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"One-Pot" Process for Fabrication of Organic-Silica Hybrid Monolithic Capillary Columns Using Organic Monomer and Alkoxysilane

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A "one-pot" process for the preparation of organic-silica hybrid capillary monolithic columns by concurrently using organic monomers and alkoxysilanes was described. In this process, the hydrolyzed alkoxysilanes of tetramethoxysilane (TMOS) and vinyltrimethoxysilane (VTMS) as precursors for the synthesis of a silica-based monolith using the sol−gel method and the organic monomer (allyldimethyldodecylammonium bromide (ADDAB) or acrylamide (AA)) with vinyl groups for free radical polymerization along with the initiator of azobisisobutyronitrile (AIBN) were concurrently introduced into a pretreated capillary; after that, the polycondensation of alkoxysilanes and the copolymerization of organic monomers and as-precondensed siloxanes were subsequently carried out within the confines of a capillary at the proper reaction conditions. Two types of organic-silica hybrid capillary monolithic columns with hydrophobic and hydrophilic properties have been fabricated, respectively, by this "one-pot" process using two different organic monomers of ADDAB and AA. The morphologies of the synthesized organic-silica hybrid monolithic columns were characterized by scanning electron microscopy (SEM). The performances of these organic-silica monolithic columns were investigated by capillary electrochromatography (CEC). The retention behaviors of the neutral and polar compounds on the resulting hydrophobic and hydrophilic organic-silica hybrid monolithic columns confirmed the successful incorporation of organic monomers in the silica monolithic matrix. In addition, the ADDA-silica hybrid capillary monolithic column was also applied in the analysis of tryptic digests of bovine serum albumin (BSA) and mouse liver extract by microliquid chromatography−tandem mass spectrometry (μLC−MS/MS) for demonstrating its potential in proteome analysis.

Monolithic columns have been widely applied in capillary liquid chromatography (CLC) and capillary electrochromatography (CEC) due to their easy preparations, unique properties, and high performances compared to the particle packed columns or the open tubular columns.[1,2] With dependence on the nature of the precursors used, the monolithic columns can be categorized into two major types: the organic polymer-based and the inorganic silica-based monolithic columns. Organic polymer-based monolithic columns, such as polystyrenes, polymethacrylates, and polyacrylamides, have good stability to pH and great flexibility to tune the surface chemistry by tailoring the compositions of porogenic solvents and monomers in the prepolymerization solutions.[1−4] However, the swelling in organic solvents and the deficiency in mechanical stability result in the short lifetime and undesirable retention reproducibility of organic polymer-based monoliths in some cases. In contrast, the inorganic silica-based monolithic columns demonstrate good solvent resistance and high mechanical stability.[5−7] Nevertheless, the surface functionalization of silica-based monolithic columns is still time-consuming and laborious.[7,8]

Recently, the organic−inorganic hybrid monolithic columns have been attracting great attention since the organic functional moieties can be incorporated into the inorganic silica monolithic matrixes via the sol−gel process to give various properties, such as good mechanical stability and solvent resistance. The trialkoxysilanes (as the organo-functionality providers) are mixed with tetra-alkoxysilanes (such as tetramethoxysilane (TMOS) or tetraethoxysilane (TEOS)) and incorporated into hydrolyzation and polycondensation to form the organo-functionalized hybrid silica-based monoliths. In the resulting organic−inorganic hybrid material, the organic moieties are covalently linked into the monolithic matrix via a Si−C bond, which avoids the further time-consuming postmodification of silica monoliths. Hayes and Malik demonstrated the incorporation of an organic sol−gel precursor,

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N-octadecyldimethyl [3-(trimethoxysilyl)propyl] ammonium chloride, into the sol solution for the preparation of a C18 functionalized organic−inorganic hybrid monolithic column using a single-step procedure for CEC separation.[9] Organic−inorganic hybrid monoliths with different organic moieties including aminopropyl, octyl, phenyl, vinyl, allyl, propyl, etc.[10−17] have also been reported with different macromesoporous structures and applied in chromatographic separations or solid phase extractions (SPEs).[18] Additionally, with the use of the vinyl functionalized hybrid monoliths, the further modification via the free radical polymerization can be performed by avoiding the time-consuming steps of introducing vinyl groups onto the silica monoliths.[7,8]

For covalently incorporation of the organic moieties into the inorganic silica-based monolithic matrixes by combining the merits of the variety of organic functional moieties and the silica monoliths into the organic−inorganic hybrid monoliths, several other approaches have been attempted besides the above-mentioned sol−gel processes of co-condensating the organotrialkoxysilanes and TMOS or TEOS. For instance, Wei et al.[19] and Jang et al.[20] reported an approach that organic monomers, such as methyl methacrylate (MMA) or vinyl acetate, were first copolymerized with 3-(trimethoxysilyl)propyl methacrylate (γ-MPS) or vinyltrimethoxysilane (VTMS) in THF and then mixed with TMOS or TEOS and hydrolyzed at acidic conditions to form the hybrid monoliths after drying for 7−14 days in a mold. Also, Avila-Herrera et al.[21] prepared a polymethylmethacrylate (PMMA)-silica hybrid monolith by pouring the solutions of TEOS, γ-MPS, and MMA into a spectrophotometer cell and drying and solidifying in a conventional oven for 7 days to obtain the transparent hybrid organic−inorganic monoliths. These methods were interesting approaches to introduce the organic moieties into the silica-based monolithic matrixes. However, the preparation time of the hybrid organic−inorganic monoliths was long, and these approaches have not been applied in the preparation of monolithic columns yet.

