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Columns switch recycling size exclusion chromatography for high resolution protein separation

Huiming Yuan[a,b], Lihua Zhang[a,*], Weibing Zhang[c], Zhen Liang[a], Yukui Zhang[a]

[a] National Chromatographic R.&A. Center, Key Laboratory of Separation Science for Analytical Chemistry, Dalian Institute of Chemical Physics, The Chinese Academy of Sciences, 457 Zhongshan Road, Dalian 116023, China
[b] Graduation School of Chinese Academy of Sciences, 19 Yuquan Road, Beijing 100049, China
[c] Department of Chemistry, School of Chemistry and Molecular Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China

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ABSTRACT

Columns switch recycling size exclusion chromatography (csrSEC) was proposed to achieve high resolution protein separation with good biocompatibility. Proteins were firstly separated by two serially coupled SEC columns, and fractions were in sequence switched back to the first column by two-position valves for further separation in terms of close-loop recycling until satisfactory resolution was achieved. Compared to SEC, the separation window was broadened by increasing column length via cycling without further increase on back pressure. Compared to recycling SEC (rSEC), the overtaking of later eluted components by early eluted ones after several cycles could be avoided for complex sample analysis, by parking fractions in the second SEC column before transferred in turn back to the first one for cycling ordinally. In our experiments, the baseline separation of five proteins with molecular weight ranging from 10 kDa to 80 kDa was achieved by csrSEC. Furthermore, a multidimensional csrSEC–RPLC platform was constructed, and peak capacity up to 3600 was obtained for protein separation. All these results demonstrated that csrSEC is a promising protein separation mode with good biocompatibility, broadened separation window and improved resolution.

1. Introduction

Size exclusion chromatography (SEC) is an entropically controlled technique that relies on the differences in molecular weight and the ability to penetrate into pores of column packing materials. Although due to good biocompatibility, SEC has been widely used for biomolecule separation [1–3], the poor resolution, limited separation efficiency, narrow separation window and low peak capacity prevent its application in high resolution purification [4,5] and separation of complex biomolecules [6].

The most commonly used approach to improve the separation efficiency and resolution of SEC is to increase column length or decrease particle size [7–8]. However, these methods might result in high back pressure, leading to the instability of packing materials and apparatus. Therefore, recently, improvement in the separation modes of SEC has attracted more attention. Stol et al. [9] introduced a novel size exclusion electrochromatography (SEC), and optimized the experimental parameters to obtain the best performance. Tan et al. applied this technique for the separation of proteins, in which an oscillatory low-voltage electric field perpendicular to the mobile-phase streamline was added. By this approach, gel-excluded proteins (BSA/IgG) and gel-permeable proteins with similar Mw (myoglobin/lysozyme) were partially separated [10]. Colvin et al. [11] reported a recycling size exclusion chromatography (rSEC) for the separation of nanomaterials. By this technique, three kinds of nanocrystals with similar size could be separated in terms of close-loop recycling mode. However, for the biological samples with wide Mw distribution, after several times recycling, the early eluted components might catch up with the late eluted ones when separated in a close-loop, resulting in poor resolution.

For complex protein sample analysis, single-dimension high performance liquid chromatography (HPLC) can hardly meet the requirements of high peak capacity and high resolution. Therefore, multidimensional liquid chromatography is a prerequisite. As a result of easy operation and low demands on equipments, some off-line multidimensional HPLC approaches have been developed [12–14]. However, by such systems, samples might be lost or contaminated, and the operation is usually time-consuming. To solve these problems, recently, on-line 2D-HPLC has been paid much attention. 2D-ion exchange chromatography (IEC)-reversed phase chromatography (RPLC) platform has been widely used for protein analysis [15–17]. However, the peak capacity and resolving power are still not comparable to those obtained by 2D-PAGE, by

* Corresponding author. Tel.: +86 411 84379720; fax: +86 411 84379779.
E-mail address: lihuazhang@dicp.ac.cn (L. Zhang).

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which at least 2000 proteins could be resolved in a single run [18]. Although SEC is also an orthogonal separation mode to RPLC, the narrow separation window limited its application in continuous flow 2D-HPLC. Therefore, either increase in SEC running time or the analysis speed of RPLC is indispensable. Opiteck and Jorgenson [7] connected six preparative SEC columns in tandem, and applied them as the first dimension for the separation of tryptic digests. However, when these SEC columns were used, the back pressure of the whole system might be very high. In addition, ultrahigh-pressure RPLC system [19] or high-temperature RPLC [20] could also be used as the second dimension to fasten the analysis speed. Nevertheless, the high cost of such equipments prevents their wide application.

In our present work, to the best of our knowledge, for the first time, with common HPLC apparatus, columns switch recycling size exclusion chromatography (csrSEC) is proposed with the combination of serially coupled SEC, sample parking technique and rSEC. Through the separation of protein mixture with wide distribution of molecular weight, improved separation window and resolution were obtained by csrSEC. Furthermore, due to the orthogonal separation mechanisms with RPLC and broadened separation window by cycling, an on-line multidimensional csrSEC-RPLC platform was established to achieve high resolution and peak capacity for complex protein mixtures.

2. Experimental

2.1. Chemicals and materials

Myoglobin (horse heart), chicken egg albumin, apo-transferrin (horse), ribonuclease (bovine pancreas), cytochrome c (bovine heart), hemoglobin (bovine), catalase (bovine liver), β-lactoglobulin (bovine milk), carbonic anhydrase (bovine erythrocytes) and trifluoroacetic acid (TFA) were purchased from Sigma (St. Louis, MO, USA). Bovine serum albumin (BSA) was obtained from Shanghai Milk Company (Shanghai, China). HPLC-grade acetonitrile (ACN) was ordered from Merck (Darmstadt, Germany). SEC *SRT*-150 Å and SEC *nanofilm*-250 Å particles were purchased from Sepax Technologies Inc. (Newark, NJ, USA). C8 particles (5 μm, 300 Å) were obtained from Dalian Elite Analytical Instrument Co. Ltd. (Dalian, China). C8 Macrotrap columns were purchased from Michrom Bioresources (Auburn, CA, USA). Water was purified by a Milli-Q system (Millipore, Milford, USA). All other chemicals and solvents were analytical-grade.

