Journal of Pharmaceutical and Biomedical Analysis 140 (2017) 190–198

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Mesoporous silica nanoparticles incorporated hybrid monolithic stationary phase immobilized with pepsin for enantioseparation by capillary electrochromatography

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ARTICLE INFO

Article history:
Received 9 March 2017
Accepted 9 March 2017
Available online 19 March 2017

Keywords:
Mesoporous silica nanoparticles
Enantioseparation
Hybrid monolithic column
Capillary electrochromatography

ABSTRACT

In this study, a novel mesoporous silica nanoparticles incorporated chiral hybrid monolithic stationary phase was developed. The stationary phase was firstly prepared by an in situ copolymerization of amino-modified mesoporous silica nanoparticles (NH_2-MSN), glycidyl methacrylate (GMA), and ethylene dimethacrylate (EDMA) and then functionalized with pepsin as chiral selector. The incorporated mesoporous silica nanoparticles provided additional interactions sites, and in turn yielded different enantioselectivity thus enhancing the overall separation. The column was successfully employed for enantioseparation of fifteen basic chiral drugs in capillary electrochromatography. Effects of nanoparticles percentage, pepsin concentration, the pH of running buffer and the applied voltage were investigated. All the analytes could be eluted in less than ten minutes and nine of them could achieve baseline separation. Satisfactory repeatabilities with relative standard deviations less than 4.2% were achieved through intraday, interday, column-to-column and batch-to-batch investigations. These results indicated that the simultaneous utilization of the unique properties of mesoporous silica nanoparticles and versatile features of monoliths could be a promising strategy for enantioseparation.

1. Introduction

The need for fast and effective analytical tools for chiral separations is continuously growing because that understanding the pharmacodynamic and pharmacokinetic effects of single enantiomer on the metabolism of chiral drugs is key for the development of novel drugs [1]. Combining the high selectivity of high-performance liquid chromatography (HPLC) with the high efficiency of capillary electrophoresis (CE), chiral capillary electrochromatography (CCEC) is a powerful microanalytical technique as it provides fast and efficient separation with short analysis time, high enantioresolution, and low consumption of expensive and exotic chiral stationary phase. As one category of capillary columns in CEC, the monolithic capillary column gains its popularity in the field of chiral separation due to its high permeability, fast mass transfer and easy preparation [2–6].

The use of monolithic column for chromatographic separation has been recently reviewed [7,8] and in both of these reviews the application of nanoparticles (NPs) to fabricate capillary monolithic columns has improved separation performance in terms of efficiency and selectivity, especially for polymer monolithic column. Due to their large surface-to-volume ratio and specific physical and chemical properties, NPs including single-walled carbon nanotube [9], gold nanoparticles [10,11] as well as graphene oxide [12] have begun to be used in capillary monolithic columns for enantiomeric separation and demonstrated great potential in chiral discrimination.

In recent years, silica nanoparticles (SiNPs) have received a great deal of attention because of their intriguing properties, such as high surface area, good biocompatibility, high organic solvent resistance and easy postmodification with different functional groups. For chromatographic enantioseparation, silica nanoparticles are usually functionalized firstly and then added to background electrolyte solution or dynamically coated on the inner of the capillary to alter EOF, increase column capacity and improve separation selectivity and column efficiency in CE or CEC [13–15]. However, one drawback of this method is that the dynamic coating layer is unstable and another limitation is the requirement to form a stable, uni-

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http://dx.doi.org/10.1016/j.jpba.2017.03.013
0731-7085/

form suspension of nanoparticles in the electrolyte. In order to obtain a stable stationary phase, it's a good choice to immobilize NPs on the capillaries. Incorporating SiNPs into monolithic column is perceived as an effective method to immobilize SiNPs, which is due to the fact that monolithic column is ideal support for holding NPs thus allowing the exploitation of the widely differing selectivity offered by the unique properties of NPs [16,17]. This is evidenced by Zhang [18,19] and Liu et al. [20]. Nevertheless, these studies were only focused on the separation of small molecules, proteins as well as peptides, and no further study for chiral separation has been investigated. In addition, Dong et al. [21] immobilized the MCM-41 mesoporous silica nanoparticles onto the inner wall of a bare fused-silica capillary and subsequently coated cellulose tris(3,5-dimethylphenyl-carbamate) (CDMPC) as chiral selector. Compared with a bare fused-silica capillary column coated with CDMPC under the same coating procedure, this CDMPC-coated MCM-41 open-tubular (OT) capillary column offered significantly higher enantioselectivity for the separation of eight enantiomers, but the analysis time was long and some enantoimers even were eluted as long as in 42 min which is not meeting the requirements of fast separation.

In this study, for the purpose of obtaining a rapid, efficient and stable enantiomeric separation media, we fabricated a novel NH_2-MSN incorporated chiral hybrid monolithic column by immobilizing pepsin as coated ligand (pepsin-based poly(GMA-EDMA-NH_2-MSN) monolithic column). Pepsin, as a kind of aspartic protease, is usually used as a bioreactor and less attention has been paid to its chiral discrimination properties. The first chiral stationary phase based on pepsin was introduced by Haginaka et al. [22] in 1995 and a variety of basic and uncharged enantiomers were resolved. However, the scope of enantiomers which pepsin can separate remain nearly constant and its enantioseparation capacity has hardly improved after that [23]. In 2014, our group tried to immobilize pepsin on capillary silica monolith and polymer monolith, but the results were not well and there were only four enantiomers could be baseline or partially separated [24,25]. Because the chiral separation performance of protein-immobilized monolith is positively correlated to the protein bonding quantity [26,27], this phenomenon may due to the limited binding sites for pepsin on the capillary monolith. NH_2-MSN not only possess the stability necessary for enantiomeric separation media but also provide function groups to immobilize pepsin, which can enhance the capacity for chiral resolution. The pepsin-based poly(GMA-EDMA-NH_2-MSN) monolithic column was successfully applied to the enantioseparation of fifteen pairs of basic chiral drugs in ten minutes by CEC, of which eight pairs were not chiral separated by pepsin in previous literatures [22–25]. Finally, the prepared column was also applied for analysis of pharmaceutical formulations of amlodipine.