In this work, a simple "one-pot" approach for preparing the organic−inorganic hybrid monolithic capillary columns was developed. When the vinyl-organic monomers and initiator of azobisisobutyronitrile (AIBN) were mixed with hydrolyzed TMOS and VTMS, the resulting homogeneous mixture was introduced into a fused capillary for the subsequent polycondensation and polymerization at a certain condition to form the organic−inorganic hybrid monolithic capillary column. With the use of this strategy, two types of organic-silica hybrid monolithic capillary columns including hydrophobic and hydrophilic were successfully prepared in about 24 h. Compared to the traditional methods in the preparation of organic−inorganic hybrid monoliths, this "one-pot" reaction manner would represent a new way to prepare the organic-silica hybrid monoliths by using a variety of organic monomers.

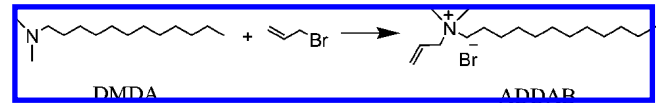

Figure 1. Scheme for the synthesis of ADDAB.

EXPERIMENTAL DETAILS

Materials. Allyl bromide, *N*,*N*-dimethyl-*N*-dodecylamine (DMDA), vinyltrimethoxysilane, and poly(ethylene glycol) (PEG, M_n = 10 000) were purchased from Aldrich (Milwaukee, WI). Acrylamide (AA) was purchased from Acros (NJ). Tetramethoxysilane was obtained from Chemical Factory of Wuhan University (Wuhan, China). 2,2-Azobisisobutyronitrile was purchased from Shanghai Chemical Plant (Shanghai, China) and recrystallized in ethanol before use. Fused-silica capillary with 75 μm i.d. and 375 μm o.d. was purchased from the Reafine Chromatography Ltd. (Hebei, China). Trypsin was obtained from Promega (Madison, WI). Bovine serum albumin (BSA) was purchased from Sigma (St. Louis, MO). DL-Dithiothreitol (DTT) and iodoacetamide were purchased from Sino-American Biotechnology Corporation (Beijing, China). Glu-fibrinopeptide B (GFP) and MassPREP digestion standard (MPDS) mixtures were obtained from Waters Corporation (Manchester, U.K.). HPLC-grade acetonitrile (ACN) was used for the preparation of mobile phases. Water used in all experiments was doubly distilled and purified by a Milli-Q system (Millipore Inc., Milford, MA). Other chemical reagents were of analytical grade.

Synthesis of Allyldimethyldodecylammonium Bromide (ADDAB). Allyl bromide (9.5 mL, 0.11 mol) was added dropwise to a solution of 27.5 mL of DMDA (0.1 mol) in ethanol (80 mL), which was stirred for 20 min at room temperature. Then, the obtained solution was heated to 55 °C and stirred for 36 h. After the solvent removal of the stirred solution by a rotary evaporator under vacuum, the resulting yellow oil-like liquid was recrystallized in 80 mL of ethyl acetate/hexane (20/80, v/v), followed by filtration and vacuum drying to give 32.1 g of white solid product (ADDAB) with 96% yield. The schematic synthesis of ADDAB was shown in Figure 1. The product was characterized by IR, ^{1}H NMR, and matrix-assisted laser desorption-time-of-flight (MALDI-TOF) mass spectrometry with results as follows: IR (KBr), 2956, 2853, 1624, 1425, 886 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$), δ = 0.88 (t, J = 6.3 Hz, 3H, CH_3), 1.25 (m, 14H, CH_2), 1.34 (m, 4H, CH_2), 1.75 (m, 2H, CH_2), 3.38 (s, 6H, N−CH_3), 3.49(m, 2H, N−CH_2), 4.38 (d, J = 6.3 Hz, 2H, CH_2−CH), 5.72 (d, J = 9.6 Hz, 1H, CH=CH_2), 5.83 (d, J = 16.5 Hz, 1H, CH=CH_2), 5.97 (m, 1H, CH=CH_2); MALDI-TOF MS, m/z at 254.4 [M^+].

Preparation of the ADDA-Silica Hybrid Monolithic Column. Before the preparation of the ADDA-silica 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

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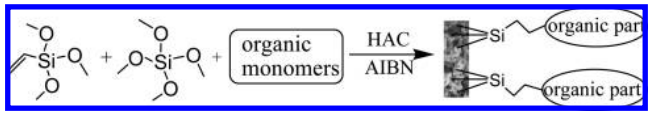

Figure 2. Schematic incorporation of organic monomers with alkoxysilanes for the preparation of the organic-silica hybrid monolith.

water for another 30 min, respectively, which was then dried by a nitrogen stream at room temperature. For the preparation of the ADDA-silica hybrid monolithic column, a prehydrolyzed mixture was prepared by mixing and stirring acetic acid (0.01 M, 5.0 mL), PEG (10 000 MW, 540 mg), TMOS (1.8 mL), and VTMS (600 μL) for 1 h at 0 °C to form a homogeneous solution. Then, 30 mg of ADDAB and 2 mg of AIBN were added into 0.5 mL of the resulting hydrolyzed mixture with 10 min sonication. After that, the mixture was manually introduced into the pretreated capillary to an appropriate length with a syringe. Both ends of the capillary were sealed with two pieces of rubber, and the capillary was incubated at 40 and 60 °C for 12 h, respectively, for condensation and polymerization. The obtained ADDA-silica hybrid monolithic capillary column was then flushed with water and methanol to remove the PEG and other residuals. The schematic synthesis of the ADDA-silica hybrid monolith was illustrated in Figure 2. For comparison, an ADDA-silica hybrid monolithic column without adding AIBN was also prepared with the same procedure.