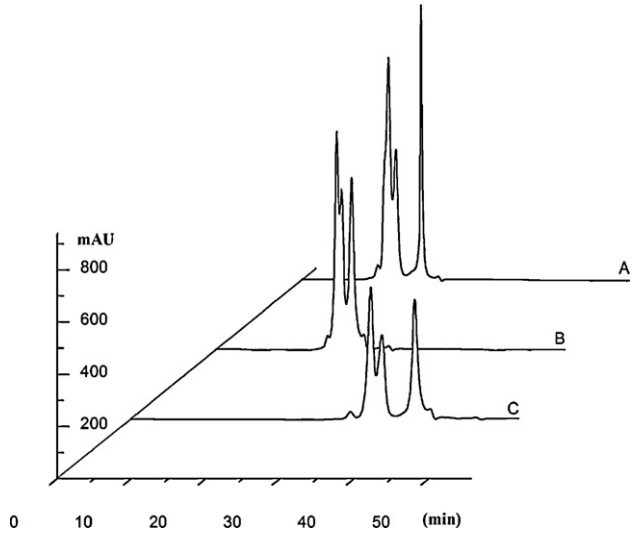

Fig. 1. Separation of a 4-protein mixture by *SRT*-150 Å column (A), *nanofilm*-250 Å column (B) and serially coupled columns by *SRT*-150 Å and *nanofilm*-250 Å (C). Experimental conditions: columns: (a) SEC *SRT*-150 Å column (150 mm × 4.6 mm I.D., 5 μm, 150 Å); (b) *nanofilm*-250 Å column (250 mm × 4.6 mm I.D., 5 μm, 250 Å); (c) coupled columns by *SRT*-150 Å and *nanofilm*-250 Å; mobile phase: 10 mM Tris–HCl containing 150 mM NaCl and 5% (v/v) ACN (pH 8.0), flow rate: 150 μL/min; injection: 20 μL; UV detection: 215 nm. Samples: β-lactoglobulin, myoglobin, ribonuclease and cytochrome c with the concentration of 0.25 mg/mL for each protein

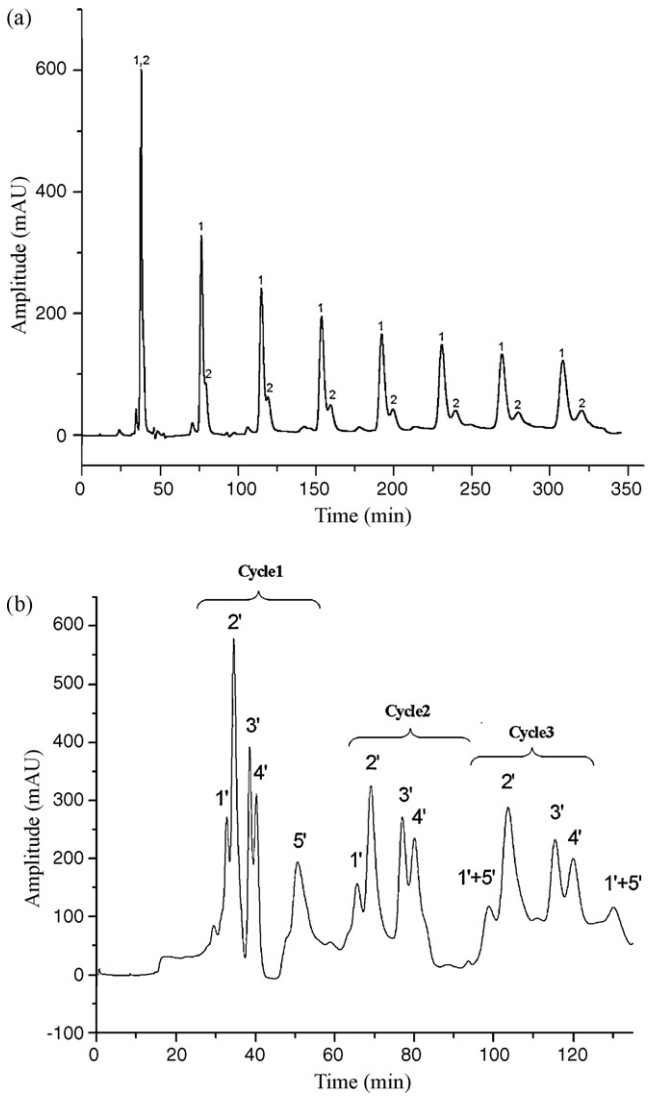

Fig. 2. Separation of protein mixtures by rSEC with serially coupled columns. Experimental conditions were the same as those shown in Fig. 1. Samples: (a) 0.5 mg/mL of myoglobin (1) and ribonuclease (2); (b) 0.2 mg/mL of apo-transferrin (1′), chicken egg albumin (2′), myoglobin (3′), ribonuclease (4′) and cytochrome c (5′).

