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Scale-up of protein purifications using aqueous two-phase systems: Comparing multilayer toroidal coil chromatography with centrifugal partition chromatography☆

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ABSTRACT

Two different laboratory scale liquid–liquid extraction processes using aqueous two-phase systems (ATPS) are compared: centrifugal partition chromatography (CPC) and multilayer toroidal coil chromatography (MTCC). Both use the same phase system, 12.5% (w/w) PEG-1000:12.5% (w/w) K_2HPO_4, the same flow rate of 10 mL/min and a similar mean acceleration field of between 220 × g and 240 × g. The main performance difference between the two processes is that there is a continuous loss of stationary phase with CPC, while for MTCC there is not – even when sample loading is increased. Comparable separation efficiency is demonstrated using a mixture of lysozyme and myoglobin. A throughput of 0.14 g/h is possible with CPC despite having to refill the system with stationary phase before each injection. A higher throughput of 0.67 g/h is demonstrated with MTCC mainly due to its ability to tolerate serial sample injections which significantly reduces its cycle time. While CPC has already demonstrated that it can be scaled to pilot scale, MTCC has still to achieve this goal.

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1. Introduction

Ever since Albertsson's pioneering work with aqueous two-phase systems (ATPS) in the early 1960s [1] there has been an interest in their use for the separation of proteins. Albertsson devoted over 10 pages of his book to this subject and supported the development of multistage unit gravity [1] and centrifugal [2] counter-current distribution systems to enhance their separation. Since then a number of groups internationally have been using ATPS for protein separations using a variety of different counter-current chromatography (CCC) methods to achieve multistage separations: centrifugal partition chromatography (CPC) [3–5], toroidal coil chromatography (TCC) [6–10], non-synchronous CCC [11–15], cross-axis CCC [16–29]; locular CCC [30], spiral disk CCC [31–36] and conventional multilayer CCC [37–39]. These methods often used ATPS but occasionally use single physiological saline solutiuons [40] or other types of phase system [41–44].

Scalable protein separations using centrifugal partition chromatography (CPC) have been demonstrated [5] using aqueous two-phase systems comprising 12.5% (w/w) PEG-1000:12.5% (w/w) K_2HPO_4 and a model sample mixture of lysozyme and myoglobin. CPC comprises a series of interconnecting cells (Fig. 1a) which are rotated in a centrifugal force field to retain the stationary liquid phase against the flow of mobile phase. While optimisation at laboratory scale (500 mL) was demonstrated and rapid scale up to a pilot scale (6.25 L) CPC achieved with 7.6% stage efficiency [10] and throughputs of 1.6 and 40 g/day respectively, rotating seals were used and there was considerable loss of stationary phase.

A new larger scale dynamic extraction process has now been developed based on multilayer toroidal coil chromatography (MTCC), which involves multilayer toroidal coils wound on a drum (Fig. 1b) which rotates in planetary motion [8–10]. The process is a continuous flow hydrostatic one with cascade mixing, similar to CPC, but instead of a uniform centrifugal force field and large mixing chambers with small bore interconnecting tubes (Fig. 1a), MTCC involves a continuous tube with no rotating seals and a variable force field. Preliminary protein purification feasibility studies were performed [8] as part of a Biotechnology and Biological Sciences Research Council (BBSRC) research project which encouraged the scale-up to larger bore tubing [9] which showed stage efficiencies rising to as high as 25% as rotation speed was increased to give a mean *g* field, measured at the centre of the planetary bobbin,

