Journal of Chromatography A, 1317 (2013) 138–147

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Separation of intact proteins by using polyhedral oligomeric silsesquioxane based hybrid monolithic capillary columns

Zheyi Liu[a,b], Junjie Ou[a], Zhongshan Liu[a,b], Jing Liu[a,b], Hui Lin[a,b], Fangjun Wang[a,*], Hanfa Zou[a,*]

[a] Key Laboratory of Separation Sciences for Analytical Chemistry, National Chromatographic R&A Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China
[b] University of Chinese Academy of Sciences, Beijing 100049, China

ARTICLE INFO

Article history:
Received 12 April 2013
Received in revised form 2 September 2013
Accepted 2 September 2013
Available online 7 September 2013

Keywords:
Hybrid monolithic capillary column
Intact protein
Reversed phase liquid chromatography
High speed separation

ABSTRACT

High-efficient separation of intact proteins is still a huge challenge in proteome analysis of complex biological samples by using capillary columns. In this study, four POSS-based hybrid monolithic capillary columns were prepared and applied in nano-flow liquid chromatography (Nano-LC) separation of intact proteins. It was observed that the POSS-based hybrid monolithic columns exhibit high permeability, good LC separation reproducibility and column efficiency for intact protein separation. The effects of different LC separation conditions such as flow rate, gradient steepness, column length and mobile phase additives on the LC separation efficiency of the POSS-based hybrid monolithic column were systematically examined. Finally, fast LC separation of 7 proteins mixture was realized in 2.5 min by using the optimized conditions on the 100 μm i.d. POSS-based hybrid monolithic capillary column.

1. Introduction

Mass spectrometry-based proteomics is greatly developed in recent years and attracts more and more interests in biological study and biomarker discovery [1,2]. High-efficient separation of intact proteins is extremely important in both "bottom–up" and "top–down" proteomics due to the complexity of biological samples, such as body fluid and tissue or cell lysate [3–7]. In order to improve the performance of proteome analysis, it is essential to develop new liquid chromatography (LC) technologies with high separation capability and throughput. So far, a wide variety of liquid chromatography methods have been developed to reduce the complexity of the intact protein samples, such as ion exchange chromatography (IEC) [8], hydrophilic interaction chromatography (HILIC) [9], reversed-phase liquid chromatography (RPLC) [10] and so on. Among them, RPLC is the most popular one due to its high separation efficiency and compatibility to the MS detection. Due to the multiple charge states distribution of the intact proteins in the MS process and strong affinity to adducts, the MS based intact protein analysis always show a low sensitivity and require a large quantity of starting material [11]. High performance capillary columns with LC separation flow rate less than 1 μL/min have already been widely applied for peptides mixture separation to increase the MS detection sensitivity. Therefore, it is important to develop high performance capillary column that suitable for intact proteins separation. The traditional capillary columns packed with porous particles always suffer a significant peak broadening when operating at a high flow rate due to the low diffusivity of the large proteins, while the capillary columns with non-porous particles always show a low sample loading capacity [12]. What's more, the solubility of protein sample is usually lower than peptides sample, and the capillary column packed with small particles is easy to be blocked during the separation of intact protein sample.

Emerged at the end of the 1980s, because of the merits such as ease of preparation, high sample loadability, versatile surface modification and high permeability, monolithic column has become an attractive alternative for the traditional packed column in high-efficient LC separation [13]. Polymer based monolithic columns have been well examined and applied to the RPLC separation of intact proteins, such as poly(styrene-divinylbenzene) (PS-DVB) organic polymer monolithic column [14–17], poly(butyl methacrylate-co-ethylene dimethacrylate) monolithic column [18] and octadecyl derived polymethacrylate monolithic column [19]. However, given the increasing attention on the high-efficient separation of intact proteins in bio-analysis, such as top–down proteomics [20], developing new monolithic columns with high separation efficiency and throughput is still highly needed. The hybrid monolithic columns combine the advantages of the polymer based and silica based monolithic columns, and usually exhibit ease of preparation good pH and mechanical stability and much

* Corresponding authors. Tel.: +86 411 84379610; fax: +86 411 84379620.
E-mail addresses: wangfj@dicp.ac.cn (F. Wang), hanfazou@dicp.ac.cn (H. Zou).

0021-9673/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.chroma.2013.09.004

Table 1
Detail ingredients for preparation of POSS-based monolithic columns.

Monolithic columns	POSS-MA_8 (mg)	SMA	BeMA	LMA	1-Propanol (μL)	PEG400 (μL)	AIBN (mg)
LMA-POSS	30	–	–	5 μL	90	50	1
BeMA-POSS	30	–	5 μL	–	84	56	1
SMA-POSS	30	5 μL	–	–	110	30	1
BeMA-SMA hybrid	30	3 μL	3 μL	–	89	51	1

more homogenous morphology, and have shown good separation efficiency for the small molecules [21,22]. However, the RPLC separation of intact proteins using the hybrid monolithic columns has little been done.

In our previous work, a cage-like silsesquioxane-polyhedral oligomeric silsesquioxane (POSS) was introduced as a cross-linker for "one-pot" preparation of hybrid monolithic columns, which embodies a truly inorganic–organic hybrid architecture with an inner inorganic framework [23–25]. These POSS-based hybrid monolithic columns with homogeneous morphology exhibit good column stability and reproducibility. Thus the application of POSS-based hybrid monolithic capillary columns to separation intact proteins was investigated. Due to the widely usage of RPLC in the biological sample analyses, the analogical POSS-based hybrid monolithic columns LMA-POSS, BeMA-POSS and SMA-POSS were prepared for intact protein separation. Based on the different separation selectivity of SMA-POSS and BeMA-POSS hybrid monolithic columns, a two monomers POSS-based monolithic column BeMA-SMA-POSS was developed. The performance of this POSS-based hybrid monolithic column in intact protein separation was evaluated and good LC separation performance was obtained at low operating back pressure. The effect of flow rate, gradient elution time, column length and the mobile phase additives on separation efficiency were also systematically studied. It was demonstrated that the POSS-based hybrid monolithic columns are suitable for intact proteins separation with both good resolution and reproducibility.

2. Materials and methods

2.1. Chemicals and materials

POSS-methacryl substituted ($N = 8$, POSS-MA_8) was purchased from Acros (NJ, USA). Acetonitrile (ACN, HPLC grade) was purchased from Merck (Darmstadt, Germany). γ-Methacryloxypropyltrimethoxysilane (γ-MAPS), triflouroacetic acid (TFA), formic acid (FA), stearyl methacrylate (SMA), benzyl methacrylate (BeMA), lauryl methacrylate (LMA) and polyethylene glycol 400 (PEG400) were all obtained from Sigma (St. Louis, MO, USA). 1-Propanol was purchased from Tianjin Kermel Chemical Plant (Tianjin, China). Azobisisobutyronitrile (AIBN) was obtained from Shanghai Chemical Plant (Shanghai, China) and re-crystallized in ethanol before use. A fused-silica capillary with inner diameter (i.d.) of 100 μm was purchased from Reafine Chromatography Ltd. (Hebei, China). Water used in the following experiments was purified by a Milli-Q system (Millipore Inc., Milford, MA).

Ribonuclease B (bovine pancreas), myoglobin (horse heart), serum albumin (bovine), ovalbumin (chicken egg white), lysozyme (chicken egg white), insulin (bovine) were all purchased from Sigma (St. Louis, MO, USA). Cytochrome c (bovine heart) was purchased from Aladdin (Shanghai, China). The standard protein mixture is comprised of the above seven proteins. The final concentration of each protein is 5–7 μg/mL in the water.

