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Large-bore particle-entrapped monolithic precolumns prepared by a sol–gel method for on-line peptides trapping and preconcentration in multidimensional liquid chromatography system for proteome analysis

Xue Gu, Yan Wang, Xiangmin Zhang *

Department of Chemistry, Fudan University, 220 Handan Road, Shanghai 200433, China

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Abstract

The present report describes the preparation and characterization of large-bore particle-entrapped monolithic precolumns, which are suitable for incorporation into a two-dimensional liquid chromatography (2D-LC) system for proteome analysis. The fritless precolumns with different inner diameter (i.d.) (320 and 530 μm) were rapidly and successfully prepared by entrapping octadecylsilica (ODS) particles (5 μm, 300 Å) prepacked into fused silica capillaries with a sol–gel network, which was formed by hydrolysis and polycondensation of methyltriethoxysilane (MTES). By optimizing the composition of the sol solution, the resulting large-bore monolithic precolumns of 5 mm length allow a flow rate of 20 μL/min loading buffer at a reasonable low back pressure of 25 bar or less and are capable of withstanding up to 300 bar inlet pressure. Scanning electron micrograms of the precolumns profile showed that the evolving sol–gel network joined particles to each other and onto the column wall, and no cracking or shrinkage of the column bed was observed even in 530 μm-i.d. capillary. The performance of the particle-entrapped monolithic precolumns used for preconcentration and desalting of proteolytic digest was evaluated by on-line coupling the large-bore precolumns with a capillary reversed-phase liquid chromatographic (RPLC) column followed by UV detection. The laboratory-made monolithic precolumns with 320 and 530 μm i.d. were characterized by using BSA tryptic digest or peptide standards as the analytes with respect to sample loading capacity, linearity, recovery and reproducibility, etc. The results indicate that the large-bore and short precolumns (5 mm × 320 μm i.d. or 5 mm × 530 μm i.d.) allow sample fast loading at a flow rate of 30 or 60 μL/min. The precolumns also have a mass loading capacity for BSA peptides of about 70 μg and for standard peptides of about 80 μg. Good linear calibration curves ($R^2 > 0.99$) were obtained and the limits of detection (signal-to-noise ratio, S/N = 3) were improved by more than 60-fold and were between 0.53 and 1.32 ng/μL even with a UV absorbance detector. The total recovery was found to be approximately 90–100% for BSA digest and standard peptides. The day-to-day relative standard deviation (RSD) values for recoveries of BSA peptides on a single precolumn ranged from 4.66 to 7.56% and 2.68 to 3.05% for precolumn back pressure, while the column-to-column RSD values were 3.51–6.13% and 1.22–1.26% for recoveries of BSA peptides and precolumn back pressure, respectively. With good precolumn reproducibility, no significant degradation or decrease in precolumn performance was showed even after ∼150 preconcentration/desorption cycles. The precolumns also proved to be resistant to salt buffer with high concentration and low-pH mobile phase. The large-bore particle-entrapped monolithic precolumns will be further used in a high-throughput 2D-LC array system coupled with tandem matrix assisted laser desorption/ionization-time of flight–time of flight–mass spectrometry (MALDI-TOF–TOF–MS) detection for proteome analysis.

Keywords: Peptide preconcentration; Monolithic precolumns; Large-bore; Sol–gel; Capillary LC

1. Introduction

Chromatography-based methods for the analysis the components of complex protein mixtures have provided

* Corresponding author. Tel.: +86 21 65643983; fax: +86 21 65641740.
E-mail address: xmzhang@fudan.edu.cn (X. Zhang).

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a promising alternative approach to two-dimensional gel electrophoresis (2D-gel) for proteome analysis [1]. Single-dimensional liquid chromatography (1D-LC) and two-dimensional liquid chromatography (2D-LC) coupled with tandem mass spectrometry (MS/MS) have been currently developed as powerful tools for characterizing proteomic mixtures. However, improving the sensitivity and robustness of methods remains a challenge [2].

On-line sample preconcentration by using a large-bore precolumn prior to separation is an important and widely used sample pretreatment technique, which offers both the enrichment of analytes and the reduction of interfering components. Its combination with electrophoretically based or chromatographically based techniques has gained increasing attention. On-line incorporation the precolumns into 1D- or 2D-LC platform has proven to be an attractive technique of allowing robust operation and facilitating achieving lower detection limits with increasing sample mass and solution volume processing capability in proteomics analyses [2–4]. Sample preconcentration was performed by using column-switching technology or not in different manners including preconcentration-reversed phase liquid chromatography (RPLC) [2,4–7], strong cation exchange (SCX)-preconcentration-RPLC [1,3,8] and preconcentration-SCX-RPLC [9]. In addition, this technique has also shown its versatility with the aid of different types of sorbent material in preconcentrator-cartridge. Not only RPLC packing materials, which was the most commonly used, but also many specific materials were employed as trapping material for any purpose, such as SCX resins [10], immunoaffinity resins [11], restricted access materials (RAM) [12,13] and molecularly imprinted polymers materials (MIP) [13], etc.

