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Intact-protein trapping columns for proteomic analysis in capillary high-performance liquid chromatography

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ABSTRACT

A new type of monolithic trapping columns with high mechanical strength was prepared by thin-layer sol–gel coating method and applied to trapping intact proteins for on-line capillary liquid chromatography. Monolithic trapping columns were fabricated by entrapping C8 reversed-phase particles into the capillary columns through a sol–gel network, which was formed by hydrolysis and polycondensation of methyltriethoxysilane. Hundreds times of trapping/untrapping for intact proteins were carried out. The trapping columns showed long-term stability up to 300 bar. Recovery, loading capacity and reproducibility of trapping columns were evaluated using four proteins. The recovery of four protein mixtures for the C8 monolithic trapping columns was 99.3% on average. The loading capacity of 5 mm × 320 μm i.d. C8 trapping columns for the protein mixtures was 30 μg. Day-to-day relative standard deviation (RSD) values for recoveries of protein mixtures on the same C8 trapping column ranged from 2.34 to 5.87%, column-to-column RSD values were from 3.01 to 6.81%. The C8 trapping columns were used to trap normal mouse liver intact proteins in a capillary liquid chromatography system. Results demonstrated high efficiency of the monolithic trapping columns for trapping intact proteins for proteomic analysis in on-line capillary liquid chromatography system.

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1. Introduction

"Bottom-up" and "top-down" strategies are two main approaches for the analysis of complex biological sample in proteomic research [1]. Bottom-up proteomic approaches commonly refer to shotgun proteomic technologies, which have been widely used in proteome in recent years [2,3]. Although shotgun proteomic strategies play an important role in large scale identification of unknown proteins, they provide very limited molecular information about intact proteins and this poses a great challenge for the scan rate of mass spectrometry (MS) to analyze a complex mixture of hundreds of thousands of tryptic digest peptides.

Top-down proteomics, in which intact proteins rather than peptides are measured, can generate high sequence coverage and provide excellent molecular level information of intact proteins (e.g., molecular weight and post-translational modifications (PTMs)) [4–6]. We could make full use of peptide mass fingerprint information to study intact proteins. Knowing the intact mass of the protein that gave rise to a specific proteolytic peptide would be useful in determining its identity and whether it is a form related to a pathological stimulus. Understanding how proteins vary in quantity between different states is an integral goal of proteomic science. Studying protein–protein interactions provides us with a better understanding of the biological function of specific proteins and the regulation of cell signaling events. All this information could be obtained by studying intact proteins with a top-down proteomic strategy. In order to acquire full potential of top-down approaches, effective separation of intact proteins prior to MS could decrease sample complexity and increase the dynamic range of detection.

For the separation of complex protein mixtures, two-dimensional liquid chromatography (2D-LC) is a promising alternative to two-dimensional gel electrophoresis (2D-PAGE) due to its advantages of automation, versatility, high throughput and high sensitivity [7,8].

In micro- and nano-scale high-performance liquid chromatography (HPLC), a trap column is often used in front of the analytical column to increase sensitivity, sample loading amount and minimize loading time.

Most commercially available precolumns need on-column end-frits or in-line filters to retain packing materials in the capillaries. The frit is not easy to be fabricated in a reproducible manner, and column reproducibility in terms of mechanical strength and permeability is difficult to accomplish.

One solution of avoiding frits is to making monolithic columns. The monoliths can easily be prepared in capillaries through in situ polymerization reaction or the sol–gel process. Generally, mono-

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lithic materials can be classified into three types: silica-based [9–11], polymer-based [12–17], and particle-fixed monoliths [18]. Tanaka et al. [19–22] prepared various silica-based and polymer-based monolithic columns and evaluated their chromatographic properties in HPLC. Some of them [21,22] showed promising advantages for separation of peptides and proteins. The application of monoliths as trapping columns for enrichment of small molecules and peptides was also found in HPLC.

Trace environmental contaminants were preconcentrated by monolithic C18 capillary columns in microcolumn HPLC via large volume injection [23,24]. Wang et al. [25] prepared a strong cation-exchange (SCX) monolithic trap column within a 150 μm i.d. capillary, which was coupled with a reversed-phase column for on-line multidimensional separation of tryptic digest of yeast proteins. Schley et al. [26] used monolithic columns based on poly-(styrene-divinylbenzene) (PS-DVB) both for preconcentration and analytical separation of peptides in micro-scale HPLC. A similar research was carried out by Marcus et al. [27]. Gu et al. [28] developed a large-bore particle-fixed monolithic precolumn for on-line peptides trapping in multidimensional liquid chromatography.