2.2. Preparation of hybrid monolithic capillary columns

The fused-silica capillary was washed with 0.1 M NaOH for 2 h, and then rinsed with 0.1 M HCl for another 12 h. After rinsing with water until the pH of the outlet up to 7, the capillary was washing with methanol and then dried with nitrogen gas at room temperature. In order to modify the inner surface of the capillary with γ-MAPS, the capillary was filled with a solution of γ-MAPS and methanol (50/50, v/v) and kept at 60 °C in the water bath for 12 h with both ends sealed by rubber stoppers. After this, the capillary was again rinsing with methanol and dried with nitrogen stream.

LMA-EDMA monolithic column was prepared according to the previous work [26]. And the other four POSS-based hybrid monolithic columns were prepared as the following procedures.

The pre-polymerization mixture was consisted of POSS-MA_8, 1-propanol, PEG400, AIBN and the corresponding functional monomers, and the detail ingredients see Table 1. After ultrasonic assisted homogenization, the pre-polymerization mixture was filled into a 30 cm-long capillary by a syringe and kept at 55 °C in the water bath for 12 h with both ends sealed by a rubber stopper. Finally, the prepared monolithic column was rinsing with methanol to remove the residuals and then kept in water for usage.

The Pepswift nanoViper monolithic PS-DVB column (100 μm i.d. × 25 cm) is provided by the Thermo Fisher Scientific as a gift.

2.3. Escherichia coli culture and protein isolation

Escherichia coli (*E. coli*) strain was obtained from Takara (Dalian) and cultured in liquid Luria broth (LB) (containing 10 g/L tryptone, 5 g/L yeast extract and 5 g/L NaCl, pH = 7.4) for 14 h at 37 °C. Then the culture mediums were centrifuged for 20 min at 2500 × *g* at 4 °C and the supernatant was discarded. The pellets were washed with ice-cold PBS three times to remove the residual medium. Then the cleaned cell pellets were lysed in ice-cold PBS by sonicated three times for 6 min (200 W) with at least 3 s between two pulses. The cell lysates were centrifuged at 25,000 × *g* for 20 min at 4 °C, then the supernatants were collected and the protein concentration was determined by the Bradford assay. Finally, the protein was desalted by Supelclean LC-4 SPE columns, and re-dissolved in the buffer A before LC separation.

2.4. Instrument and methods

Separation of intact proteins was performed on an Eksigent one dimensional (1D) Plus Nano-HPLC system (Eksigent, Dublin, CA) coupled with a UV detector K-2520 from Knauer (Berlin, Germany). The mobile phase is consisted of buffer A (0.05% TFA in water) and buffer B (0.05% TFA in ACN). The flow rate was set at 500 nL/min. Gradient elution was used to separate the standard protein mixture from 20% to 60% buffer B in 30 min. 1 μL of the standard protein mixture was directly loaded to the analytical column using 100% buffer A before gradient elution. The detection was performed using a 5 nL flow cell with the detection wavelength set at 214 nm. All the chromatography data were collected and analyzed by Ekisgent Control Software. Peak capacity was calculated by dividing gradient elution time with the average baseline peak width.

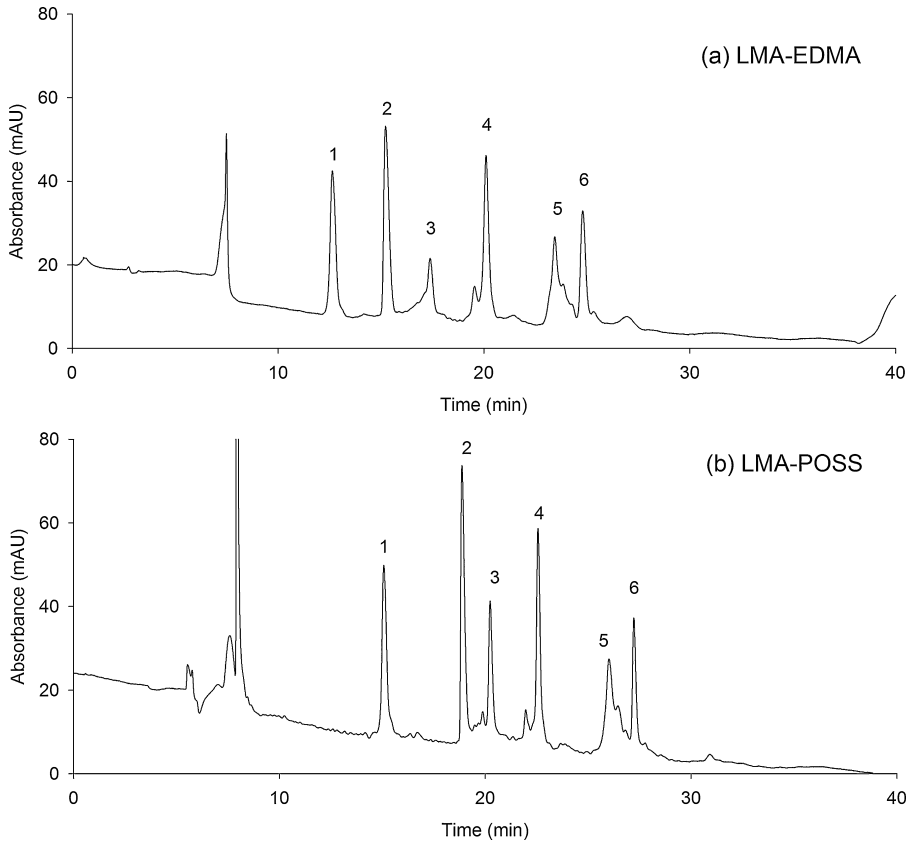

Fig. 1. Chromatograms for separation of intact proteins on the different monolithic capillary columns. (a) LMA-EDMA monolithic column, (b) LMA-POSS monolithic column. Solute of standard protein mixture: (1) ribonuclease B, (2) cytochrome c, (3) insulin, (4) lysozyme, (5) BSA, (6) myoglobin. Injection, 5–7 ng of each tested protein; flow rate, 500 nL/min; gradient, 20–60% B with 0.1% TFA in 30 min; column, 100 μm i.d. × 25 cm long of different monolithic stationary phases; detection wavelength, 214 nm.

3. Results and discussion

3.1. LC separation performance of POSS-based hybrid monolithic capillary columns

Firstly, the RPLC separation performance of POSS-based hybrid and polymer-based organic monolithic columns with the same C12 functional monomer LMA was compared. LMA-EDMA organic monolithic column was already applied for the peptides separation [26,27], and this organic monolith exhibited abundant mesopores and macropores, which may be suitable for the intact protein separation [26,28]. A total of 18 monolithic capillary columns with 100 μm i.d. were prepared in three different batches, 9 for LMA-POSS hybrid monolithic columns and 9 for LMA-EDMA organic monolithic columns. The typical flow rate for optimal intact protein separation within a 100 μm i.d. capillary column is about 500 nL/min, and a moderate gradient about 1% buffer B per minute was selected [29]. Due to the bad stability of the ovalbumin, a mixture of other 6 proteins was used to investigate the LC separation efficiency and preparation reproducibility of the monolithic capillary columns under identical separation conditions. It was observed that all of the 6 proteins could be well separated on the two types of monolithic capillary columns with good peak shape and identical elution order (Fig. 1). Comparing with the polymer-based LMA-EDMA organic monolithic column, the LMA-POSS hybrid monolithic column exhibited slightly stronger retention to the protein analytes (Fig. 2) and the average peak capacity was 61.2 and 68.1 for LMA-EDMA and LMA-POSS capillary columns, respectively. This is due to the different cross-linkers that used in the two monolithic capillary columns to obtain different types of monoliths (Fig. 3). Both LMA-EDMA and LMA-POSS capillary columns exhibited good run-to-run reproducibility in LC separation, and the relative standard deviations (RSDs, $n = 3$) were all less than 0.3% (Table S1). And the column-to-column reproducibility (nine columns in three batches) was 0.91% and 2.93% for the LMA-POSS hybrid capillary columns and the LMA-EDMA organic capillary columns, respectively (Table 2). The POSS-based hybrid monolithic