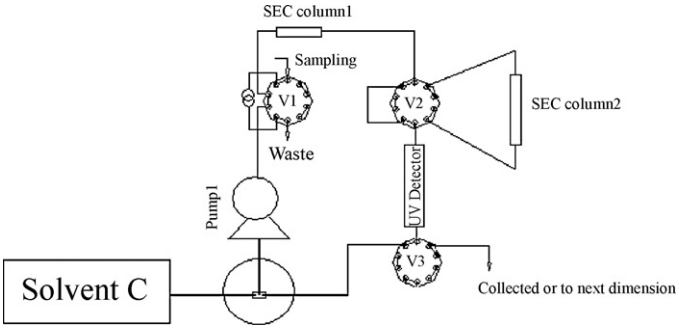

Fig. 3. Schematic diagram of csrSEC platform.

2.2. Experimental apparatus

SEC experiments were performed on a MAGIC MS4 HPLC system (Michrom, CA, USA) equipped with a UV detector (Knauer, Berlin, Germany). RPLC experiments were performed on a P230 high-pressure gradient HPLC system (Dalian Elite Analytical Instrument Co. Ltd., Dalian, China). Two-position, high-speed ten-port valves were purchased from Valco Instruments (Houston, TX, USA). Speed vac concentrator was from Thermo Fisher Scientific (San Jose, CA, USA). Bruker Autoflex MALDI TOF MS was used for protein identification (Bruker, Bremen, Germany).

2.3. Column packing

SEC *SRT*-150Å-5 μm and SEC *nanofilm*-250Å-5 μm particles were slurried in a mixture of water and alcohol (v/v = 2:1), and then packed into steel tubes (150 mm × 4.6 mm I.D. or 250 mm × 4.6 mm I.D.) under a constant pressure of 4000 psi.

2.4. SEC operation

Proteins were separated by home-packed SEC *SRT*-150 Å column (150 mm × 4.6 mm I.D., 5 μm, 150 Å) and SEC *nanofilm*-250 Å column (250 mm × 4.6 mm I.D., 5 μm, 250 Å). The mobile phase was composed of 10 mM Tris–HCl containing 150 mM sodium chloride and 5% (v/v) ACN (pH 8.0). Proteins were eluted isocratically at the flow rate of 150 μL/min, and the effluents were detected by UV at 214 nm.

2.5. Protein identification

For MALDI TOF MS analysis, collected protein fractions were desalted with a C8 macrotrap precolumn, which was firstly activated by mobile phase B containing 98% (v/v) ACN and 0.1% (v/v) TFA, and then equilibrated with mobile phase A containing 2% (v/v) ACN and 0.1% (v/v) TFA at flow rate of 0.5 mL/min. Samples were loaded onto the precolumn, and then washed with mobile phase A at 0.5 mL/min to remove salts. Finally, proteins were eluted with 1 mL of 80% (v/v) ACN, and then concentrated by a speed vac

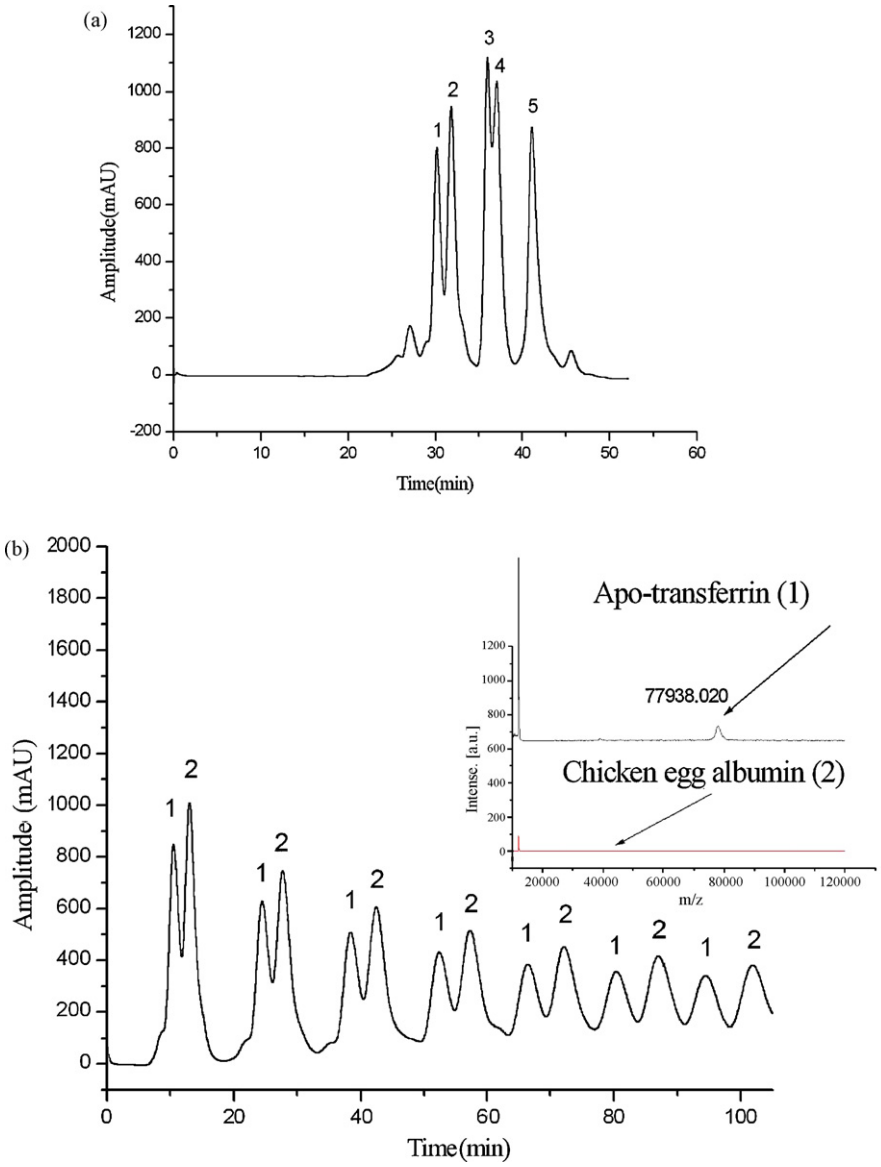

Fig. 4. Separation of a five-protein mixture by csrSEC. Experimental conditions were the same as those shown in Fig. 1. Sample: (1) apo-transferrin, (2) chicken egg albumin, (3) myoglobin, (4) ribonuclease, and (5) cytochrome c.

