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Online Multidimensional Separation with Biphasic Monolithic Capillary Column for Shotgun Proteome Analysis

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Abstract: A biphasic monolithic capillary column with 10 cm segment of strong-cation exchange monolith and 65 cm segment of reversed-phase monolith was prepared within a single 100 μm i.d. capillary. Separation performance of this column was evaluated by a five-cycle online multidimensional separation of 10 μg tryptic digest of yeast proteins using nanoflow liquid chromatography coupled with tandem mass spectrometry, and it took 12 h for whole separation under the operating pressure only ~900 psi. Totally, 780 distinct proteins were positively identified through assignment of 2953 unique peptides at false-positive rate less than 1%. The good separation performance of this biphasic column was largely attributed to the good orthogonality of the strong-cation exchange monolith and reversed-phase monolith for multidimensional separation.

Keywords: Biphasic monolithic column • shotgun proteome analysis • multidimensional separation • μHPLC−MS/MS

Introduction

Multidimensional separation is the most powerful approach to increase the separation performance in liquid chromatography (LC) analysis, and the separation peak capacity could be improved greatly by combining two dimensions of separations with orthogonal mechanisms.[1,2] Though off-line multidimensional separation can provide more sufficient prefractionation for analytes,[3,4] online multidimensional separation displays advantages such as higher sensitivity, minimal loss of sample, no vial contamination, and no sample dilution effect.[5–8] Especially in capillary liquid chromatography−tandem mass spectrometry (μHPLC−MS/MS), online multidimensional separation exhibits one of the best solutions for analysis of the limited amount of biological samples. Multidimensional protein identification technology (MudPIT) developed by Yates and coworkers has been successfully applied in shotgun proteome analysis for its prominent separation performance and good compatibility to mass spectrometry (MS) detector.[9–11] In the classical mode of this technology, strong-cation exchange (SCX) and reversed-phase (RP) particles are packed in sequence within a single capillary, and peptides binding initially onto the SCX segment are fractionated with stepwise elution of salt buffer followed with gradient separation on the RP segment before MS detection. However, as a packed column is used, the packing length of the both segments is limited by the operating pressure of the LC system. Therefore, the sample loading capacity, as well as separation capability of this type of biphasic packed column, is hard to increase unless ultrahigh pressure system is used.[12]

Monolithic columns with polymeric stationary phases are widely applied in LC analysis due to their advantages such as pH stability, good permeability, inertness to biomolecules, absence of deleterious effects from silanol, and facility for modification.[13–15] Recently, Jmeian et al. applied tandem affinity monolithic microcolumns to deplete the high-abundance proteins in serum samples, and zero dead volume (ZDV) union was used to connect the monolithic columns in sequence.[16] We have prepared a SCX monolithic column based on the functional monomer ethylene glycol methacrylate phosphate (EGMP)[17] to trap and fractionate peptides in multidimensional separation, and this type of phosphate monolithic column has higher dynamic binding capacity than column packed with commercially available Polysulfethyl A. A neutrally hydrophobic monolithic column based on the functional monomer lauryl methacrylate (LMA) was also prepared for capillary electrochromatography (CEC) separation of ionic compounds driven by electrophoretic mobility.[18] It can be expected that these two types of monolithic columns should have good orthogonality in retention mechanism for multidimensional separation. Therefore, after optimizing the polymerization mixture constitution for preparation of the LMA-based monolith, a biphasic monolithic column was synthesized with 10 cm segment of SCX monolith and 65 cm segment of RP monolith in a single 100 μm i.d. capillary in this study. This biphasic monolithic column was applied in online multidimensional separation for shotgun proteome analysis under relatively low operating pressure of ~900 psi, and good separation performance of this biphasic column was demonstrated.

Experimental Section

Materials. Fused silica capillaries were purchased from Yongnian Optical Fiber Factory (Hebei, China). Azobisisobutyronitrile (AIBN) was obtained from Shanghai Fourth Reagent Plant (Shanghai, China). Other materials were purchased from Sigma (St. Louis, MO).