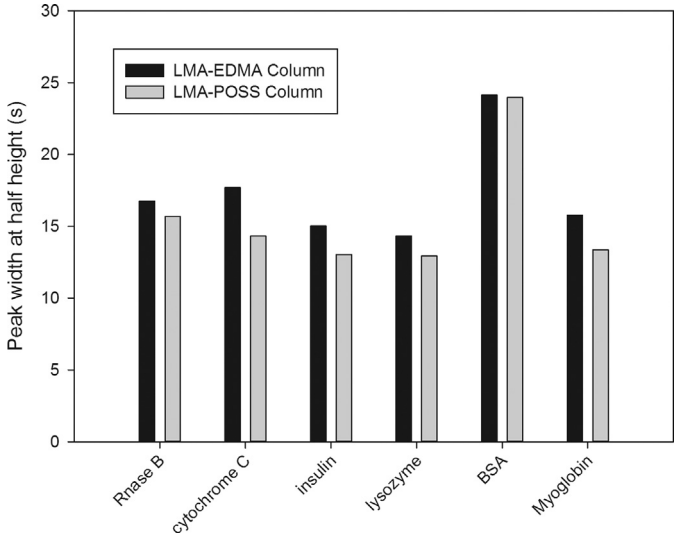

Fig. 2. Mean values of peak width at half height of separated proteins on the LMA-EDMA and LMA-POSS monolithic capillary columns ($n = 27$). Injection, 5–7 ng of each tested protein; flow rate, 500 nL/min; gradient, 20–60% B with 0.1% TFA in 30 min; column, 100 μm i.d. × 25 cm.

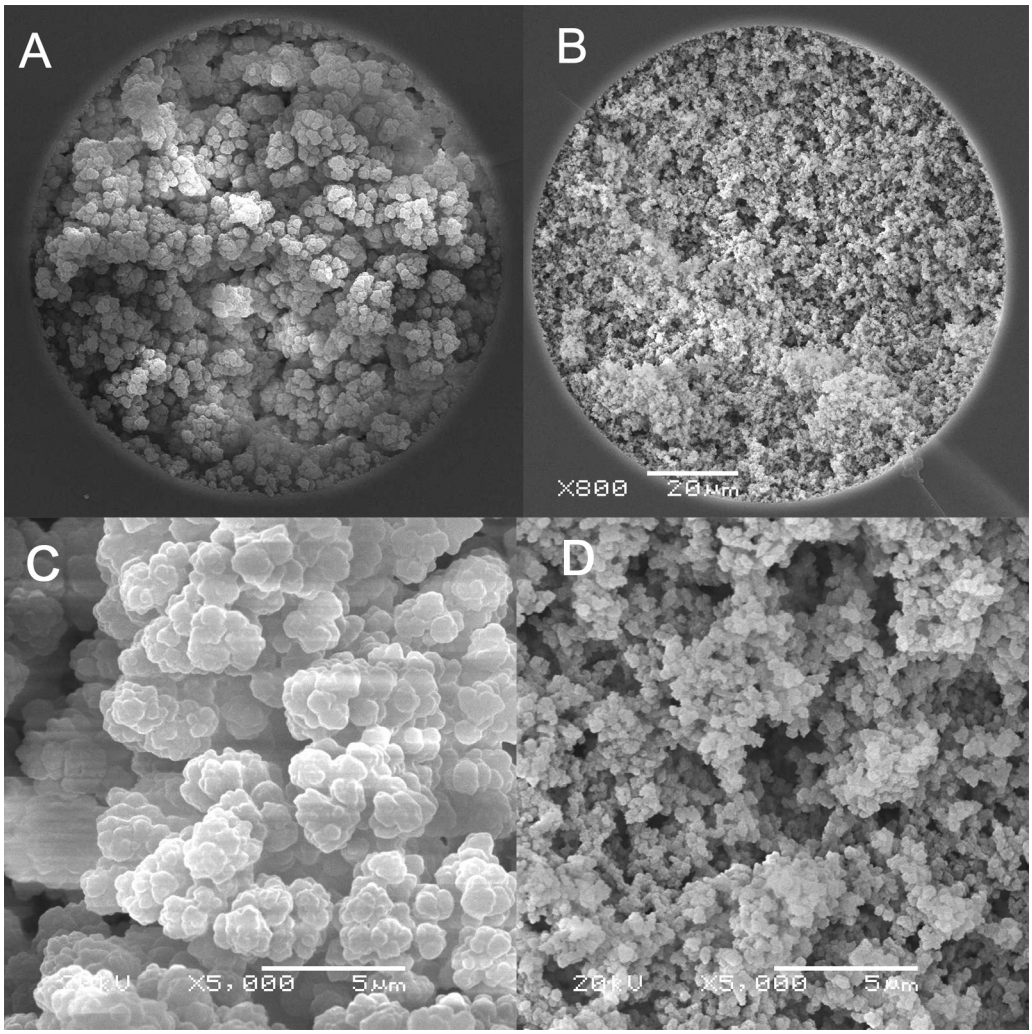

Fig. 3. SEM images of (A) and (C) LMA-EDMA and (B) and (D) LMA-POSS monolithic columns. Magnification: (A) and (B), 800× (C) and (D), 5000×.

columns embodies a inorganic–organic hybrid architecture with an inner inorganic framework, which eliminates the extra swelling or shrank of the LMA-EDMA organic monolithic columns and show a better stability [21].

Due to the wide usage of C18 packed columns and PS-DVB organic monolithic columns in protein analyses, the analogical SMA-POSS and BeMA-POSS hybrid monolithic columns were also developed to introduce C18 and benzyl RP groups, respectively (Fig. 4(a) and (b)). Comparing to LMA-POSS hybrid monolithic columns, SMA-POSS hybrid monolithic columns exhibit similar separation efficiency but slightly stronger retention and better resolutions because of the higher hydrophobicity of the C18 group (Figure S1, Table 3). When compared to the BeMA-POSS capillary columns, the SMA-POSS capillary columns also exhibited stronger LC retention to the proteins due to its higher hydrophobicity (Fig. 4(a) and (b)). Further, the BeMA-POSS and SMA-POSS capillary columns show slightly different LC separation selectivity to the intact protein analytes. Comparing to SMA-POSS columns, the BeMA-POSS columns exhibited lower LC separation resolutions for most of the neighbor analytes except for BSA and myoglobin (Table 3). This indicated that hydrophobicity is not the only effect that responsible for the LC separation behavior of the intact protein on the POSS-based capillary columns. The π–π stacking interaction introduced by the BeMA function monomer may exert positive effect on the LC separation of some types of intact proteins. In order to combine the advantages of hydrophobicity and π–π stacking interaction, another POSS-based hybrid monolithic column was developed with an equal weight of functional monomers SMA and

Table 2
Retention times of the 18 different monolithic columns of two different types.

	LMA-EDMA				LMA-POSS			
	Mean (n = 9)	Max	Min	RSD (n = 9) (%)	Mean (n = 9)	Max	Min	RSD (n = 9) (%)
Rnase B	13.05	12.58	13.68	3.29	15.00	14.84	15.28	1.11
Cytochrome c	15.69	15.12	16.49	3.20	18.70	18.44	18.98	1.16
Insulin	17.83	17.30	18.59	2.98	20.07	19.87	20.33	0.87
Lysozyme	20.58	19.95	21.44	2.82	22.42	22.18	22.75	0.86
BSA	23.99	23.37	24.84	2.67	25.90	25.59	26.20	0.73
Myoglobin	25.33	24.66	26.27	2.60	27.07	26.85	27.41	0.73

Conditions: injection, 5–7 ng of tested proteins; flow rate, 500 nL/min; gradient, 20–60% B with 0.1% TFA in 30 min; column, 100 μm i.d. × 25 cm long of different monolithic stationary phases.