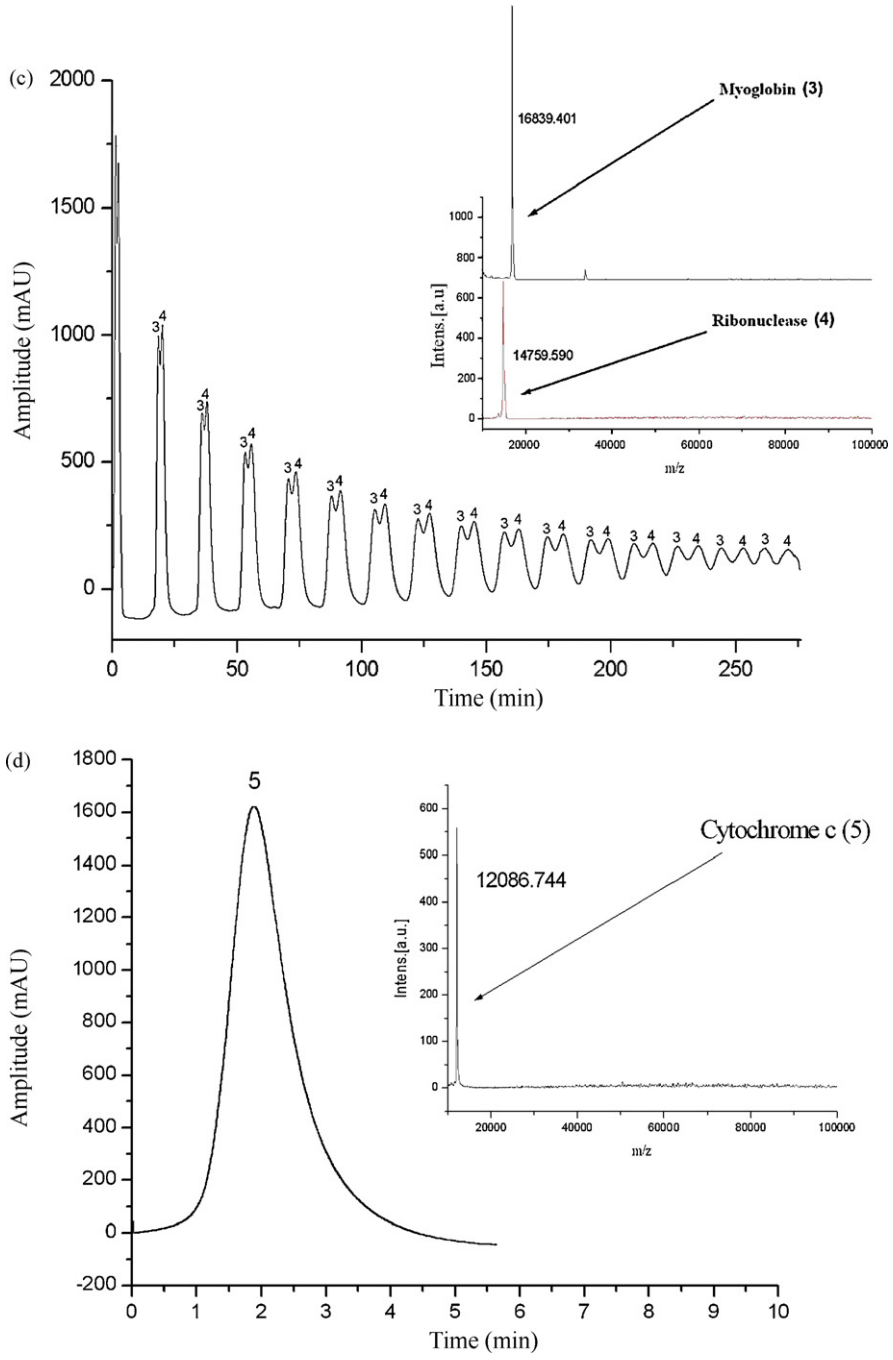

Fig. 4. (*Continued*).

concentrator. The residues were dissolved by 0.1% (v/v) TFA before analyzed by MALDI-TOF MS.

MALDI-TOF MS experiments were performed on Bruker Autoflex instrument equipped with a nitrogen laser (λ = 337 nm), and its available accelerating potential is in the range of +20/−20 kV. The analytical range of laser energy was adjusted to slightly above the threshold to obtain good resolution and signal-to-noise ratio. External mass calibration was obtained using two points that bracketed the mass range of interest. All mass spectra were obtained in the positive ion detection mode.

2.6. csrSEC-RPLC operation

The solvents for csrSEC were composed of 10 mM Tris–HCl containing 150 mM NaCl and 5% (v/v) ACN (pH 8.0) (C) and Water (D). Proteins eluted by SEC under isocratic mode with the flow rate of 150 μL/min were captured by C8 precolumns (10 mm × 4.6 mm I.D, 5 μm, 300 Å), and then separated by a C8 column (50 mm × 4.6 mm I.D, 5 μm, 300 Å). The mobile phases for RPLC were composed of 0.1% (v/v) TFA in both water (A) and ACN (B), and the binary gradient elution was set as follows: 0%B (0 min) → 0%B (1 min) → 20%B (3 min) → 80%B (19 min) → 80%B (20 min) with a flow rate of 2 mL/min. After each RPLC run, the column was equilibrated with the initial mobile phase for 5 min.

