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Preparation of capillary hybrid monolithic column with sulfonate strong cation exchanger for proteome analysis

Zhenbin Zhang[a,b,1], Fangjun Wang[a,1], Bo Xu[a,b], Hongqiang Qin[a,b], Mingliang Ye[a], Hanfa Zou[a,*]

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

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ABSTRACT

Strong cation exchange (SCX) chromatography is one of the most important separation modes in liquid chromatography and SCX column is widely applied in high resolution separation or fractionation of various samples. In this work, a sulfonate SCX hybrid monolithic column was successfully prepared by "one-pot" strategy and the hybrid monolith is well optimized to obtain homogenous structure. It was observed that this sulfonate SCX hybrid monolithic column had ~7 times permeability (in water) and ~3 times sample loading capacity (tested by dipeptide Gly-Tyr) comparing to particulate SCX column packed with commercial available material. Then, it was used as trap column for fast sample loading of the enriched phosphopeptides. Comparing to phosphate SCX polymer monolithic column, the number of identified phosphopeptides increased ~19% due to the sulfonate group has higher retention strength than phosphate group for peptide cations. And the coverage of phosphoproteome obtained by sulfonate SCX hybrid monolithic column is similar to particulate packed SCX column, because they had identical sulfonate group to retain the peptide cations. Finally, the sulfonate SCX hybrid monolithic column was used as enzyme reactor for online protein digestion. Comparing to particulate SCX packed column, the identified peptides number increased 40% and the protein coverage increased 10%. This might be ascribed to the high porous structure and relative high surface area that elevated the digestion efficiency.

1. Introduction

Strong cation exchange (SCX) column is widely applied in liquid chromatography (LC) for separation of drugs, peptides, proteins, and so on [1–3]. Direct separation of these compounds by SCX column is usually coupled with UV detector under isocratic or gradient elution and its separation capacity is usually inferior to reversed phase (RP) separation for most of the compounds [4]. However, SCX column is widely used to couple with RP column for multidimensional separation due to the good orthogonality between SCX and RP separation mechanisms. And the SCX-RP system is also the most popular system for multidimensional separation in large scale proteome analysis [5–7].

The first step of SCX-RP multidimensional separation in proteome analysis is loading sample onto the SCX column. Therefore, the loading capacity of SCX column is critical for high resolution separation or fractionation [8,9]. For large amount of protein samples, SCX column with higher binding capacity can provide better resolution in offline fractionation [6,10]. Gygi and coworkers applied offline SCX fractionation for 10 mg tryptic digest of protein sample extracted from mouse liver and *Drosophila melanogaster* embryos, followed with IMAC enrichment of the phosphopeptides, finally 5635 and 13 720 phosphorylation sites were identified [11,12], respectively. However, excessive sample loss will be introduced by offline fractionation for minute amount of samples, and online SCX fractionation by capillary column is a good choice to achieve much higher detection sensitivity and analysis throughput [13,14]. Multidimensional protein identification technology (MudPIT) with online SCX fractionation was firstly established for proteome analysis, which was based on a biphasic column by packing with RP and SCX particulates sequentially within the same capillary column. The samples loaded onto the SCX segment is online fractionating to the RP separation column by stepwise salt solution automatically, and high sensitivity and proteome coverage can be both obtained by this method [15,16]. However, the packing amount of SCX material is limited due to the back pressure of the LC system, thus the sample loading amounts and fractionation resolution are also limited [9]. One way to increase the packing amount of SCX materials is applying ultra-high pressure LC systems [17]. However, these types of equipments are expensive and difficult to operate. Another good alternative is

* Corresponding author. Tel.: +86 411 84379610; fax: +86 411 84379620.
E-mail address: hanfazou@dicp.ac.cn (H. Zou).
[1] The first two authors contributed equally to this work.

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to develop new type of SCX materials with both high binding capacity and permeability. Monolithic columns are considered as the new generation of LC stationary phases, which are synthesized by in situ preparation of polymer-based organic and silica-based inorganic monoliths with various specific functionalities arising from the versatile in situ chemical reactions and/or post-modifications, and exhibit high porous structure and mass transfer rate [18,19]. Both organic and inorganic SCX monolithic columns were prepared for different purposes in LC separation [20,21]. Organic sulfonate SCX columns are more convenient to prepare than inorganic ones and the binding capacity can be increased by adjusting the amount of functional monomer in the prepolymerization mixture [22,23] or by increasing grafting time in the post-modification process [24]. However, the sulfonate organic monolith exhibited excessive shrinking and swelling in different types of separation buffer [22] and there is always some RP retention mechanism mixed into the sulfonate organic monolith [20].

In our previous work, we have developed a phosphate SCX polymer monolithic column, which exhibited 10 times higher permeability and comparable binding capacity over commercial available SCX packing materials. It was utilized as trap column for automated sample injection and online multidimensional separation of protein digested samples [9], and the usage time is as long as two months due to its high rigidity and stability. Although the phosphate SCX monolithic column could be applied in high efficient online multidimensional separation for different proteome analysis [25,26], the retention strength of phosphate group is actually weaker than sulfonate group for peptide cations. It is still necessary to develop new types of sulfonate SCX monolithic columns with high rigidity to increase the retention strength. And this is extremely important for some low SCX retention compounds, such as phosphopeptides, which usually have more negative charges than non-phosphopeptides [27]. Alternatively, the organic-silica hybrid monolith has attracted great attentions since it could combine the merits of both organic polymer-based and silica-based monoliths, such as ease of preparation, pH stability and good mechanical stability. We have recently developed a simple "one-pot" approach for preparing of the organic-silica hybrid monolithic capillary columns, which represented a new way by using a variety of organic functional monomers in the preparation of organic-silica hybrid monoliths [28,29]. In this work, we successfully prepared a silica-based sulfonate SCX hybrid monolithic column. This column had similar porous structure as silica-based monolith and exhibited much higher permeability and loading capacity than the column packed with commercial available SCX material. Then, it was used as trap column for phosphoproteome analysis and comparable retention strength could be obtained when comparing with particulate packed SCX column. This sulfonate SCX hybrid monolithic column was also used as enzyme reactor for automated and rapid protein digestion, and 10% higher protein coverage could be achieved due to its porous structure and high mass transfer rate for enzyme digestion of proteins. To the best of our knowledge, this is the first time that hybrid SCX monolithic column was prepared and applied into proteome analysis.