2. Experimental

2.1. Chemicals and materials

Commercial mesoporous silica nanoparticles (150 nm diameter, 1100–1200 m^2/g) were obtained from XFNANO Materials Tech Co. Ltd. (Nanjing, China). GMA, EDMA, 2,2′-azobisisobutyronitrile (AIBN), propanol, 1,4-butanediol, γ-methacryloxypropyltrimethoxylsilane (γ-MAPS), 3-aminopropyltriethoxysilane (APTES) and glutaraldehyde (GA) were purchased from Aladdin Chemistry (Shanghai, China). HPLC-grade methanol was from Jiangsu Hanbon Science & Technology Co. Ltd. (Nanjing, China). Thiourea, toluene, ammonium hydroxide, acetic acid and hydrochloric acid were commercially available from Nanjing Chemical Reagent Co. Ltd. (Nanjing, China). Ammonium acetate and sodium hydroxide were from Xilong Chemical Co. Ltd. (Shantou, China). Pepsin from porcine stomach mucosa was from Sigma–Aldrich (Shanghai, China). Deionized water was used in all of experiments, including synthetic reaction and mobile phase preparation. (±) Azelastine and (±) salmeterol were obtained from Jiangsu Hengrui Medicine Co. Ltd. (Lianyungang, China). (±) Atenolol and (±) clenbuterol were obtained from National Institutes for Food and Drug Control (Beijing, China). Other racemic drugs were from Dinghui Chemical Industrial Co. Ltd. (Wuhan, China). Amlodipine Besylate Tablets (5 mg) and Levamlodipine Besylate Tablets (2.5 mg) were obtained from local drugstore.

The morphology of the prepared monolithic column was investigated by SEM (Hitachi 3400N, Japan). The surface content of NH_2-MSN on the monolithic column was characterized by energy dispersive X-ray spectrometry (EDS, Hitachi 3400N, Japan). Fourier-transform infrared spectra were collected on FTIR-8400S (Shimadzu, Japan) using KBr pellet. Porous properties were obtained by nitrogen adsorption on Micromeritics ASAP 2020 V4.00 (Micromeritics, USA). Bare fused-silica capillaries (75 μm i.d. × 365 μm o.d.) were supplied by Yongnian Rui-feng Chromatographic Devices Co. Ltd. (Handan, China).

2.2. Sample and mobile phase preparation

The mobile phase of ammonium acetate-acetic acid buffer with pH from 4.2 to 5.8 was prepared by dissolving an exact amount of ammonium acetate in deionized water and adjusted by acetate. The chiral drugs used in this study were dissolved in deionized water or methanol and diluted to a proper concentration. The pharmaceutical formulation of amlodipine was crushed and suspended in methanol/H_2O (v/v, 50/50). All above solutions were filtered through nylon membrane filters of 0.22 μm pore size prior to experiments and stored at 4 °C when not in used.

2.3. Preparation of pepsin-based poly(GMA-EDMA-NH_2-MSN) monolithic column

2.3.1. Synthesis of poly(GMA-EDMA-NH_2-MSN) monolithic column

Modification of the mesoporous silica nanoparticles with amino groups was carried out by reaction with APTES, according to the previously reported method [28]. Fused-silica capillary was pretreated with 1 mol L^{-1} NaOH for 1 h, 0.1 mol L^{-1} HCl for 0.5 h, washed with water to neutral and then with methanol for 10 min, followed by a drying step with nitrogen at 120 °C for 2 h. Then, the pretreated capillary was silanized by γ-MAPS (50%, v/v) in methanol and reacted at 50 °C overnight in order to provide covalent attachment of polymer. Afterwards, the capillary was rinsed with methanol and dried with nitrogen. For the fabrication of NH_2-MSN incorporated monolithic column, NH_2-MSN were added in propanol to create a white and homogeneous dispersion at a concentration of 10 mg mL^{-1} under vortex for 3 min and ultrasonication for 1 h. A mixture of 21.96% (v/v) GMA, 7.47% (v/v) EDMA, 8.19% (v/v) 1,4-butanediol, 62.38% (v/v) propanol containing NH_2-MSN and 0.32% (m/v) AIBN was introduced into the preconditioned fused-silica capillary to a length of 22 cm with another 13 cm empty and reacted at 46 °C for 12 h. Finally, to remove the unreacted monomers and porogens, the obtained monolithic column was immensely washed with methanol and water. The synthetic step of monolithic column containing NH_2-MSN was shown in Fig. 1A.

2.3.2. Pepsin immobilization

As presented in Fig. 1B, pepsin was covalently immobilized onto the surface of monolithic column by GA method. First, 25% (m/v) ammonium hydroxide was pumped into the monolithic column and reacted at 40 °C for 5 h. After being rinsed with water to wash the unreacted ammonium hydroxide the col-