Preparation of the Polyacrylamide (PAA)-Silica Hybrid Monolithic Column. The preparation procedure of PAA-silica hybrid monolithic column was similar to that of the ADDA-silica hybrid monolithic column except the use of acrylamide instead of ADDAB. Briefly, AA (30 mg) and AIBN (2 mg) were added into the hydrolyzed mixture (0.5 mL) of acetic acid, PEG, TMOS, and VTMS as described in Preparation of the ADDA-Silica Hybrid Monolithic Column. After sonication for 10 min, the mixture was manually introduced into the pretreated capillary to an appropriate length by a syringe. The PAA-silica hybrid monolithic column could then be obtained by going through the same other steps for the ADDA-silica hybrid monolithic column.

Capillary Electrochromatography. All CEC experiments were carried out on a capillary electrophoresis (CE) instrument-P/ACE MDQ system (Beckman, Fullerton, CA) equipped with a UV detector with the temperature set at 25 °C and detection wavelength set at 214 nm. A detection window was made by removing the polyimide coating of a fused-silica capillary with a razor blade in the empty section of the capillary at the edge of the hybrid silica continuous bed. The total length of the prepared capillary column was 30 cm with an effective length of 20 cm. The monolithic column was first preconditioned by running buffer for at least 30 min with a manual syringe pump and then equilibrated on the instrument by applying a low voltage (10 kV, ramping time for 15 min) until a stable current was obtained. All data obtained were based on three runs. The retention factor (k') was defined as $(t_r - t_0)/t_0$, where t_r and t_0 represent the retention times of an analyte and an unretained compound in this work, respectively.

Tryptic Digestion of BSA and Mouse Liver Extract. BSA (6.6 mg) was dissolved in 1 mL of denaturing buffer containing 8 M urea and 50 mM ammonium bicarbonate. After the addition of 20 μL of DTT (50 mM), the mixture was incubated at 60 °C for 1 h to reduce the disulfide bonds of the protein; subsequently, 40 μL of IAA (50 mM) was added, and the mixture was then incubated at room temperature in dark for 30 min;. Finally, the mixture was diluted 10-fold with 50 mM ammonium bicarbonate buffer (pH 8.2) and digested at 37 °C for 16 h with trypsin at the ratio of enzyme-to-substrate of 1:40 (w/w). After digestion, the pH value of the obtained tryptic digestion solution was adjusted to 2.7 by 10% trifluoroacetic acid (TFA) aqueous solution. Followed by a solid-phase extraction of the BSA tryptic digest with a homemade C18 cartridge, the collected peptides were dissolved into a 0.1% formic acid aqueous solution, which was stored in a −20 °C freezer before μLC−MS/MS analysis. The procedure for preparing protein extract from mouse liver was described elsewhere previously.[22] The protein concentration was determined by Bradford protein assay, and the procedure of tryptic digestion was the same as that of BSA.

LC−MS/MS Analysis of Tryptic Digests. The BSA tryptic digest was separated on a microultraperformance (μUPLC) system (Waters Corp., Milford, MA). Buffer A was water (0.1% formic acid) and buffer B was 100% ACN (0.1% formic acid). An online C18 trap column (2 cm × 180 μm i.d., 5 μm C18 particulate, Waters Corp.) was used for sample injection. BSA tryptic digest was automatically injected onto the trap column at a flow rate of 5 μL/min for 3 min with buffer A. After that, the trapped peptides were then separated at a flow rate of 250 nL/min on a 40 cm × 50 μm i.d. ADDA-silica hybrid monolithic column that was coupled with a homemade C12 monolithic ESI emitter[23] (7 cm × 25 μm i.d., with a 5 μm tip) by a stainless steel union.

The MS/MS detection of peptides was carried out on a Q-TOF Premier mass spectrometry system (Waters Corp., Milford, MA), which was operated in positive-ESI V-mode with typical resolving power of at least 8 000 and calibrated by GFP in m/z range from 50 to 1950. Data acquisition was operated in the data dependent analysis mode. The method included a full MS scan (m/z 400−1600, 0.9 s) and three subsequent MS/MS scans (m/z 50−1950, 1.2 s each scan) on the three most intense ions presented in the full MS scan. The radio frequency (rf) offset applied to the mass analyzer was adjusted such that the LC−MS data were effectively acquired from m/z 400 to 2000. GFP (200 fmol/μL) was used as LockSpray, which was delivered through the reference sprayer and scanned every 30 s. Electrospray voltage was set at 2.8 kV. The MS acquisition was started after the gradient was actuated for 15 min due to the delay of gradient elution caused by void volume of the LC system.