Preparation of the Tryptic Digest of Yeast Proteins. The yeast protein extract was prepared in a denaturing buffer

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containing 50 mM Tris/HCl (pH 8.1) and 8 M urea according to procedures previously reported.[17,20] The protein concentration was determined by BCA assay. The protein sample was reduced by DTT at 37 °C for 2 h and alkylated by iodoacetamide in dark at room temperature for 40 min. Then the solution was diluted to 1 M urea with 50 mM Tris/HCl (pH 8.1). Finally, trypsin was added with weight ratio of trypsin to protein at 1/25 and incubated at 37 °C overnight. Then, the tryptic digest was purified with a homemade C18 solid phase cartridge and exchanged into buffer A (0.1% formic acid−water solution). Finally, the samples were stored at −20 °C before usage.

Preparation of the Biphasic Monolithic Column. We developed a two-step procedure to prepare the biphasic monolithic capillary column. First the empty capillary with 75 cm × 100 μm i.d. was pretreated with γ-MAPS, then one end of the empty capillary was synthesized to prepare 10 cm length of phosphate SCX monolith according to the procedures previously reported.[17] Briefly, 80 μL of EGMP, 60 mg of methylene bis acrylamide, 270 μL of dimethylsulfoxide, 200 μL of dodecanol, 50 μL of *N*, *N*′-dimethylformamide, and 2 mg of AIBN were mixed and sonicated for 20 min to obtain a homogeneous solution and then purged with nitrogen for 10 min. After the pretreated capillary was filled with 10 cm length of plug of this SCX polymerization mixture by siphon, both ends of the capillary were sealed with rubber stoppers. The sealed capillary was submerged into a water bath and allowed to react for 12 h at 60 °C. The resultant capillary column with 10 cm length of SCX monolith was washed with methanol using an HPLC pump to remove unreacted monomers and porogens followed with drying by nitrogen gas. Second, the RP polymerization mixture containing 100 μL of LMA, 100 μL of ethylene dimethacrylate, 147 μL of 1-propanol, 73 μL of 1, 4-butanediol, and of 1 mg AIBN was pushed into the capillary column from the other end using a pneumatic pressure cell at constant nitrogen gas pressure of about 5 psi. The flow of the RP polymerization mixture was monitored under a microscope to carefully control the front of polymerization mixture to exceed the fringe of the SCX monolith by 2–3 cm. Then, the capillary column was sealed at both ends with rubber stoppers again and reacted in water bath at 60 °C for 12 h. Methanol was flushed from the SCX monolithic section to RP monolithic section to remove unreacted monomers and porogens as described above. Finally, the biphasic monolithic column was flushed with water, and an integrated electrospray tip was directly pulled from the end of RP section as described by Xie et al.[19]

μHPLC−MS/MS System. The HPLC system (ThermoFinnigan, San Jose, CA) consisted of a degasser and a quaternary Surveyor MS pump. The three buffer solutions used for the quaternary pump were 0.1% formic acid aqueous solution (buffer A), ACN with 0.1% formic acid (buffer B), and 500 mM NH_4Ac aqueous solution at pH 3 (buffer C). Capillary with 50 μm i.d. was used for eluent splitting, and the flow rate of eluent for separation after splitting was adjusted to about 300 nL/min. The LTQ linear ion trap mass spectrometer was equipped with a nanospray source, a six-port/two-position valve (Thermo, San Jose, CA). The temperature of the ion transfer capillary was set at 200 °C. The spray voltage was set at 1.82 kV, and the normalized collision energy was set at 35.0%. One microscan was set for each MS and MS/MS scan. All MS and MS/MS spectra were acquired in the data-dependent mode. The mass spectrometer was set such that 1 full MS scan was followed by 6 MS/MS scans on the 6 most intense ions. The dynamic exclusion function was set as follows: repeat count 2, repeat duration 30 s, and exclusion duration 90 s. System control and data collection were done by Xcalibur software version 1.4

Online Multidimensional μHPLC−MS/MS Analysis. Tryptic digest of 10 μg of yeast proteins was manually loaded onto this biphasic column using a homemade pneumatic pressure cell at a pressure of 580 psi. After sample loading, the biphasic column was connected into the μHPLC−MS/MS system. Operating pressure of ~900 psi was applied to generate a separation flow rate at about 300 nL/min. A binary RP separation gradient with buffers A and B was developed to separate the peptides retained onto the RP segment, and one separation cycle lasted 120 min (from 0 to 10% buffer B for 2 min, from 10% to 35% for 90 min, and from 35% to 80% for 5 min; after hold at 80% buffer B for 10 min, the whole system was re-equilibrated by buffer A for 18 min). Four stepwise elutions with salt concentrations of 100, 200, 300, and 500 mM NH_4Ac generated by buffers A and C were used to gradually elute peptides from SCX segment onto the RP segment. Each salt elution lasted 10 min, except the last one which lasted 20 min, followed with a 10 min equilibration with buffer A before the RP gradient separation described above was started. The data acquisition of MS was begun immediately after the start of RP gradient separation.

