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A simple tool for tubing modification to improve spiral high-speed counter-current chromatography for protein purification

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Abstract

A simple tool is introduced which can modify the shape of tubing to enhance the partition efficiency in high-speed countercurrent chromatography. It consists of a pair of interlocking identical gears, each coaxially holding a pressing wheel to intermittently compress plastic tubing in 0 – 10 mm length at every 1 cm interval. The performance of the processed tubing is examined in protein separation with 1.6 mm ID PTFE tubing intermittently pressed in 3 mm and 10 mm width both at 10 mm intervals at various flow rates and revolution speeds. A series of experiments was performed with a polymer phase system composed of polyethylene glycol and dibasic potassium phosphate each at 12.5% (w/w) in deionized water using three protein samples. Overall results clearly demonstrate that the compressed tubing can yield substantially higher peak resolution than the non-processed tubing. The simple tubing modifier is very useful for separation of proteins with high-speed countercurrent chromatography.

Keywords

Simple tubing modifier; Spiral high-speed counter-current chromatography; Protein separation; Spiral tube assembly; Polymer phase system; Intermittently pressed tubing

1. Introduction

Countercurrent chromatography (CCC) is liquid-liquid partition chromatography without solid support [1]. The method retains the liquid stationary phase in the column utilizing unit gravity or centrifugal force fields. In the past a variety of CCC models have been introduced [2, 3]. Among those high-speed CCC yields the high partition efficiency and was widely

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used for the separation of natural and synthetic products [4, 5]. It utilizes a multilayer coiled column which consists of a long piece of plastic tubing coaxially wound around the cylindrical column holder forming multiple coiled layers. Under an Archimedean screw effect produced by a J-type planetary motion this multilayer coil column can produce satisfactory levels of stationary phase retention for many types of two-phase solvent systems with high to moderate hydrophobicity such as hexane-ethyl acetate-methanol-water at various volume ratios. However, when it is applied to highly polar solvent systems such as polymer phase systems [6] for separation of bioactive compounds, the system suffered from emulsification of solvent followed by steady carry-over of the stationary phase resulting in low partition efficiency. In order to solve this problem, spiral column configurations have been introduced in our laboratory. This modified column configuration has substantially improved the retention of stationary phase by utilizing the radially acting centrifugal force in addition to the Archimedean screw effect. This spiral disk assembly has been devised and successfully applied for the separation of proteins and nucleic acid components [7–13]. It consists of a stack of several plastic disks each with a set of 4 interwoven spiral grooves connected in series which is sealed by a Teflon sheet to form a long continuous spiral channels. This spiral channel can be easily modified into various shapes and also it allows inserting mixers such as glass beads to improve the partition efficiency. Recently this mixer-settler column assembly has been successfully used for separation of proteins [11, 13] and single-wall carbon nanotubes [14]. However, this spiral column has a risk of leakage of solvent through seals at high column pressure. In order to prevent leakage, another type of the spiral system called the spiral tube assembly has been introduced. It was made by inserting a long piece of plastic tubing into a set of interwoven spiral grooves formed on a plastic disk [14–20]. It has advantages over the spiral disk assembly such as minimal risk of solvent leakage and the use of various tubing sizes for a given tube support. The experimental conditions including composition of solvent systems, flow rates, revolution speeds, sample size and volume, elution modes, diameter and length of the tubing, etc. were well tested to improve the separation of polar compounds. Possibility of further improvement of the system is also explored by modifying the shape of tubing. Performances of flat tubing [20], flat-twisted tubing [19] and cross-pressed tubing [18] are so far tested by manually modifying the shape of tubing with a pair of pliers. However, making these tubing modifications is tedious and time-consuming and also lacks accuracy and reproducibility. Recently, Englert and Vetter have introduced a tubing crimping system to improve the partition efficiency in the multilayer coil HSCCC. It consists of a pair of rotating wheels with periodical crimping extrusions to pinch the tubing at a given intervals. They reported that such tubing improves the retention and separation of compounds in organic and aqueous two-phase solvent systems if applied to the conventional multilayer coil separation column [21]. We have constructed a simple tubing modifier to form various shapes of tubing such as pressed at various length at a given intervals, flattened, and flat-undulated or twisted. This paper introduces the design of this versatile tubing modifier in detail and reports the performance of the intermittently compressed tubing in the separation of test protein samples by spiral HSCCC.

