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Capillary Trap Column with Strong Cation-Exchange Monolith for Automated Shotgun Proteome Analysis

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A 150 μm internal diameter capillary monolithic column with a strong cation-exchange stationary phase was prepared by direct in situ polymerization of ethylene glycol methacrylate phosphate and bisacrylamide in a trinary porogenic solvent consisting dimethylsulfoxide, dodecanol, and *N*,*N*′-dimethylformamide. This phosphate monolithic column exhibits higher dynamic binding capacity, faster kinetic adsorption of peptides, and more than 10 times higher permeability than the column packed with commercially available strong cation-exchange particles. It was applied as a trap column in a nanoflow liquid chromatography−tandem mass spectrometry system for automated sample injection and online multidimensional separation. It was observed that the sample could be loaded at a flow rate as high as 40 μL/min with a back pressure of ∼1300 psi and without compromising the separation efficiency. Because of its good orthogonality to the reversed phase separation mechanism, the phosphate monolithic trap column was coupled with a reversed-phase column for online multidimensional separation of 19 μg of the tryptic digest of yeast proteins. A total of 1522 distinct proteins were identified from 5608 unique peptides (total of 54 780 peptides) at the false positive rate only 0.46%.

Current proteome analyses always deal with complicated samples due to the big protein dynamic expression range as well as the large diversities of proteins.[1−3] For large-scale protein identification, powerful separation strategies coupled to mass spectrometry are usually applied. Nanoflow LC−MS/MS is the dominant platform in shotgun proteome analysis due to its high sensitivity.[4] There are usually two approaches to improve the separation efficiency in nanoflow LC. One is to improve the one-dimensional separation by using longer columns and/or smaller packing materials. Such systems are typically operated at ultrahigh pressure and can only be implemented using advanced HPLC instrumentation.[5−8] The other one is multidimensional separations,[9] which are typically achieved by off-line[10−12] or on-line[13−17] coupling of the two columns with orthogonal retention mechanisms. It is reported that by combining two orthogonal dimensions, the separation peak capacity could improve from ∼10^2 to ∼10^3.[18,19] Strong cation-exchange (SCX) chromatography is the most widely used for peptide or protein prefractionation among all the modes due to its good orthogonality to reversed phase (RP) chromatography.

The most prominent strategy for online multidimensional separation is often referred to as multidimensional protein identification technology (MudPIT), which is typically achieved by a biphasic capillary column packed with SCX and RP materials in sequence.[20−22] Peptides binding initially onto the SCX materials are eluted stepwise to the RP segment by a flushing salt step, where each fraction is further separated on the RP segment of

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the column and detected by MS. This online 2D-LC is fully automated and displays advantages such as minimal loss of sample, no vial contamination, and no sample dilution. However, the sample loading capacity is limited because of the small amount of SCX packing materials packed in the same column with C18 materials. In order to load enough of the analytes for a multidimensional separation, overloading could easily occur. In addition, since the biphasic capillary column with an internal diameter of 100 μm is applied in nanoflow LC (μHPLC) to increase the detection sensitivity of MS, it is time-consuming and labor intensive to manually load samples with a volume varying from several to hundreds of microliters onto the capillary column.

A monolithic column is a good alternative to columns packed with particles as they have extremely low back pressure in liquid chromatography.[23−26] Polymer-based monolithic columns with SCX stationary phases are usually used due to their pH stability, inertness to biomolecules, absence of deleterious effects from silanol, and facility for modification.[23,24] In the preparation of a SCX capillary column, adsorption of surfactants,[28,29] grafting of the pore surface,[30,31] modification of the reactive monoliths,[27] and copolymerization of the cross-linker and the monomer containing the functional group[32−35] are most widely used to introduce active groups (sulfonate groups) into the monolith backbone.[24] Among them, copolymerization of the cross-linker and the functional monomer is the most straightforward strategy and the dynamic binding capacity of these SCX columns can be controlled by adjusting the amount of functional monomer in the polymerizing mixture.[35]

We have developed a method for the preparation of a phosphate monolithic capillary column by direct copolymerization of an ethylene glycol methacrylate phosphate (EGMP) and bisacrylamide in a trinary porogenic solvent consisting of dimethylsulfoxide, dodecanol, and *N*,*N*′-dimethylformamide.[36] It was demonstrated that this phosphate monolithic column was able to specifically isolate phosphopeptides after Zr^{4+} was loaded onto the column. In this study, the phosphate monolithic capillary column was prepared in a 150 μm i.d. capillary column and applied as the trap column in an automated shotgun proteome analysis. When this phosphate monolithic capillary column (150 μm i.d., 7 cm) is coupled to a C18 packed capillary column (75 μm i.d., 12 cm) with an integrated ESI tip, this system exhibited good separation performance as well as high proteomic coverage in both one- and multidimensional μHPLC−MS/MS analysis. Because of the low back pressure of the phosphate monolithic column, automatic sample injection at a high flow rate of 40 μL/min for μHPLC−MS/MS analysis could be easily realized. It was demonstrated that this type of phosphate monolithic column provided a reliable alternative to the particulate SCX columns for automated shotgun proteome analysis.