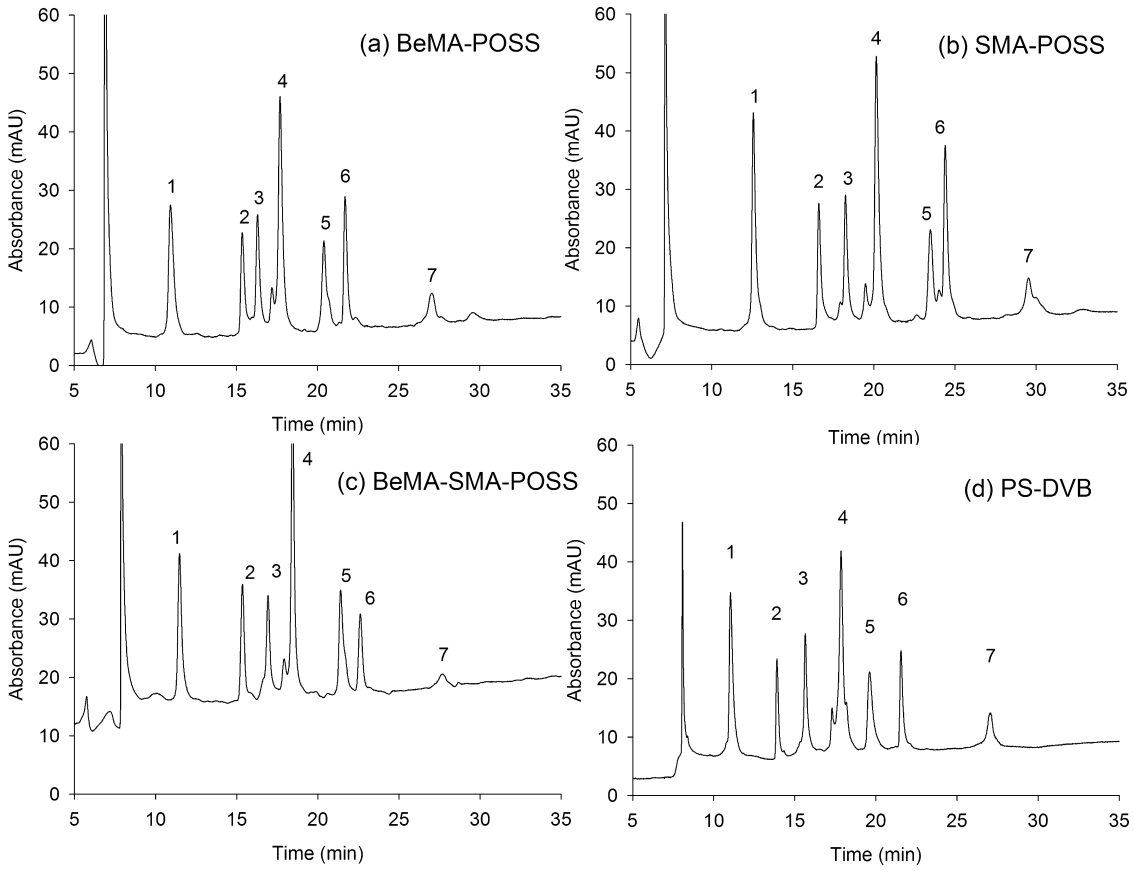

Fig. 4. Chromatograms for separation of intact protein mixture on the different monolithic capillary columns. (a) SMA-POSS hybrid monolithic column, (b) BeMA-POSS hybrid monolithic column, (c) SMA-BeMA hybrid monolithic column, (d) commercial PS-DVB monolithic column. Solute of standard protein mixture: (1) ribonuclease B, (2) cytochrome c, (3) insulin, (4) lysozyme, (5) BSA, (6) myoglobin, (7) ovalumin. Injection, 1 μL of the standard protein mixture; flow rate, 500 nL/min; gradient, 20–60% B with 0.05% TFA in 30 min; column, 100 μm i.d. × 25 cm; detection wavelength, 214 nm.

BeMA. Finally, all of the proteins were baseline separated by using the SMA-BeMA hybrid monolithic capillary column and unbiased LC separation resolutions were obtained for all of the intact proteins (Fig. 4c), which may more suitable for LC separation of complex protein samples. Therefore, the separation selectivity of the POSS-based hybrid monolithic column can be adjusted by combining different types of function monomers to some extent, which may be an alternative method to improve the LC separation capability.

The PS-DVB monolithic column has been widely used in the purification, fractionation and LC separation of the intact proteins [30–34]. Thus, a commercial PS-DVB monolithic capillary column has also been investigated for comparison (Fig. 4d). The same type of the commercial PS-DVB monolithic column with 200 μm i.d. and same length was always operated at 2 μL/min for the separation of the intact protein mixtures with optimal separation efficiency [17,35], thus flow rate 500 nL/min was selected for the 100 μm i.d. column. It was observed that the SMA-BeMA capillary column exhibits lower peak capacity but comparable run to run reproducibility to the PS-DVB capillary column (Table 4 and Table S2). However, the back pressure of SMA-BeMA hybrid monolithic column was just the half of PS-DVB monolithic column at identical flow rate (Figure S2). Therefore, the SMA-BeMA hybrid monolithic column has good separation efficiency with relative low operating back pressure, which may facilitate the fast separation of the protein mixture in high throughput analysis.

Table 3
The resolution of proteins on the different columns: (1) ribonuclease B and cytochrome c; (2) cytochrome c and insulin; (3) insulin and lysozyme; (4) lysozyme and BSA; (5) BSA and myoglobin.

Monolithic columns	1	2	3	4	5
LMA-POSS	9.2	2.4	3.1	6.4	2.1
SMA-POSS	10.7	4.3	4.5	6.8	1.9
BeMA-POSS	8.7	2.3	3.2	5.8	3.1
BeMA-SMA hybrid	9.5	3.9	4.0	7.1	2.7

Conditions: injection, 1 μL of the standard protein mixture; flow rate, 500 nL/min; gradient, 20–60% B with 0.05% TFA in 30 min; column, 100 μm i.d. × 25 cm long of different monolithic stationary phases.

Table 4
Mean values of peak width at half height and peak capacities of the different columns ($n = 3$).

Monolithic columns	Ribonuclease B (s)	Insulin (s)	Cytochrome c (s)	Lysozyme (s)	BSA (s)	Myoglobin (s)	Peak capacity
SMA-POSS	17.9	15.2	16.1	15.9	20.4	16.2	62.4
BeMA-POSS	16.2	13.6	13.4	16.6	20.1	12.9	68.4
SMA-BeMA	15.8	14.8	13.0	14.8	19.7	15.6	67.8
PS-DVB	14.2	9.0	11.1	14.4	20.2	11.7	78.8

Conditions: injection, 1 μL of the standard protein mixture; flow rate, 500 nL/min; gradient, 20–60% B with 0.05% TFA in 30 min; column, 100 μm i.d. × 25 cm long of different monolithic stationary phases.