2. Design of the tubing modifier

Fig. 1 A shows the photograph of the simple tubing modifier. It is composed of a pair of identical gears of 7.6 cm (3 inch) pitch diameters approximately 2 mm thick with 57 spur teeth. Each gear has a coaxial pressing wheel of 5 mm thick and 7.5 cm diameter which is indented every 1 cm to compress the tubing in 1 cm width at 1 cm intervals. A handle is attached to one of the gears to manually rotate the pair of gears each in the opposite direction. The tubing is inserted from a side hole to reach the pressing wheels, and the rotation of the gears will compress and automatically send out the tubing continuously until the rotation is stopped. It was found that when the interlocking position of the gears is shifted, the length of the tubing compression is reduced to sub-cm at the same 1 cm intervals. This pressing tool can be applied to the tubing with the wall thickness of around 0.4 mm and is suitable to modify the PTFE (polytetrafluoroethylene) tubing of SW20 (0.85 mm ID, analytical), SW14 (1.6 mm ID, semi-preparative) and SW 10 (2.6 mm ID, preparative) all available from Zeus Industrial Products, Orangeburg, SC, USA. Photographs of the standard 1.6 mm ID tubing, and intermittently pressed 3 mm (IP 3 mm) and 10 mm (IP 10 mm) tubing are shown in Fig. 2B. When the gears are rotated one round, about a 24 cm length of the tubing is pressed out, and at 30 rpm of gear rotation 100 m of tubing (about the typical semi-preparative column length) is pressed within 15 min, compared with several hours required for manually pressing the tubing with a pair of pliers. However, more importantly, the present tool will give high accuracy and reproducibility.

3. Experimental

3.1. CCC Apparatus

High-speed CCC separation was performed by a type-J coil planet centrifuge (P.C. Inc., Potomac, MD, USA, production discontinued). It holds a single separation column and its counter-weight symmetrically on the rotary frame at a distance of 10 cm from the central axis of the apparatus. The separation column was prepared from a spiral tube support (STS) custom-made from CCBiotech, Rockville, MD, USA. It is 16 cm in diameter and 5 cm in height made with a laser sintering method from Nylon powder. This STS has 4 interwoven spiral grooves to accommodate PTFE tubing of 1.6 mm ID (SW 14, Zeus Industrial Products, Orangeburg, SC, USA) intermittently pressed by the above modification tool (Figure 1A) at 3 mm and 1 cm widths both at 1 cm intervals (Figure 1 B). Each column has 8 - 9 spiral layers with the total capacity of about 80 ml. For comparative studies, the same STS was filled with standard (non-pressed) tubing of the same ID which formed 10 spiral layers with a total capacity of 114 ml.

3.2. Reagents

All chemicals used to prepare the two-phase solvent system and sample proteins including polyethylene glycol 1000, dibasic potassium phosphate, cytochrome c (from equine heart), myoglobin (from equine skeletal muscle), and lysozyme (from chicken egg white) were purchased from Sigma and Aldrich (St. Louis, MO, USA). Reverse osmosis deionized water (Hydro, Research Triangle Park, NC) was used to prepare two-phase solvent system and sample solutions.

3.3. Solvent system and sample solution

The polymer two-phase solvent system was prepared by dissolving polyethylene glycol 1000 and dibasic potassium phosphate each at the concentration of 12.5% (w/w) in degassed deionized water. Sample solution was prepared from three stable protein samples of cytochrome c, myoglobin and lysozyme by dissolving each at a suitable concentration in the upper phase used for separation.

3.4. Procedure of HSCCC separation

The separation column was first completely filled with the upper phase followed by injection of the sample solution with a glass syringe. Then the column was rotated at a given speed (800 or 1000 rpm) while the lower phase was eluted through the column through the inner end of spiral tubing in a tail to head elution mode at a desirable speed (1–3 ml/min). The effluent from the outlet of the column was continuously monitored at 280 nm with a UV spectrophotometer (Uvicord IIs, LKB Instruments, Bromma, Sweden) and elution curve was recorded with a strip-chart recorder (Millipore, Boston, MA, USA). In order to improve the elution curve, the effluent was diluted with deionized water through a dialyzer (Spectrum, California, USA) inserted on-line near the inlet of the monitor at 1/10 of the mobile phase flow rate using an HPLC pump (model LC10 AD, Shimadzu Corporation, Kyoto, Japan). After all three peaks were eluted, the column rotation was stopped and the column contents were pushed out with pressurized N₂ gas into a graduated cylinder to determine the volume of the stationary phase retained in the column.

3.5. Analysis of partition efficiency

The performance of each column was evaluated in terms of theoretical plate number (N) of each peak and peak resolution (Rs) between the 1st – 2nd and 2nd – 3rd peaks as well as % retention of the stationary phase (Sf) and separation time. N and Rs were computed from the chromatogram according to the following conventional equations:

$$N=(4t_R/W)^2 \quad (1)$$

$$Rs=2(t_{R2} - t_{R1})/(W_1+W_2) \quad (2)$$

where t_R is the retention time, and W, the peak width at base where substrates indicate peak numbers. In order to make a fair comparison in separation efficiency between the standard and two modified columns, the N, Rs and separation times were all normalized to a 100 ml column volume.