EXPERIMENTAL SECTION

Materials. Daisogel ODS-AQ (5 μm, 12 nm pore) was purchased from DAISO Chemical Co., Ltd. (Osaka, Japan). Magic C18AQ (5 μm, 20 nm pore) was purchased from Michrom BioResources (Auburn, CA), and polysulfoethyl aspartamide (5 μm, 20 nm pore) was a gift from PolyLC Inc. (Columbia, MD). PEEK tubing, sleeves, microtee, microcross, and mini-microfilter assembly (with a filter capsule, porosity 2 μm) were obtained from Upchurch Scientific (Oak Harbor, WA). Fused silica capillaries with 75 and 150 μm i.d. were purchased from Polymicro Technologies (Phoenix, AZ), and 50 μm i.d. fused silica capillaries were purchased from Yongnian Optical Fiber Factory (Hebei, China). All the water used in experiments was purified using a Mill-Q system from Millipore Company (Bedford, MA). Dithiothreitol (DTT) and iodoacetamide were both purchased from Sino-American Biotechnology Corporation (Beijing, China). Ethylene glycol methacrylate phosphate (EGMP), bisacrylamide, dimethylsulfoxide, dodecanol, *N*,*N*′-dimethylformamide, and γ-methacryloxypropyltrimethoxysilane (γ-MAPS) were obtained from Sigma (St. Louis, MO). Azobisisobutyronitrile (AIBN) was obtained from Shanghai Fourth Reagent Plant (Shanghai, China). Trypsin was obtained from Promega (Madison, WI). Synthetic peptide was purchased from Serva (Heidelberg, Germany), and Tris was from Amersco (Solon, Ohio). Formic acid was obtained from Fluka (Buches, Germany). Acetonitrile (ACN, HPLC grade) was from Merck (Darmstadt, Germany).

Sample Preparation. The yeast protein extract was prepared in a denaturing buffer containing 50 mM Tris/HCl (pH 8.1) and 8 M urea according to the procedures previously reported.[13] 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 the 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 a 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 Particle Packed Columns. Particulate columns were packed using a homemade pneumatic pressure cell at a constant nitrogen gas pressure of about 580 psi with a slurry packing method.[13,38] For the preparation of the analytical column, one end of a 75 μm i.d. fused silica capillary was first manually pulled to a fine point of ~5 μm with a flame torch, and then the

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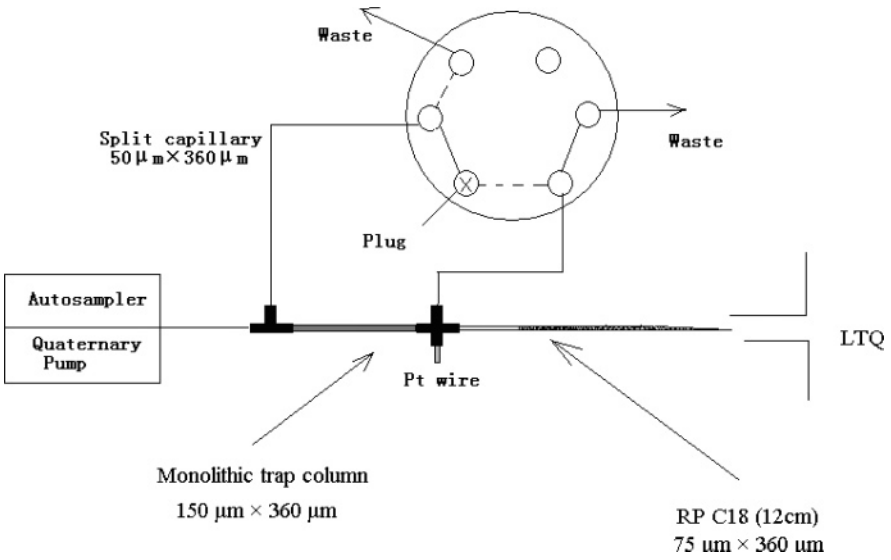

Figure 1. Schematic diagram of the automated sample injection system. Solid line represents loading the sample onto the trap column, and the dashed line represents the sample eluting to the analytical column and gradient RPLC analysis.

C18 particles (5 μm, 12 nm pore) were packed until the packing section reached a length of 12 cm. After a mini-microfilter assembly (with a filter capsule, porosity 2 μm) was placed on one end of a 150 μm i.d. capillary, a 1 or 2 cm length of SCX or C18 resin (5 μm, 20 nm pore) was packed into the column to prepare the particulate SCX or C18 column. After the column was packed with the appropriate length, it was connected to a LC pump and equilibrated with a 0.1% formic acid water solution at ~1500 psi for at least 30 min before usage. In our following experiments, the 2 cm long particulate SCX column was used to measure the dynamic binding capacity of PolySulfoethyl A and the 1 cm long particulate SCX and C18 trap columns were used in permeability measurement.