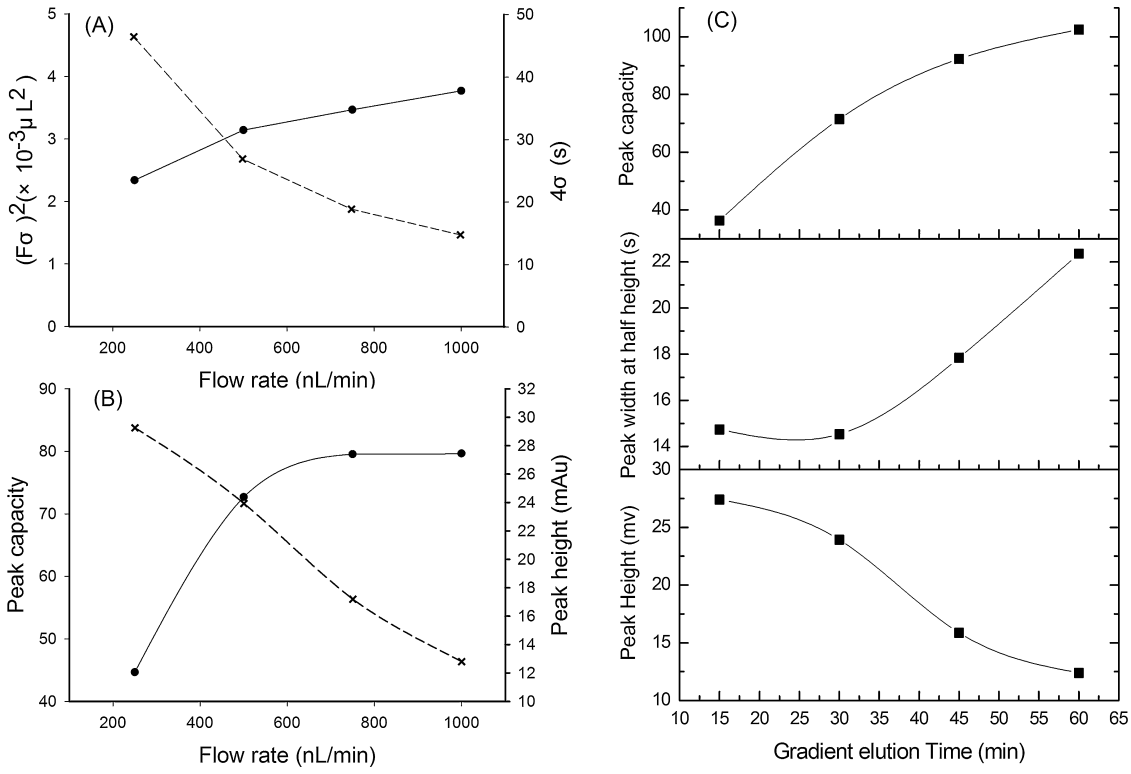

Fig. 5. The effect of the flow rate and gradient elution time on the separation efficiency. (A) The relationship between flow rate and $(\sigma F)^2$ (●)or peak with (4σ) (×) at a constant gradient steepness. (B) The peak capacity (●) and average peak height (×) as a function of the flow rate at a constant gradient elution time. (C) the effect of the gradient elution time on the peak height, peak width at half height and peak capacity at a fixed flow rate and gradient window.

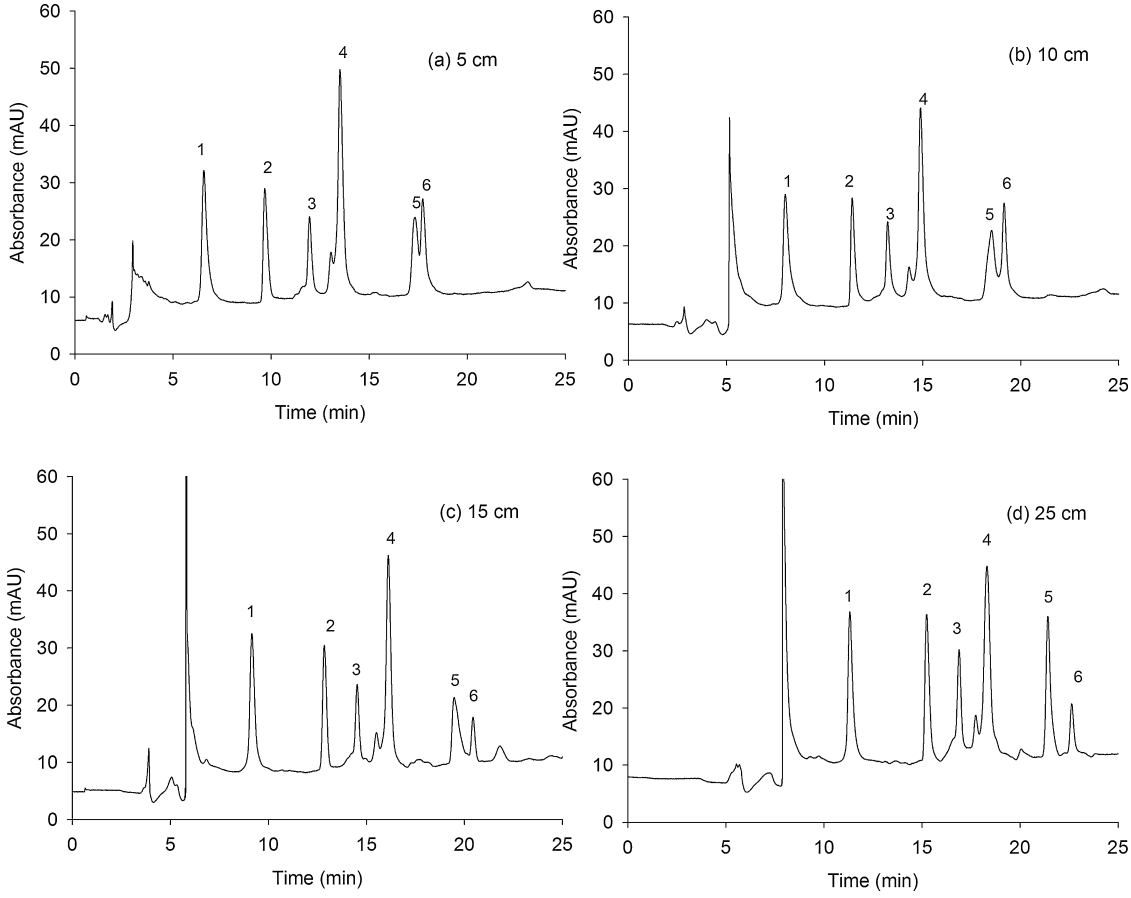

Fig. 6. Chromatograms obtained on different column lengths of 100 μm i.d. SMA-BeMA hybrid monolithic capillary column. Solute: (1) ribonuclease B, (2) cytochrome c, (3) insulin, (4) lysozyme, (5) BSA, (6) myoglobin. Injection, 1 μL of the standard protein mixture; flow rate, 500 nL/min; gradient, 20–60% B with 0.05% TFA in 30 min; detection wavelength, 214 nm.