Compared to peptide trapping and enrichment, there are seldom reports about intact protein on-line trapping technologies. With the growing interest in separation of intact proteins by micro- and nano-scale HPLC, developing an effective method for trapping and desalting of intact proteins is an essential task. Obviously, adopting monolithic columns is still an elegant way. Particle-fixed monolithic columns are prepared by immobilizing conventional spherical packing materials inside a fused-silica capillary. There is a wide application in capillary electrochromatography field [29,30]. They inherit the versatility of well-developed packing materials. Various types of packing materials can be entrapped by sol–gel, forming a variety of continuous-bed columns. Different approaches have been developed to fix the particles in the capillary, and the obtained columns can be classified as particle-sintered [31], particle-loaded [32,33], particle-bonded [34] and particle-entrapped monoliths [30,35]. Every method has its merits and shortcomings. For the particle-entrapped monoliths, sol–gel as entrapping solution is used to "glue" adjacent particles and capillary wall together. The preparation method is simple and reliable, column-to-column and batch-to-batch have good reproducibility. Large-bore particle-entrapped monolith has large sample loading capacity. As a trapping column, this type of monolith is an ideal choice.

In our previous study, particle-entrapped monolithic precolumns have been developed for peptide preconcentration in the capillary liquid chromatography. Peptides were recovered effectively. However, its application to trapping proteins was not satisfactory. Recoveries for some proteins were less than 20%. The low recovery of proteins attributes to thick sol–gel coatings on the surface of particles and high back pressure. Thus, great efforts were taken to improve the procedure of making monoliths suitable for trapping proteins. An effective method was to decrease the thickness of sol–gel coatings on the surface of particles.

The main aim of our present study was to develop a new type of particle-entrapped monoliths with thin sol–gel layer for effectively trapping intact proteins. Formula was optimized, process conditions were improved, and packing materials were selected. Thin-layer sol–gel coatings on the surface of particles were obtained and the mechanical strength of the trapping columns was still high. Permeability was improved and recovery of intact proteins was increased. C8 monolithic trapping columns were prepared and applied to trapping standard proteins. By setting up a C8 monolithic trapping column-capillary reverse phase liquid chromatography (RPLC) system for on-line preconcentration, desalting and separation of proteins, various parameters affecting protein preconcentration were investigated and optimized. The home-made monolithic trapping columns were applied to trap mouse liver intact proteins. Effective recovery of intact proteins approved excellent performance of monolithic trapping columns for its future application in 2D-LC platform.

2. Experimental

2.1. Materials and chemicals

Fused-silica capillaries (320 μm i.d., 450 μm o.d.) were purchased from Yongnian Optical Fiber Factory (Yongnian, Hebei, China). Packing materials of C18 particles (Kromasil spherical silica, 5 μm, 300 Å) and spherical silica gel (5 μm, 300 Å) were obtained from AKZO NOBEL Company (Sweden) and Beijing Giant-Carrier Co., Ltd. (Beijing, China) respectively. Packing materials of C4 (cleanert XBP-C4, 5 μm, 300 Å), C8 (H-ASB-C8, 5 μm, 300 Å) particles were purchased from Agela Technologies (Tianjin, China).

Phenylmethanesulfonyl fluoride (PMSF), methyltriethoxysilane (MTES), sodium orthovanadate (Na_3VO_4), trifluroacetic acid (TFA) and protein standards were purchased from Sigma–Aldrich (St. Louis, MO, USA). The water used was Milli-Q grade (Millipore, Molsheim, France). HPLC grade acetonitrile (ACN) was provided by Mallinckrodt Baker (Phillipsburg, USA).

2.2. Sample preparation

Standard protein solutions were prepared by dissolving ribonuclease B, cytochrome *c*, myoglobin and albumin from chicken egg white in pure water at a concentration of 10 μg/μL, respectively as the stock solution.

Healthy rat liver was cut into small pieces and cleaned with cold physiological saline solution (0.9% NaCl) to remove blood and some possible contaminants. Then, 0.4 g of tissue debris was rapidly mixed with 10 mL lysis buffer, containing 1 mM PMSF, 0.2 mM Na_3VO_4, 1 mM sodium fluoride, complete protease inhibitor cocktail (Roche, Basel, Switzerland). Tissue sample was homogenized in an ice bath. The resulting homogenate was swirled for 30 min and centrifuged for 15 min at 14,000 × *g*. The supernatant was collected. Protein concentration of the sample was 4 mg/mL by the modified Bradford method described by Qu et al. [36].

2.3. Fabrication of particle-fixed intact-protein trapping columns

The monolithic trapping columns were prepared by thin-layer sol–gel coating method.

Prior to column packing, the capillaries (150 mm × 0.32 mm i.d.) were rinsed with methylene chloride (CH_2Cl_2) and then were dried by passage of nitrogen gas. On-column frits were fabricated by sol–gel technology according to previously reported method [37]. High pressure slurry packing method was adopted to pack capillaries. C8-bonded particles in methanol were introduced into capillaries with 320 μm inner diameters using an LC-100 constant flow pump (Shanghai Wufeng Scientific Instrument Company, China). During the packing process, capillaries were sonicated for 0.5 h under the 300 bar of packing pressures to acquire homogeneous and tight column bed. Finally they were depressurized overnight and then dried by an oven at 100 °C for 10 h to ensure that no dilution of the entrapment solution occurred.