Most precolumns are commercially available products, especially those used for 2D-LC system. Certainly, there are several approaches of making precolumns in laboratory. The commonly used and laboratory-made trap is virtually a short slurry-packed capillary LC column, which means two frits or something similar to these (i.e., in-line filters) are required to retain the packed stationary phase [10,14–16]. Retaining frits are also known as a source of band-broadening, especially for short precolumns. In addition, the procedure of preparation is time-consuming and it is difficult to make the short packed columns have same characteristics. An alternative to avoid making frits is to making porous polymer and silica monolith, which have been prepared in capillaries [17–19] or even within the channel of a microfluidic device [20] for sample enrichment. However, one drawback associated with polymer monolith is its tendency of solvent swelling and pressure deformation of column bed, while silica monolith is prone to shrinking and cracking during column drying. Therefore, reproducibility and durability of the resulting columns could not be controllable precisely. Moreover, large-bore precolumn shows advantages on sample enrichment because of higher sample loading capacity, however, till now, the preparation of porous monolithic capillary columns are mainly designated for narrow-bore capillaries (50–200 μm). Although some publications [21–23] have already successfully demonstrated the possibility of making porous monolithic columns of large diameters to improve sample loadability, there are not many reports dealing with the preparation of these columns and their use for sample preconcentration.

Apart from porous polymer and silica monolith, there is a special particle-entrapped monolithic column which is prepared by entrapping the conventional LC packing materials inside a fused silica capillary using sol–gel technology [24–29]. Here, the sol–gel solution serves as a “glue” to create a bridge between adjacent particles, as well as the capillary wall and particles in its vicinity, thereby eliminating the need for retaining frits. The procedure of column making is so simple and reliable that the resulting columns provide good column-to-column reproducibility and remarkable stability. Another marked advantage of the “sol–gel-glued” monolithic column is that the presence of entrapped packing particles helps to largely alleviate the shrinking and cracking on the sol–gel matrix by decreasing the stress within the matrix during drying, and thus the procedure of column making has promising possibility of applying to large-bore capillaries. However, to our knowledge, this type of monolithic column is narrow-bore and till now, no attempt is cited in the literature for the fabrication of this kind of column with large inner diameter. Furthermore, it is mainly used in the range of capillary eletrochromatography (CEC) separations without having been employed as preconcentration column in multidimensional LC system.

Our aim of the work described in this report was to investigate the feasibility of preparation of large-bore particle-entrapped monolithic column and check the applicability of it as a preconcentration column for on-line peptides enrichment. In our previous study [30], we reported the on-column frit making by bonding bare silica gel or octadecylsilica (ODS) particles with sol–gel in the capillary in situ. The frits proved to be mechanically strong, permeable and reproducible. In the present study, firstly we have successfully prepared the ODS particle-entrapped monolithic columns with much larger inner diameter (320 and 530 μm) by similar use of methyltriethoxysilane (MTES) alone as precursor. And then, validations of the large-bore monolithic precolumns were performed by developing an on-line preconcentration of peptides system, in which the precolumn was followed by capillary RPLC–UV analysis via a switching valve. Various parameters affecting peptides preconcentration on the precolumns were investigated and optimized. The laboratory-made monolithic precolumns with 320 and 530 μm inner diameter (i.d.) are demonstrated to be able to enhance sample processing capabilities and separation efficiency in the analysis of peptides and both of them proved to be very suitable for incorporation into 2D-LC platform.

2. Experimental

2.1. Materials and chemicals

Fused silica capillaries (250 μm i.d., 380 μm o.d.; 320 μm i.d., 450 μm o.d.; 530 μm i.d., 690 μm o.d.) were purchased from Yongnian Optical Fiber Factory (Yongnian, Heibei, China). Packing materials of C_{18} particles (Hypersil, 5 μm, 300 Å) and spherical silica gel (Zorbax BP-SIL, 7 μm, 80 Å) were obtained from Thermo Hypersil-Keystone (Runcorn, Cheshire, UK) and DuPont (Wilmington, DE, USA), respectively. MTES was from Institute of Rubber Product (Shanghai, China). Peptide standards (lecucine enkephalin, oxytocin), bovine serum albumin (BSA) and trypsin were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile (ACN) and trifluroacetic acid (TFA) were provided by Merck (Darmstadt, Germany).

2.2. Sample preparation

BSA was dissolved in 100 mmol/L NH_4HCO_3 buffer at a concentration of 25 μg/μL and boiling for 15 min. The protein solution was digested overnight at 37 °C with trypsin at a radio of 25:1 (w/w).

Standard peptide solutions were prepared by dissolving lecucine enkephalin and oxytocin in loading buffer (5% ACN–H_2O/0.1% TFA) at a concentration of 20 μg/μL, respectively.

2.3. Capillary columns packing

Previously described procedures [30] were used to manufacture packed capillaries with different inner diameter (530, 320 and 250 μm). Briefly, on-column frits were fabricated by sol–gel technology using Zorbax BP-SIL particles. A high-pressure slurry packing procedure was employed to pack the capillaries with 5 μm Hypersil C_{18} particles. After packing, the capillary columns were conditioned in an ultrasonic bath (model SCQ 50, 220 V/50 Hz, Shanghai Shenbo Ultrasonic Co., Ltd.) for 0.5 h under the packing pressures to stabilize the packing bed.

2.4. Preparation of large-bore particle-entrapped monolithic precolumns

ODS particle-entrapped monolithic columns were prepared in a manner similar to that given in ref. [27], except that the formula of sol solution was completely different. The optimized composition of our sol solution was prepared as follows: 30 μL MTES (for 320 μm-i.d. precolumn) or 36 μL MTES (for 530 μm-i.d. precolumn), 100 μL methylene chloride, 50 μL TFA and 5 μL water were thoroughly vortex-mixed in a plastic vial, which was subsequently introduced into a home-made gas pressure-operated capillary filling/purging device. The packed capillary column with inner diameter of 320 or 530 μm was inserted into the sol solution, and then a pressurized inert gas was used to fill the capillary 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 GC oven and cured at 100 °C for 24 h. Then the frits of ODS particle-entrapped monolithic column were removed and a 5 mm long monolithic column was cut off to be precolumn.