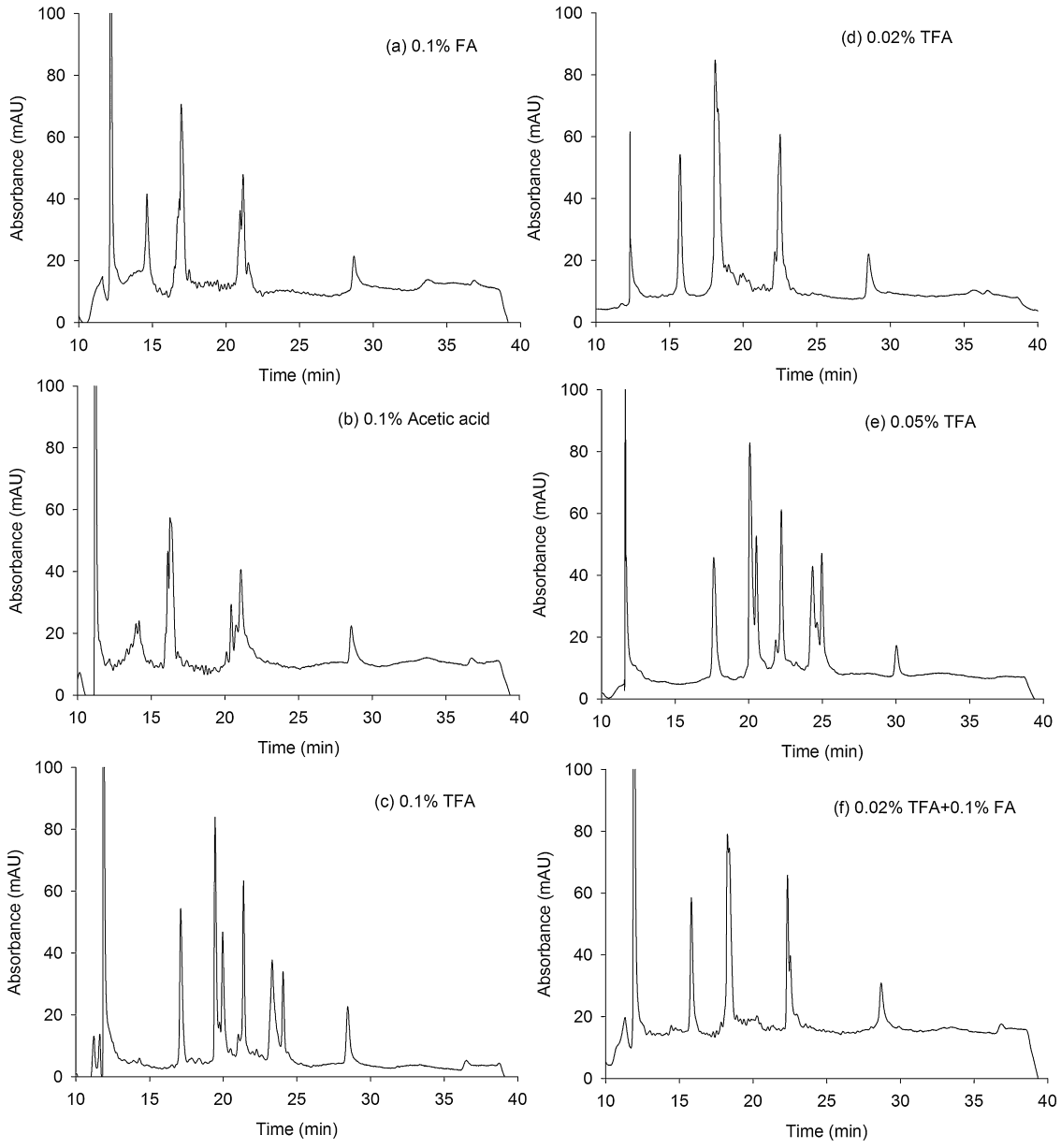

Fig. 7. Effects of mobile phase additives and concentration of the TFA on the separation of standard protein mixture. Injection, 1 μL of the standard protein mixture; flow rate, 500 nL/min; gradient, 0–60% B in 30 min; column, 100 μm i.d. × 25 cm long of SMA-BeMA hybrid monolithic capillary column; detection wavelength, 214 nm.

3.2. The effect of the flow rate, separation gradient and column length on separation performance

Rapid and efficient LC separation at high flow rate is needed for high throughput analysis of a large number of protein samples, which could significantly reduce the total analysis time. What's more, the flow rate has a great influence on the column efficiency. Usually, the effects of the eddy diffusion and the longitudinal diffusion are insignificant to the peak broadening under normal chromatographic condition. The only parameter that contributes to the peak broadening is mass transfer coefficient, which is related to the diffusivity of the analytes. However, due to the significant low diffusivity of intact proteins, they are more sensitive than small molecules toward the change of the flow rate [12]. Thus, different flow rates ranged from 250 to 1000 nL/min were investigated for the 100 μm i.d. × 25 cm long SMA-BeMA hybrid monolithic column. Because the hold up time decreases with the increase of the flow rate, the gradient time was normalized to keep gradient steepness as a constant. To assess the effect of flow rate on the plate-height (H), the following equation was used [35]:

$H = \frac{(\sigma\mu_0)^2}{L(1+k_e)^2} \sim (\sigma F)^2$ which was derived from $W = 4\sigma = \frac{4(1+k_e)\sqrt{H}\sqrt{L}}{\mu_0}$ where μ_0 is the velocity of the un-retained components, which is directly proportional to the flow rate (F). When the gradient steepness is a constant, the gradient retention factor (k_e) has nothing to do with flow rate [36], and column length (L) was kept same, thus the peak height (H) is proportional to the $(\sigma F)^2$. As shown in Fig. 5A, the $(\sigma F)^2$ increased slightly with the increase of the flow rate, which demonstrates the fast mass transfer rate of the hybrid monolithic columns otherwise the peak height (H) would dramatically increase due to the significant low diffusivity of the proteins [12]. The increase of the plate height would cause the peak bonding, but the peak width was dominated by the flow rate [35]. The peak width (4σ) decreased dramatically as the increase of the flow rate (Fig. 5A). Because the peak capacity is the only factor to be considered when determining the optimal

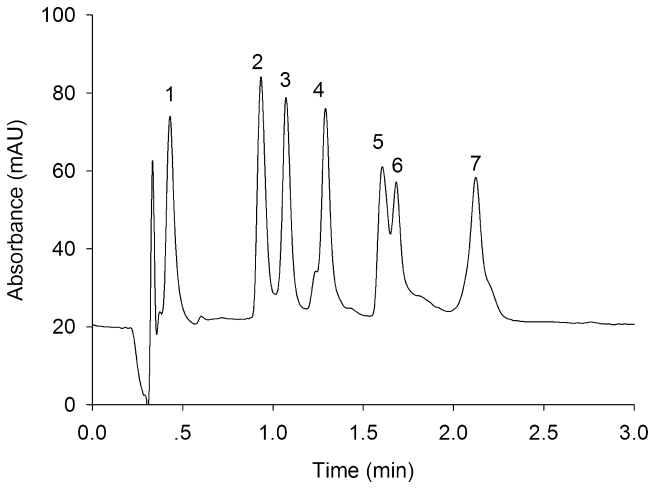

Fig. 8. Rapid separation of the standard protein mixture. Solute of standard protein mixture: (1) ribonuclease B, (2) cytochrome c, (3) insulin, (4) lysozyme, (5) BSA, (6) myoglobin, (7) ovalumin. Injection, 1 μL of the standard protein mixture; flow rate, 3000 nL/min; gradient, 30–75% B with 0.1% TFA in 3 min; column, 100 μm i.d. × 25 cm BeMA-SMA-POSS hybrid monolithic capillary column; detection wavelength, 214 nm.

flow rate [37], the effect of the flow rate on the peak capacity was also examined (Fig. 5B). The peak capacity was increased along with the flow rate at a constant gradient elution time, and the maximum peak capacity was about 80. The retention time of each protein decreased along with the increase of the flow rate. However, the decreasing trends greatly slowed down after the flow rate >750 nL/min (Figure S3), where the retention time of the proteins was mainly dominated by the gradient, this is consistent to the 'on–off' mechanism. As the UV detector is a concentration detector, the response is proportional to the concentration of the solutes. When a high flow rate is employed, the concentration of the solutes decreases significantly due to dilution effect, so as the UV response (Fig. 5B). Thus, high flow rate would have a negative effective on the sensitivity, and the 750 nL/min is the optimal separation flow rate for the BeMA-SMA hybrid monolithic column. It can be concluded that high separation flow rate is suitable for high-efficient separation of intact proteins by using the hybrid monolithic columns.