After drying, the column was cooled to room temperature, particle-fixed monolithic trapping columns were prepared by thin-layer sol–gel coating method. A sol solution composed of 35 μL MTES, 120 μL CH_2Cl_2, 65 μL TFA and 5 μL H_2O was vortexed in a plastic vial, which was subsequently introduced into a home-made gas pressure-operated capillary filling/purging device. The packed capillary column was inserted into the sol solution, and then a pressurized nitrogen gas was used to flush through the capillary column

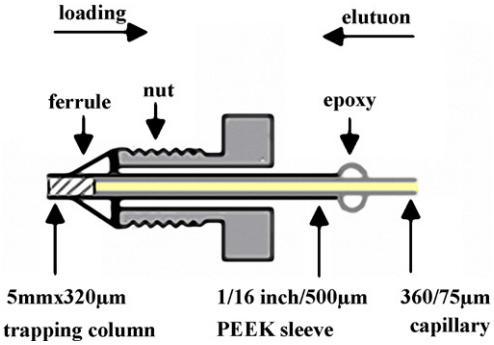

Fig. 1. Holder for a 5 mm × 0.32 mm monolithic trapping column.

with the sol solution. The sol-filled packed capillary was stored at room temperature for at least 2 h for the conversion of the sol to a gel, and for aging of the resulting wet gel. Finally, the columns were placed in oven and cured at 100 °C for 24 h. Then the temporary frits of particle-entrapped monolithic columns were removed, the choice of column length is quite flexible. In the experiment, monoliths were cut into 5 mm long in store.

The morphology of the monolithic trapping columns was examined by scanning electron microscopy (SEM, Philips XL30, Eindhoven, The Netherlands).

2.4. On-line trapping-capillary RPLC experiments setup

Because of the geometry restriction of column connections, the 5 mm long laboratory-made monolithic trapping column, instead of a commercial trap column, is experimentally difficult to connect to the standard unions and fittings. We designed a special column holder to facilitate leak-tight connections (Fig. 1). To minimize the void volume between trapping column and the analytical column, a trapping column and a 75 μm i.d. fused-silica capillary of 20 cm in length were inserted into a 3 cm × 500 μm i.d. polyether ether ketone sleeve (PEEK) tubing from two ends. They were placed head-to-head inside this 3-cm long PEEK tubing. On the outlet-end of PEEK tubing, the trapping column was fixed tightly with PEEK tubing using stainless steel ferrule. A standard microtight fitting (nut) was used to mount the trapping column to the valve. Finally, epoxy glue was applied around the outside of the PEEK/fused-silica capillary boundaries to avoid leakage. After epoxy was dry, the trapping column and the separation column were connected to valve 2 in a way that the trapped analytes were transferred from the trapping column to the separation column in back-flush mode.

Fig. 2 illustrates the instrumental setup of on-line preconcentration-capillary RPLC system. The system consists of a shimadzu isocratic pump (LC-10Ai, Shimadzu, Kyoto, Japan), an Agilent 1100 series capillary pump (Agilent Technologies, Inc., Palo Alto, CA), an Agilent 1200 series multi-wave UV detector with an 80-nL flow cell, a 6 port 2-pos manual injection valve (Valco Instruments Co., Inc., USA) (valve 1) fitted with a 2 or 20 μL loop,

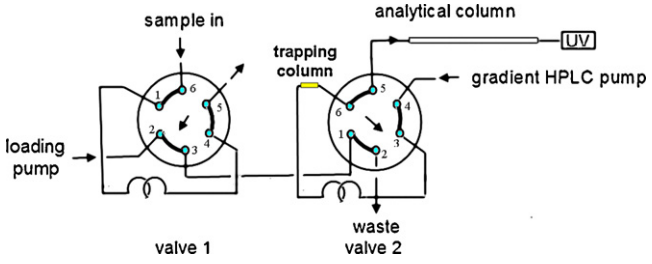

Fig. 2. Illustration of on-line trapping system.

a 6-port switching valve (Valco Instruments Co., Inc., USA) (valve 2), a particle-fixed monolithic trapping column (5 mm × 320 μm i.d.), a capillary analytical RPLC column (Zorbax 300 SB-C18, 5 μm, 150 mm × 0.3 mm, Agilent).

The trapping column with the holder was directly mounted to port 6 of valve 2. Valve 1 and valve 2 were connected by a 15 cm × 65 μm i.d. PEEK tubing. Separation column was connected to port 5 of valve 2. The column outlet was connected to the UV detector.