2.7. 1D-RPLC operation

To compare with the results obtained by 2D-csrSEC-RPLC platform, the same protein mixture was also separated by 1D-RPLC. The solvents for RPLC were the same as those described in

Section 2.6, and the optimized gradient for 1D-RPLC separation was set as follows: 0%B (0 min) → 0%B (5 min) → 20%B (10 min) → 80%B (70 min) → 80%B (75 min) with a flow rate of 1 mL/min. After each RPLC run, the column was equilibrated with the initial mobile phase for 20 min.

3. Results and discussion

Due to good biocompatibility, SEC has been widely used for biomolecule separation and preparation. However, due to the poor resolution and narrow separation window, the separation of complex samples by SEC is still of great challenges. Therefore, to achieve high resolution separation, in our present study, csrSEC was developed, and further used to construct a multidimensional csrSEC-HPLC platform to further improve peak capacity.

3.1. Evaluation of csrSEC

To improve the separation resolution, SEC with serially coupled columns, SEC-150 Å column (15 cm) and SEC-250 Å column (25 cm), were used for protein separation, not only with increased column length, but also with different pore sizes of packing materials for protein separation. As shown in Fig. 1, compared to the separation with single SEC column, the resolution of proteins was improved with serially coupled columns. However, the baseline separation was still not achieved, which indicated that SEC with limited column length could hardly meet the requirement of complex sample analysis.

Recycling size exclusion chromatography (rSEC) was also a practical method for SEC to increase separation column length without further increase on back pressure, by which samples were separated in a close-loop system by repeated separation till satisfactory resolution was achieved [11]. In our experiments, rSEC with coupled columns was used to separate myoglobin and ribonuclease, with 2500 Da difference on Mw. From Fig. 2a it could be seen that the baseline separation of such two proteins was achieved after 7 cycles. However, for complex sample analysis, the early eluted components in the cycle ($n+1$) might catch up and overlap with the late eluted ones in the cycle n. As shown in Fig. 2b, for the separation of a 5-protein mixture, with Mw ranging from 10 kDa to 80 kDa, after three cycles run, cytochrome c was overlapped with apo-transferrin, and the improved resolution was lost. All these results demonstrate that rSEC with coupled columns is applicable only for the analysis of simple samples with narrow Mw distribution.

To improve the resolution and peak capacity of SEC for complex samples, csrSEC was proposed with the combination of serially coupled SEC, sample parking technique and rSEC. As shown in Fig. 3, in such a system, by switching valve 1, proteins were injected into csrSEC system, and then proteins were separated by two serially coupled SEC columns through the control of valve 2. To ensure all fractions eluted from column 1 could be parked in column 2, a-25 cm-long column (250 mm × 4.6 mm I.D.) was chosen, which was much longer than column 1 (150 mm × 4.6 mm I.D.). By valve 2, a part of protein fractions with similar Mw were transferred from column 2 back to column 1, and repeatedly separated by column 1 till satisfactory resolution was achieved. With valve 3 switched, the components separated completely were collected or sent to the next dimension, respectively. Subsequently, other fractions maintained in column 2 were in sequence switched back to rSEC through the control of valve 2, and separated by column 1 till all components were analyzed.

Five proteins with Mw ranging from 10 kDa to 80 kDa, which could not be separated by rSEC with coupled columns (shown in Fig. 2b), were analyzed by csrSEC. As shown in Fig. 4a, the protein mixture was firstly separated by two serially coupled SEC columns, and all components parked in column 2 were divided into 3 fractions, and in turn switched back to column 1 to perform rSEC. By this technique, the first fraction was separated into apo-transferrin and albumin after 7 cycles (Fig. 4b), and the second fraction was separated into myoglobin and ribonuclease after 16 cycles (Fig. 4c), and cytochrome c was found as the unique protein in fraction 3 (Fig. 4d). Proteins except chicken egg albumin in each fraction were identified by MADLI TOF MS after collection. The absence of MS signal of chicken egg albumin might be caused by the low protein concentration and difficulty to be ionized. Compared with traditional SEC and rSEC, although prolonged separation window, higher resolution and improved peak capacity could be obtained by csrSEC, its disadvantages, such as sample dilution and band broadening caused by recycling separation, should still be further overcome.

3.2. Construction of csrSEC-RPLC platform

Due to the orthogonal separation mechanisms of SEC and RPLC, a multidimensional platform (csrSEC-RPLC) was constructed for protein analysis. As shown in Fig. 5, proteins were injected into csrSEC

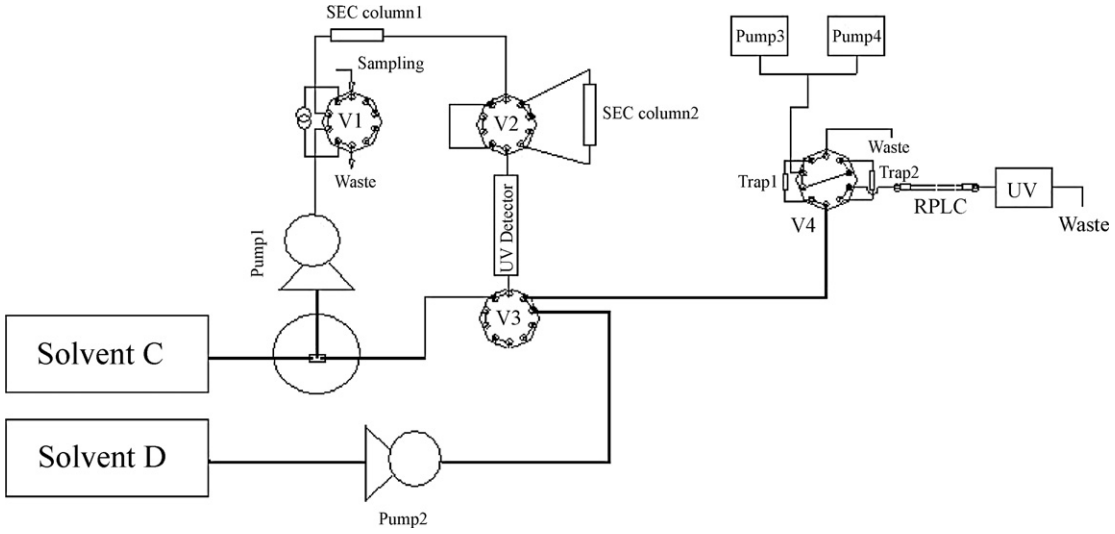

Fig. 5. 2D-csrSEC-RPLC platform.