Moreover, a short length of the monolithic column was cut off and sputtered with a gold coating for observation using a scanning electron microscope (Philips XL30, Eindhoven, The Netherlands).

2.5. On-line preconcentration-capillary RPLC experiments

On-line preconcentration-capillary RPLC system is displayed in Fig. 1. The system consists of a Waters isocratic pump (Waters, Milford, MA, USA), an Agilent 1100 series capillary pump (Agilent Technologies. Inc., Palo Alto, CA), a 6-port 7725i injection valve (Rheodyne, Cotati, CA, USA) (valve 1) fitted with a 50 μL loop, a 6-port switching valve (Valco Instruments Co. Inc., Houston, TX) (valve 2), a particle-entrapped monolithic precolumn, a capillary analytical RPLC column, and a Waters 484 tunable absorbance detector (Waters, Milford, MA, USA) with a modification for on-column detection.

The precolumn was directly connected, using a 7 cm × 100 μm-i.d. fused-silica capillary, to two ports of valve 2 at the sample loop. A 50 μm-i.d. fused-silica capillary of 15 cm length was used to connect valves 1 and 2. The analytical capillary column was connected to valve 2 and the column outlet was connected to the UV detector.

The Waters isocratic pump was used to deliver loading buffer (5% ACN/0.1% TFA) at a flow rate of 30 or 60 μL/min. Samples were loaded onto the monolithic precolumn (5 mm × 320 μm i.d. or 5 mm × 530 μm i.d.) by using valve 1. After flushing the precolumn with the loading buffer for 10 min, valve 2 was switched to connect the precolumn on-line with the capillary RPLC column (25 cm × 250 μm i.d.), preconcentrated peptides on the precolumn were back-flushed to the analytical column where the separation took place.

Capillary RPLC separation experiments were performed on the Agilent 1100 series capillary pumping system. Binary solvents of A (5% ACN/0.1% TFA) and B (80% ACN/0.1% TFA) were used in elution. Gradient elution for BSA digested sample was as follows: 0–40% B in 40 min, and further increased to 80% B in 20 min, and maintained for 10 min. Isocratic elution for standard peptides was performed by using 70% B as mobile phase. The flow rate was 2 μL/min. On-column UV detection was carried out using the Waters 484 tunable absorbance detector at 214 nm. The on-column detection window was made by using an electric heating wire to burn off the capillary polyimide coating. Data acquisition

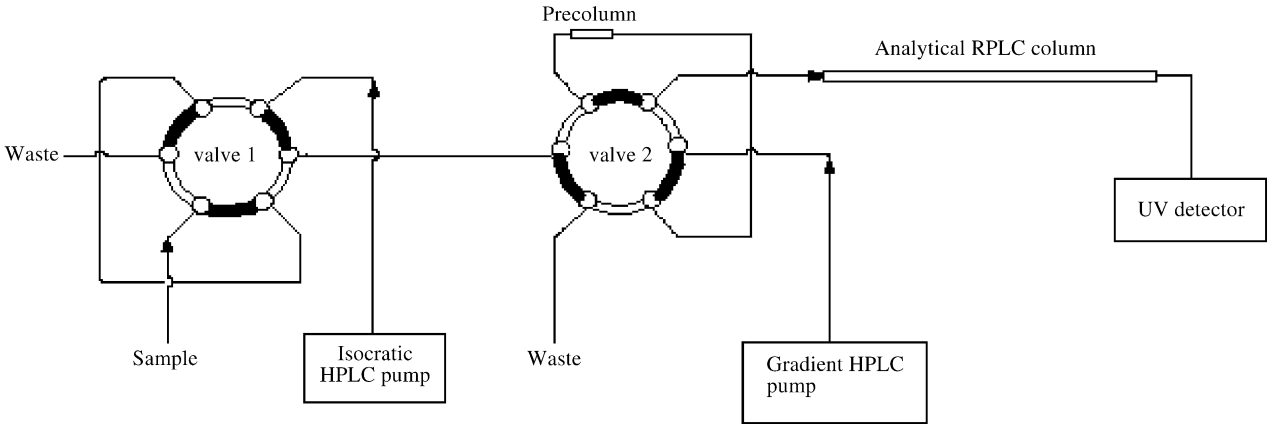

Fig. 1. Schematic representation of on-line preconcentration system.

and processing was carried out by an Echrom98 Chromatographic Workstation (Elite, Dalian, China).

3. Results and discussion

3.1. Large-bore monolithic precolumns preparation

The precolumns, having larger inner diameter and shorter length than the capillary analytical separation columns, can be compatible with a high sample loading flow rate and desorbing of the loaded sample at a moderate back pressure. Therefore, the inner diameter of precolumn was chosen to be 320 μm or even up to 530 μm relative to 250 μm (or more smaller) of analytical separation column, and short length was easily obtained by cutting the prepared monolithic column to any desired length with no constraint of frits.

Entrapment of ODS particles using sol–gel technique requires well-controlled conditions, especially for the large inner diameter columns, which are pone to serious shrinking and cracking. This may lead to the creation of excessively large cavities within the large-bore capillary column. Fortunately, pioneer articles describing sol–gel immobilization procedures within narrow-bore capillaries revealed the critical issues in successful manufacturing of high quality, reproducible and crack-free monolithic columns: homogeneity of the initial chromatographic bed, moderate interaction between the sorbent material and immobilization sol–gel solution [27]. Therefore, in this experiment, large-bore precolumn preparation was accomplished in a two-step process, where the particles were first packed into a capillary and then entrapped by sol–gel. In first step, maximum packing pressure (500 bar) and sufficient ultrasonic conditioning time (0.5 h) were applied during slurry-packing to make the particles highly compact and uniformly distributed.