The shimadzu isocratic pump was used to deliver loading buffer (pure water) at a flow rate of 10 μL/min. Samples were loaded onto the monolithic trapping column using valve 1. After flushing the trapping column with the loading buffer for 5 min, valve 2 was switched to connect the trapping column on-line with the capillary RPLC column, trapped proteins were desorbed and transferred to the analytical column for chromatographic separation.

Capillary RPLC separation experiments were performed on the Agilent 1100 series capillary pumping system. Binary solvents of A (5% ACN/0.05% TFA) and B (90% ACN/0.05% TFA) were used in elution. Gradient elution for standard protein mixtures was as follows: 10 min linear gradient from 0 to 25% B, 20 min linear gradient from 25 to 45% B, then 5 min linear gradient from 45 to 95% B, and 5 min linear gradient from 95 to 90% B, after 5 min back to 0% B. Gradient elution for normal mouse liver proteins was as follows: 45 min linear gradient from 0 to 80% B, 10 min linear gradient from 80 to 95% B, after 10 min back to 0% B. The flow rate was 4 μL/min. UV detection was carried out at 215 nm. Chromatographic data acquisition and processing were performed by an Agilent Chromatographic Workstation.

3. Results and discussion

3.1. Preparation of intact-protein trapping columns

The thickness of sol–gel coatings on the particles plays an important role in molecules' diffusion. Peptides are similar to small molecules, they diffuse through the pores of the stationary phase easily. In contrast to peptides, proteins are more complicated. They have different physicochemical properties, space structures, and may include a wide variety of PTMs. It is difficult for large proteins to diffuse into and out of the pores of the stationary phase freely.

To effectively trap intact proteins prior to separation and obtain high recovery, monoliths should offer thin sol–gel film coatings, high mechanical strength and good permeability. Thin layer of film enables proteins pass through pores of the particles easily, mass transfer performance quickly. Moreover, the macropores around the silica skeleton must be sufficiently large to facilitate free transport of proteins through sol–gel network. Formula and preparation process were improved to prepare a new type of monolithic trapping columns by thin-layer sol–gel coating method.

As far as formula is concerned, when precursor volume percentages are higher than 18.0%, particles were coated completely by multilayer sol–gel, which caused formation of thick film and poor permeability (Fig. 3a). When precursor volume percentages are lower than 12.0%, weak mechanical strength and poor durability happened (Fig. 3b). By comparison, 15.5% (v/v) precursor was adopted in this work. A thin layer of film on the surface of the particles was formed and mechanical strength of the monoliths was retained (Fig. 3c) via the new formula.

In terms of preparation procedure, the sol–gel solution in an ice bath was pushed into the packed capillary quickly by nitrogen at 0.5 MPa inlet pressure in case sol solution became viscous. After the capillary was full of the entrapping mixtures, frit end of the capillary was plugged with silicon rubber. Under 0.5 MPa of nitrogen flow, sol solution reacted with the inner walls of the capillary and particles