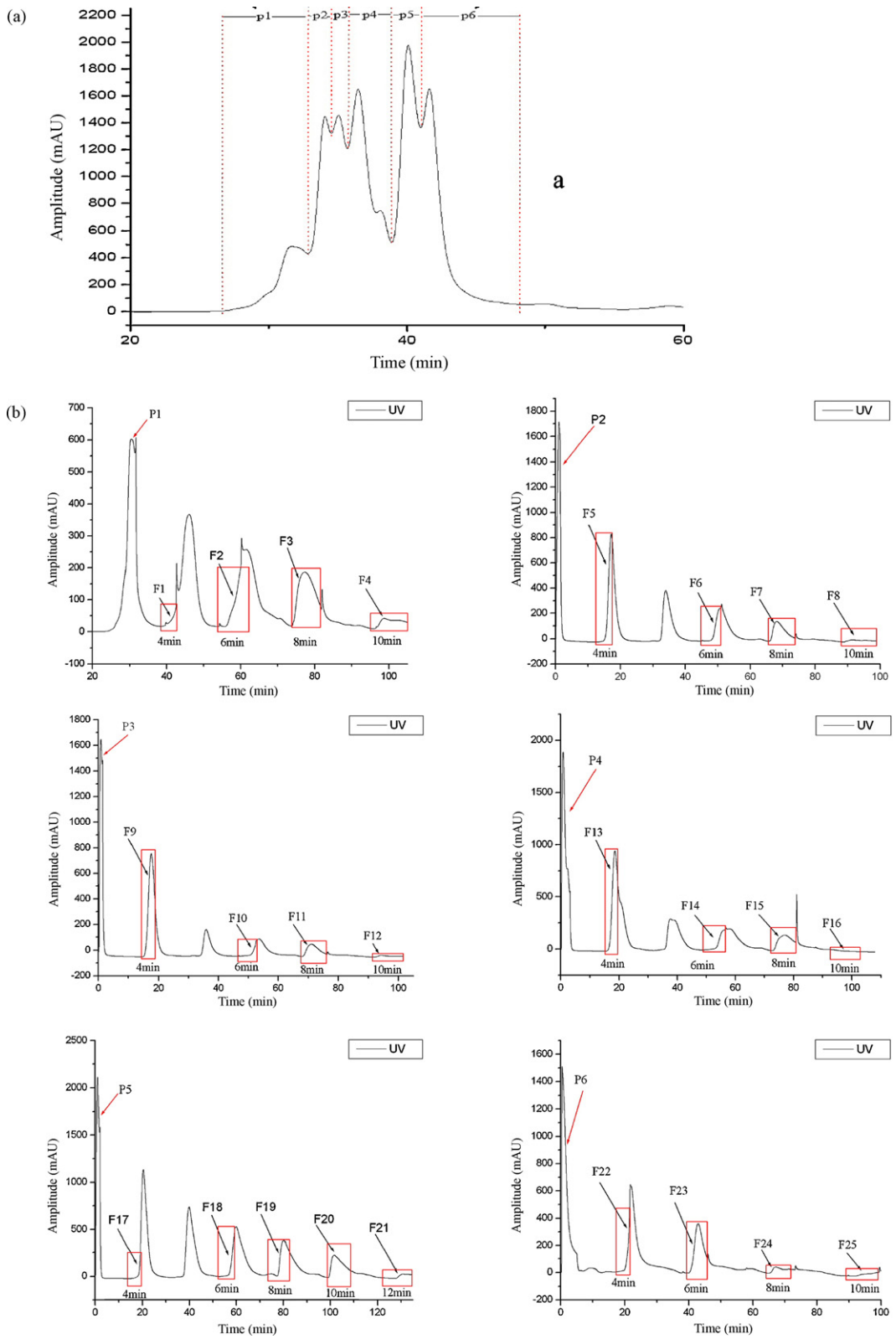

Fig. 6. Separation of an eight-protein mixture by csrSEC-RPLC. (a) Chromatograms of an eight-protein mixture separated by serially coupled SEC columns; P1, P2, . . ., P6, represented peaks. (b) Chromatograms of each peak switched from column 2 back into column 1 for rSEC separation; F1, F2, . . ., F25 represented fractions that were transferred into C8 trap columns for RPLC separation. (c) Chromatograms of each fraction from csrSEC separated by RPLC. (d) Chromatogram of an eight-protein mixture separated by 1D-RPLC. Experimental conditions: csrSEC: same as those shown in Fig. 1. RPLC: Mobile phase A: 0.1% TFA + water; mobile phase B: 0.1%TFA + ACN; column: C8 column (50 mm × 4.6 mm I.D., 5 μm, 300 Å); flow rate: 2 mL/min. The gradient conditions in the reversed-phase separation were set as follows: 0%B (0 min) → 0%B (1 min) → 20%B (3 min) → 80%B (19 min) → 80%B (20 min). UV detection: 225 nm. Experimental conditions of 1D-LC: mobile phase A: 0.1% (v/v) TFA + water; mobile phase B: 0.1% (v/v) TFA + ACN; column: C8 column (50 mm × 4.6 mm I.D., 5 μm, 300 Å); flow rate: 1 mL/min. The gradient conditions in the reversed-phase separation were set as follows: 0%B (0 min) → 0%B (5 min) → 20%B (10 min) → 80%B (70 min) → 80%B (75 min). UV detection: 225 nm. Samples: β-lactoglobulin (1), apo-transferrin (2), BSA (3), myoglobin (4), hemoglobin (5), catalase (6), carbonic anhydrase (7), and chicken egg albumin (8) with the concentration of 1.25 mg/mL for each protein.