Raw data of MS acquisition were processed using Proteinlynx GlobalServer (PLGS) v2.3 and the resulting MS/MS data set was exported to *pkl data file format. The peptide identification was performed using an in-house version of Mascot v2.2 (Matrix Science, London, U.K.). The MS/MS data were searched against the SwissPort database for BSA and the Mouse International Protein Index (IPI) protein database (v3.17) for mouse liver extract. Cysteine residues were searched as a static modification of 57.0215 Da and methionine residues as variable modification of 15.9949 Da. Peptide identifications were restricted to tryptic

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Table 1. Effects of Synthesis Parameters on the Formation of ADDA-Silica Hybrid Monolith[a]

Column**	TMOS (mL)	VTMS (mL)	Temp. (°C)	ADDAB (mg/mL)	Back pressure (MPa)	Morphology	Optical microscopy image***
A[a]	1.8	0.4	40	60	~2.1	Slack, opaque, detached	
B[a,b,c]	1.8	0.6	40	60	~5.7	Homogeneous, semitransparent	
C[a]	1.8	0.8	40	60	>17	Homogeneous, semitransparent	
D[b]	1.8	0.6	35	60	<2.1	Seriously detached	
E[b]	1.8	0.6	45	60	Too hard to pump through	Homogeneous, transparent	
F[c]	1.8	0.6	40	40	Too hard to pump through	Homogeneous, semitransparent	
G[c]	1.8	0.6	40	80	~3.0	Opaque, slight detached	

[a] Flushed with methanol; flow rate, 2 μL/min; column length, 15 cm; optical microscopy magnification at ×100. **: (a) the effect of VTMS content, (b) the effect of temperature, (c) the effect of ADDAB content. ***: high-resolution images of optical microscopy are illustrated in the Supporting Information.

peptides with no more than two missed cleavages. The mass tolerances were 20 ppm for precursor ions and 0.1 Da for fragment ions. To evaluate the false discovery rate (FDR) of peptides identification, a decoy database created by Mascot was applied to the database search, and the FDR was controlled at <1% by setting the Mascot score ($p < 0.05$).

RESULTS AND DISCUSSION

Preparation of ADDA-Silica Hybrid Monolithic Column. The formation of the hybrid ADDA-silica monolithic column involves two major reactions: the polycondensation of hydrolyzed precursors of TMOS and VTMS, and the copolymerization of the precondensated siloxanes and vinyl organic monomers. For investigation of the amount of VTMS in the reaction mixture on the formation of the hybrid ADDA-silica monolith, the ratios of TMOS to VMTS (v/v) from 4:1 to 2:1 were evaluated by referring to values from the literature.[13,15] The obtained columns A, B, and C corresponding to the ratio of TMOS to VTMS at 4:1, 3:1, and 2:1 were displayed in Table 1, respectively, with their synthesis parameters and resulting optical microscope images. As seen from these optical microscope images, the lower content of VTMS (as for column A) in the reaction mixture would result in the slack monolith with opaque aggregation inside the capillary; the higher content of VTMS (as for columns B and C) could result in the homogeneous and semitransparent monolithic matrixes within the confines of the capillary. The backpressure for these synthesized monolithic columns was measured by pumping methanol through the column at a flow rate of 2 μL/min. For columns A, B, and C, the backpressure was ~2.1, 5.7, and >17 MPa, respectively, which was consistent with the observed tightness of the monolithic matrixes by optical microscopy. So, the careful adjustment of silane monomers in the reaction mixture will be necessary for obtaining the desirable monolithic column.

Since the polycondensation is temperature sensitive, the condensation temperature was thus examined in the synthesis of hybrid monolithic column. In this work, the polycondensation was performed at a relatively low temperature (35, 40, and 45 °C), and the copolymerization was performed at 60 °C as usually adopted for the preparation of organic monolithic columns with AIBN as the initiator. The morphology and permeability of resultant monolithic columns (columns D, B, and E) condensed at different condensation temperatures (35, 40, and 45 °C) were displayed in Table 1. The optical microscopy images clearly showed that the temperature of 35 °C was not appropriate for the co-condensation of VTMS and TMOS with other synthesis parameters illustrated in Table 1 for column D, in which the monolithic matrix was seriously detached from the inner capillary wall due to the incomplete co-condensation of silane monomers. With the increase of the condensation temperature, the obtained monolithic matrixes were homogeneous and fully filled in the capillary. However, on the basis of the investigation of column permeability, the column synthesized at 45 °C showed bad permeability since it was even hard to pump methanol through the monolith. However, the column synthesized at 40 °C (column B) showed the rational backpressure, which indicated the acceptable column permeability. These results confirmed that the co-condensation of the silane monomers was temperature dependent, and the increase of the temperature would accelerate the co-condensation of silane monomers. In this experiment, we also found that the change of the amount of silane monomers in the reaction mixture would

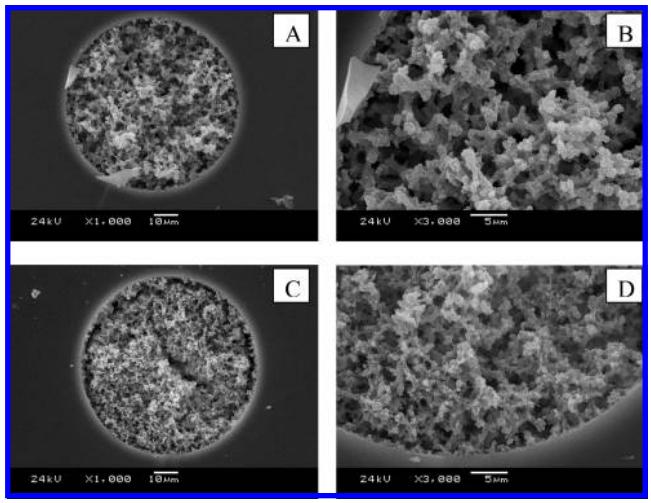

Figure 3. SEM photographs of ADDA-silica hybrid monolith: (A) ×1000 and (B) ×3000. PAA-silica hybrid monolith: (C) ×1000 and (D) ×3000.

require the corresponding change of condensation temperature for obtaining the homogeneous and permeable hybrid monoliths.