4. Results

Fig. 2 shows a set of chromatograms obtained from semi-preparative tubing of the standard (A), pressed at 3 mm width (B) and pressed at 10 mm width (C) at various flow rates (1 – 3 ml/min) and revolution speeds (800 and 1000 rpm). In all chromatograms the separation was

performed with a sample solution containing 1 mg of cytochrome c, 10 mg each of myoglobin and lysozyme dissolved in 1 ml of the upper phase. Partition efficiencies expressed in terms of theoretical plate number (N), peak resolution (Rs), % stationary phase retention (Sf) and separation time normalized at a 100 ml column capacity are listed in Tables 1 – 3. It clearly indicates that the spiral column assembly with pressed tubing can yield substantially higher peak resolution (Rs) than that with the non-pressed tubing, especially with 10 mm length compression at 1000 rpm. The highest Rs values (2.86 between peaks 1 & 2 and 2.64 between peaks 2 & 3) were obtained from IP 10 mm at a flow rate of 2 ml/min where 51% of the stationary phase was retained in the column. It is interesting to note that the pressed tubing also gives much higher theoretical plate numbers than the non-compressed tubing. The retention of the stationary phase in the processed tubing was similar to that in the non-processed tubing..

5. Discussion

High-speed CCC has been extensively used for separation of small molecules derived from natural and synthetic produces with organic/aqueous two-phase solvent systems using a multilayer coil separation column. It has been found, however, that the conventional multilayer coiled column fails to retain the polymer phase systems which can be applied for separation of polar macromolecules such as proteins, polysaccharides and nucleic acids. In order to separate these bioactive compounds, we must satisfy two major requirements, i. e., a satisfactory level of stationary phase retention and high partition efficiency. Each requirement is discussed in the following sections.

5.1. Stationary phase retention

The retention of the stationary phase in the column is the first and most important requirement for CCC separation, since the peak resolution in CCC is strongly affected by the retention of the stationary phase. The level of stationary phase retention is determined with the physical property of the two phase solvent system such as viscosity, interfacial tension and difference in density between the two phases in addition to the applied experimental conditions such as a flow rate of the mobile phase and revolution speed of the column. Therefore, it is difficult to predict the retention level before actual separation. However, it has been found that the retention of the stationary phase is strongly correlated with the settling time of the two phases in the test tube in unit gravity [22]. If the settling time of the two-phase solvent system is over 30 sec, the conventional multilayer coil cannot hold enough amount of the stationary phase in the column. The polymer phase systems such as polyethylene glycol/phosphate buffer and dextran/polyethylene glycol give long settling times due to their high viscosity, extremely low interfacial tension and small difference in density between the two phases. Therefore, the conventional multilayer coil separation column cannot be efficiently used for protein separation with polymer phase systems by high-speed CCC. However, we found that this problem can be solved by modifying the column configuration from the multilayer coil to a spiral tubing configuration where the centrifugal force radiating from the center of the rotation toward the periphery helps to hold the lower phase in the periphery and the upper phase in the center of the spiral to enhance the Archimedean screw effect. Obviously this beneficial effect for retaining the stationary

phase is enhanced by increasing the spiral pitch,. This can be accomplished by making interwoven multiple spirals accommodated in each spiral layer without losing the column capacity [23]. Although these spiral columns can retain over 50% of the polymer phase systems, it was found that the separation of the macromolecules such as proteins is still unsatisfactory apparently due to the poor mass transfer rate of large molecule through the interface of the two phases. And this requires further challenge for improvement of the partition efficiency in protein separation as described below.

5.2. Partition efficiency and mass transfer rate

Partition efficiency of the high-speed CCC is highly dependent on the mass transfer rate of the analyte through the interface between the two phases. And this mass transfer rate is further influenced by the moving rate of the analytes which is determined by the linear momentum (mv) of analytes, where m is the molecular mass and v is the three-dimensional velocity of the analyte molecule. In the solution, all existing molecules are continuously moving and colliding each other to keep the equal magnitude of linear momentum where a large molecule moves at a lower rate than the small molecules. For example, a large molecule such as serum albumin (M_s : 66,500) moves at a much lower rate ($<1/300$) than a small molecule such as a free amino acid (M_s : 200). Therefore, decreasing the depth of each phase is an effective way to improve the mass transfer rate for large molecules. In addition, the large molecules have more resistant to break through the interface. These phenomena have been investigated in our previous studies using a simple rotary system and a set of test samples [19, 24]. In order to accelerate the mass transfer rate of protein molecules, one may consider two different ways, i.e., increasing the interface area per volume of the two phases and reducing the thickness of each solvent layer. These two strategies are fulfilled by compressing the tubing which provides broader interface per volume of the two phases and thin layer of each phase (provided that the centrifugal force is acting in perpendicular to the flatten surfaces as in the present spiral column). As shown in Fig. 3, increasing the compressed length of the tubing from 3 mm to 10 mm substantially improved the partition efficiency measured by peak resolution of the test protein samples. Intermittently compressing the tubing may also disrupt the laminar flow of the mobile phase to prevent sample band broadening which tends to occur in the standard tubing as suggested by Englert and Vetter [21]. Also the moving rate of the mobile phase in the compressed portion of the tubing is accelerated to mix the two phases in the following non-compressed portion of the tubing to improve the partition efficiency. The present tubing modifier can be used to produce other tubing configurations such as flattened, flat-waving and flat-twisted (twisting by hand) by simply modifying the shape of the pressing wheels. The performance of these newly modified tubing shapes in protein separation will be reported in the future.