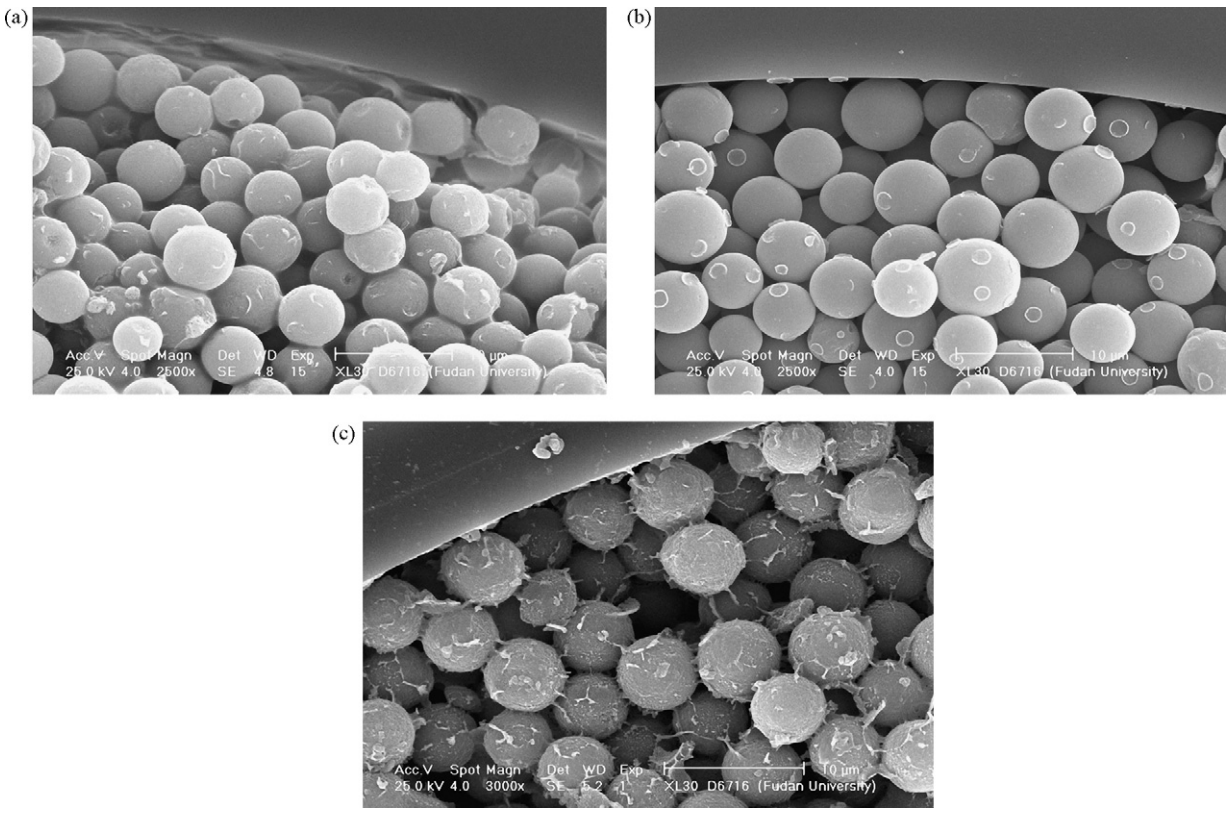

Fig. 3. Scanning electromicrograph of the monolithic trapping column packed with 5 μm C8 particles, entrapped with "sol–gel" mixture. (a) Precursor volume percentages are higher than 18.0%; (b) precursor volume percentages are lower than 12.0%; (c) precursor in the sol solution is 15.5% (v/v).

for 2 h. The excess sol solution was then flushed out under small nitrogen flow. The coated capillary column was subsequently aged and dried overnight in an oven at 100 °C. During the whole aging process, the capillary column was purged with nitrogen to further remove residual sol–gel, which leaves a thin film on the outside surface of the packing particles.

Apart from formula and preparation procedure, another key to successful column preparation is the characteristic of the packing materials themselves. In the experiment, some packing materials with excellent performance for separation of proteins resulted in poor efficiency and even complete column failure. After trial and error, we found that exposed silanol groups on the silica surface had a tremendous influence on successful preparation. Generally, residual silanol groups on the stationary phase surface were unfavorable for the chromatographic separation. Endcapping reagents were often employed to react with the surface of the stationary phase. Endcapped surface is beneficial to separation, but it is disadvantageous to sol–gel reaction. Monolithic trapping columns were prepared by entrapping stationary phase particles into the capillary columns through a sol–gel network, which was formed by hydrolysis and polycondensation of methyltriethoxysilane. Insufficient silanol groups could not link particles and wall of the capillary together through sol–gel network. There are two ways to solve this problem. One way is to treat endcapped packing materials with appropriate concentration of sodium hydroxide solution. Treated particles have more silanol groups on the surface of the stationary phase, prepared trapping columns could trap proteins effectively. However, treating procedure is time-consuming and tedious. Another way is to adopt nonendcapped packing materials.

Under the optimum conditions, nonendcapped C8 particles were adopted to prepare intact protein monolithic trapping columns. C4, C18 monolithic trapping columns were also prepared in the same way. Compared to one single commercially available precolumn with frits, a batch of reproducible monolithic trap columns with similar back pressure and permeability were obtained. And the choice of column length is quite flexible.

3.2. Permeability and mechanical strength of intact-protein trapping columns

The sol–gel network offers high permeability, high mechanical strength. Once entrapment is completed, the capillary no longer requires frits. Fig. 3c shows the SEM photograph of sol–gel entrapped C8 monolithic column. It can be seen that sol–gel network with homogeneous macropores was formed, particles were bonded to each other and to the column inner wall by the thin-layer 3D-sol–gel network, forming monoliths without cracks. So we can cut trapping columns into any desired length. For a 5 mm monolithic trapping column, the back pressure at a flow rate of 10 μL/min loading buffer was typically in the range of 10–20 bar.

Since the application of column switching involves very abrupt changes in flow rate and column pressure, mechanical strength and stability of trapping columns are quite important to its long-term use. Hundreds times of trapping/untrapping for intact proteins were carried out. The trapping columns were quite stable. We did not observe any failure of the trapping columns for more than five hundred times of switching.