In second step, a novel sol–gel entrapment method was successfully developed to make desired columns which not only have crack-free column bed even within large-bore capillary but also offer maximal mechanical robustness with minimal disruption of chromatographic properties of the original reversed-phase packing material. The latter two characteristics make the precolumns be capable of withstanding long-time gradient back-flush elution, having good permeability and preserving high enrichment efficiency. The key of the sol–gel method is the precursor type and concentration in the sol solution.

In general, tetramethoxysilane (TMOS) is commonly used as precursor, but shrinking or cracking of the gel network was often observed during drying and the silica gel network is active because of the exposed silanol groups on the gel surface. Tang et al. [25,26] selected ethyltrimethoxysilane (ETMOS) as a co-precursor with TMOS to obtain a flexible and inert gel work. Chirica and Remcho [27] compared different immobilized sol mixtures and high efficiency separations were finally obtained by using *tert*-butyl-triethoxysiliane and tetraethoxysilane as precursors to entrap column sorbents. To our knowledge, the silica gel fabricated from MTES alone results in a more flexible, strong and inert network. In our laboratory, on-column frits were also made by sol–gel method based on MTES [30]. Till now, research work rarely reported on the preparation of monolithic column containing sol–gel entrapped ODS particles by using MTES. Consequently, MTES was chosen to be the single precursor in our sol–gel solution for large-bore monolithic column making.

Based on our original formula of making sol–gel frits, various sol mixtures were tested to prepare monolithic columns. The volume percentage of the precursor in the sol solution was optimized because a high percentage of the precursor led to low permeability of the monolithic column and possible disruption of retention behavior of the packed bed, while a low percentage of the precursor resulted in weak mechanical strength and poor durability. Once prepared, a short segment of the monolithic column was cut off and flushed with acetonitrile by using LC pump to test mechanical stability of the immobilized bed. If the bed was stable, the sol solution was further diluted with methylene chloride to minimize the

amount of precursor to be added, and a new monolithic column was made. At last, 18.8% (v/v) MTES in sol solution for 530 μm-i.d. monolithic column was an optimized compromise. In the same way, a comparatively lower percentage MTES-16.2% (v/v) was required in making column of 320 μm i.d.

Column drying play a very important role in making crack-free monolithic columns. Tang et al. [25,26] employed supercritical CO_2 to replace the solvent in the sol–gel matrix and dry the column bed under elevated temperature. This approach of drying effectively shortened the drying time and produced crack-free columns. Unfortunately, few laboratories are commonly furnished with supercritical CO_2 equipment. In our work, we combined some advantages of two drying approaches presented by Tang et al. [25,26] and Chirica and Remcho [27] and successfully prepared crack-free columns with modest equipment. The packed capillary columns filled with sol were first stored at room temperature for aging and then placed in GC oven for drying. During drying, we kept low-pressure nitrogen gas instead of supercritical CO_2 passing through the whole column all the time to accelerate drying. Finally, crack-free monolithic columns were obtained after curing for 24 h, which is short relative to the time reported by Chirica et al.

Fig. 2 shows scanning electron micrographs of the resulting particle-entrapped monolithic columns with 320 and 530 μm i.d. As can be seen, the particles were bonded to each other and to the column inner wall by the 3D-sol–gel network, forming a monolith without cracks even within 530 μm-i.d. capillary.

3.2. Mechanical strength and permeability

The optimal precolumn requires maximal mechanical strength to withstand long-time flushing and minimal pressure drop across the trapping column to facilitate the use of longer capillary LC separation column.

The monolithic precolumns were tested for mechanical stability by acetonitrile flushing. The 5 mm precolumn of 530 μm i.d. can provide high mechanical strength for withstanding up to 250 bar inlet pressure. Such a precolumn for 320-μm-i.d. capillary can resist higher pressure, up to 300 bar.

Enough mechanical strength was achieved by using low concentration sol–gel during column making. However, packing materials covered by a layer of silica-based gel network might change some column behaviors in various degrees, such as column permeability or original retention abilities of packing materials. To check the influencing degree of sol–gel on column, permeability tests were performed for sol–gel monolithic column and packed column together.

