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Preparation of a biomimetic polyphosphorylcholine monolithic column for immobilized artificial membrane chromatography

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ABSTRACT

The present work aims to prepare a novel phosphatidylcholine functionalized monolithic stationary phase by *in situ* co-polymerization of 12-methacryloyl dodecylphosphocholine (MDPC) and ethylene dimethacrylate (EDMA) for immobilized artificial membrane chromatography. Scanning electron microscopy, energy dispersive X-ray spectroscopy, FT-IR spectroscopy, pore size distribution analysis, ζ-potential analysis and micro-HPLC were used to evaluate the monolithic structure and physicochemical properties. Satisfactory morphology, high mechanical stability, good permeability and chromatographic performance were obtained on the optimized monolithic columns. A typical reverse-phase retention mechanism was observed over a wide range of organic solvent content (acetonitrile< 80%). The optimized poly(MDPC-*co*-EDMA) monolith exhibited good selectivity for proteins and basic drugs. Good correlation was observed between the retention on commercial IAM column (IAM.PC.DD2) and poly(MDPC-*co*-EDMA) monolith. This novel poly(MDPC-*co*-EDMA) monolith exhibited good potential for studying the drug-membrane interaction.

1. Introduction

Cell membranes provide a stable environment for protecting the organelles and also keep undesired particles from entering the cell body. It has become increasingly clear that drug-membrane interactions play an important role for drug activity, selectivity, and toxicity [1]. For a better understanding of drug-membrane interactions and its related physiological activities, artificial membranes, which have many striking similarities to biological membranes, have been developed as simple models to mimic the cell membranes' environment in various techniques [2]. For example, immobilized artificial membrane (IAM), prepared by covalently immobilizing a monolayer of phospholipids to the surface of chromatographic supports [3,4], have been applied successfully to predict drug membrane transport and protein purification [4–6]. The first silica based IAM column, which was referred to as IAM.PC, was prepared by linking phosphatidylcholine (PC) analogue covalently to silica-propylamine through their ω-carboxylic group on the C2 fatty acid chain by Charles Pidgeon in 1989 [3]. Because of the steric hindrance of phospholipid groups, some residual free propylamine groups still remain on the silica surface and further decrease the chemical stability of IAM.PC surfaces [7,8]. In order to improve the chemical stability of IAM.PC surfaces, the residual free propylamines are end-capped with glycidol/methyl glycidol (MG) or decanoic/propionic anhydride [9]. Both surface end-capped IAM.PC stationary phases are commercially available now and called IAM.PC.MG and IAM.PC.DD2, respectively. A single-chain phosphatidylcholine functionalized silica column with the commercial name IAM.PC.DD was also prepared through the reaction between 11-carboxylundecylphosphocholine and propylamine functionalized silica to increase the surface density of phospholipids and stability under acidic conditions [10]. However, the unreacted silanol groups and residual amino groups still exist on end-capped IAM surfaces [8,11], which could elicit silanophilic interactions and hydrolysis of amide bonds. The preparation processes is not trivial and is laborious [3,10,12], which limited their application for self-lab preparation. Therefore, it is of great interest to develop a novel preparation process of IAM stationary phases. Recently, Verzele et al. prepared a sphingomyelin functionalized IAM column using the amide to substitute the esters of IAM.PC through a solid phase synthesis method for the first time [13], which not

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only enhanced column lifetime and reliability of IAM columns but also provided an *in vitro* prediction model for blood-brain barrier permeability of drugs. In addition to the traditional covalent immobilization strategies, a few of the non-covalent immobilization methods have also been reported. For instance, Krause et al. coated the ditetradecanoyl-sn-glycero-3-phosphocholine onto the C18 stationary phase through hydrophobic interactions [14]. Liposomes can also be immobilized onto the surface of various beaded matrices through hydrophobic interaction [15,16]. However, poor stability and the possible loss of phospholipid remain major issues for these non-covalent immobilization approaches.

Monolithic columns have been extensively studied and applied since it was introduced to micro-HPLC in 1989 by Hjerten et al. [17,18]. Compared to silica-based packing columns, monolithic columns exhibit several advantages, such as easy preparation, good permeability, biocompatibility and pH tolerance. Few studies have been focused on IAM monolithic columns. Previously, Jiang and colleagues prepared a zwitterionic polyphosphorylcholine monolithic column for hydrophilic interaction chromatography through thermal co-polymerization of 2-methacryloyloxyethyl phosphorylcholine (MPC) and ethylene dimethacrylate (EDMA) within 100 μm I.D. capillaries [19]. Although this column could not effectively mimic the drug-membrane interaction because of the weak hydrophobic interaction, it demonstrated the possibility to prepare IAM monolithic columns. Subsequently, a mixed phospholipids functionalized monolithic column, through a facile one-pot copolymerization of a single-chain phosphatidylcholine and phosphatidylserine containing monomers, was developed. The resulting columns exhibited great prediction accuracy for drug induced phospholipidosis risk [20].

In this study, a long alkyl chain phosphatidylcholine functionalized monolithic column was prepared and evaluated for IAM chromatography. 12-Methacryloyl dodecylphosphocholine (MDPC) and EDMA were used as functional monomer and crosslinker, respectively. The composition of the polymerization mixture was carefully optimized in order to obtain satisfactory performance with respect to column permeability, efficiency and selectivity. The morphology, surface chemistry, stability, permeability and reproducibility of the monolithic columns were systematically evaluated using scanning electron microscopy (SEM), energy dispersive X-ray spectroscopy (EDX), FT-IR spectroscopy, pore size distribution analysis, ζ-potential analysis and micro-HPLC. The optimized phosphatidylcholine functionalized monolithic columns were subsequently applied for protein and basic drugs separation. In order to further evaluate the applicability of poly(MDPC-*co*-EDMA) monolith for studying the drug-membrane interaction, a series of 71 commercial drugs with diverse chemical structure were selected as test compounds. Their retentions on both poly(MDPC-*co*-EDMA) monolith and commercial IAM.PC.DD2 column were measured and compared.