3.3. Effects of the C8 monolithic trapping columns on trapping standard proteins

The applicability of the monolithic preconcentration columns to trapping intact proteins was studied by injecting a mixture of four proteins including: ribonuclease B, cytochrome *c*, myoglobin and albumin from chicken egg white onto the monolithic trapping columns. After back-flush elution, the proteins were separated

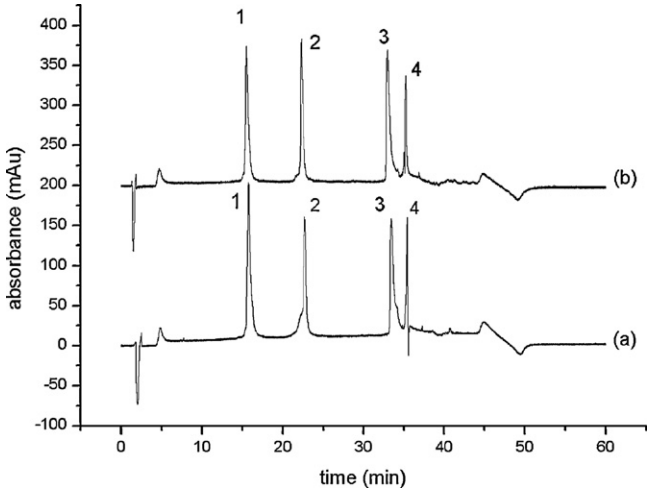

Fig. 4. Capillary RPLC separation chromatograms of 0.5 μg protein mixtures. Proteins: (1) ribonuclease B; (2) cytochrome *c*; (3) myoglobin; (4) albumin from chicken egg. (a) Proteins directly injected to the capillary analytical column; (b) proteins trapped by a 5 mm C8 trapping column; loading flow, 10 μL/min; trapping time, 5 min; detection wavelength, 215 nm.

in separation column and detected at 215 nm. We calculated the recoveries of four proteins for the three different trapping columns. 98.9, 99.3, and 99.0% of recoveries were obtained respectively for C4, C8 and C18 monolithic trapping columns. Fig. 4 shows the capillary chromatogram of the protein mixtures after trapping by the C8 trapping column including peak assignments. Every protein was recovered effectively.

3.3.1. *Effect of sample loading flow rate on trapping standard proteins*

We carried out the experiments in two aspects. First, we investigated the effect of loading volume on recovery of proteins at a constant loading flow rate of 10 μL/min, loading time ranged from 1 to 10 min in 2.0 min intervals. It was found that when loading volume exceeded 60 μL, recoveries of proteins decreased, partial proteins probably came off the trap column before the valve was switched. Therefore, 50 μL of loading volume was chosen for the following experiment.

At a constant loading volume, we investigated the influence of loading flow rate on protein recovery. 0.5 μg of four protein mixtures were loaded on the trapping columns when flow rates changed from 3 to 40 μL/min, as indicated in Fig. 5. Interestingly, it was observed that slow flow rate is preferable for recovery of proteins. From 3 to 25 μL/min, with the increase of loading flow rate, the eluted peak areas decreased slightly, the recoveries changed from 99.3 to 95.6%. This might be because proteins are larger than peptides, under fast loading flow, the dynamic equilibrium between proteins and the stationary phase was not complete, which resulted in partial loss of the analytes and drop of the recovery. From 25 to 40 μL/min, the eluted peak areas tended to drop obviously. In view of analysis speed, we chose 10 μL/min of loading flow rate in the experiment.

3.3.2. *Loading capacity of the monolithic trapping columns*

Sample loading capacity is one of the important characteristics for on-line preconcentration. It is particularly crucial to determine the maximum sampling amount allowed to avoid any analyte loss. The mixture of ribonuclease B, cytochrome *c*, myoglobin and albumin from chicken egg was used to test the loading capacities of a 5 mm × 320 μm i.d. trapping column. A series of solutions with increasing concentration were injected onto the trapping column, as shown in Fig. 6. Under sampling amount of 30 μg, the signal

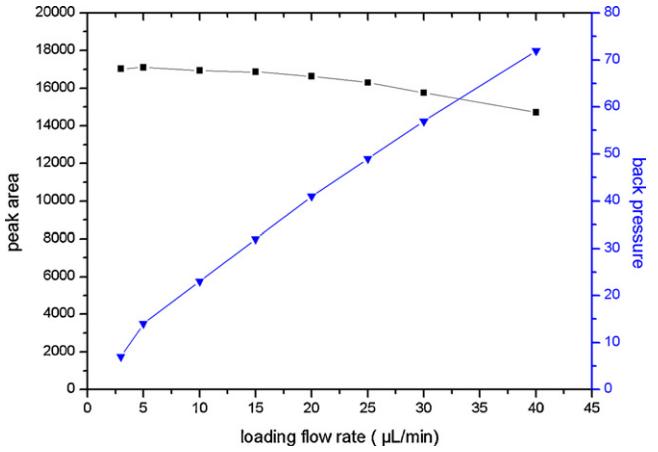

Fig. 5. Effects of loading flow rate on the trapping efficiency of the protein mixtures. Sample volume, 2 μL; sample concentration, 0.25 μg/μL; detection wavelength, 215 nm; ■, peak area; ▼, back pressure.

increased almost linearly in relation to the injected amount. However, on increasing injected amount from 37.5 to 75 μg, no further increase in the signal was observed. The maximal loading capacity of the trapping column (5 mm × 320 μm i.d.) was 30 μg of standard protein mixtures.