Preparation of the Phosphate Monolithic Capillary Column. Prior to the polymerization, the capillary was pretreated with γ-MAPS as described elsewhere.[28,34] The phosphate monolithic capillary column was prepared with the following procedures.[36] Briefly, the reaction mixture consisting of ethylene glycol methacrylate phosphate (80 μL, ~100 mg), bisacrylamide (60 mg), dimethylsulfoxide (270 μL), dodecanol (200 μL), *N*,*N*′-dimethylformamide (50 μL), and AIBN (2 mg) was sonicated for 20 min to obtain a homogeneous solution and then purged with nitrogen for 10 min. After the pretreated capillary was completely filled with the mixture, it was sealed at both ends with rubber stoppers. The sealed capillary was submerged into a water bath and allowed to react for 12 h at 60 °C. The resulting monolithic capillary column was washed with methanol for 2 h using an HPLC pump to remove unreacted monomers and porogens. Then this phosphate monolithic capillary column could be directly used as the strong cation exchange trap column for proteome analysis without any other pretreatments.

Scanning electron microscopy (SEM) images of the monolithic column were obtained using a JEOL JSM-5600 scanning electron microscope (JEOL Company, Japan).

Binding Capacity Measurement of the Phosphate Monolithic Capillary Column. For measurement of the dynamic binding capacity, a synthetic peptide Leu−Trp−Met−Arg−Phe−NH_2·HCOOH (MW: 798.42) was used to saturate the phosphate monolithic column (150 μm i.d., 7 cm) by frontal analysis. Briefly, 10 mg of synthetic peptide was dissolved into 20 mL of buffer containing 0.1% formic acid and 10% acetonitrile (pH ~2−3), which was pumped through the phosphate monolith column at a constant flow rate of 20 μL/min by a LC-10ADvp pump (Shimadzu, Kyoto, Japan). The solution from the monolithic column flowed into a 500 nL 1100-DAD micro cell UV detector (Agilent, Hewlett-Packard, Waldbronn, Germany). A 50 mM benzonic acid water solution containing 0.1% formic acid and 10% acetonitrile was used to estimate the void time of this system at the same flow rate because benzonic acid with considerable UV absorption is neutral in acidic solution.

To provide comparable data, the dynamic binding capacity of a particulate SCX column with a 150 μm i.d. × 2 cm was also measured following the same procedures.

Automated Sample Injection. The configuration for automated sample injection using the phosphate monolithic trap column was shown in Figure 1. During sample injection, the switching valve was switched to close the splitting flow and the flow through from the trap column was switched to waste (solid line mode in Figure 1). We adjusted the time of sample injection at different flow rates to ensure the volume that flows though the trap column was three sample volumes for system equilibrium and removing contaminants. The sample solutions were loaded at flow rates of 2, 6, 10, 15, 20, 30, and 40 μL/min by setting loading times at 30, 10, 6, 4, 3, 2, and 1.5 min, respectively, when 20 μL sample was injected. After sample loading, the valve was switched to the dashed line mode and the gradient separation was automatically started.

One-Dimensional Separation. The three buffer solutions used for the quaternary pump were a 0.1% formic acid water solution (buffer A), ACN with 0.1% formic acid (buffer B), and 1000 mM NH_4Ac at pH 3 (buffer C). The flow rate after splitting was adjusted to optimize the separation at ~180 nL/min. The binary gradient with buffer A and buffer B for reversed phase separations was developed 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 flushing 80% buffer B for 10 min, the separation system was equilibrated

by buffer A for 13 min. In the one-dimensional separation, the peptides retained on the trap column were all eluted onto the analytical column by flushing with buffer C (containing 1000 mM NH_4Ac at pH 3) for 10 min. Then, after the system was re-equilibrated with buffer A for 10 min, the binary separation gradient described above was started.

Multidimensional Separation. An amount of 19 μg of the tryptic digest of yeast protein was loaded automatically onto the phosphate monolithic trap column. Then a series stepwise elution with salt concentrations of 25, 50, 75, 100 , 125, 150, 175, 200, 225, 250, 300, 350, 400, 450, 500, and 1000 mM NH_4Ac was used to gradually elute peptides from the phosphate monolithic column onto the C18 analytical column. Each salt step lasts 5 min except for the last one which lasts for 10 min. After the whole system was re-equilibrated for 10 min with buffer A, the binary gradient elution described in the section One-Dimensional Separation was applied to separate peptides prior to MS detection in each cycle.

Mass Spectrometric Analysis. 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 that one full MS scan was followed by six MS/MS scans on the six 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 (Thermo).

Data Analysis. The acquired MS/MS spectra were searched on the database using Turbo SEQUEST in the BioWorks 3.2 software suite (Thermo). The yeast database was downloaded from a website (ftp://genome-ftp.stanford.edu/yeast/data_download/sequence/genomic_sequence/orf_protein/orf_trans.fasta.gz). Reversed sequences were appended to the database for the evaluation of the false positive rate. Cysteine residues were searched as a static modification of 57.0215 Da, and methionine residues as a 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 were higher than 1.9 for a singly charged peptide, 2.2 for a doubly charged peptide, and 3.75 for a triply charged peptides. The ΔC_n cutoff value was set to control the false positive rate of peptide identification <1%, determined by the calculation based on the reversed database.[12]

RESULTS AND DISCUSSION

Characterization of the Phosphate Monolithic Trap Column. Sulfonate groups are usually introduced in the organic polymer monolithic column for cation exchange chromatography. However, increasing the active binding sites as well as increasing the stability of the SCX monolith is still a challenge.[35] As the sulfonate-containing monolith is believed to swell excessively in aqueous buffer,[27,32,33] we prepared a phosphate monolithic capillary column in this study. This type of phosphate monolith is relatively hydrophilic and biocompatible due to the use of the cross-linker bisacrylamide.[36] In order to increase the sample loading capability as well as the sample loading rate, we synthesized the phosphate monolith in a confined 150 μm i.d. capillary, and the obtained monolith was characterized by a scanning electron micrograph as shown in Figure 2. It can be seen that the monolithic bed linked to the pretreated capillary wall and the macropores are feasibly formed by the trinary porogenic solvent.