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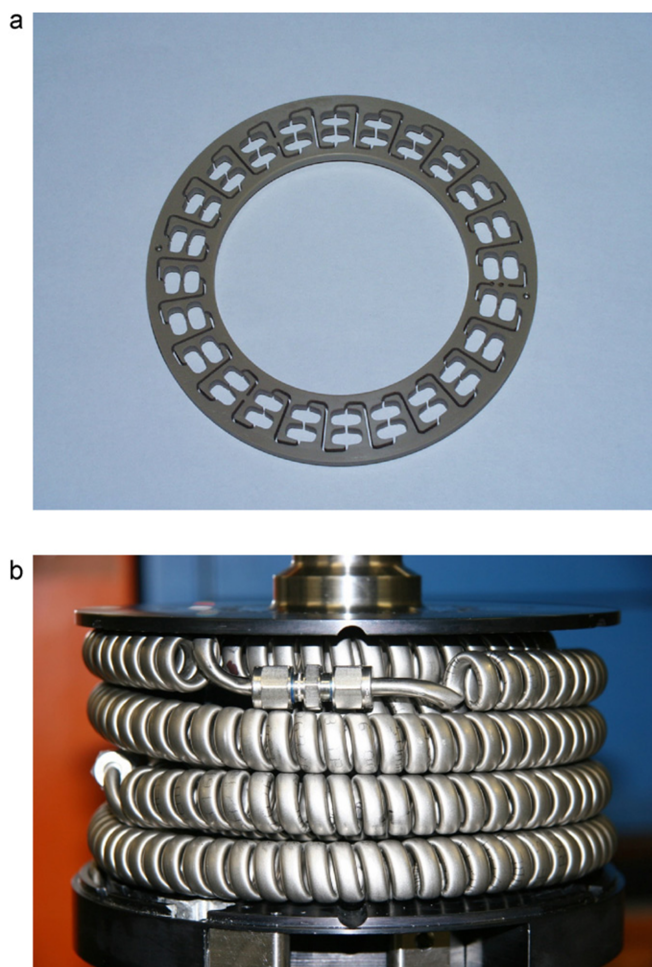

Fig. 1. (a) A typical prototype rotor disk configuration reproduced from [5] is shown comprising 24 double chambers per disk. The Armen CPC unit at Brunel was equipped with two 500 mL rotors each containing 42 stacked discs with a total of 1008 cells (chambers). Note only one of the rotors was used in the study described in [5] and (b) details of one of the prototype multilayer toroidal coil chromatography bobbins reproduced from [9], each with a volume of 334 mL, 5 mm bore and 404 toroidal loops. Note also that only one of these bobbins was used in this study.

of 240 × *g* with no loss of stationary phase. The aim of this article is to compare the results obtained with this new toroidal coil arrangement with those obtained [5] under identical conditions with centrifugal partition chromatography (CPC) and to speculate on their use in industrioal scale-up of protein purifications using ATPS.

2. Experimental

2.1. Reagents

Myoglobin (horse skeletal muscle, cat. No. M0630), lysozyme (chicken egg white, cat. no. L6876) and polyethylene glycol 1000 (cat. no. P3515) were obtained from Sigma–Aldrich. Dipotassium hydrogen phosphate (cat. no. P/5240/65) and HPLC grade solvents were supplied by Fisher Chemicals (Loughborough, UK). Deionised water and HPLC grade water were purified by a Purite Select Fusion pure water system (Thame, UK).

2.2. Apparatus

Centrifugal partition chromatography (CPC) separations were reproduced from [5] using the cell arrangement shown in Fig. 1a. Multilayer toroidal coil chromatography (MTCC) separations were performed on a single coil of a prototype twin bobbin preparative scale stainless steel toroidal column (Fig. 1b – 334 mL coil volume; 5 mm bore and 404 loops each column [9]) mounted in a commercial high performance Midi rotor (supplied by Dynamic Extractions, Slough, UK) capable of 1400 rpm (240 × *g*). Upper and lower phases were pumped with a Knauer K-1800 HPLC pump (250 mL head volume) and UV detection of the eluant at 220 nm was using a K-2501 Knauer spectrophotometer.

HPLC was performed on a Waters Alliance 2695 separations module (Empower software) connected to a Waters 2996 photodiode array (DAD) detector (210–800 nm) (Waters, Milford, MA, USA) using a YMC-Pack ODS-AQ C18 column suitable for the analysis of proteins (cat. no. AQ20S05-1546WT, particle size: 5 μm, pore 20 nm, column size: 150 mm × 4.6 mm I.D.).