3.3.3. *Trapping factor of the monolithic trapping columns*

Capillary chromatography separation of the four proteins mixture is shown in Fig. 7. Fig. 7a was obtained when 2 μL volume of 0.0625 μg/μL of ribonuclease B, cytochrome *c*, myoglobin and albumin from chicken egg white respectively was injected into the system, while Fig. 7b was obtained when 20 μL of 6.25 ng/μL of the same mixture was enriched by a monolithic trapping column before it was separated by the analytical column. There is not any loss in resolution and recoveries, the result proved enrichment ability of the trapping column with a dilute sample. The enrichment factor was 10.

3.3.4. *Effect of salt concentration on trapping standard proteins*

In ion exchange chromatography-RPLC platform, trapping column functions to trap the analytes eluted from the first dimension by the salt buffer and simultaneously to remove excess solvent, salts to waste for the second dimensional separation. We studied the influence of salt concentration on the enrichment efficiency of proteins. Protein mixtures in the different concen-

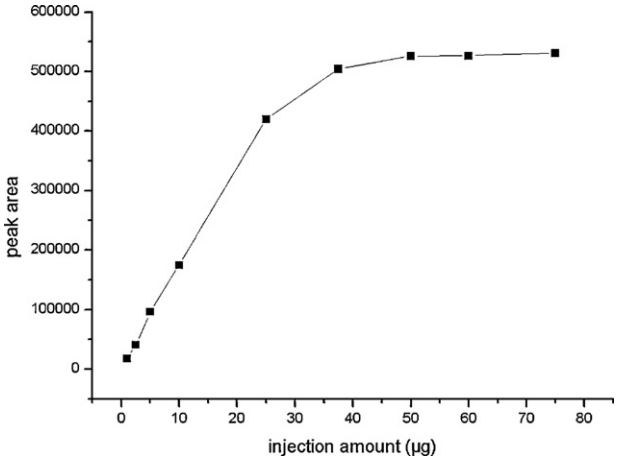

Fig. 6. Sample mass loading measurement curves for the four protein mixtures. Loading flow, 10 μL/min; trapping time, 5 min.

Table 1
Reproducibility of properties of C8 trapping columns.

$n = 6$	RSD% of recovery of four standard proteins				RSD % of back pressure
	RB	Cyto *c*	Myo	Albumin	
Same trapping column					
Run-to-run	1.26	3.02	1.83	5.60	1.02
Day-to-day	2.34	3.45	4.21	5.87	1.53
Different trapping columns					
One batch	3.01	4.19	5.20	6.34	2.07
Batch-to-batch	4.92	5.13	6.62	6.81	2.35

trations of ammonium acetate (NH_4Ac) buffer ranging from 200 to 1000 mmol/L in 20 μL volume were injected to the system. We did not observe any influence on the recovery of proteins caused by the high concentration of salt buffer. We also demonstrated salt durability of the trapping column to NaCl solution in 2D–LC. The trap column was applied to trap and desalt of one randomly selected weak anion exchange (WAX) fraction of mouse liver extract. Mouse liver proteins on the WAX column were eluted by salt step with mobile phases (A) 10 mM Tris–HCl (pH 7.5) and (B) 10 mM Tris–HCl/500 mM sodium chloride (pH 7.5). Experimental results demonstrated home-made trap column was durable to high concentration of NaCl buffer. It also approved promising application of monolithic trapping columns in 2D-LC platform.

3.3.5. Recovery and reproducibility

By calculating the sum of peak areas upon combining a trapping column with an analytical column as compared with a setup using an analytical column only, recoveries of the standard proteins for the C8 trapping columns were calculated. The total recovery was 99.3% for the standard proteins at the optimum loading flow rate.

Table 1 lists column-to-column, batch-to-batch reproducibility of performance parameters of the C8 monolithic trapping columns for enriching standard proteins. Day-to-day RSD values for recoveries of standard proteins on the same C8 trapping column ranged from 2.34 to 5.87%, column-to-column RSD values were from 3.01 to 6.81%. Good reproducibility for both the recovery and the back pressure demonstrated that the performance of trapping columns was quite reliable and stable in application.

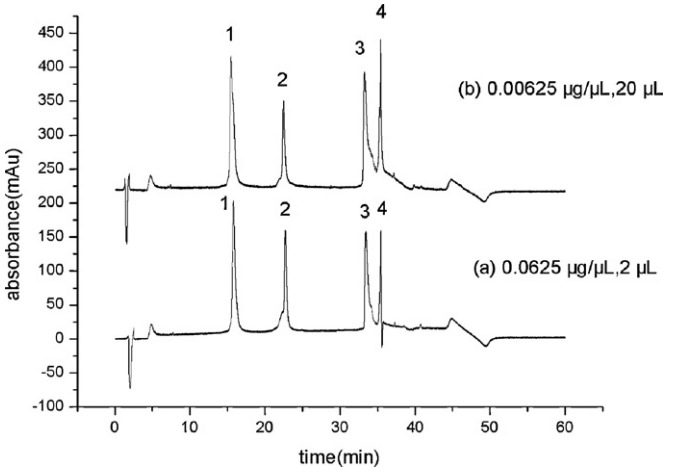

Fig. 7. Capillary RPLC separation chromatograms of 0.5 μg four standard protein mixtures. (a) Proteins with sample volume of 2 μL directly injected without enrichment; (b) proteins with sample volume of 20 μL concentrated by the trapping column. Protein peak assignments and other conditions are the same as shown in Fig. 4.