For preparation of the organic-silica hybrid monolithic column, the organic monomer was simultaneously mixed with the silane monomers in this experiment as with the "one-pot" manner. Thus, the amount of the organic monomer of ADDAB in the reaction mixture should be examined because the existence of ADDAB would affect the formation of monolith. The precondensation solutions with different concentration of ADDAB (40, 60, 80 mg/mL) were prepared, and the obtained hybrid monolithic columns were displayed in Table 1 as columns F, B, and G. It showed that the higher amount of ADDAB (80 mg/mL) in the precondensation solution would result in the large through-pore matrix (column G), and the lower amount of ADDAB (40 mg/mL) would result in the small through-pore matrix (column F). For column F, the column permeability was poor due to the small through-pore structure of the resulting monolithic matrix. Also for column G, there was a slight detachment of the monolithic matrix from the capillary wall, which seemed to result in the relatively lower backpressure with a comparison to column B. Additionally, the longer gelation time was also observed when the higher ADDAB content was used in the precondensation solution. This observation indicated that the existence of ADDAB in the precondesation solution did affect the gel formation in the sol−gel process. On the basis of the above investigations, the TMOS to VTMS ratio of 3:1, the ADDAB concentration of 60 mg/mL in the precondensation solution, and the condensation temperature of 40 °C were adopted in the preparation of the ADDA-silica hybrid monolithic column.

Characterization of the ADDA-Silica Hybrid Monolithic Column. Following the investigations above, the prepared ADDA-silica hybrid monolithic column was then characterized. The SEM images of the ADDA-silica hybrid monolithic column were shown in parts A and B of Figure 3. As shown in Figure 3B, a uniform organic-silica hybrid monolithic matrix with large through-pores was obtained. Also, in Figure 3A, it can be seen that the formed organic-silica monolithic matrix was well attached to the inner wall of the capillary. This was because the silanol groups at the

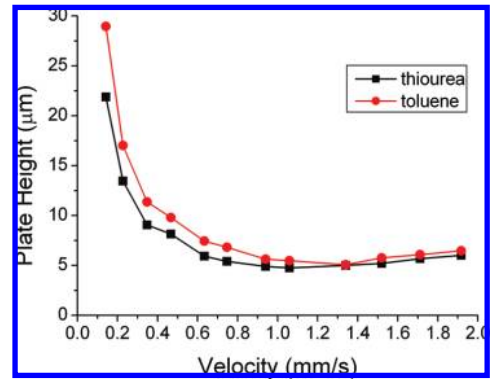

Figure 4. Dependence of the plate height of analytes on the linear velocity of the mobile phase by the ADDA-silica hybrid monolithic capillary column. Experimental conditions: monolithic capillary column, effective length of 20 cm × 75 i.d., total length of 30 cm; mobile phase, 10 mM Na_2HPO_4−citric acid buffer at pH 3 containing 40% ACN; test compounds, thiourea and toluene; applied voltage, −20 kV; injection, −5 kV for 1 s; detection wavelength, 214 nm.

inner wall of the capillary had taken part in the polycondensation of the monolithic preparation.[13]

Additionally, the mechanical stability of the obtained ADDA-silica hybrid monolithic column was examined by connecting the column (length, 25 cm) to a μUPLC pump (Waters) with the flow rate ranged from 0.1 to 2.9 μL/min (mobile phase, 40% ACN). The measured backpressure was linearly ($R = 0.997$) increased from 0.3 to 13.5 MPa as the flow rate was increased. This indicated that the ADDA-silica hybrid monolith possessed good mechanical stability under the pressure of 13.5 MPa. Using Darcy's Law[24] of permeability $B_0 = F\eta L/(\pi r^2 \Delta P)$, where F is the flow rate of the mobile phase, η is the viscosity of the mobile phase (the value of 0.801 cP was from ref 25), L is the effective length of column, r is the inner radius of the column, and ΔP is the pressure drop of the column. The permeability of the monolithic column was calculated as 1.73×10^{-13} m^2, which indicated the good permeability of the prepared monolithic column.[26]

The column efficiency of the ADDA-silica hybrid monolithic column was evaluated in CEC by changing the applied voltage from 5 to 29 kV. The relationship between the flow velocity and the plate height of thiourea and toluene was demonstrated in Figure 4. The lowest plate height of ∼5 μm was obtained, which corresponded to a column efficiency (theoretical plates, N) of ∼200 000 plates/m. Also, it can be seen that the column remained at high efficiency in CEC with the linear velocity ranging from 0.8 to 1.5 mm/s. The run-to-run reproducibility was evaluated on a single capillary monolithic column, and the relative standard deviations (RSDs) for electroosmotic flow (EOF) and retention time of analytes on the capillary monolithic column were less than 2.9% for 5 runs in CEC. Both column-to-column and batch-to-batch reproducibilities for the preparation of monolithic columns were also evaluated in term of the RSDs of EOF and retention times of analytes, which were less than 4.3 ($n = 3$) and 6.9% ($n = 3$), respectively. These results indicated that the prepared ADDA-silica hybrid monolithic columns had good stability and reproducibility.

