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Silica-based polypeptide-monolithic stationary phase for hydrophilic chromatography and chiral separation

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ABSTRACT

Glutathione (GSH)-, somatostatin acetate (ST)- and ovomucoid (OV)-functionalized silica-monolithic stationary phases were designed and synthesized for HILIC and chiral separation using capillary electrochromatography (CEC). GSH, ST and OV were covalently incorporated into the silica skeleton via the epoxy ring-opening reaction between their amino groups and the glycidyl moiety in γ-glycidoxypropyltrimethoxysilane (GPTMS) together with polycondensation and copolymerization of tetramethyloxysilane and GPTMS. Not only could the direction and electroosmotic flow magnitude on the prepared GSH-, ST- and OV-silica hybrid monolithic stationary phases be controlled by the pH of the mobile phase, but also a typical HILIC behavior was observed so that the nucleotides and HPLC peptide standard mixture could be baseline separated using an aqueous mobile phase without any acetonitrile during CEC. Moreover, the prepared monolithic columns had a chiral separation ability to separate DL-amino acids. The OV-silica hybrid monolithic column was most effective in chiral separation and could separate DL-glutamic acid (Glu) (the resolution R = 1.07), DL-tyrosine (Tyr) (1.57) and DL-histidine (His) (1.06). Importantly, the chiral separation ability of the GSH-silica hybrid monolithic column could be remarkably enhanced when using gold nanoparticles (AuNPs) to fabricate an AuNP-mediated GSH-AuNP-GSH-silica hybrid monolithic column. The *R* of DL-Glu, DL-Tyr and DL-His reached 1.19, 1.60 and 2.03. This monolithic column was thus applied to separate drug enantiomers, and quantitative separation of all four R/S drug enantiomers were achieved with R ranging from 4.36 to 5.64. These peptide- and protein-silica monolithic stationary phases with typical HILIC separation behavior and chiral separation ability implied their promise for the analysis of not only the future metabolic studies, but also drug enantiomers recognition.

1. Introduction

With the advantages of excellent permeability, versatile surface modification and ease of preparation, the monolithic stationary phase has attracted increasing attention since the 1990s [1,2]. As an alternative to the microparticle-based technique, it is used in capillary electrochromatography (CEC) and capillary liquid chromatography for the separation of small molecules and large biomolecules [3,4]. Rapid progress has been made in column efficiency, modulation of selectivity and applications of the state-of-the-art monoliths [5]. The surface chemistry of the monolithic stationary phase can be adjusted by the selection of monomers [6–12] and cross-linkers [13–16], as well as further modification of the nanoparticles [17–26] which differ in hydrophobicity, hydrophilicity and ionizability under a certain mobile phase, but, in general, one type of the stationary phase currently used emphasizes a given chromatographic mode. Mixed-mode chromatography employing multifunctional stationary phases often provides resolution that far exceeds that observed with a single-mode process [27–33], for example, the separation of basic chitonoligosaccharides, strongly acidic carrageenan oligosaccharides and standard peptides on cation-exchange chromatography (CEX) were much improved on HILIC/CEX [30]. In addition to target analytes, the separation efficiencies of a monolithic column are largely dependent on the physicochemical properties of the precursors that

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form the column structure and control the Kinetics and thermodynamic properties. Peptides and/or proteins are naturally zwitterionic and chiral compounds with hydrophilic or hydrophobic properties and various spatial structures dedicated by their corresponding amino acid compositions and sequence. They could be modified on the monolithic columns by post-modification [16,34–37] or incorporated in monolithic skeleton by one-pot strategy [31,38–41]. However, systematic evaluation of both HILIC behavior and chiral separation ability of such monolithic columns is very scarce. We aimed to design and synthesize a monolithic stationary phase using tetramethyloxysilane (TMOS) and γ-glycidoxypropyltrimethoxysilane (GPTMS) as co-precursors to build the silica monolithic skeleton, while peptide such as glutathione (GSH) and/or somatostatin (ST) or a protein such as ovomucoid (OV) as exemplified was decorated simultaneously in the silica monolithic skeleton via the epoxy ring-opening reaction between the glycidyl in GPTMS and the amine of the peptide and/or protein, alongside polycondensation and copolymerization of TMOS and GPTMS in the so-called one-pot manner. These monolithic stationary phases designed and synthesized were expected to demonstrate the pH-switchable characteristics of electroosmotic flow (EOF) direction and magnitude, HILIC behavior and chiral separation ability during CEC. Moreover, gold nanoparticles (AuNPs) were used to fabricate an AuNP-mediated GSH-AuNP-GSH monolithic stationary phase via the strong interaction between sulfhydryl and Au to improve its chiral separation ability.