2. Experimental

2.1. Chemicals and materials

EDMA, azobisisobutyronitrile (AIBN), 1,4-butanediol, *iso*-propanol, 3-methacryloxypropyltrimethoxysilane (γ-MAPS), ammonium acetate, sodium hydroxide, methacryloyl chloride, 2-chloro-1,3,2-dioxaphospholane-2-oxide, pyridine, triethylamine, acetic acid, tetrahydrofuran, trifluoracetic acid, ammonium hydroxide were all purchased from Aladdin Chemicals (Shanghai, China). Lysozyme egg, ribonuclease, cytochrome C, bovine serum albumin were purchased from Sigma–Aldrich (St. Louis, MO, USA). HPLC-grade methanol (MeOH) and acetonitrile (ACN) were obtained from Merck (Shanghai, China). All compounds listed in Table S1 were purchased from Sigma-Aldrich or provided by other labs. The fused silica capillaries with an internal diameter of 100 μm (375 μm O.D.) were purchased from Yongnian Optic Fiber Plant (Hebei, China). Distilled water was filtered through a 0.22 μm membrane before use. Phospholipid monomer MDPC was synthesized in our lab.

2.2. Instrumentation

Mass spectra (MS) were carried out on a Shimadzu LC-MS-2010A instrument with an ESI or APCI mass selective detector. ^{1}H and ^{13}C NMR spectra were performed using tetramethylsilane (TMS) as the internal standard in CD_3OD or $CDCl_3$ with a Bruker-AVANCE III Digital NMR Spectrometer (Karlsruhe, Germany) at 400 MHz and 100 MHz, respectively. FT-IR spectra of the samples prepared as KBr pellets in the range from 4000 to 400 cm^{-1} were recorded at room temperature with an Equinox55 spectrometer (Bruker, Billerica, MA, USA). The pore size distribution was measured using a Quantachrome PoreMaster 60G Instrument Mercury Porosimetry (Florida, USA). A Jinghong DK-S22 water bath (Shanghai, China) was used for thermal initiated co-polymerization. All SEM and EDX experiments were carried out using an ultra-high resolution Hitachi S-4800 SEM equipped with an attached EDX system (Tokyo, Japan) at an acceleration voltage of 3 or 10 kV. All micro-HPLC experiments were performed on a Dionex Ultimate 3000 RSLC nano system equipped with an Ultimate 3000 Binary Rapid Separation nano flow pump, an Ultimate 3000 RS variable wavelength detector with a 3 nL flow cell, an Ultimate 3000 RS autosampler and a four-port injection valve with 20 nL internal loop or a six-port injection valve with 1000 nL sample loop (Houston, TX, USA). Data acquisition and date handling were performed using Chromeleon 6.8. All HPLC experiments were performed on an Agilent 1260 HPLC system (Agilent Technologies, Waldbromm, Germany) equipped with a diode-array detector. All chromatograms were converted into .txt files and then redrawn using Microcal Origin 8.5. A Regis IAM.PC.DD2 column (100 mm × 4.6 mm I.D., 10 μm particle size) was purchased from Regis Technologies Inc. (Morton Grove, Illinos, USA).

2.3. Synthesis of MDPC

MDPC was synthesized according to previously reported method with proper modification [21] (Fig. 1). After obtaining the intermediate 2 according to [20], MDPC was prepared as following:

A dry Schlenk-flask was charged with 5.8 g of **2** (21.5 mmol), 60 mL of dry tetrahydrofuran and 3.0 mL of triethylamine (21.5 mmol). After cooling to −30 °C, 2.0 mL of 2-chloro-1,3,2-dioxaphospholane-2-oxide (45.4 mmol) were added dropwise over 1 h. The temperature was held at −30 °C for 10 h. Then 2.0 mL (53.5 mmol) of separately condensed anhydrous trimethylamine were quickly added via syringe. The resulting solution was stirred vigorously for 10 min at −30 °C and then heated to 60 °C for 35 h. The turbid solution was allowed to cool down to room temperature, followed by storage for 24 h at 4 °C and 24 h at −20 °C. The precipitate was filtered under argon and washed with a small amount of cold ACN. Then the precipitate was purified by flash column chromatography on silica gel (CH_2C1_2/MeOH/H_2O, 65/25/5, v/v/v) to give **3** (4.0 g, 9.2 mmol, 43%) as a white solid. ESI-MS *m*/z: 436.2 $[M+H]^+$. ^{1}H NMR (400 MHz, CD_3OD) δ 6.07–6.01 (m, 1H), 5.60–5.50 (m, 1H), 4.26–4.12 (m, 2H), 4.07 (t, 2H), 3.81 (m, 2H), 3.61–3.53 (m, 2H), 3.18 (s, 9H), 1.89–1.83 (m, 3H), 1.66–1.50 (m, 4H), 1.38–1.19 (m, 16H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 167.52, 136.49, 125.17, 66.20, 65.61, 64.79, 59.18, 54.21, 30.95, 29.72, 29.59, 29.29, 28.59, 25.98, 25.92, 18.32.