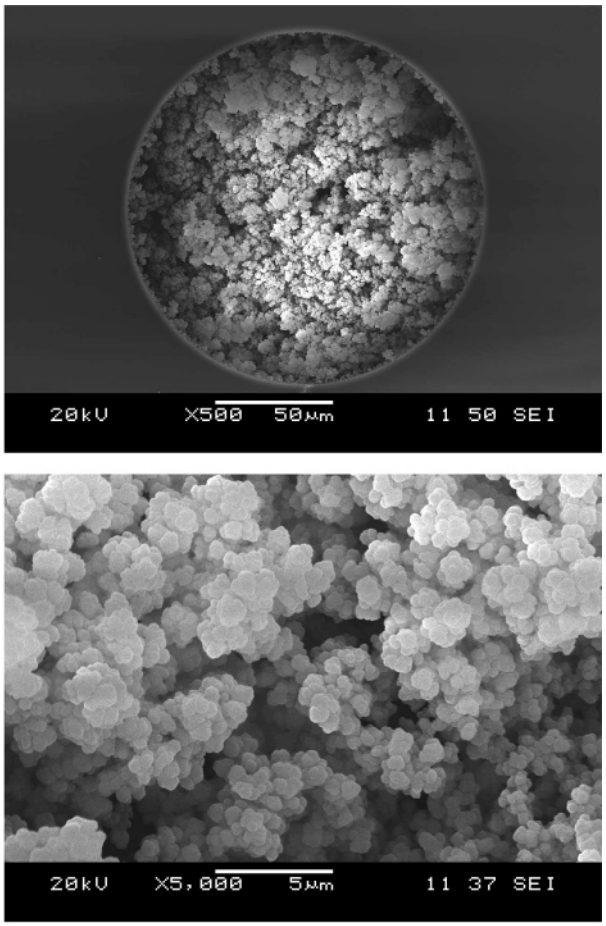

Figure 2. Scanning electron microscopy photographs of phosphate monolith inside a capillary with 150 μm i.d. at magnification of (a) 500× and (b) 5000×.

An ideal monolithic column used in proteome analysis should be stable enough in different separation buffers and not exhibit extra swelling or shrinking, which always influences the permeability of the monolith. Therefore, we measured the permeability of the phosphate monolithic column as well as the particulate SCX and C18 columns under the solutions of ACN and water, and the obtained results are shown in Table 1. It is obvious that the permeability of the phosphate monolithic column is 15 times higher than that of particle packed columns with acetonitrile containing 0.1% formic acid and about 10 times higher with 0.1% formic acid in water. It is reported that the sulfonate monolith swells in more polar solvents and shrinks in less polar solvents;[35] similar phenomena were also observed in our experiments. However, the permeability decreased only 44% when the flushing solution was changed from acetonitrile to water for the phosphate monolithic column, and a relatively low back pressure of ~1300 psi was observed under the high flow rate of water at 40 μL/min. Though slight swelling in aqueous buffer and shrinking in organic buffer will occur, no detachment of the monolith from the capillary wall was observed at any conditions

Table 1. Permeability of Different Trap Columns

column (150 μm i.d.)	flushing solution	viscosity η (cP)[a]	back pressure ΔP (psi)	flow rate F(μL/min)	permeability k ($\times 10^{-14}$ m^2)[b]
phosphate monolithic (7 cm)	acetonitrile	0.369	594.5	80	47.5
	water	0.890	1290.5	40	26.4
particulate SCX (1 cm)[c]	acetonitrile	0.369	1483.4	80	2.7
	water	0.890	2128.6	40	2.3
particulate C18 (1 cm)[c]	acetonitrile	0.369	1348.5	80	3.0
	water	0.890	1679.1	40	2.9

[a] Viscosity data were from reference 35. [b] Permeability was calculated by Darcy's Law,[41] $k = \eta LF/(\pi r^2 \Delta P)$, where η is the viscosity, L is the column length, F is the solvent flow rate, r is the radius of the trap column, and ΔP is the column back pressure. [c] The packed length of the particulate trap columns was kept at 1 cm due to the pressure limit at high flow rate.

applied in our experiments. The phosphate monolith was stable enough for continuous usage of ~3−4 weeks with a constant back pressure.