HPLC method, CPC apparatus and setup was described previously [5].

2.3. Preparation of ATPS phase systems and sample solutions

The phase system comprising 12.5% (w/w) PEG-1000:12.5% (w/w) K_2HPO_4 ATPS was prepared by dissolving dipotassium hydrogen phosphate (1250 g) in deionised water (5000 g) at 60 °C. With stirring PEG-1000 (1250 g, previously melted in an oven at 60 °C) was added. The total mass was increased to 10 kg by addition of deionised water. The resultant solution was shaken vigorously, equilibrated overnight at room temperature, re shaken and finally separated. The sample was prepared by mixing either 2.2 mg/mL or 10 mg/mL each of lysozyme and myoglobin in equal quantities of upper and lower phase. The 10 mg/mL solution was placed in an ultrasound bath for 5 min to assist dissolution. Sample solutions were filtered to remove any particulates before injection.

2.4. Separation of proteins by MTCC

The column was initially filled with the upper stationary phase at 100 mL/min. The coil was rotated (1400 rpm, 240 × *g*) and the column was equilibrated by flowing the mobile lower phase at 11 mL/min in a direction opposing the rotation of the column. Immediately after equilibration, the mobile phase flow rate was reduced to 10 mL/min. Volumes of upper and lower phases eluted from the column were monitored to determine the stripping profile of the MTCC coil. The sample was injected (16.8 mL sample loop, 5% coil volume) and fractions were collected every 1 min for HPLC analysis.

3. Results and discussion

The stationary phase equilibrium characteristics for CPC and MTCC and compared in Fig. 2. The flow rates are the same (10 mL/min). The CPC column has a volume of 500 mL, but 71 mL of the volume is interconnecting pathways between each chamber resulting in a working column volume of 429 mL. The MTCC column has a volume of 334 mL comprising toroidally wound multilayer tubing of 5 mm bore as described in [9,10]. The total pumped volume and volume of stationary phase displaced from the column is plotted against time for (a) the CPC and (b) the MTCC. It can be seen that after the initial breakthrough at 27 min, stationary phase continues to elute for the next 50 min (Fig. 2a) – i.e., equilibrium is not established. In contract, the MTCC elution is at 29 min, but once breakthrough occurs there is no further elution of stationary phase and equilibrium is established (Fig. 2b).

The chromatograms for the purification of lysozyme and myoglobin are given in Fig. 3 for (a) the CPC and (b) the MTCC. Both runs were at 10 mL/min with 5% of the coil volume used as the sample injection volume. The CPC used 2.2 mg/mL concentration while