Data Analysis. The acquired MS/MS spectra were searched on the database using the Turbo SEQUEST in the BioWorks 3.2 software suite (Thermo). Reversed sequences were appended to the database for the evaluation of false-positive rate. Cysteine residues were searched as static modification of 57.0215 Da, and methionine residues as variable modification of +15.9949 Da. Peptides were searched using fully tryptic cleavage constraints, and up to two internal cleavages sites were allowed for tryptic digestion. The mass tolerances were 2 Da for parent masses and 1 Da for fragment masses. The peptides were considered as positive identification if the Xcorr values were higher than 1.9 for singly charged peptides, 2.2 for doubly charged peptides, and 3.75 for triply charged peptides. ΔC_n cutoff value was set to control the false-positive rate of peptides identification <1%.

Results and Discussion

To prepare a biphasic column with no void volume between SCX and RP monolithic segments, we initially tried to fill a 75 cm × 100 μm i.d. capillary with 10 cm plug of SCX polymerization mixture and 65 cm plug of RP polymerization mixture in sequence using a syringe followed with simultaneous reaction of both polymerization mixtures under the same temperature. Unfortunately, diffusion would occur in the border section between the two absolutely different polymerization mixtures, and a colloid-type polymer was easily formed between two types of monoliths after reaction, which could not be permeated with solution even at pressure over 6000 psi. Therefore, a two-step procedure as described in Experimental Section has been developed to prepare the biphasic monolithic column. The scanning electron microscopy photographs of the EGMP-based SCX and LMA-based RP monolith segments in the same biphasic monolithic capillary column are shown in Figure 1A,B. Obviously the pore structure in the SCX monolith is much larger than that in the RP monolith, which is controlled intentionally for the two types of monoliths due to their different function in multidimensional separation. In preparation of the LMA-based RP monolith, the ratio of porogens was optimized to generate a morphology structure with relatively small size of pass-through pore for increasing the surface area