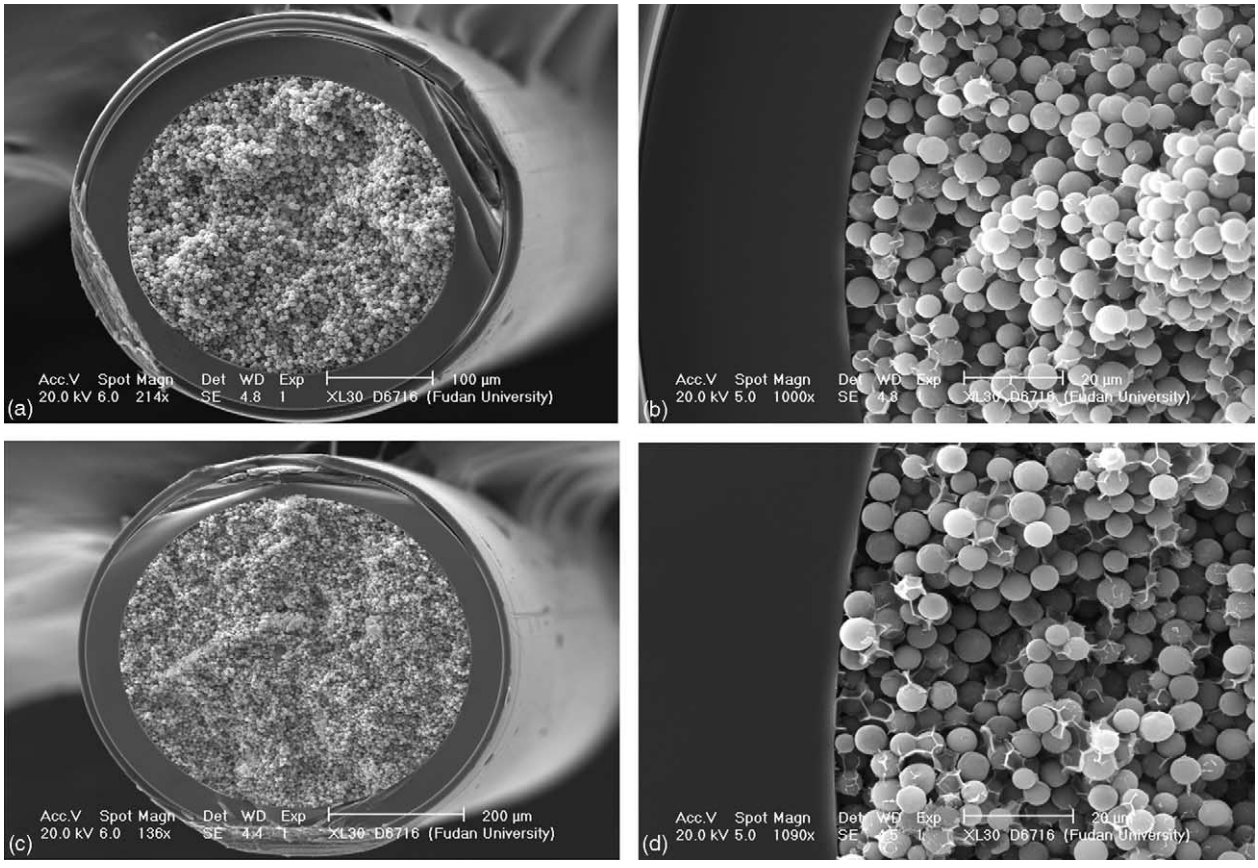

Fig. 2. Scanning electron micrographs of the sol–gel monolithic precolumns. (a) and (b) for 320 μm-i.d. precolumn; (c) and (d) for 530 μm-i.d. precolumn.

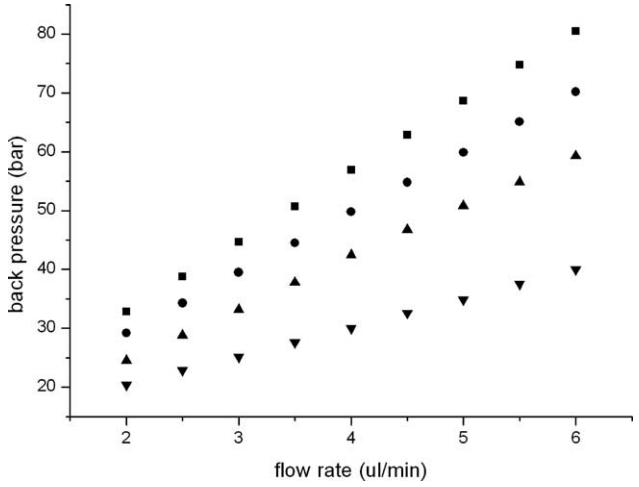

Fig. 3. Plots of back pressure vs. mobile phase (100% ACN) flow rate. (■) 320 μm-i.d. sol–gel monolithic column, (●) 530 μm-i.d. sol–gel monolithic column, (▲) 320 μm-i.d. packed column, and (▼) 530 μm-i.d. packed column.

Permeability was calculated by following the equation [26,28,29]

$$B_0 = \frac{u \times \eta \times L}{\Delta P}$$

where B_0 is the column permeability; u, the linear velocity; η, the viscosity; L, the column length; and ΔP is the back pressure.

Fig. 3 shows the plots of back pressure against the flow rate using acetonitrile as mobile phase. Based on the equation and the relationship between flow rate and linear velocity, B_0 is achievable. In the case of sol–gel monolithic column of 320 μm i.d., 1.29×10^{-14} m^2 was obtained for B_0, while its corresponding value of conventionally packed column with same length was 2.01×10^{-14} m^2. Meanwhile, for the 530 μm-i.d. monolithic column and its packed column, the permeability values were 1.89×10^{-14} m^2 and 2.53×10^{-14} m^2, respectively. Thus, in general, the sol–gel entrapped ODS monolithic columns have lower column specific permeability than the packed column. The reason for this result might be that the interstitial voids between particles in the bed decrease when the packed particles are entrapped within a sol–gel matrix. The results are also in accordance with by Tang et al. [26].

In fact, sol–gel column with a little lower permeability does not seriously influence its application, because it has been cut to be a very short segment when it is applied to preconcentration and then there is an acceptable pressure drop when the precolumn is on-line integrated with the analytical RPLC column during gradient elution. The back pressure in a 5 mm monolithic precolumn at a flow rate of 20 μL/min loading buffer was typically in the range of 20–25 bar.

The effects of sol–gel on the original retention abilities of packing materials in precolumns are also negligible which have been verified by the following discussion about evaluation of enrichment performance of the precolumns.

3.3. Optimization of the on-line preconcentration system

In this study, BSA tryptic digest was mainly used to be model sample to test concentration efficiency of the precolumn because tryptic digestion of BSA can generate numerous peptides and the diversity of the peptides presents a challenge for validation of the on-line method. Two additional standard peptides (lecucine enkephalin and oxytocin) were also chosen to be auxiliary testing sample.

