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

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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

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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.