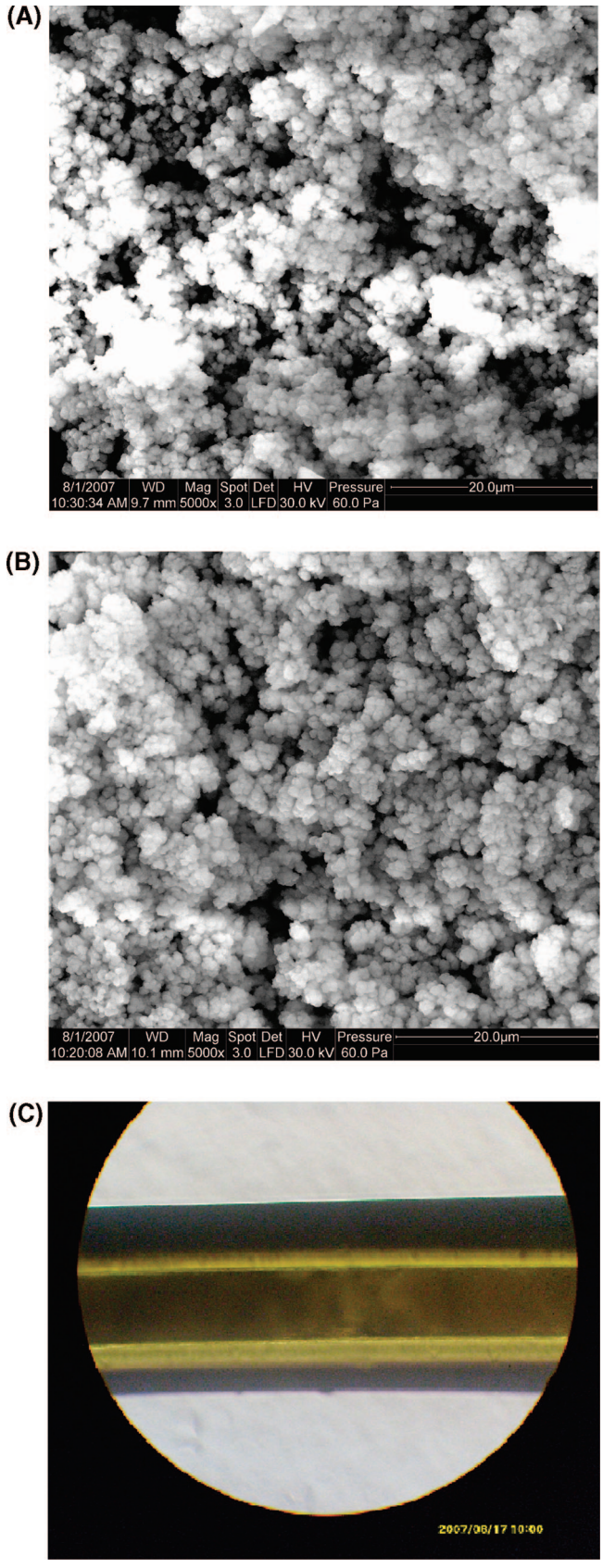

Figure 1. Scanning electron microscopy photographs of the biphasic monolithic column with 100 μm i.d. at magnification of 5000× for the (A) SCX segment and (B) RP segment. (C) The border of the two types of monolith in the biphasic monolithic capillary column.

of monolith and RP separation performance of the monolithic column. And phosphate SCX monolith with much larger size of pass-through pore and higher permeability was prepared because its function was just trapping and stepwise fractionation of the peptides in multidimensional separation, and the SCX monolith with this pore structure was enough to generate high sample dynamic binding capacity.[17] The border between two types of monoliths in the biphasic column is also shown in Figure 1C, and the phosphate SCX monolith is on the left and RP monolith on the right. It can be seen that the border section between two types of monoliths is a little incompact but the difference is not so obvious. The length of the incompact section is about 1 mm, and the corresponding volume of this border section is less than 10 nL. Therefore, the void volume resulted from the incompact monolith section would be much smaller than that in the connection of two types of monolithic columns even with a ZDV union.

To demonstrate the separation performance of the biphasic monolithic column, tryptic digest of 10 μg of yeast proteins was analyzed on the prepared column by online multidimensional μHPLC−MS/MS, and the obtained base peak chromatograms were shown in Figure 2. It takes only about 12 h for such a five-cycle online multidimensional separation by including 460 min for binary gradient separation, which was similar to one-dimensional separation on ultralong capillary columns in some cases.[21,22] The acquired MS/MS spectra were searched on the database with filtering criteria described previously.[17,20] Finally, 780 distinct proteins were positively identified from 2953 unique peptides (total 15 512 peptides) with false-positive rate at 0.85% by setting ΔC_n at 0.35 (Xcorr: 1.9, 2.2, and 3.75 for singly, doubly, and triply charged peptides). When conventional criteria by setting ΔC_n at 0.1 with the same Xcorr values were adopted,[9−11,19] then 1449 proteins were positively identified with 4380 unique peptides (total 20 849 peptides). However, the false-positive rate was 10.29% under these filtering criteria. These results were inferior to what we have reported by identification of 2989 proteins from assignment of 10 504 unique peptides (total 89 137 peptides) with false-positive rate at 10.52%, which was obtained under 17 cycles of multidimensional separation by taking about 40 h analysis.[17] Thus, the good separation performance of this biphasic monolithic column can still be concluded by considering the four salt elution cycles and short time applied for RP gradient separation in this case (five cycles, 12 h). Recently, Yates et al.[12] reported the identification of 742 yeast proteins with two or more unique peptides by setting ΔC_n to control the false-positive rate at 5%. According to their criteria, 579 yeast proteins were positively identified in our case, though this number of proteins identified in our case is less than that obtained by Yates et al. even under the same gradient volume of 1.4 (gradient length/RP column length, 92/65). However, their results were obtained with total analysis time of 27.5 h and operating pressure of ∼15 kpsi, and our results were obtained with analysis time of 12 h and operating pressure of ∼900 psi applied. Therefore, it can be expected that the comparable results should be obtained with this biphasic monolithic column by prolonging separation time or taking more number of salt elution cycles. The good separation performance of this biphasic monolithic column might be largely contributed from the good orthogonality of the SCX and RP monoliths in column. Loaded peptides are first trapped onto the SCX segment of the phosphate SCX monolith by electrostatic interaction. Then, trapped peptides are stepwisely eluted with salt buffer from SCX monolithic segment to