Binding capacity is one of the most important properties of an ion-exchange column, which determines the resolution, column loadability, and gradient elution strength. A synthetic peptide Leu−Trp−Met−Arg−Phe−NH_2·HCOOH (MW: 798.42), which bears a +2 charge at pH ~2−3, was used to determine the dynamic binding capacity of the phosphate monolithic column (150 μm i.d., 7 cm) as described above. With the use of 0.5 mg/mL concentration of peptides, a sharp increase in the baseline could be observed when the column was saturated. After the phosphate monolithic column was saturated with the peptides, it was flushed with 120 μL of 1000 mM NH_4AC buffer by a syringe manually. Then, it was reconnected into the system, and the capacity was measured again. In our experiment, the binding capacity was measured 3 times with the same column. The void time, estimated by flushing 50 mM benzonic acid in the same column, was subtracted from the total time consumed for saturating the phosphate monolithic column. And finally, the average time for saturation was 17.29 min in three measurements (RSD, 1.6%). As the flow rate was 20 μL/min, the dynamic binding capacity of the phosphate monolithic column was 140 mg/mL, corresponding to 175 μequiv/mL. Binding capacity of the particulate SCX column with 150 μm i.d. × 2 cm was also measured following the same procedures. The average time for saturating the particulate SCX column was 4.41 min in three time measurements (RSD, 13.6%), and the dynamic binding capacity was 125 mg/mL, corresponding to 156 μequiv/mL. However, the packed length of the particulate column was not further increased due to the limitation of the system pressure.

It can be seen that the phosphate monolith in unit volume has a larger dynamic binding capacity than the PolySulfoethyl A. Figure 3 shows the frontal analysis curves of the synthetic peptide on the phosphate monolithic column and the column packed with PolySulfoethyl A particles. It is obvious that the frontal analysis curve of the phosphate monolithic column increases more sharply, which indicates faster kinetic adsorption of the peptide on the phosphate monolith than on the PolySulfoethyl A particles. Furthermore, the phosphate monolith exhibits the SCX mechanism because only one plateau was observed in the frontal analysis curve.

Automated Sample Injection in μHPLC−MS/MS Analysis. As the nanoliter flow rate is optimum for separation in μHPLC−MS/MS, it is time-consuming to manually load sample with

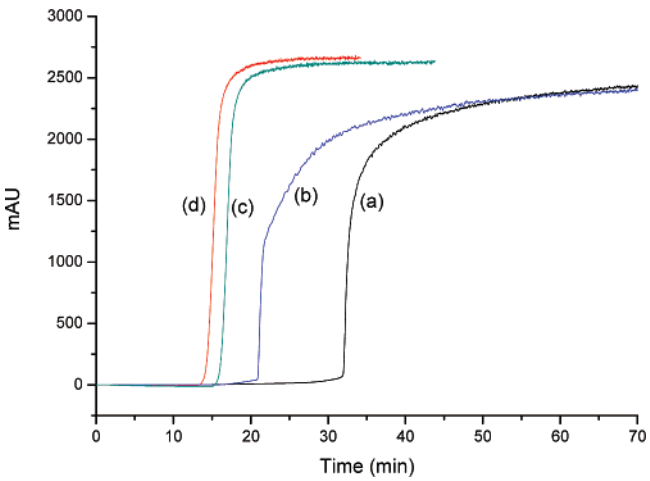

Figure 3. Dynamic adsorption curves of frontal analysis with (a) 7 cm long phosphate monolithic column and (b) 2 cm long packed PolySulfoethyl A column. The void time was estimated by flushing 50 mM benzonic acid for (c) packed SCX column and (d) phosphate monolithic column. The UV absorption wavelength was set at 280 nm.

volumes commonly varying from several to hundreds of microliters onto a capillary column under the optimum flow rate for separation.[4] To overcome these limitations, automated and rapid sample injection for μHPLC−MS/MS is required, which is usually realized by different instrument configurations with using a short and larger internal diameter trap column coupled to an analytical column. Peptide mixtures of big volumes are first loaded onto the trap column at a high flow rate in a short time, and the adsorbed peptides are eluted from the trap column to a reversed phase analytical column after equilibrium. When the C18 trap column is used for rapid sample injection, the void volume between the trap and analytical columns inevitably leads to degradation of separation performance. Many efforts have been made to decrease the void volume.[38−40] We found that automation of the sample injection using the SCX trap column can alleviate the influence of the void volume on separation performance and proteomic coverage in μHPLC−MS/MS, and it is a good choice for construction of a void volume insensitive automated sample injection system.[13,37]

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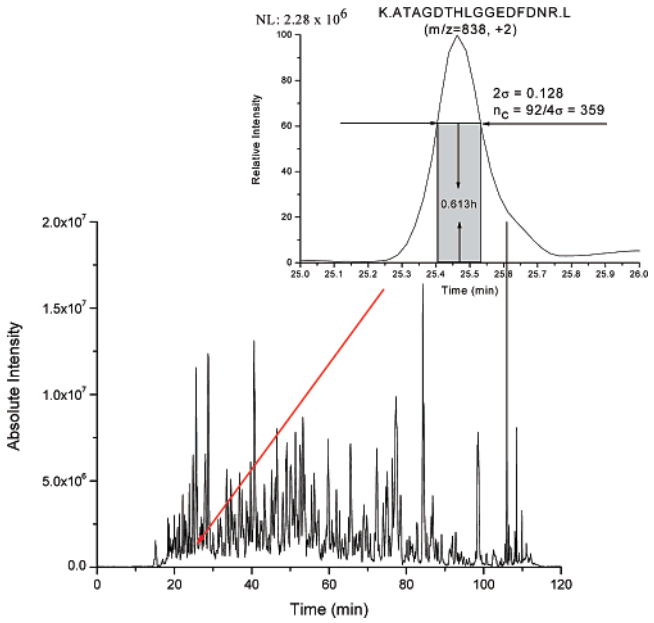

Figure 4. Base peak chromatograms for μHPLC−MS/MS analysis of 0.95 μg of SPE cleaned tryptic digest of yeast proteins on the automated sample injection system using phosphate monolithic trap column. Peptides were separated using a 2 min gradient from 0 to 10% buffer B, a 90 min gradient from 10 to 35% 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 13 min. The peak capacity of this system was evaluated for the separation window, which was about equal to the effective separation time of 92 min.