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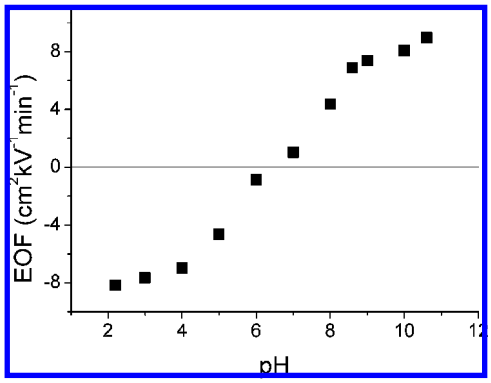

Figure 5. Dependence of EOF on the pH value of the mobile phase on the ADDA-silica hybrid monolithic column. Experiment conditions: mobile phase, 10 mM Na_2HPO_4−citric acid buffer (pH 2.2−8) and 10 mM glycine−NaOH buffer (pH 8.6−10.6) containing 40% ACN with various pH values; applied voltage, −20 kV for pH < 6.5 and 20 kV for pH > 6.5; injection, −5 kV for 1 s at pH < 6.5, 5 kV for 1 s at pH > 6.5; void time marker of EOF, thiourea. Other conditions are the same as in Figure 4.

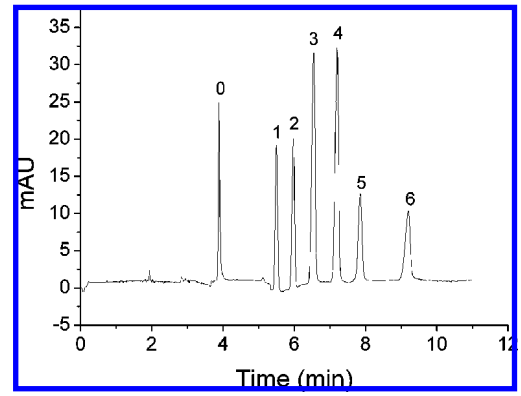

Figure 6. Separation of neutral aromatic compounds on the ADDA-silica hybrid monolithic column. Experimental conditions: mobile phase, 10 mM Na_2HPO_4−citric acid buffer containing 40% ACN at pH 3.0. Other conditions are the same as in Figure 4 Solutes: (0) thiourea, (1) benzene, (2) toluene, (3) *p*-xylene, (4) 1,3,5-trimethylbenzene, (5) 1,2,4,5-tetramethylbenzene, and (6) 2,7-dimethylnaphthalene.

Generation of EOF on the ADDA-Silica Hybrid Monolithic Column. In CEC, electroosmotic flow is generated by applying a voltage at both ends of a column. Monolithic columns bearing positively charged or negatively charged groups could generate anodic or cathodic EOF, which could be affected by the pH change of the running buffer. Figure 5 showed the relationship between the pH of the running buffer and the EOF generated on the hybrid monolithic column in CEC, where the negative and positive values of EOF denote cathodic EOF and anodic EOF, respectively. As seen in Figure 5, the anodic EOF was obtained with the maximum of (−)8.17 $cm^2\ kV^{-1}\ min^{-1}$ at pH 2.2, and the anodic EOF decreased with the increase of the pH value of the running buffer. This is due to the ionization of the quaternary amine of ADDA incorporated into the hybrid monolithic matrix and suppression of the silanol ionization at a low pH value. With an increase of the pH, the suppression of the silanol ionization and the ionization of the quaternary amine of ADDA would tend to decrease. As a result, the anodic EOF turned to the cathodic EOF when the pH was greater than ~6.5, and which reached up to (+)8.96 $cm^2\ kV^{-1}\ min^{-1}$ when the pH value of running buffer was 10.6. The flip-flop of the EOF generated on the ADDA-silica hybrid monolithic column as the pH value changed from low to high would be very useful in the separation of acidic and basic solutes in CEC by simply changing the pH value of mobile phases.[27]

On the other hand, the generation of the anodic EOF on the ADDA-silica hybrid monolithic column also indicated that the ADDA had been well incorporated into the silica monolithic matrix and leads to the positively charged surface of the monolith at low pH conditions. For the further confirmation of the incorporation of ADDA in the silica monolith, a silica monolithic column prepared through the same procedure and with the same reactants except the absence of AIBN in the reaction mixture for the ADDA-silica monolith was obtained for comparison, which was then applied in CEC under the condition of pH 2.2. It was found that the EOF on the obtained monolithic column without using AIBN in its preparation was very weak at the low-pH condition. This indicated that the ADDA could not be copolymerized into the monolithic matrix and would be easily flushed out after the copolymerization procedure by water and methanol when AIBN was not used. Shortly, the simultaneous incorporation of the vinyl organic monomer into the vinyl-silica monolithic matrix could not be achieved without using AIBN. Whereas, with the use of AIBN, the ADDA would be covalently copolymerized into the silica monolithic matrix to generate the strong enough EOF in CEC as observed in this experiment. These results confirmed that the "one-pot" approach was feasible for the preparation of the organic−inorganic hybrid monolithic column by simply mixing the vinyl organic monomers, the vinyl alkoxysilane (VTMS), and the regular tetra-alkoxysilane (such as TMOS, TEOS) using AIBN as the free radical initiator at a certain temperature.