2. Experimental

2.1. Chemicals and materials

3-Sulfopropyl methacrylate potassium salt, vinyltrimethoxysilane (VTMS), urea and poly(ethylene glycol) (PEG, M_n = 10 000) were purchased from Aldrich (Milwaukee, WI). Tetramethoxysilane (TMOS) was obtained from Chemical Factory of Wuhan University (Wuhan, China). Daisogel ODS-AQ (5 μm, 12 nm pore) was purchased from DAISO Chemical Co., Ltd. (Osaka, Japan), and PolySulfoethyl Aspartamide (5 μm, 20 nm pore) was a gift from PolyLC Inc. (Columbia, MD, USA). PEEK tubing, sleeves, microtee, microcross, and mini microfilter assembly (with a filter capsule, porosity 2 μm) were obtained from Upchurch Scientific (Oak Harbor, WA, USA). Fused silica capillaries with 75 and 200 μm I.D. were purchased from Polymicro Technologies (Phoenix, AZ, USA). Dithiothreitol (DTT), iodoacetamide (IAA), trypsin, tris(hydroxymethyl)aminomethane (Tris), formic acid (FA) and γ-methacryloxypropyltrimethoxysilane (γ-MAPS) were all obtained from Sigma (St. Louis, MO, USA). Azobisisobutyronitrile (AIBN) was obtained from Shanghai Fourth Reagent Plant (Shanghai, China). Synthetic peptide was purchased from Serva (Heidelberg, Germany). Acetonitrile (ACN, HPLC grade) was from Merck (Darmstadt, Germany). All the water used in experiments was purified using a Mill-Q system (Millipore, Bedford, MA, USA).

2.2. Columns preparation

The sulfonate SCX hybrid monolithic column was prepared in the similar way as we reported before with a minor modification [28,29,40]. Before the preparation of the sulfonate SCX hybrid monolithic column, the fused-silica capillary was pretreated and rinsed by 1.0 M HCl for 12 h, water for 30 min, 1.0 M NaOH for 12 h, and water for another 30 min, respectively, which was then dried by nitrogen stream at room temperature. For the preparation of the sulfonate SCX hybrid monolithic column, a prehydrolyzed mixture was prepared by mixing and stirring acetic acid (0.01 M, 5.0 mL), PEG (Mn = 10 000, 540 mg), urea (450 mg), TMOS (1.8 mL) and VTMS (0.6 mL) for 1 h at 0 °C to form a homogeneous solution. Then, 35 mg of 3-sulfopropyl methacrylate potassium salt and 2 mg of AIBN were added into 0.5 mL of the resultant hydrolyzed mixture with 10 min sonication. After that, the mixture was manually introduced into the pretreated capillary to an appropriate length with a syringe. By sealing the both ends of the capillary with two pieces of rubbers, the capillary was incubated at 45 °C and 60 °C for 12 h, for condensation and polymerization, respectively. The obtained sulfonate SCX hybrid monolithic capillary column was then flushed with water and methanol to remove the residuals. The schematic synthesis of sulfonate SCX hybrid monolith was illustrated in Fig. 1A. Scanning electron microscopy (SEM) images of the hybrid monolithic column were obtained using a JEOL JSM-5600 scanning electron microscope (JEOL Company, Japan).

For the preparation of separation column, one end of a 75 μm I.D. fused silica capillary was first manually pulled to a fine point of ~3 μm with a flame torch, and then the C18 particulates (3 μm, 12 nm pore) were packed with a slurry packing method until the packing section reached the length of 12 cm. For the preparation of particulate packed SCX trap column, one 1 mm-long phosphate monolithic frit was prepared at the end of one 200 μm I.D. fused silica capillary [9], then SCX particulates (5 μm, 20 nm pore) was packed to appropriate length. For the preparation of C18 trap column, one 1 mm-long C12 monolith frit was prepared at the end of one 200 μm I.D. fused silica capillary [41], then C18 particulates (5 μm, 12 nm pore) were packed to appropriate length.

2.3. Permeability and binding capacity measurements

The column permeability and stability were examined by connecting the columns (3.5 cm-long for monolithic column and 2 cm-long for packed column) to a μUPLC pump (Waters) with the flow rate ranged from 20 to 40 μL/min (mobile phase, ACN or water with 0.1% FA) at 25 °C. For measurement of the dynamic binding capacity, a dipeptide Gly-Tyr (MW: 238.2) was used to saturate the sulfonate SCX hybrid monolithic column (200 μm I.D., 3.5 cm) by frontal analysis. Briefly, 10 mg of Gly-Tyr was dissolved into 20 mL of buffer containing 0.1% FA and 10% ACN (pH 2.3), which was