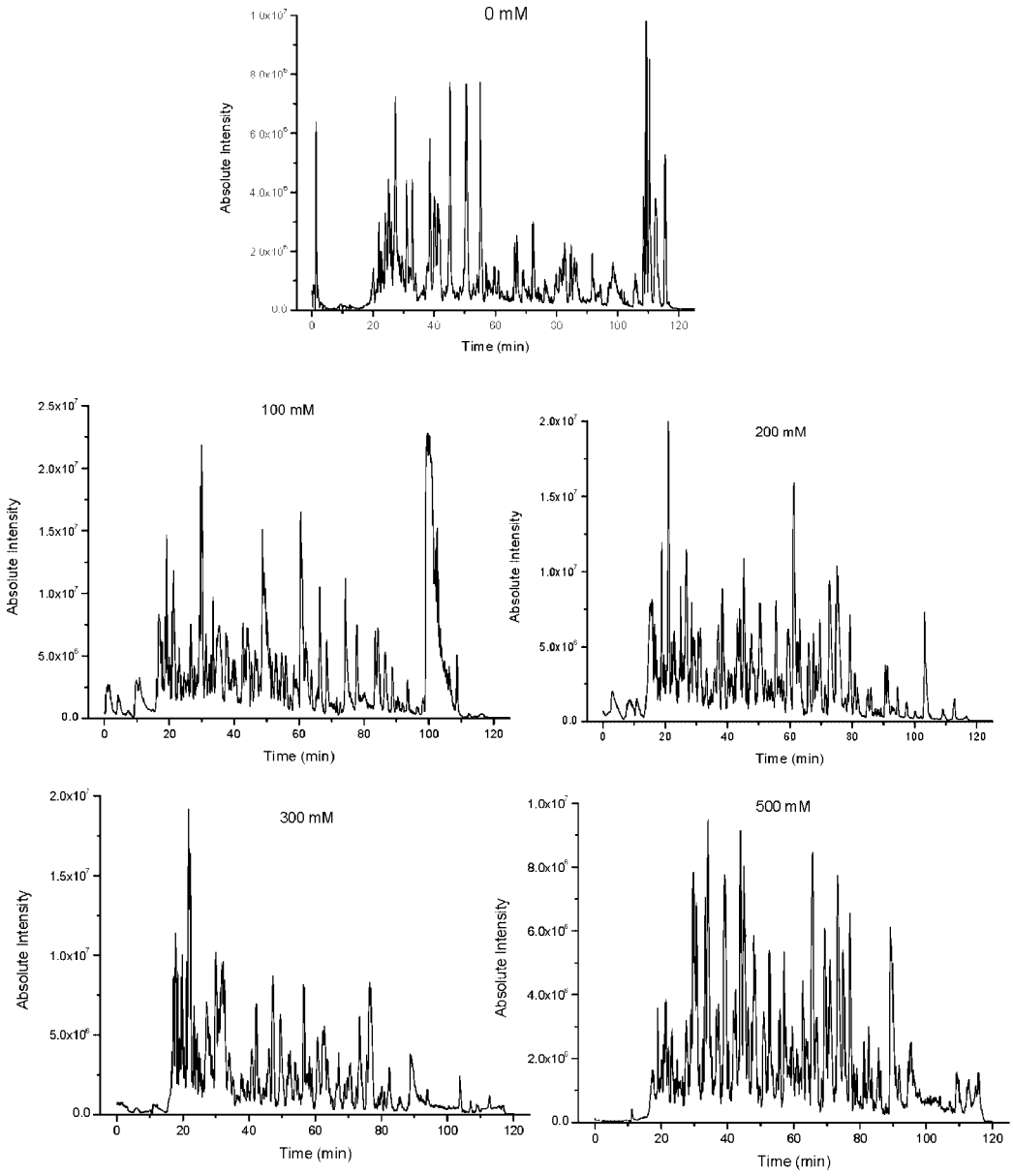

Figure 2. Base peak chromatograms of the five-cycle online multidimensional separation of tryptic digest of 10 μg of yeast proteins. Cycle 1 consisted of a 2-min gradient from 0 to 10% buffer B, a 90-min gradient from 10 to 35% buffer B, and a 5-min gradient from 35 to 80% buffer B; after a 10-min hold at 80% buffer B, the system was equilibrated with 100% buffer A for 18 min. Each of the next 3 cycles was 145 min with the following procedures: 10 min of *X*% buffer C, 10 min of 100% buffer A, then the separation gradient was just the same as cycle 1. The 10-min buffer C in cycles 2–4 was as follows: cycle 2, 20%; cycle 3, 40%; cycle 4, 60%. Cycle 5 consisted of a 20-min 100% buffer C wash followed by a 10-min 100% buffer A wash, and the separation gradient was also the same as cycle 1.

RP monolithic segment, and concentrated onto the front of the latter one, followed with RP gradient separation. Figure 3 shows that the total numbers of the newly identified proteins and unique peptides increase along with the separation cycles during this online multidimensional separation, which also indicate the good orthogonality of the SCX and RP monoliths for online multidimensional separation.

This biphasic monolithic column can be easily prepared and applied in online multidimensional separation conveniently. The two types of monoliths synthesized in the capillary column have both been demonstrated to be stable enough in the separation buffers for longtime usage.[17,18] Comparing to the conventional MudPIT type of biphasic-packed column, this biphasic monolithic column has the following advantages. First, the prepared biphasic column has much lower operating backpressure than the particles-packed column and can be applied in