2. Materials and methods

2.1. Chemicals and reagents

TMOS, GPTMS, polyethylene glycol (PEG, Mw = 10,000 Da), HPLC peptide standard mixture (Gly-Tyr, Val-Tyr-Val, Met enkephalin, Leu enkephalin and angiotensin II), OV (Type III-O, free of ovoinhibitor, 186 amino acids, p*I* 4.71, polarity −0.29 (http://web.expasy.org/protparam/), Mw = 20476.7 Da), tris(2-carboxyethyl)phosphine (TCEP) and HPLC-grade acetonitrile (ACN) were purchased from Aldrich (Milwaukee, WI, USA). GSH (p*I* 5.93, polarity −0.49 (http://www.hilic.com/), Mw = 307.3 Da), adenosine 5′-monophosphate (AMP), cytidine 5′-monophosphate (CMP), guanosine 5′-monophosphate (GMP), uridine 5′-monophosphate (UMP), tyrosine (Tyr), glutamic acid (Glu) and histidine (His) were purchased from Aladdin (Shanghai, China). Somatostatin acetate (ST, a cyclopeptide containing 14 amino acids A-G-C-K-N-F-F-W-K-T-F-T-S-C, p*I* 8.91, Mw = 1637.9 Da, polarity 0.029) was purchased from Sangon (Shanghai, China). Chloroauric acid ($HAuCl_4$), trisodium citrate, sodium borohydride and disodium hydrogenphosphate were obtained from the Sinopharm Chemical Reagent Corporation (Shanghai, China). Drug enantiomers: nimodipine (Yabao Phamaceutical Ltd., Shanxi, China), amlodipine and lansoprazole (Kailun New Chemical Materials Ltd., Hubei, China), and omeprazole (Huaxin Phamaceutical Ltd., Guangxi, China) were gifted by Prof. Hui Zhang. All other reagents used in this study were of at least analytical grade. Water used in all experiments was purified using a Milli-Q system (Millipore, Milford, MA, USA). Fused-silica capillary with 75 μm i.d. and 375 μm o.d. was purchased from Refine Chromatography Ltd. (Hebei, China).

2.2. Instruments

CEC experiments were carried out on a P/ACE MDQ system (Beckman, USA) equipped with a UV–vis detector at 25 °C. A HITACHI S-4800 SEM and energy dispersive X-ray spectrometry (EDS) instrument (Hitachi, Japan) was used to study column morphology and to determine the elemental content of Au and S. A JEM-1400 TEM instrument (JEOL, Japan) was used to study gold nanoparticle morphology. The UV–vis spectra were recorded on a UV-2550 (Shimadzu, Japan). All IR measurements were carried out on a Nicolet IR200 Spectrometer (Thermo Electron, USA). A Vario EL III (Elementar, Germany) was used for elemental analysis. The macropore size distribution of the monoliths synthesized was measured on a Poremaster 60 mercury intrusion apparatus (Quantachrome, Boynton Beach, FL, USA), and the mesopore diameter and Brunauer-Emmett-Teller surface area were determined on a Micromeritics Tristar 3000 (Norcross, GA, USA) through nitrogen adsorption/desorption.

2.3. Electrochromatography procedures

Prior to CEC, the monolithic column placed in the CE cartridge was preconditioned with an appropriate running buffer for 1 h with a syringe pump (Unimicro Technologies, Pleasanton, CA, USA), and then equilibrated on the CE instrument by applying a low voltage of 5 kV until the current was stable before sample introduction. Both sampling and separation were performed at 25 °C. The ACN-aqueous mobile phase was prepared by mixing the desired amount of phosphate solution or ammonium formate buffer and ACN. All the mobile phases were filtered through 0.45 μm membranes and degassed under sonication before use. All the hybrid monolithic columns used had a total length of 30 cm (effective length 20 cm). A detector window (2 mm) was created by removing the polyimide coating of a fused-silica capillary with concentrated sulfuric acid.

2.4. Preparation of the peptide- and protein-silica hybrid monolithic columns

First, in order to clean and activate the inner surface of the capillary for effective attachment of the silica skeleton, it was pretreated by rinsing with 1 M HCl for 12 h, water for 30 min, 1 M NaOH for 12 h, water for 30 min and acetone for 1 h in sequence using a syringe pump, and then dried using a nitrogen stream at room temperature overnight. Subsequently, a prehydrolyzed mixture was prepared by mixing appropriate amounts of TMOS, GPTMS, HAc and PEG for 1 h under an ice bath to form a homogeneous solution. The molar ratio of TMOS and GPTMS monomer was optimized at 4:3 under HAc/PEG = 1:2 and 25:36, and used for preparing each type of hybrid monolithic column. Then, appropriate amounts of GSH, ST and OV as well as NaOH solution, which was to adjust the pH and increase the peptide/protein solubility in the hydrolyzed mixture, were added into the hydrolyzed mixture (0.5 mL), followed by sonicating for 10 min. Afterwards, the resulting mixture was manually injected into the pretreated capillary with a syringe, and both ends of the capillary sealed with rubber septa. The filled capillary was placed in a GC oven and kept at 40 °C for polycondensation (12 h) and then at 80 °C for copolymerization (12 h). The obtained monolithic columns were flushed with ACN/water (70:30, v/v) with an HPLC pump in order to remove the unreacted residuals. Because of the different physical and chemical properties of GSH, ST and OV, the amounts of the porogens HAc and PEG used were not the same for each type of monolithic column synthesized. The final preparation conditions are listed in Table 1. The prepared hybrid monolithic columns were named GSH-, ST- and OV-silica hybrid monolithic columns, and were stored in a refrigerator at 4 °C to preserve them from possible bacterial growth before use. In parallel, the corresponding bulk hybrid monoliths were prepared in a centrifuge tube under the same conditions for characterization of the hybrid silica monoliths prepared. The bulk hybrid monolith was cut into smaller pieces, extracted with ethanol overnight in a Soxhlet apparatus and then dried at 80 °C overnight for further measurements.