It is well known that the gradient steepness has a great importance on the efficiency of the RPLC separation [31,38]. The effect of gradient elution time on LC separation performance of the intact proteins was studied by applying 15, 30, 45 and 60 min RP binary separation gradients of 20–60% buffer B. The retention time of each protein is nearly direct proportion to the gradient elution time (Figure S4). The LC separation resolution can be greatly improved in a long gradient elution time. Although the peak width is broader and the peak height is lower in a longer separation gradient, the LC separation capacity is feasibly increased along with the increase of LC separation gradient elution time (Fig. 5C). Therefore, the increase of the LC separation gradient elution time is an effective way to improve the LC separation performance of intact proteins.

Then, intact protein separation by using different column lengths at identical chromatographic condition was further studied (Fig. 6). Obviously, the decrease of the column length can slightly reduce the retention time of each protein, so as the total analysis time. And the standard protein mixture is well separated in all the four columns with different column lengths. The peak capacity slightly increased from 62 to 70 when the column length increases from 5 cm to 25 cm of the SMA-BeMA hybrid monolithic capillary column. BSA and myoglobin is well resolved in the 25 cm long monolithic column with a separation resolution of 3.8. However, the separation resolution was decreased to 0.8 when the 5 cm long hybrid monolithic column was utilized. This is indicated that the separation of proteins on the reversed phase stationary is not strictly following the on–off mechanism. Otherwise, the short column would have a similar or better separation efficiency because of less eddy diffusion and longitudinal dispersion, this result is consistent with the Eeltink's work [35]. Therefore, increasing the column length has a positive effect on the peak capacity and resolution, which may benefit the separation of complex protein samples.

3.3. The effect of mobile phase additives

It is well known that mobile phase additives have a great influence on the retention of intact proteins, and different columns show different dependences of the mobile phase additives [39]. Therefore, the species and the concentration of the mobile phase additives in LC separation for POSS-based monolithic capillary columns were also investigated.

Three types of mobile phase additives, TFA, FA and acetic acid were investigated in the same concentration of 0.1% (v/v). It was observed that the standard protein mixture is well separated only when TFA was adopted (Fig. 7c), which is related to the strong ion-pairing effect between TFA and proteins. Intact proteins are well separated when 0.05% or 0.1% TFA was used, but a narrow peak width and slightly improvement on the separation resolution was observed for 0.1% TFA. However, when the TFA concentration was decreased to as low as 0.02%, some proteins cannot well separated with each other, such as the lysozyme cannot be well separated from cytochrome c and insulin, meanwhile myoglobin and BSA are

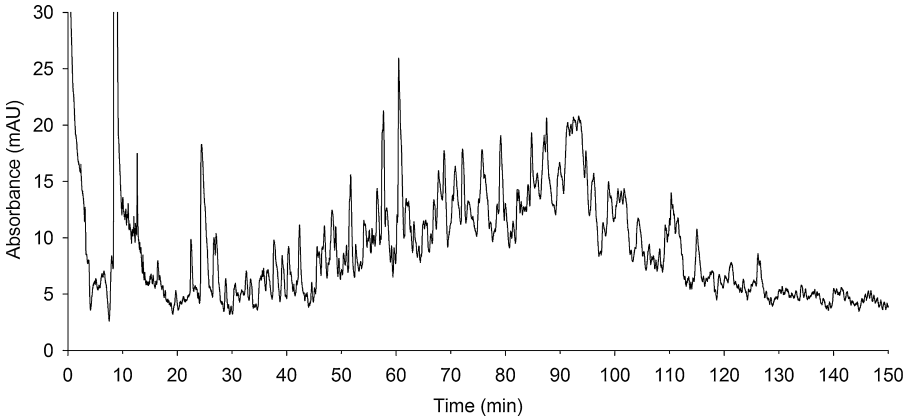

Fig. 9. Separation of *E. coli* proteins. Injection, 1 μg of *E. coli* proteins; flow rate, 750 nL/min; gradient, 27–40% B with 0.1% TFA in 50 min 40–55% B in 100 min; column, 100 μm i.d. × 25 cm BeMA-SMA-POSS hybrid monolithic capillary column; detection wavelength, 214 nm.

almost overlapped (Fig. 7d). Although TFA can improve the LC separation performance as a mobile phase additive, it prevents the ionization of proteins when coupled to the MS due to the strong ion-pairing effect with intact proteins, which will compromise the MS detection sensitivity [40]. A mixture of TFA and FA is usually adopted when RPLC is coupled to the mass spectrometry [41–43]. When a mixture of 0.02% TFA and 0.1% FA was applied, no improvement of the separation efficiency was found compared to the 0.02% TFA alone (Fig. 7f). Therefore, at least 0.05% TFA is needed to obtain good LC separation performance for the POSS-based hybrid monolithic columns.

3.4. The application of the POSS-based monolithic capillary columns

Monolithic columns are considered as an good stationary phase for fast LC separation due to its high permeability and fast mass transfer rate [44]. BeMA-SMA hybrid monolithic column was used for the fast separation due to its unbiased LC separation selectivity. A 10 cm-long SMA-BeMA hybrid monolithic capillary column was used for fast separation of intact proteins with a flow rate of 3000 nL/min and a 3 min binary gradient elution for 30%–75% buffer B with 0.1% TFA. Before each analysis, the column was equilibrated of 25% buffer B for 1 min. As shown in Fig. 8, the 7 intact proteins are well separated within 2.5 min. Therefore, it was confirmed that POSS-based hybrid monolithic capillary column can be applied for fast separation of intact proteins with high flow rate and rapid separation gradient elution.

The separation of complex sample was also conducted using *E. coli* lysates. At the optimal condition, 1 μg of *E. coli* proteins was well separated with a 150 min gradient of 27%–55% buffer B and 0.1% TFA as mobile phase additive at a flow rate of 750 nL/min (Fig. 9). This further demonstrated the good performance of the POSS-based hybrid monolithic column for the intact protein separation.

4. Conclusion

LC separation with capillary column and flow rate less than 1 μL/min has been widely applied in peptides mixture separation to increase the MS detection sensitivity in shotgun proteome analysis. However, high-efficient LC separation of intact protein samples by using capillary columns is still a big challenge. It is necessary to develop new LC separation technologies to improve the LC separation capability and shorten the analysis time for intact protein samples. Due to simplicity of preparation, excellent robustness and a wide variety of functional monomers, monoliths are considered as the promising LC stationary phases, especially the hybrid monolith with the advantages of both inorganic and organic monoliths. Therefore, it is important to investigate the performance of hybrid monolithic capillary columns in LC separation of intact proteins.

Four POSS-based hybrid monolithic capillary columns were developed by using LMA, SMA and BeMA as functional monomers and POSS as the cross-linker through “one-pot” process. Good reproducibility was achieved in both run-to-run and column-to-column analyses for these POSS-based hybrid monolithic capillary columns. It was observed that the combination of two functional monomers SMA and BeMA in preparation of the POSS-based hybrid monolithic columns has a better LC separation selectivity than using one type of functional monomer alone. And good LC separation performance of the SMA-BeMA hybrid monolithic capillary column was obtained at low operating back pressure. Further, the separation efficiency of the POSS-based hybrid monolithic capillary column is not significant decreased with higher separation flow rate and shorter column length. Therefore, the hybrid monolithic column has a great potential for high throughout separation of intact proteins.

In conclusion, the optimized hybrid monolithic column might be a promising alternative for high performance LC separation of intact proteins, which might play an important role in top down proteomics in the future.

Acknowledgement

The authors wish to thank Thermo Fisher Scientific for providing the PS-DVB monolithic columns. H.Z. would like to acknowledge the financial supports from the China State Key Basic Research Program Grant (2013CB911203, 2012CB910601, and 2012CB910101), the financial support from the NSFC (21021004 and 21235006), and the Analytical Method Innovation Program of MOST (2012IM030900). J.O. would like to acknowledge the financial support from the NSFC (21175133).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2013.09.004.