common HPLC systems without any difficulties. In this study, though a 75 cm long biphasic column was used, the operating pressure was only ~900 psi, which makes the operation of the whole system very easy and robust. Second, as relatively long SCX and RP segments of this biphasic column can be adopted, the sample loading capacity and separation peak capacity can be improved correspondingly.[23] Third, as the biphasic column can easily be prepared, it is possible to prepare this type of biphasic column in capillary with much smaller internal diameter (such as 10 μm), which will increase the sensitivity of the whole μHPLC−MS/MS system greatly as the MS is a concentration-dependent detector.[24]

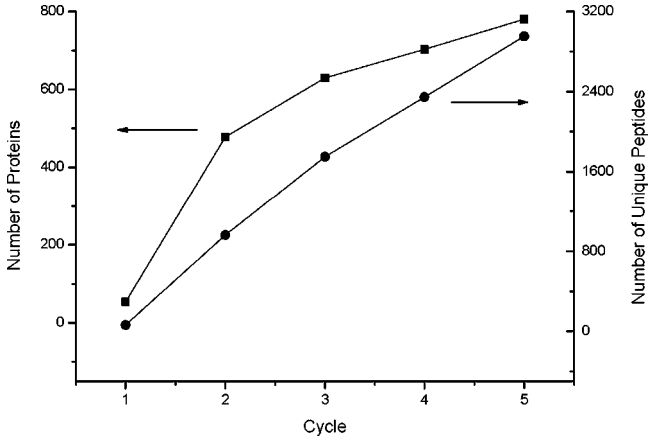

Figure 3. Total number of the newly identified proteins (left) and unique peptides (right) along with the separation cycles during online multidimensional separation.

Conclusion

In this study, two types of monoliths were synthesized in a single capillary column with 75 cm × 100 μm i.d. in sequence. As the two types of monoliths inside the capillary had high permeability, the 75 cm length of biphasic monolithic column was operated with conventional HPLC instrument under relatively low pressure of ~900 psi. The separation performance of this new column was demonstrated by online multidimensional μHPLC−MS/MS analysis of tryptic digest of yeast proteins within a total analysis time of 12 h and five separation cycles. As a result, totally, 780 distinct proteins were positively identified through assignment of 2953 unique peptides at false-positive rate less than 1%. The development of this novel monolithic column might have some potential for application in shotgun proteome analysis.

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