3.4. Trapping and separation of mouse liver intact proteins

Under the optimized conditions, the on-line C8 trapping column system for trapping proteins was applied to enrich and analyze 20 μL volume of healthy mouse liver tissue extract. 15 μg mouse liver proteins were trapped and separated by the on-line trapping column-capillary RPLC system, as shown in Fig. 8 chromatogram. In direct injection mode (Fig. 8a), sample was eluted through 20 μL loop-analytical column. In trapping-separation mode (Fig. 8b), sample was eluted through 75 μm i.d. × 20 cm capillary (0.9 μL)-5 mm trap column-analytical column. Void volume difference between two modes was close to 19 μL. Analytical flow rate was 4 μL/min, time delay was 4.75 min. Therefore, as shown in Fig. 8b, proteins were eluted faster. In addition, compared to injection directly to the separation column (Fig. 8a), before 25 min, a small fraction of salts and hydrophilic proteins were slightly lost, but after

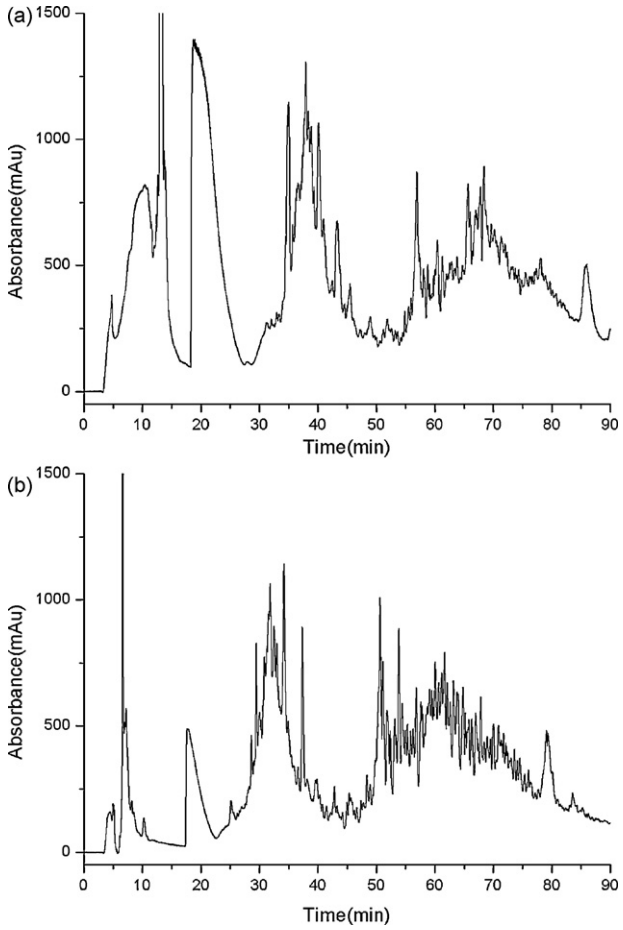

Fig. 8. Capillary RPLC separation chromatograms of 15 μg mouse liver proteins. (a) 20 μL directly injected without enrichment; (b) 20 μL injected after concentrated by the C8 trapping column.

25 min, proteins were enriched effectively (Fig. 8b). The resolution was better when the trapping column was used. The peak shape was improved. Sharp chromatographic peaks were due to high relative concentration of the eluting proteins. For the lost hydrophilic components, hydrophilic trapping columns might be more suitable. Trapping experiment confirmed high efficiency of coupling a monolithic trapping column to RPLC for trapping and desalting of intact proteins from real sample.

4. Conclusions

In this paper, a novel type of particle-entrapped monolithic trapping columns was prepared by the thin-layer sol–gel method. The performance of the trapping columns was evaluated using four standard proteins. The C8 trapping columns were applied to trap healthy mouse liver proteins. It demonstrated high efficiency of setting up a monolithic trapping column-capillary RPLC system for on-line trapping, desalting and separation of intact proteins.

We will employ the top-down strategy for separation of intact proteins on a high-throughput 2D-LC array system [38] using WAX chromatography as the first dimension and parallel RPLC as the second dimension. Reproducible octylsilica monolithic trapping columns prior to RPLC will undoubtedly play an important role for trapping and desalting of intact proteins eluted from the first dimension.

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