6. Conclusions

The simple tubing modifier devised in our laboratory was introduced. It can change the shape of tubing by manually rotating a pair of interlocking gears each equipped with pressing wheel. It can precisely produce compressions intermittently on the PTFE tubing at a high speed. The effect of intermittent compression of the tubing was tested with separation of a set of three test protein samples with a polymer phase system composed of PEG1000

and dibasic potassium phosphate each at 12.5% (w/w) in deionized water in high-speed CCC. The overall results indicated that the intermittently compressed tubing yields substantially higher partition efficiency in terms of both theoretical plate number and peak resolution, where the longest compression width (10 mm) produced the best separation. The present tubing modifier can produce other tubing configurations such as flattened, flat-waving and flat-twisting which may be tested for further improving the partition efficiency in protein separation.

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PTFE 1.6 mm tubing

Fig. 1.

The design of the tubing modifier. A: photogram of the prototype; B: photographs of the standard and intermittently pressed tubing (IP) (1.6 mm ID)

A. Standard 1.6 mm ID tubing

B. 3 mm pressed 1.6 mm ID tubing

C. 10 mm pressed 1.6 mm ID tubing

Fig. 2.

Sets of HSCCC Chromatograms obtained from standard and intermittently pressed tubing.

A: Protein separation by the standard tubing of 1.6 mm ID; B: Protein separation by the intermittently pressed tubing in 3 mm length at 1 cm intervals; C: protein separation by intermittently pressed tubing in 10 mm length at 1 cm intervals. Experimental conditions: apparatus: the J-type planetary centrifuge. It holds a spiral tube assembly at 10 cm from the central axis of the centrifuge. Solvent system: PEG 1000 and dibasic potassium phosphate each 12.5% (W/W) in deionized water; sample: cytochrome c (1 mg), myoglobin (10 mg) and lysozyme (10 mg) in 1 ml upper phase.

Fig. 3.
Comparison in partition efficiency between the non-processed and 3 mm & 10 mm
intermittently pressed tubing.

Table 1

Performance of the standard column normalized to 100 ml capacity.

Revolution (rpm)	flow rate (ml/min)	N*			R _s **			Sf***	time
		Peak 1	Peak 2	Peak 3	Peaks 1-2	Peaks 2-3		(%)	
800	1	290	27	47	1.31	1.22		58	254
	2	550	84	56	1.62	1.20		48	123
	3	388	65	48	0.95	1.31		42	79
1000	1	225	33	70	1.66	2.02		68	450
	2	690	116	92	1.69	1.88		50	114
	3	626	128	62	1.45	1.62		49	79

* N: Theoretical Plate number;

** R_s: Peak resolution;

Sf: Stationary phase retention.

Table 2

Performance of the column pressed at a width of 3 mm & normalized to 100 ml capacity.

Revolution (rpm)	flow rate (ml/min)	N*		R _s **			Sf***	time
		Peak 1	Peak 2	Peak 3	Peaks 1-2	Peaks 2-3	(%)	
800	1	420	60	59	1.82	2.20	68	288
	2	1581	176	128	2.68	2.35	53	110
	3	395	217	78	1.55	1.60	36	73
1000	1	320	41	85	1.81	2.24	72	232
	2	645	160	94	2.67	2.05	58	195
	3	1762	312	82	1.66	1.75	43	72

* N: Theoretical Plate number;

** R_s: Peak resolution;

Sf: Stationary phase retention.

Table 3

Performance of the column pressed at a width of 10 mm & normalized to 100 ml capacity.

Revolution (rpm)	flow rate (ml/min)	N*			R _s **			Sf***	time
		Peak 1	Peak 2	Peak 3	Peaks 1-2	Peaks 2-3		(%)	
800	1	312	38	67	1.47	1.90		71	256
	2	488	90	46	2.20	1.63		50	122
	3	275	312	57	1.31	1.61		40	68
1000	1	538	62	89	2.28	2.24		68	232
	2	488	176	78	2.86	2.64		51	171
	3	373	211	98	2.16	1.89		48	73

* N: Theoretical Plate number;

** R_s: Peak resolution;

Sf: Stationary phase retention.