Retention Behavior of Neutral Solutes on the Hybrid ADDA-Silica Monolithic Column. For investigation of the performance of the ADDA-silica hybrid monolithic column in CEC, the obtained ADDA-silica column was applied for the separation of neutral compounds at an acidic condition. Figure 6 showed the resultant electrochromatogram of neutral aromatic compounds on this ADDA-silica hybrid monolithic column with the pH of mobile phase at 3.0. The analytes were eluted in the order of thiourea < benzene < toluene < *p*-xylene < 1,3,5-trimethylbenzene < 1,2,4,5-tetramethylbenzene < 2,7-dimethylnaphthalene that was corresponding to the hydrophobicities of these analytes from low to high. The retentions of the analytes in CEC were investigated by changing the ACN content in the mobile phase. In Figure 7, the retention factors (k') of these neutral aromatic compounds decreased with the increase of the ACN content in the mobile phase, which indicated a typical reversed-phase chromatographic property of the ADDA-silica hybrid monolithic stationary phase toward the neutral hydrophobic solutes.[11] These results confirmed that the long alkyl chain of the organic monomer ADDA was successfully bonded to the monolithic matrix, which thus provided the hydrophobicity of the hybrid monolithic stationary phase to the neutral aromatic compound in the reversed-phase chromatography mode in CEC.

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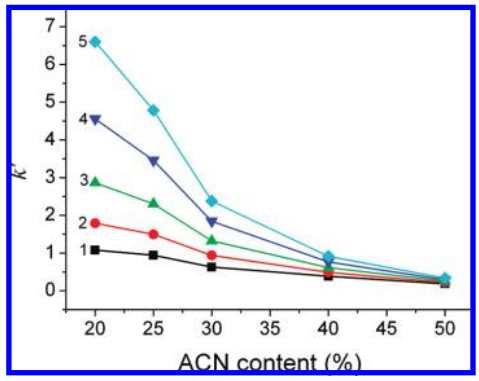

Figure 7. Effect of ACN content in the mobile phase on the retention of neutral aromatic solutes. Experimental conditions: mobile phase, 10 mM Na_2HPO_4–citric acid buffer with different ACN content at pH 3.0. Other conditions are the same as in Figure 4. Solutes: (1) thiourea, (2) benzene, (3) toluene, (4) *p*-xylene, (5) 1,3,5-trimethylbenzene, and (6) 1,2,4,5-tetramethylbenzene.

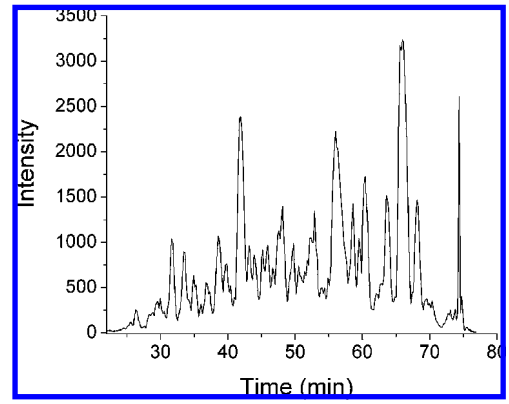

Figure 8. Base peak chromatogram of μRPLC–MS/MS analysis of 1 μg of tryptic digest of mouse liver extract. Experimental conditions: 40 cm × 50 μm i.d. ADDA-silica hybrid monolithic column with 60 min separation gradient at operating pressure ~19.4 MPa. The RP separation gradient was buffer B from 0% to 35% in 60 min, and from 35% to 90% in 5 min. After the system was flushed with 90% buffer B for 5 min, the separation system was equilibrated with buffer A for 25 min.

The LC–MS/MS Analysis of Protein Tryptic Digests with the ADDA-Silica Hybrid Monolithic Column. Silica-based monolithic columns have hierarchical meso- and macroporous structures, which lead to the fast mass transfer kinetics and lower backpressure during separation.[6] These unique properties have made the monolithic column the emerging choice toward the conventional particulate-packed columns for the analysis of complex samples such as peptides and proteins. Therefore, instead of the separation of the above-mentioned neutral solutes in CEC, the separation of peptide mixtures derived from the tryptic digestion of BSA and mouse liver extract were attempted on the obtained ADDA-silica hybrid monolithic column by a μUPLC system with Q-TOF-MS as the analyzer for further investigation of the potential use of the hybrid monolithic column in the analysis of complex samples. As confirmed by CEC, this hybrid monolithic column did exhibit the sufficient hydrophobicity due to the in situ incorporation of ADDA moiety in the silica monolithic matrix. Therefore, the different amounts (250, 500, 750, 1000, and 2500 fmol) of BSA tryptic digest were, respectively, loaded on a capillary monolithic column for the μUPLC-Q-TOF mass spectrometry analysis in RP mode with a gradient elution of ACN from 0–35% ACN within 60 min. On the basis of the database search of the obtained chromatogram of BSA tryptic digest, 19 unique peptides were positively identified and the protein coverage was 35.1% (RSD = 7.7%, n = 3) for the loading amount of BSA tryptic digest at 250 fmol. As the loading amount of BSA tryptic digest on the capillary monolithic column was increased, the numbers of positively identified unique peptides were increased to 26, 31, 32, and 35 corresponding to the loading amount of 500, 750, 1000, and 2500 fmol, respectively; and the protein coverage was also increased from 35.1% to 48.6%, 50.9%, 51.2%, and 54.0% for loading amount of BSA tryptic digest from 250 fmol to 500, 750, 1000, and 2500 fmol, respectively. However, the average peak widths (at 0.613 peak height) of three selectively extracted peptide ion peaks (SHCIAEVEK, ETYGDMADCCEK, and LGEYGFQNALIVR) were changed from 0.56 to 0.64, 0.63, 0.67, and 0.88 with the increase of the loading amount of BSA tryptic digest on the capillary monolithic column, which indicated an estimated 35.6% decrease of column capacity when the loading amount was increased from 250 to 2500 fmol. For further investigation of the potential of the prepared capillary monolithic column for the analysis of complex protein samples rather than a standard protein of BSA, 1 μg of tryptic digest of mouse liver extract was analyzed by the capillary monolithic column. The base peak chromatogram of the mouse liver extract was illustrated in Figure 8. After a database search, 71 (RSD = 7.9%, n = 3) mouse liver proteins were positively identified with FDR < 1%. These results were comparable to that obtained from a particulate-packed commercial column (15 cm × 75 μm i.d., 1.7 μm particles, Waters Corp.), where 22 unique peptides (RSD = 4.2%, n = 3) and 37.8% protein coverage (RSD = 2.1%, n = 3) for a 750 fmol tryptic digest of BSA and 73 distinct proteins (RSD = 2.8%, n = 3) for a 1 μg tryptic digest of mouse liver extract were obtained under the same μUPLC–Q-TOF MS analysis conditions. These results also indicated that this is a promising approach to introduce the organic-functional groups into the silica-monolithic matrix via this "one-pot" process to provide the desirable functionalities for the separations of some complex samples.

