minimum and that re-equilibration of the emulsion occured. Consideration of the levels of total protein in all the aqueous streams indicated that

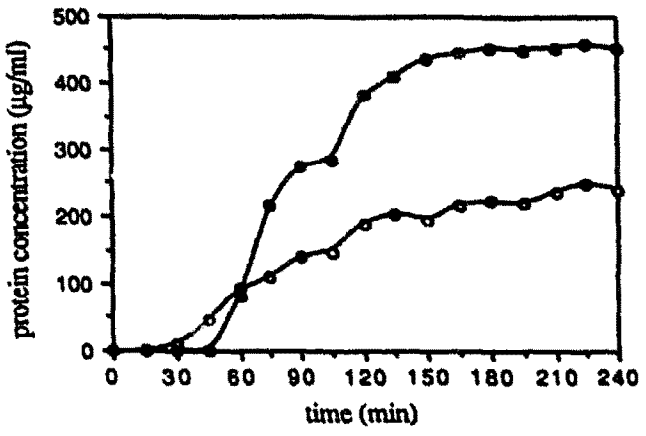



Fig. 7. Continuous purification of HSA from plasma using flocculated C.I. Reactive Blue 2 perfluorocarbon affinity emulsion and PERCAS. Plasma (diluted 1 in 100 with 20 m*M* sodium acetate buffer, pH 5.0) was pumped into PERCAS stage 1 at 0.508 ml/min. Purified HSA was collected from stage 3 after elution of the emulsion with 0.5 *M* sodium thiocyanate in 100 m*M* sodium phosphate buffer, pH 8.0 at a flow-rate of 0.39 ml/min. Assays performed as described under Experimental. ○ = Flow through; ● = eluted.

recovery of total protein was 89% with the recovery of HSA at 81%. The overall yield for the process was calculated by dividing the eluted HSA rate by the input HSA rate and gave 71%. Table III shows that HSA was recovered at 91% purity which corresponded to a purification factor of 1.52 (theoretical maximum purification fold in the sample used is

TABLE III

CONTINUOUS PURIFICATION OF SERUM ALBUMIN FROM PLASMA USING FLOCCULATED C.I. REACTIVE BLUE 2 PERFLUOROCARBON AFFINITY EMULSION

Albumin was purified from dilute plasma (1 in 100) in a continuous fashion using the PERCAS unit and flocculated C.I. Reactive Blue 2 perfluorocarbon affinity emulsion. Settled emulsion volume in PERCAS, 30 ml; running and washing buffer (in stages 1, 2 and 4), 20 m*M* sodium acetate, pH 5.0; eluting buffer (stage 3), 0.5 *M* sodium thiocyanate in 100 m*M* sodium phosphate buffer, pH 8.0. Flow-rate of applied plasma stream, 0.508 ml/min, flow-rate of product stream, 0.39 ml/min. Protein measured by Pierce Coomasic assay, albumin detected by the bromocresol green assay.

| Stream | Flow-rate (ml/min) | Protein concentration (mg/ml) | Protein flow-rate (mg/min) | HSA concentration (mg/ml) | HSA flow-rate (mg/min) | Albumin content (%) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|---|---|---|
| 5 — in (Plasma) | 0.508 | 0.75 | 0.381 | 0.457 | 0.232 | 61 | (100) | (1) |
| 1 — out | 0.510 | 0.24 | 0.122 | 0.036 | 0.018 | – | – | |
| 2 — out | 1.10 | 0.021 | 0.023 | <0.005 | <0.005 | – | – | |
| 3 — out | 0.39 | 0.45 | 0.175 | 0.42 | 0.164 | 91 | 70 | 1.52 |
| 4 — out | 1.24 | 0.015 | 0.018 | 0.01 | 0.018 | – | – | |