The desorbing process of peptides provides an elution graph, so the sum of all peak areas is the quantitative indicator of evaluation performance. The reversed-phase separations of BSA peptides were shown in Fig. 4. Fig. 4a and b were obtained when the sample was preconcentrated by large-bore monolithic precolumns before separation, while Fig. 4c was obtained when the sample was directly injected onto the top of separation column without enrichment. Signal enhancement is obvious in the separation chromatograms. However, no significant performance differences were observed between the precolumns with 320 and 530 μm i.d. The degradation of separation efficiency maybe due to an increase in the total dead volume when a relatively larger i.d. preoclumn is used,

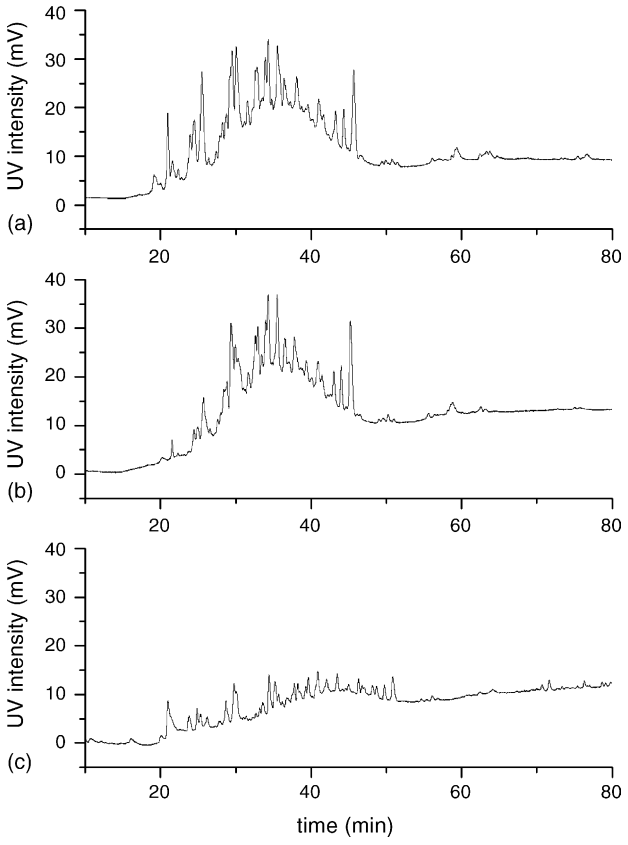

Fig. 4. Capillary RPLC separation chromatograms of 2.3 μg BSA tryptic digest. (a) peptides with sample volume of 50 μL concentrated by the 5 mm precolumn with 530 μm i.d.; (b) peptides with sample volume of 50 μL concentrated by the 5 mm precolumn with 320 μm i.d.; (c) peptides with sample volume of 1 μL directly injected without enrichment; UV detection wavelength, 214 nm. For chromatographic conditions, see Section 2.

because the void volume of precolumn and the dead volume between the precolumn and separation column increase with enlarging the inner diameter of precolumn.

3.3.1. Effect of sample loading flow rate

The flow rate during sample loading onto the monolithic precolumn is potentially a crucial factor, because the analyte requires sufficient time to distribute and adhere efficiently to the solid phase. The loading flow rate is mainly dependent on the amount of the adsorbents immobilized in the precolumn and the diameter of the precolumn. So large-bore precolumns evidently allow much faster loading than precolumns with smaller inner diameter.

A segment of ODS particle-entrapped monolithic column (5 mm × 320 μm i.d.) was firstly checked to be the trapping-column for effectiveness evaluation. We investigated the influence of loading speed on peptide retention by loading 50 μL of a 0.23 μg/μL BSA digest on the precolumn at various rates ranging from 5 to 80 μL/min, as indicated in Fig. 5. After quantitatively comparing the gradient elution peak areas of the peptides at different rates, we fixed the sample loading rate on 30 μL/min for the short monolithic precolumn with 320 μm i.d. When the 530 μm-i.d. precolumn was placed in the on-line preconcentration system, the suitable loading speed was found to be up to 60 μL/min. The large-bore monolithic precolumns allow very fast and effective loading of peptides.

3.3.2. Sample mass loading capacity

Sample mass loading capacity is one of the important characteristics for on-line preconcentration. It is particularly crucial to determine the maximum sampling mass allowed to avoid any analyte loss. For determine the sample capacity of 320 μm-i.d. precolumn, breakthrough mass was measured by a series of injection BSA peptides with increasing concentration onto the precolumn, as shown in Fig. 6. Up to

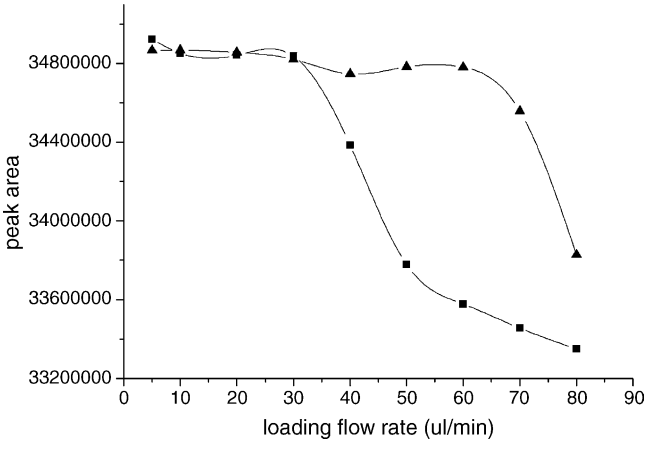

Fig. 5. Effects of loading flow rate on the preconcentration efficiency of BSA tryptic digest. (■) 320 μm-i.d. precolumn and (▲) 530 μm-i.d. precolumn; sample volume, 50 μL; sample concentration, 0.23 μg/μL; UV detection wavelength, 214 nm. For chromatographic conditions, see Section 2.