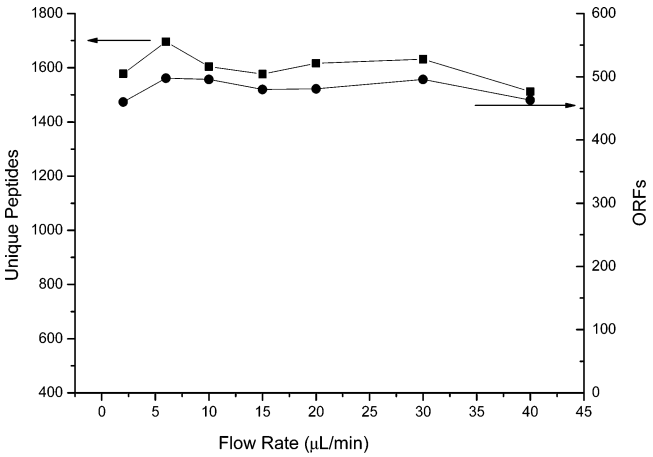

Figure 5. Effect of sample loading flow rate on a number of unique peptides and distinct proteins identified with the automated sample injection system using the phosphate monolithic trap column. The binary separation gradient was the same as in Figure 4.

Table 2. Run-to-Run Reproducibility of Six Consecutive μHPLC−MS/MS Analyses for 0.95 μg of the Tryptic Digest of Yeast Protein by Using the Phosphate Monolithic Trap Column for the Automated Sample Injection[a]

	retention time (min)							RSD
mass	run 1	run 2	run 3	run 4	run 5	run 6	av	(%)
1017.57	17.84	18.40	18.28	18.41	18.53	18.60	18.34	1.4
883.52	29.20	29.72	29.38	29.22	29.59	29.46	29.43	0.6
1416.72	35.75	36.29	36.38	36.10	36.42	36.44	36.23	0.7
925.61	43.06	43.70	43.85	43.54	43.87	44.01	43.67	0.7
1752.79	48.32	49.00	49.01	48.71	49.26	49.18	48.91	0.6
1351.75	56.22	56.92	56.70	56.67	56.88	56.99	56.73	0.4
2259.23	68.55	69.19	69.17	68.92	69.00	69.02	68.97	0.3
2388.20	76.51	76.57	76.69	76.30	76.66	76.66	76.56	0.2
2831.45	83.39	83.56	83.65	83.59	83.76	83.70	83.61	0.1
1821.92	88.40	89.35	89.08	89.13	89.36	89.32	89.11	0.4

[a] Peptides are randomly selected across the elution, and separation conditions are the same as for Figure 4.

The phosphate monolithic column exhibits the pure SCX mechanism, considerable dynamic binding capacity, and 10 times higher permeability than the particulate columns (shown in Table 1). Therefore, it should be a good choice for the trap column to further speed up the flow rate of the sample injection. A 7 cm long phosphate monolithic column was used as the trap column in μHPLC−MS/MS analysis. At first, 0.95 μg of the tryptic digest of yeast proteins was automatically loaded onto the phosphate monolithic trap columns at a flow rate of 2 μL/min followed by μHPLC−MS/MS analysis. The obtained base peak chromatograms are shown in Figure 4. By examination of the elution profile of a moderately intense peptide of KATAGDTHLGGEDFDNRL (m/z = 838, +2 charge state) extracted from the separation chromatograms, a peak capacity of 359 could be calculated with the ~92 min separation window.[26] After a database search with the spectra filtered with the above criteria, a total of 1578 peptides were positively assigned for identification of 460 distinct proteins from the yeast proteome.

Furthermore, we systematically investigated the influence of the sample loading rate on the separation performance and proteomic coverage. Figure 5 shows the unique peptides identified by this automated sample injection system using the phosphate monolithic trap columns at the sample loading rate varied from 2 to 40 μL/min. It is obvious that the sample injection rate has little influence on the number of identified peptides. Correspondingly, the number of identified proteins is also insensitive to the sample loading rate as shown in Figure 5.

The reproducibility of the automated sample injection system using the phosphate monolithic trap column was also investigated by six consecutive analyses of 0.95 μg of the tryptic digest of yeast proteins. The retention times of 10 peptides were determined, and their relative standard deviations (RSD) were calculated. As shown in Table 2, the RSD for most peptides was less than 1.0%, which indicated reproducible separation could be obtained by this automated sample injection system. The average numbers of unique peptides and distinct proteins identified in six consecutive runs were 1556 (RSD 2.0%) and 452 (RSD 0.7%), respectively. This also demonstrated that consistent results for proteome analysis could be obtained by using the phosphate monolithic trap column.