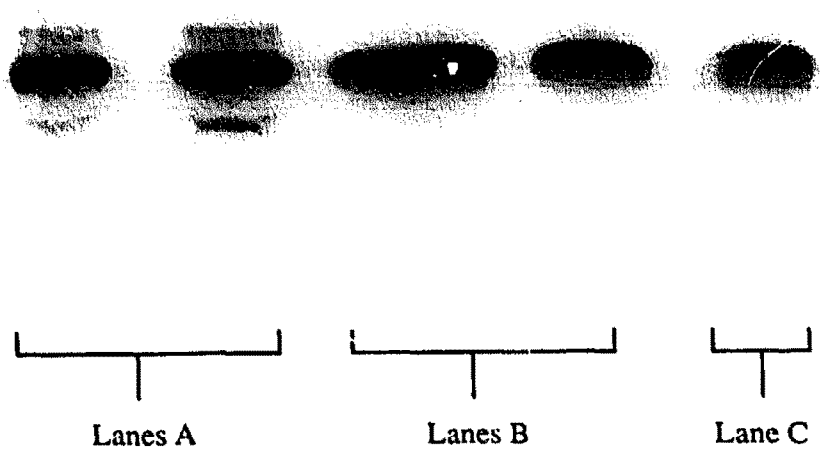



Fig. 8. SDS-PAGE analysis of the continuous purification of serum albumin from blood plasma using a four-stage mixer-settler. Lanes: A = crude human plasma; B = purified stream, flocculated C.I. Reactive Blue 2 perfluorocarbon affinity emulsion; C = pure human serum albumin, fraction V, Sigma.

1.64). Fig. 8 shows the SDS-PAGE analysis of the purification and clearly demonstrates the high purity of HSA in the eluted stream (B) compared to a commercially available pure HSA sample (C).

## CONCLUSIONS

The importance of downstream processing in biotechnology has been evident now for a number of years. General downstream processing flowsheets ultimately contain a cascade of fractionation techniques of varying selectivity. The final yield of purified product depends heavily on the number of steps required for purification, as does its selling price. In order to reduce the number of steps required for purification, the inclusion of a high resolution technique as early on in the flowsheet as possible is desirable. Ideally, this high resolution technique should be able to operate efficiently in the presence of cells, cell debris, cell culture constituents etc. If this process were also able to work continuously then the process economics would be more favourable. Continuous processes are better suited to scale up and optimisation than batch processes and are more easily integrated into a production that is itself run continuously. The inventory of bioseparation processes that fit into this ideal category is, at present, somewhat limited. Expanded bed adsorption [2] has the advantage that crude feedstocks can be processed efficiently; however, its mode of operation is not continuous since washing, elution and regeneration of the bed has to be carried out before another cycle of adsorption can take place. CARE [1] is a relatively new concept that uses standard adsorbent particles in continuous stirred tank reactors. Contacting between adsorbents and solu-

tions is carried out in continuous stirred tank reactors which permits the processing of particulate containing streams. The adsorbent is pumped as a slurry from stage to stage and is kept within the unit by use of a macroporous filter. The operating principle of PERCAS is very similar to that of CARE; however, the use of deformable emulsion flocculates ensures that no attrition is encountered as could be observed with solid particles leading to breakup and loss of ligand. The separation principle behind PERCAS relies solely on gravity settling, therefore forgoing the need for filters to retain the adsorbent; these may become blocked with extended use.

This report demonstrates the feasibility of a truly continuous bioseparation procedure using novel perfluorocarbon affinity emulsions and a simple four stage mixer-settler reactor. The relative instability of discrete emulsions has been increased by flocculation, which not only, imparts greater stability, but also faster settling rates and greater capacity. Experiments carried out consisting of recirculating the affinity emulsions through a peristaltic pump at flow-rates of 10 ml/min for 24 h have shown that the emulsion is very stable with no coalescence being observed. Indeed, in the course of our experiments we have not witnesed any emulsion breakdown after many cycles of repeated use and the emulsion can be readily autoclaved. We are now extending our work to direct broth extraction of enzymes from *Saccharomyces cerevisiae* homogenates. The rapid settling times of the adsorbent compared to cells and cell debris should ensure that clarification, as well as purification, of the enzyme takes place.

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