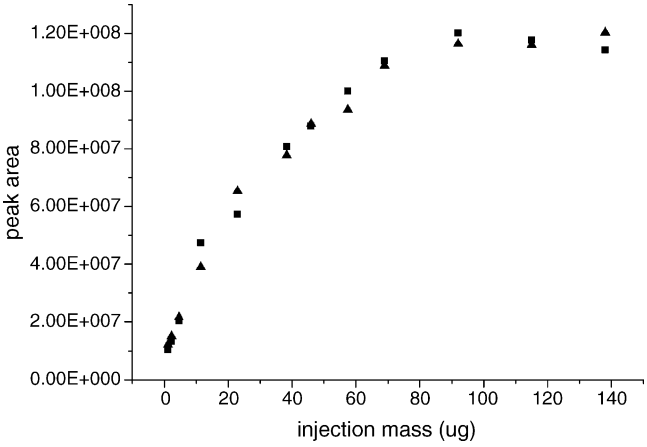

Fig. 6. Sample mass loading measurement curves for BSA tryptic digest. (■) 320 μm-i.d. precolumn and (▲) 530 μm-i.d. precolumn.

sampling mass of about 70 μg, the signal increased almost linearly in relation to the injected mass. However, on increasing injected mass from 90 to 138 μg, no further increase in the signal was observed. The loading capacity of the precolumn (5 mm × 320 μm i.d.) was then estimated to be about 70 μg of tryptic BSA digest.

When 530 μm-i.d. precolumn was used for measurement, very similar trend-line was obtained. Although the amount of entrapped ODS particles and the larger inner diameter of column give the 530 μm-i.d. precolumn the advantage of trapping more peptides, in practice, the precolumn also began to be saturated when the injection mass got to about 70 μg. This is also maybe due to the degradation of separation efficiency when larger i.d. precolumn is applied.

On the other hand, in fact, a fraction of hydrophilic peptides from BSA digest were not captured by the C_{18} precolumn so that the actual loading capacity for BSA peptides is lower than 70 μg. Standard individual peptides (lecucine enkephalin and oxytocin) were also injected into the precolumn to test mass loading respectively. The results show that loading capacity is about 80 μg for lecucine enkephalin and 85 μg for oxytocin. Therefore, the sample loading capacity is large enough for the precolumns to trap diluted peptides without any loss.

3.3.3. Effect of salt concentration

In the 2D-LC platform, peptides eluted with salt buffer from SCX column were desalted and refocused by the precolumn for further reversed-phase separation, while the salt was washed away and directed to waste. To elucidate whether the salt concentration has any influence on the retention behavior of peptides on the monolithic trapping columns, a series of salt (NH_4Ac) concentration ranging from 10 to 1000 mmol/L in 20 μL salt plug were checked to investigate the relationship between the salt concentration and the elution peak area, as shown in Fig. 7. It was found that high concentration salt buffer did not exhibit noticeable influence on the binding for both the monolithic precolumns. This suggests that the

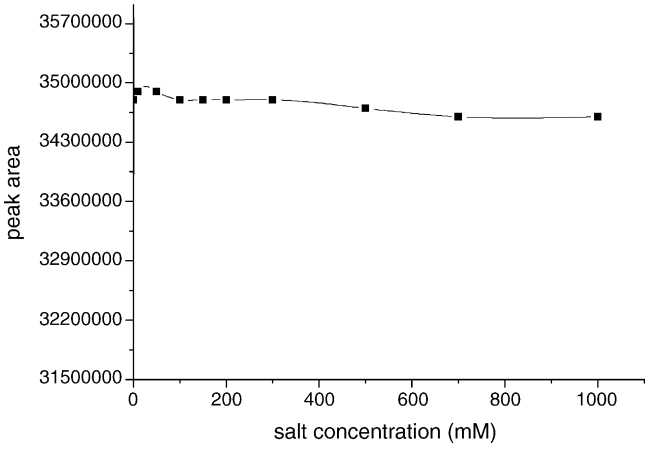

Fig. 7. Effects of salt concentration on the preconcentration efficiency of BSA tryptic digest. Conditions are identical to Fig. 5.

precolumns prepared are durable to high concentration salt buffer.

3.3.4. Calibration curve and preconcentration factor

To determine the linear dynamic range for the BSA peptides, each of BSA peptide samples with different concentration was loaded to the precolumn and separated on the analytical capillary column. For 320 μm-i.d. precolumn, a concentration calibration curve ($R^2 = 0.9942$) gave a linear response across a concentration range of 0.046–1.40 μg/μL (50 μL injection) with slope of 1.58×10^8. For the UV detection system used in the study, the concentration limit of detection (CLOD) was studied by six replicate measurements of 9.2 ng/μL BSA digest (50 μL). A low CLOD value of 1.32 ng/μL at a signal-to-noise ratio (S/N) of 3 was obtained by linear extrapolation. Equally good linear relationship ($y = 1.02 \times 10^7 + 1.62 \times 10^8 x$, $R^2 = 0.9936$) and detection limit (1.27 ng/μL) were also obtained with the 530 μm-i.d. precolumn.