system via valve 1, and then through the control of valve 2, proteins were separated into several fractions by serially coupled SEC columns. With valve 2 switched, the first fraction in column 2 with similar Mw proteins was transferred for rSEC separation. When satisfactory resolution was achieved, proteins were switched out of csrSEC by valve 3, and in sequence trapped on two C8 parallel precolumns by valve 4, followed by further separation by RPLC, during which the residual fractions in column 2 were ordinally switched for rSEC separation through the control of valve 2, and then repeated above-mentioned processes till all proteins were analyzed. Although peak broadening effect became serious with the increased cycle times, it could be compensated by increasing the sampling frequency from the first to the second dimensional separation. To obtain high two-dimensional resolution, Murphy et al. [21] proposed that each peak from the first dimension should be sampled at least 3 times into the second dimension for further separation. However, to avoid excessively short run time in the second dimension separation, Horie et al. [22] suggested that modulation periods were more favorable under realistic chromatographic conditions when they were adjusted to about 2.2–4 times

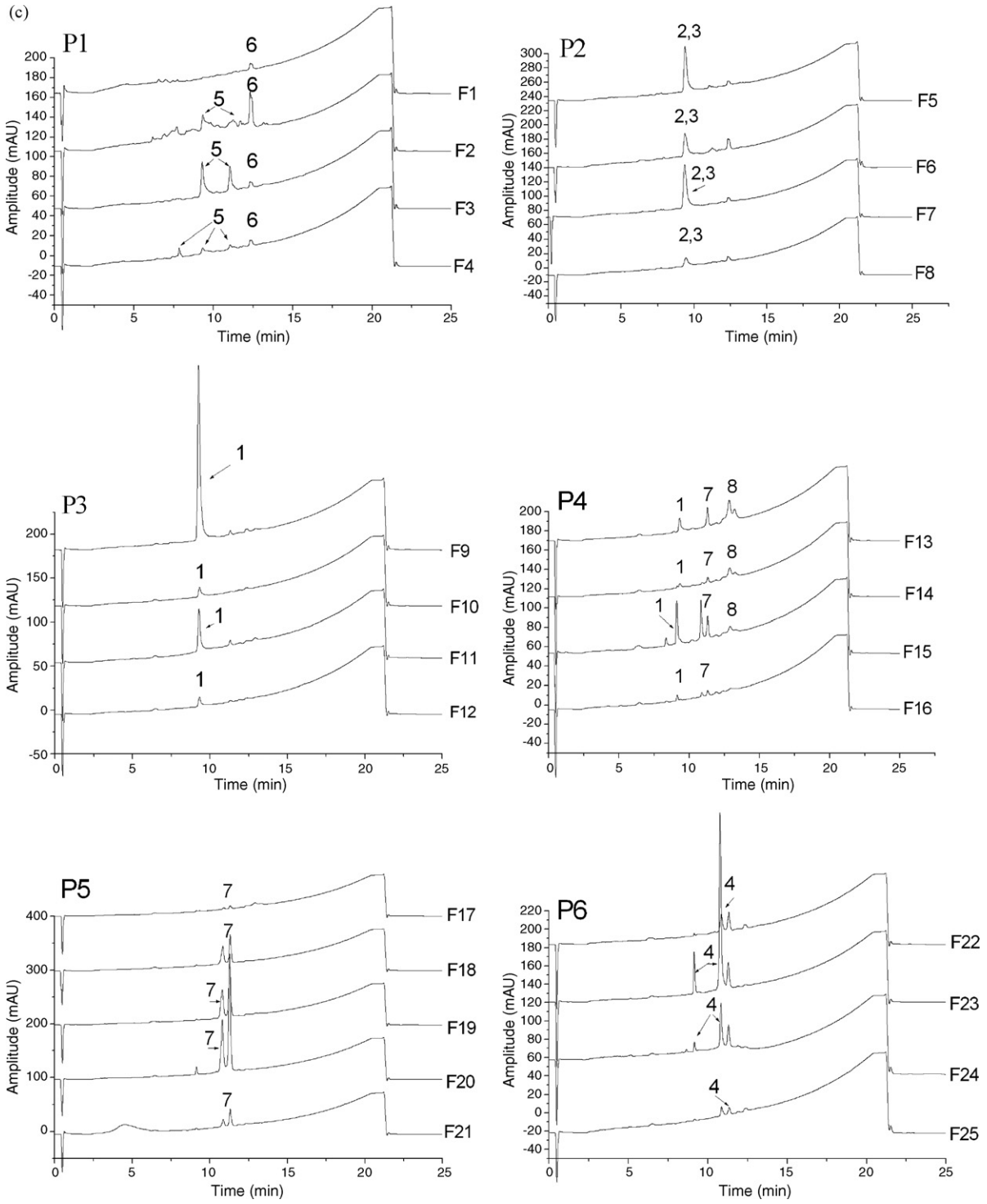

Fig. 6. (*Continued*)