Separation of Polar Solutes on a Hydrophilic PAA-Silica Hybrid Monolithic Column. For further demonstration of the success of the "one-pot" approach in the preparation of the organic-silica monolithic hybrid column, a PAA-silica hybrid monolithic column was also prepared using AA as the organic monomer instead of the ADDAB for the ADDA-silica hybrid monolithic column. The SEM images of the PAA-silica hybrid monolithic column were shown in parts C and D of Figure 3. Because of the incorporation of the hydrophilic monomer of AA in the silica monolithic matrix, the resultant PAA-silica hybrid monolithic column was thus applied for the separation of neutral hydrophilic analytes, and the obtained electrochromatogram was illustrated in Figure 9. As expected, the analytes were eluted out in the order of toluene < dimethylformamide < formamide < thiourea according to the polarity of these compounds from low to high. As increasing the content of ACN in the mobile phase from 80% to 95%, the stronger retention and better resolution of these polar analytes were obtained, which was a typical phenomenon for polar compounds on hydrophilic stationary phases in CEC. This result also indicated the successful incorporation of the hydrophilic

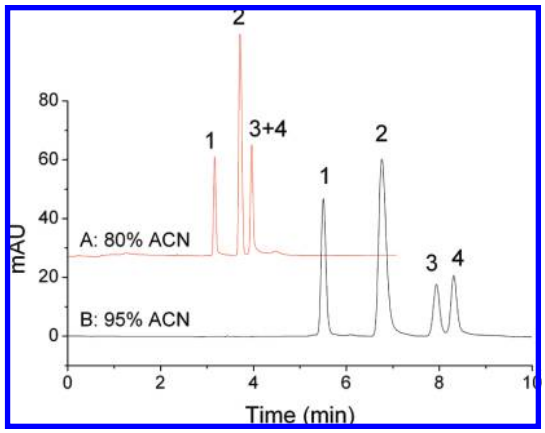

Figure 9. Separation of polar analytes on the PAA-silica hybrid monolithic column. Experimental conditions: mobile phase, 10 mM phosphate buffer containing (A) 80% ACN and (B) 95% ACN at pH 6.8; applied voltage, 20 kV; injection, 5 kV for 1 s. Other conditions are the same as in Figure 4. Solutes: (1) toluene, (2) *N,N*-dimethylformamide, (3) formamide, and (4) thiourea.

organic monomer in the silica monolithic matrix through this "one-pot" approach.

CONCLUSION

A simple approach for the incorporation of organic moieties into the silica monolithic matrix was developed, where the polycondensation and the polymerization of the organic and inorganic monomers were subsequently carried out within the confines of a capillary at proper conditions. Both the hydrophobic and hydrophilic organic-silica hybrid monolithic columns have been successfully fabricated by using ADDAB and AA as the hydrophobic and hydrophilic monomers in the "one-pot" process. On the basis of the CEC investigation of the hybrid organic-silica monolithic column, it was confirmed that the retention mechanisms of the neutral or polar solutes were consistent with the chemical properties of the incorporated organic monomers in the hybrid monolithic matrixes. The separation of the protein tryptic digests on the ADDA-silica hybrid monolithic column in reversed-phase mode by the μLC−MS/MS system also indicated the solid potential of the "one-pot" process for introducing the desirable functional groups into the silica monoliths by combining the merits of the meso- and macroporous structure of silica monoliths and the great availability of functional chemicals. Additionally, with the use of this approach, the limitation and difficulty of using or synthesizing the functional organic-trialkoxysilanes could be circumvented by simply using the organic monomers in the "one-pot" manner. This in situ process of incorporating functional groups into silica monolith is opening the avenue for the preparation of the organic-silica hybrid monolithic column. Although the prepared organic-silica hybrid monoliths were only applied for the separation in CEC and capillary liquid chromatography, we believe that this approach will be a promising method to introduce the organic moieties into the inorganic materials for other purposes.

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