Therefore, a relatively long phosphate monolithic column with a high sample loading capacity can be successfully applied as the trap column in an automated sample injection to obtain satisfactory proteomic coverage, and a high sample loading rate of 40 μL/min can be easily adopted at a back pressure of ~1300 psi (shown in Table 1). With the increase of the sample loading rate from 2 to 40 μL/min, the sample loading time decreased from 30 to 1.5 min as described. We can prepare the phosphate monolithic capillary column at a length of several meters at one time, and an appropriate length is cut during usage for appropriate sample loading capacity. After 3−4 weeks of

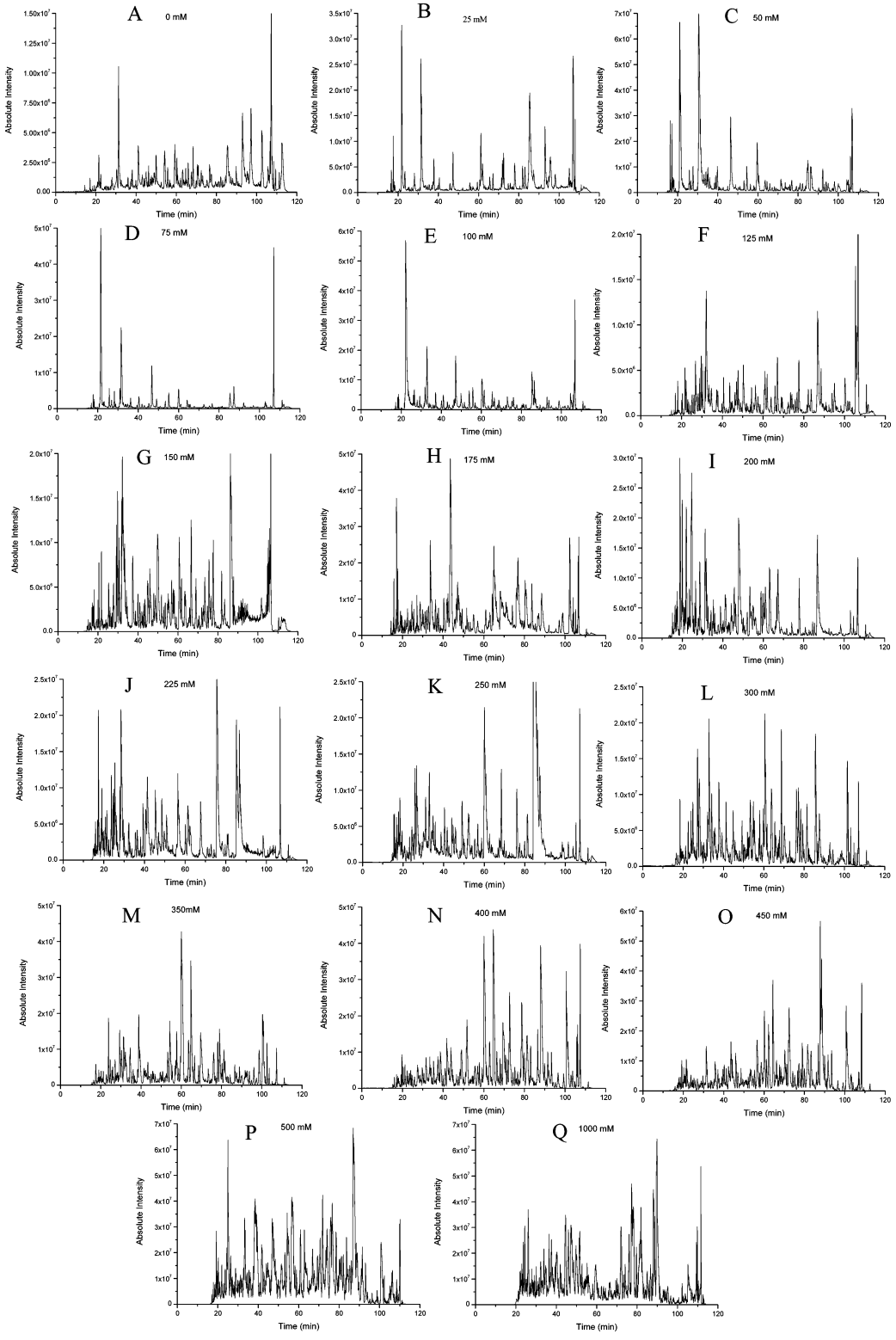

Figure 6. Chromatograms of a 17 cycle online multidimensional analysis of the tryptic digests of 19 μg of yeast proteins. The three buffer solutions used for the chromatography were 0.1% formic acid aqueous solution (buffer A), 0.1% formic acid−acetonitrile solution (buffer B), and 1000 mM ammonium acetate at pH 3 (buffer C). Cycle 1 (A) 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 13 min. Each of the next 15 cycles was 135 min with the follow procedures: 5 min of *x*% buffer C, 10 min of 100% buffer A, then the separation gradient was just the same as in cycle 1. The 5 min buffer C wash in cycles 2−16 was as follows: (B) cycle 2, 2.5%; (C) cycle 3, 5%; (D) cycle 4, 7.5%; (E) cycle 5, 10%; (F) cycle 6, 12.5%; (G) cycle 7, 15%; (H) cycle 8, 17.5%; (I) cycle 9, 20%; (J) cycle 10, 22.5%; (K) cycle 11, 25%; (L) cycle 12, 30%; (M) cycle 13, 35%; (N) cycle 14, 40%; (O) cycle 15, 45%; (P) cycle 16, 50%. Cycle 17 (Q) consisted of a 10 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.

continuous use, the phosphate monolithic trap column would become flimsy and could be replaced conveniently.