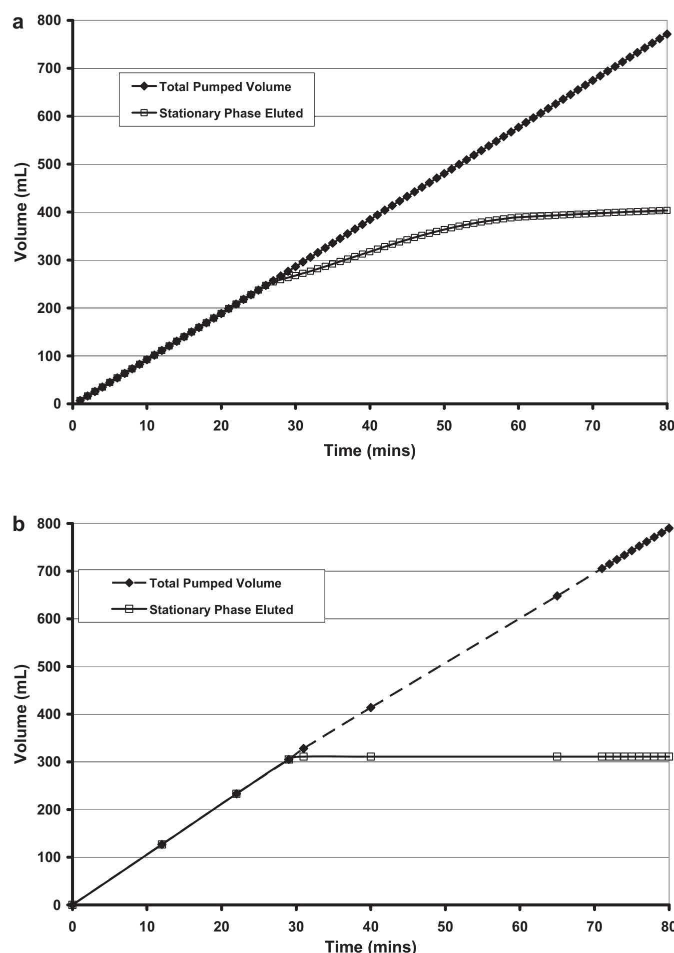

Fig. 2. The variation of total pumped volume and stationary phase eluted against time for (a) CPC from [5] and (b) MTCC using a 12.5% (w/w) PEG-1000:12.5% (w/w) K_2HPO_4 aqueous two-phase system.

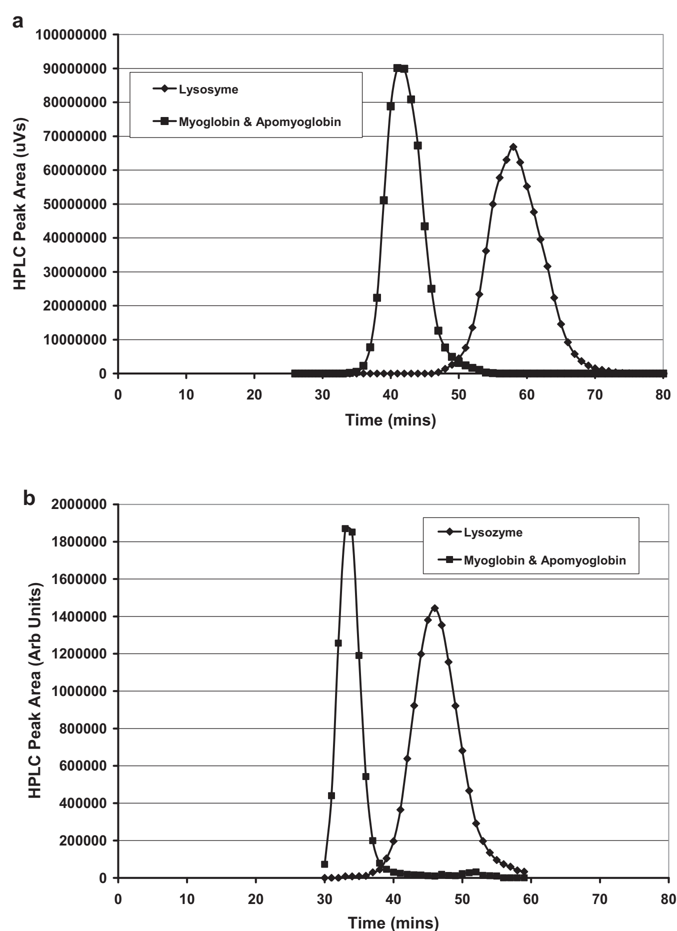

Fig. 3. Fractograms of HPLC peak area against time for the separation of lysozyme and myoglobin for: (a) CPC from [5] and (b) MTCC using a 12.5% (w/w) PEG-1000:12.5% (w/w) K_2HPO_4 aqueous two-phase system.

the MTCC was 10 mg/mL concentration. MTCC chromatograms at 2.2 mg/mL are available, but loading was maximised to see if the retention of stationary phase could be compromised. The resolution obtained between lysozyme and myoglobin was almost identical: 1.24 for CPC and 1.25 for MTCC.