On the other hand, a series of BSA peptide samples with increasing concentration were directly injected onto the top of RP capillary LC column for separation to make the calibration plot without preconcentration. A linear calibration curve ($y = 4.31 \times 10^6 + 2.38 \times 10^6 x$, $R^2 = 0.9952$, 1 μL injection) was observed for BSA digest in the range from 1.15 to 11.5 μg/μL with CLOD of 86.6 ng/μL. Comparing the data of the preconcentration curve and no-preconcentration curve, a 60-fold average preconcentration factor was achieved accordingly for BSA digest by using a UV detector. Similar methods were applied to check the preconcentration efficiency of peptide standards on precolumns. Six replicate measurements of 5 ng/μL standard peptide sample were also applied to calculation of CLOD values. CLOD values (S/N = 3) for lecucine enkephalin and oxytocin are 0.87 and 0.53 ng/μL, respectively. The improvement in sensitivity for the two peptides was achieved to ∼70 fold. The data demonstrate the optimum linearity and excellent enrichment ability of the in-house made precolumns.

3.3.5. Recovery, reproducibility and chemical stability

The optimal short precolumn should be used to trap all of the analytes during sample loading, but it should release them completely during gradient elution since this is a very important issue of enabling the repeated use of the precolumn. Recovery tests were carried out to further assess the monolithic precolumns. The percentage of recovery was calculated by the ratio between peak areas obtained by capillary RPLC-UV and precolumn-capillary RPLC-UV, respectively. The average recovery was determined to be about 90% for 320 and 530 μm-i.d. precolumn. On the other hand, considering insufficient retention of hydrophilic peptides of BSA digest on the precolumn, the recoveries for the standard peptides need to be tested. For peptide standards, lecucine enkephalin and oxytocin, the sample recovery was almost 100% (about 98 and 99%, respectively).

Reproducibility of precolumn parameters is a critical consideration in the field of preparation and application of columns. Table 1 lists the column-to-column and batch-to-batch reproducibility of performance parameters for BSA peptides on the large-bore monolithic precolumns with different inner diameter. The day-to-day relative standard deviation (RSD) values for recoveries of BSA peptides on a single precolumn ranged from 4.66 to 7.56% and 2.68 to 3.05% for precolumn back pressure, while the column-to-column RSD values were 3.51–6.13% and 1.22–1.26% for recover-

Table 1
Reproducibility of some properties of precolumns

	n	RSD% of recovery of BSA tryptic digest		RSD% of back pressure	
		Precolumn i.d.		Precolumn i.d.	
		320 μm	530 μm	320 μm	530 μm
Single precolumn					
Run-to-run	10	2.56	2.37	1.52	1.06
Day-to-day	10	4.66	5.24	2.68	1.54
	20	6.45	5.98	3.20	2.21
	30	7.83	7.56	3.88	3.05
Different precolumns					
One batch	6	3.51	4.17	1.22	0.83
Batch-to-batch	5	5.82	6.13	1.98	1.26

ies of BSA peptides and precolumn back pressure, respectively. Obviously, the reproducibility for both the recovery and the back pressure is satisfactory, which suggests that no unacceptable change in back pressure, flow, and adsorption characteristics were observed when the precolumn was used over a period of ∼1 month with ∼150 injections. In addition, the batch-to-batch reproducibility was also acceptable for monolithic column preparation.

Special attention was also paid to the long-term stability of the precolumn under extreme pH conditions. When the large-bore precolumn was used for enrichment and purification of peptides in 2D-LC system, the buffer commonly used for peptide loading on the precolumn is 0.1% TFA in 5% acetonitrile, the pH value of which is about 2. Therefore, the acidic resistance of precolumns to loading buffer flushing is required to be tested. From Table 1, it can be seen that the performance of the precolumns did not change significantly under continuous flushing with loading buffer for about 1 month. The good reproducibility for both the back pressure and the recovery demonstrated that the precolumns were rather stable for mechanical strength and enrichment efficiency under low-pH mobile phase flushing.

4. Conclusions and prospects

We have demonstrated the preparation and application of the ODS particle-entrapped sol–gel monolithic precolumns with 320 and 530 μm i.d. Simple manufacturing procedure and modest equipment requirement make it easy to fabricate the large-bore precolumns with strong and crack-free structure by using MTES alone as precursor. Elimination of frits, length customization and mechanically robustness facilitate its use for on-line preconcentration diluted peptides.

Furthermore, we presented much more detailed evaluation of quantitative enrichment performance of the large-bore precolumns in terms of accuracy, recovery, linearity, detection limits and precision, etc. The results suggest that the precolumns have a high loading capacity and allow for excellent recovery of minute amount peptides. The large enrichment effect improves the concentration detection limit more than 60-fold. With good stability and reproducibility, the performance of the precolumns does not vary greatly even after more than 150 preconcentration/desorption cycles.

The on-line enrichment and sample desalting capabilities make the particle-entrapped monolithic precolumns a suitable and practical tool for LC–MS/MS and LC–LC/MS/MS experiments. In our laboratory, a high-throughput on-line 2D-LC array system coupled with tandem matrix assisted laser desorption/ionization-time of flight–time of flight–mass spectrometry (MALDI-TOF–TOF–MS) detection for comprehensive proteomic analyses has been developed [31]. With a novel multi-channel and valve-free interface designed for fraction transfer. The 2D-LC array system allows the concurrent gradient elution of ten fractions displaced in the first separation dimension and therefore reduces total separation time for a proteomic sample by 10-fold. There is no doubt that implementation of high-efficient precolumns as part of the 2D-LC array platform will provide significantly greater overall efficiencies. Therefore, our next work is to incorporate ten precolumns into the multi-channel 2D-LC platform for real proteomic sample analysis, in which these precolumns were placed individually prior to each parallel capillary RP column for desalting and enrichment of peptide fractions eluted from capillary SCX column. A description of this further work is the subject of a future publication from our laboratory.

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