References

[1] A. Bensimon, A.J. Heck, R. Aebersold, Annu. Rev. Biochem. 81 (2012) 379.
[2] M. Schirle, M. Bantscheff, B. Kuster, Chem. Biol. 19 (2012) 72.
[3] H. Wang, S.G. Clouthier, V. Galchev, D.E. Misek, U. Duffner, C.K. Min, R. Zhao, J. Tra, G.S. Omenn, J.L. Ferrara, S.M. Hanash, Mol. Cell. Proteomics 4 (2005) 618.
[4] A. Bora, C. Anderson, M. Bachani, A. Nath, R.J. Cotter, J. Proteome Res. 11 (2012) 3143.
[5] W. Ying, Y. Jiang, L. Guo, Y. Hao, Y. Zhang, S. Wu, F. Zhong, J. Wang, R. Shi, D. Li, Mol. Cell. Proteomics 5 (2006) 1703.
[6] H. Zhou, Z. Ning, A. Starr, M. Abu-Farha, D. Figeys, Anal. Chem. (2011).
[7] A.L. Capriotti, C. Cavaliere, P. Foglia, R. Samperi, A. Laganà, J. Chromatogr. A (2011).
[8] P.C. Havugimana, G.T. Hart, T. Nepusz, H. Yang, A.L. Turinsky, Z. Li, P.I. Wang, D.R. Boutz, V. Fong, S. Phanse, M. Babu, S.A. Craig, P. Hu, C. Wan, J. Vlasblom, V.U. Dar, A. Bezginov, G.W. Clark, G.C. Wu, S.J. Wodak, E.R. Tillier, A. Paccanaro, E.M. Marcotte, A. Emili, Cell 150 (2012) 1068.
[9] Z. Tian, N. Tolic, R. Zhao, R.J. Moore, S.M. Hengel, E.W. Robinson, D.L. Stenoien, S. Wu, R.D. Smith, L. Pasa-Tolic, Genome Biol. 13 (2012) R86.
[10] S. Wu, N.M. Lourette, N. Tolic′, R. Zhao, E.W. Robinson, A.V. Tolmachev, R.D. Smith, L. Paša-Tolic′, J. Proteome Res. 8 (2009) 1347.
[11] A.T. Iavarone, J.C. Jurchen, E.R. Williams, J. Am. Soc. Mass Spectrom. 11 (2000) 976.
[12] J.-C. Janson, Protein Purification: Principles, High Resolution Methods, and Applications, Wiley.com, 2012.
[13] R.a. Wu, L. Hu, F. Wang, M. Ye, H. Zou, J. Chromatogr. A 1184 (2008) 369.
[14] J. Mohr, R. Swart, M. Samonig, G. Bohm, C.G. Huber, Proteomics 10 (2010) 3598.
[15] S. Zheng, C. Yoo, N. Delmotte, F.R. Miller, C.G. Huber, D.M. Lubman, Anal. Chem. 78 (2006) 5198.
[16] C. Legido-Quigley, N. Marlin, N.W. Smith, J. Chromatogr. A 1030 (2004) 195.
[17] B. Wouters, A. Vaast, A. Treumann, M. Ursem, J. Ho, M. Hornshaw, M. Raes, H. Terryn, S. Eeltink, LC GC Europe 25 (2012) 10.
[18] D. Lee, F. Svec, J.M. Fréchet, J. Chromatogr. A 1051 (2004) 53.
[19] Y. Li, J. Zhang, R. Xiang, Y. Yang, C. Horváth, J. Sep. Sci. 27 (2004) 1467.
[20] J.C. Tran, L. Zamdborg, D.R. Ahlf, J.E. Lee, A.D. Catherman, K.R. Durbin, J.D. Tipton, A. Vellaichamy, J.F. Kellie, M. Li, Nature 480 (2011) 254.
[21] Z. Zhang, F. Wang, J. Ou, H. Lin, J. Dong, H. Zou, Anal. Bioanal. Chem. 405 (2013) 2265.
[22] H. Lin, J. Ou, Z. Zhang, J. Dong, H. Zou, Chem. Commun. (Camb.) 49 (2013) 231.
[23] M. Wu, R. a. Wu, R. Li, H. Qin, J. Dong, Z. Zhang, H. Zou, Anal. Chem. 82 (2010) 5447.
[24] J. Ou, Z. Zhang, H. Lin, J. Dong, H. Zou, Anal. Chim. Acta (2012).
[25] J. Ou, Z. Zhang, H. Lin, J. Dong, M. Wu, Electrophoresis 33 (2012) 1660.
[26] F. Wang, J. Dong, M. Ye, R. Wu, H. Zou, J. Chromatogr. A 1216 (2009) 3887.
[27] X. Jiang, J. Dong, F. Wang, S. Feng, M. Ye, H. Zou, Electrophoresis 29 (2008) 1612.
[28] S. Shu, H. Kobayashi, N. Kojima, A. Sabarudin, T. Umemura, J. Chromatogr. A 1218 (2011) 5228.
[29] L. Geiser, S. Eeltink, F. Svec, J.M. Frechet, J. Chromatogr. A 1140 (2007) 140.
[30] C.G. Huber, W. Walcher, A.M. Timperio, S. Troiani, A. Porceddu, L. Zolla, Proteomics 4 (2004) 3909.
[31] W. Walcher, H. Toll, A. Ingendoh, C.G. Huber, J. Chromatogr. A 1053 (2004) 107.
[32] R. Bakry, C.W. Huck, G.K. Bonn, J. Chromatogr. Sci. 47 (2009) 418.
[33] S. Eeltink, B. Wouters, G. Desmet, M. Ursem, D. Blinco, G.D. Kemp, A. Treumann, J. Chromatogr. A 1218 (2011) 5504.

[34] C. Yoo, J. Zhao, M. Pal, K. Hersberger, C.G. Huber, D.M. Simeone, D.G. Beer, D.M. Lubman, Electrophoresis 27 (2006) 3643.
[35] F. Detobel, K. Broeckhoven, J. Wellens, B. Wouters, R. Swart, M. Ursem, G. Desmet, S. Eeltink, J. Chromatogr. A 1217 (2010) 3085.
[36] U.D. Neue, J. Chromatogr. A 1079 (2005) 153.
[37] H. Liu, J.W. Finch, M.J. Lavallee, R.A. Collamati, C.C. Benevides, J.C. Gebler, J. Chromatogr. A 1147 (2007) 30.
[38] V. Spicer, M. Grigoryan, A. Gotfrid, K.G. Standing, O.V. Krokhin, Anal. Chem. 82 (2010) 9678.
[39] M. Garcı´a, A. Hogenboom, H. Zappey, H. Irth, J. Chromatogr. A 957 (2002) 187.
[40] M.C. Garcia, J. Chromatogr, B. Analyt, Technol. Biomed. Life Sci. 825 (2005) 111.
[41] S. Hu, P. Denny, P. Denny, Y. Xie, J.A. Loo, L.E. Wolinsky, Y. Li, J. McBride, R.R. Ogorzalek Loo, M. Navazesh, Int. J. Oncol. 25 (2004) 1423.
[42] S. Hu, Y. Xie, P. Ramachandran, R.R. Ogorzalek Loo, Y. Li, J.A. Loo, D.T. Wong, Proteomics 5 (2005) 1714.
[43] P. Yin, X. Hou, E.V. Romanova, J.V. Sweedler, Neuropeptidomics: mass spectrometry-based qualitative and quantitative analysis, in: Neuropeptides, Springer, 2011, pp. 223.
[44] H. Zou, X. Huang, M. Ye, Q. Luo, J. Chromatogr. A 954 (2002) 5.