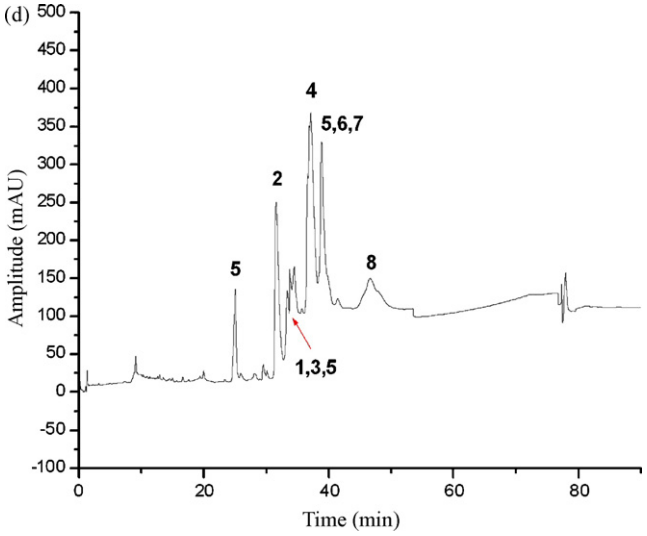

Fig. 6. (*Continued*).

the standard deviation of 1D peak. In our experiments, due to the prolonged separation window and improved resolution by csrSEC, each peak in the recycling system could be sampled about 4 times into RPLC, as shown in Fig. 6b. Therefore, it could be expected that besides the advantage of high peak capacity, multidimensional csrSEC-RPLC is of improved time compatibility with RPLC separation.

3.3. Protein separation by csrSEC-RPLC

To evaluate the performance of csrSEC-RPLC, an eight-protein mixture was separated. After separation by SEC with serially coupled columns, six main peaks were obtained, as shown in Fig. 6a, and then each peak was in turn switched from column 2 into column 1 for repeated separation, as shown in Fig. 6b. Taken the effects of cycle times on sample dilution and peak broadening into account, the sampling time of fractions from each peak obtained by csrSEC into RPLC was increased gradually. In our experiments, the sampling time was set from 4 min, to 6, 8, and 10 min respectively till all fractions from one peak were completely transferred into RPLC, which kept csrSEC analysis synchronization with RPLC. In total, 25 fractions eluted by csrSEC were separated by RPLC, which was performed under a 20 min-gradient, and most proteins, except BSA and transferrin with quite similar Mw and hydrophobicity, were separated in baseline, as illustrated in Fig. 6c. However, most of them could not be separated completely by 1D-RPLC even with a longer time gradient, as shown in Fig. 6d.

3.4. Peak capacity of csrSEC-RPLC

Since csrSEC system could be regarded as the combination of SEC with serially coupled columns and rSEC, it should be considered as multidimensional SEC. A key feature of multidimensional separation is the multiplicative peak capacity resulting from orthogonal separation. For a comprehensive multidimensional HPLC, Giddings demonstrated that the total peak capacity could be described as follows [23]:

$$p = p_{s1} \times p_{s2} \times p_{s3} \times \cdots p_{sn} (n \geq 2) \quad (1)$$

where $p_{s1}, p_{s2}, \ldots, p_{sn}$ represents the peak capacity of each dimension, which could be calculated by

$$p_s = \frac{t_p}{w_v} \quad (2)$$

where t_p is the elution time of the sample; w_v is the average peak width.

For the separation of eight proteins, the protein mixture was firstly separated by serially coupled SEC, and the peak capacity of which was calculated to be about 18, divided by the elution time of 35 min, and the average peak width, 2 min. Then each peak eluted from serially coupled SEC was repeatedly separated by rSEC, divided into several fractions, and then sampled into next dimension for further separation. As we know, both oversampling and undersampling of first dimensional peaks into the second one have disadvantageous effects on the resolution and peak capacity of 2D-LC [24–25]. For csrSEC-RPLC separation, although the undersampling of first dimension peaks would result in reduced total analysis time, the resolution and peak capacity of 2D-LC could be decreased as well. In our experiments, to compromise peak capacity with analysis time, each peak from csrSEC system was sampled 4 times into the second dimension for further separation.

For the calculation of peak capacity of rSEC, due to the existence of dead volume between the outlet of UV detector and the inlet of column 1 (V_0), resulting in the generation of dead time (t_0), as shown in Fig. 5. t_0 could be calculated according to Eq. (3):

$$t_0 = \frac{V_0}{F} \quad (3)$$

where F is the flow rate of separation. In our study, since V_0 is about 30 μL, and F is 150 μL/min, t_0 should be 12 s.

As shown in Fig. 6b, the total analysis time of a typical recycling chromatogram is estimated to be 80 min. However, due to the existence of dead time, t_0, the practical separation time should be calculated to be $(80 - nt_0)$, where n is the number of recycling in rSEC, which is 4 in our experiments. Therefore, the practical separation time of a typical recycling separation is calculated to be 79.2 min, and the average peak width is estimated to be 10 min. Therefore, the peak capacity of rSEC is calculated to be about 8.

Furthermore, the peak capacity of RPLC was 25, by dividing the separation time, 20 min, to the average peak width, about 0.8 min. Therefore, according to Eq. (1), the total theoretical peak capacity of csrSEC-RPLC should be about 3600 ($18 \times 8 \times 25$).

4. Conclusion

A novel approach for high resolution protein separation based on csrSEC has been developed, and further coupled with PRLC to achieve improved peak capacity. Compared to SEC or rSEC, not only

higher resolution can be achieved, but also the time compatibility to couple with other HPLC modes was excellent by adjusting the cycle times in csrSEC. With such a system, peak capacity for proteins as high as 3600 was obtained, comparable to that of 2D-PAGE. Therefore, it could be prospected that such a novel separation mode might play an important role in the separation of complex protein samples.

Acknowledgments

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