The fact that the MTCC instrument reaches an equilibrium where no stationary phase is lost, even when the sample was injected, meant that serial injections could be performed without replacing the stationary phase. Fig. 4 shows an absorbance chromatogram from two serial injections to establish baseline resolution with a time cycle of 30 min. This gives a throughput of 0.67 g/h which compares favourably with that obtained with CPC [5]. With the CPC the cycle time would be 80 min (10 min pumping out and refilling with stationary phase, which may be optimistic and 70 min run time) resulting in a throughput of 0.14 g/h. While operating parameters were approximately the same (i.e., flow and mean "*g*" field), the CPC column was 1.5× larger in volume and the sample mass 1.78× smaller. The most significant difference (Table 1) is in cycle time. The CPC cycle time is 2.67× longer as serial injection is not possible. It should be noted that both systems were not fully optimised – in that the effects of increasing sample concentration to 10 mg/mL on loss of stationary phase were never examined for CPC.

Scale up to pilot scale has already been demonstrated using CPC [5] where all operating parameters were increased by a factor of 12.5× – column volume from 500 mL to 6.25 L, flow from 10 mL/min to 125 mL/min resulting in an increase in throughput from 0.14 g/h to 1.65 g/h (40 g/day). Resolution was found to even increase slightly with scale up. This suggests that cascade mixing is scalable as geometrical sizes increase and that it would be feasible to further scale up the MTCC arrangement which also has cascade mixing in every coiled unit at the heart of its operation. Pilot scale multilayer CCC instruments have been built [45,46] but multilayer toroidal coils have not yet been built for these instruments.

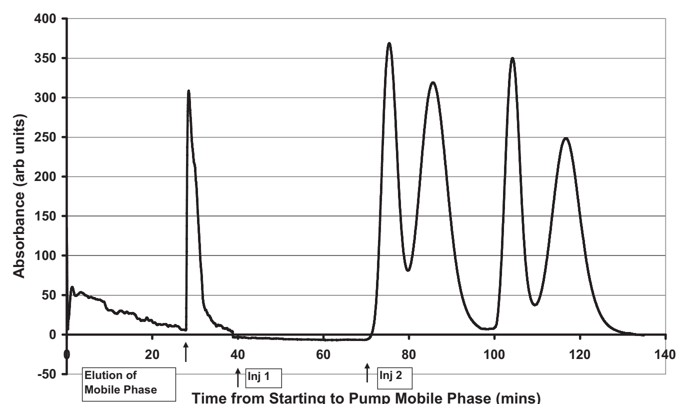

Fig. 4. Absorbance against time chromatogram showing a repeat injection of a mixture of lysosyme and myoglobin in a 12.5% (w/w) PEG-1000:12.5% (w/w) K_2HPO_4 aqueous two-phase system using the laboratory scale multilayer toroidal coil chromatography instrument demonstrating a throughput of 0.67 g/h.

Table 1
Throughput comparison between CPC and MTCC.

	Volume (mL)	Flow (mL/min)	"g" filed (×1 g)	Sample mass (mg)	Cycle time (min)	Throughput (g/h)
CPC	500	10	224	189.2	80	0.14
MTCC	333	10	241	336	30	0.67

4. Conclusions

Centrifugal partition chromatography (CPC) and multilayer toroidal coil chromatography (MTCC) have been compared at the laboratory scale and show comparable separation efficiency ($R_s \sim 1.25$) when the separation of lysozyme and myoglobin as a model system in a 12.5% (w/w) PEG-1000:12.5% (w/w) K_2HPO_4 aqueous two phase system. A throughput of 0.14 g/h has been demonstrated with CPC despite having to replace the stationary phase each cycle before each new injection. In contrast, MTCC achieved 0.67 g/h with serial injection as there was no loss of stationary phase despite quite high sample loadings. CPC has been demonstrated to be scalable up to pilot scale, but MTCC has not yet been demonstrated at this scale.

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