Online Multidimensional Separation. As the phosphate monolith column exhibits good orthogonality to the reversed phase column and its dynamic binding capacity is higher than that of column packed with PolySulfoethyl A, it can also be applied in a multidimensional separation. Therefore, 19 μg of the tryptic digest of yeast proteins was automatically injected onto the 7 cm long phosphate monolithic trap column, and 17 cycles of the salt stepwise elution of peptides from the phosphate monolithic column to the analytical column followed by μHPLC−MS/MS analysis were automatically conducted. The obtained chromatograms are shown in Figure 6. After a database search by filtering the spectra with criteria as described, and setting the ΔC_n value at 0.39 to ensure the false positive rate of peptide identifications in each fractional analysis was less than 1%, 1522 distinct proteins were positively identified from 5608 unique peptides (total of 54 780 peptides) with a total false positive rate at 0.46% (Xcorr: 1.9, 2.2, 3.75 for singly, doubly, triply charged peptides). These results are quite reliable as conservative criteria were used. If we used conventional criteria by setting the ΔC_n at 0.1 and not controlling the false positive rate,[13,20−22,26] 2989 proteins were identified with 10 504 unique peptides (total of 89 137 peptides). However, the false positive rate was 10.52% under these filtering criteria.

Though SCX monolithic columns are widely used in chromatographic separations, the application in analysis of complex proteome samples is seldom reported, which mainly results from three reasons. First, the dynamic binding sites of these columns are limited,[35] in contrast to the considerable amount of sample (vary from hundreds of nanograms to grams) that must be loaded for large scale proteome analysis. Second, many organic polymers based SCX monolithic columns are mixed modes with SCX and RP mechanisms due to the polymeric monomers always having hydrophobic groups. Though these mixed mode columns may be good at separating analytes in a chromatographic analysis,[34] they will decrease the orthogonality in the multidimensional separation in combination with SCX-RP columns, which are widely used in proteome analysis. Third, the sulfonate group is most widely introduced in the SCX monolithic column, but the sulfonate containing monolith is believed to swell excessively in aqueous buffer,[27,32,33] which limits its usage for proteome analysis greatly. In contrast to the conventional sulfonate monolith that is used for SCX chromatographic separations, the phosphate monolith prepared in this work has the following advantages. First, it has a relatively high dynamic binding capacity (higher than PolySulfoethyl A) as well as high permeability (10 times higher than particulate columns with water). Therefore, the sample loading capacity could be easily controlled by using different lengths of the phosphate monolithic column. Second, it exhibits the SCX mechanism when interacting with peptides and has a faster kinetic adsorption rate, which leads to good orthogonality to RP in a SCX−RP multidimensional separation. Third, though slight swelling and shrinking of the phosphate monolith would occur, it is stable enough in different separation conditions for long time usage.

When the phosphate monolithic trap column was applied in automated sample injection for one-dimensional μHPLC−MS/MS analysis of tryptic digests of yeast proteins, better protein/peptide identification efficiency was obtained than with both the particulate SCX and the C18 trap columns. In a multidimensional separation, the phosphate monolithic trap column exhibits good performance as well. Since the 7 cm long phosphate monolithic trap column has a considerably high sample loading capacity, overloading would not happen and high fractional resolution could be obtained during the automatic online multidimensional separation of 19 μg of yeast proteins. Thus, we expected the phosphate monolithic column might be a good choice of trap column for automated proteome analysis.

CONCLUSIONS

A phosphate monolithic capillary column prepared by direct copolymerization of as high as a 60% amount of EGMP with bisacrylamide within a confined 150 μm i.d. capillary in the presence of a trinary porogenic solvent was applied as the SCX trap column for shotgun proteome analysis. This phosphate monolithic column has higher dynamic binding capacity, faster kinetic adsorption rate with peptides, as well as higher permeability than the column packed with commercially available PolySulfoethyl A particles. Because of its good orthogonality to RP, the phosphate monolithic column was successfully applied in the automated sample injection system to alleviate the influence of void volume on separation performance. Though 7 cm length of the phosphate monolithic trap column was used, an extremely high flow rate (up to 40 μL/min) for sample injection could be easily realized at a relative low pressure of ~1300 psi. Furthermore, this phosphate monolithic column had high resolution in the stepwise fractionation when applied to the online SCX-RP multidimensional separation, and a total of 1522 distinct proteins were positively identified from 5608 unique peptides at the false positive rate of only 0.46%. With the advantages of easy preparation and good performance in chromatographic separations, the phosphate monolithic capillary column may be widely applied in large scale proteome analysis.

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