Journal of Chromatography A, xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Preparation and evaluation of a hydrophilic interaction and cation-exchange chromatography stationary phase modified with 2-methacryloyloxyethyl phosphorylcholine

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ARTICLE INFO

Article history:
Received 18 October 2017
Received in revised form 7 February 2018
Accepted 27 February 2018
Available online xxx

Keywords:
2-Methacryloyloxyethyl phosphoryl choline
Hydrophilic interaction chromatography
Weak cation exchange chromatography
Polar compounds
Protein

ABSTRACT

In this work, 2-methacryloyloxyethyl phosphorylcholine (MPC) was used as a ligand to prepare a novel mixed-mode chromatography (MMC) stationary phase by the thiol-ene click reaction onto silica (MPC-silica). It was found that this MPC-silica showed the retention characteristics of hydrophilic interaction chromatography (HILIC) and weak cation exchange chromatography (WCX) under suitable mobile phase conditions. In detail, acidic and basic hydrophilic compounds and puerarin from pueraria were separated quickly with HILIC mode. Meanwhile, six standard proteins were allowed to reach baseline separation in WCX mode, and protein separation from egg white was also achieved with this mode. In addition, reduced/denatured lysozyme could be refolded with the MPC-silica column. In the meantime, the MPC-silica has been applied for refolding with simultaneous purification of recombinant human Delta-like1-RGD (rhDll1-RGD) expressed in *Escherichia coli*. The results show that the mass recovery and purity of rhDll1-RGD could reach 63.4% and 97% by one step, respectively. Furthermore, the reporter assay results demonstrated that refolded with simultaneously purified rhDll1-RGD could efficiently activate the signalling pathway in a dose-dependent manner. In general, this MPC-silica has good resolution and selectivity in the separation of polar compounds and protein samples in different high-performance liquid chromatography (HPLC) modes, and it successfully achieved refolding with simultaneous purification of denatured protein.

1. Introduction

Mixed-mode chromatography (MMC) is a type of chromatography method in which two or more than two mechanisms of action taking place during the separation. The superiority of MMC is that it has great flexibility and versatility in the separation of a mixture sample [1,2]. Compared with traditional chromatography, MMC is advantageous due to its high loading, high resolution and unique selectivity [3–5]. Thus far, some types of MMC have been reported, such as reverse-phase liquid chromatography (RPLC)/ion-exchange chromatography (IEC) [6–8], RPLC/hydrophilic interaction chromatography (HILIC) [9–12], HIILC/IEC [13,14], RPLC/HIILC/IEC [15,16], etc.

Among them, HILIC/IEC is one of the MMC techniques that uses the hydrophilicity or the charge difference of solutes as the chromatographic mechanism to separate different solutes. Since this MMC incorporates two widely used separation mechanisms, peptides can be selectively separated according to their differences in affinity/hydrophobicity and charges [17]. Hartmann [18] compared RPLC with HILIC/CEX for the separation of amphipathic ahelical peptides with L-amino acids and D-amino acid substitutions, and the results show that although the selectivity of the two methods is different, the peptide can be effectively separated. Although RPLC is still used to separate peptides, HILIC/CEX has been equal to or even beyond the performance of RPLC in the separation of peptide mixtures [19]. A cationic hydrophilic interaction monolithic stationary phase based on the copolymerization of 2-(methacryloyloxy)-ethyltrimethylammoniummethyl sulphate (META) and pentaerythritol triacrylate (PETA) was prepared for performing capillary liquid chromatography, which mainly showed the hydrophilic characteristic when the concentration of acetonitrile in the mobile phase was above 20% [20].

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https://doi.org/10.1016/j.chroma.2018.02.059
0021-9673/

The stationary phase mainly depended on the hydrophilic interaction when separating the neutral compounds, while the hydrophilic interaction and cation exchange interaction combined to act when separating the basic nucleoside, basic group and benzoic acid derivatives so that the analyte is highly separated and no tailing phenomenon occurs. The HIILC/IEC MMC stationary phase is a significant tool to separate hydrophilic compounds [21,22].

The phosphorylcholine (PC) group is the terminal hydrophilic group in the outer layer of the cell membrane double molecule layer, which has a zwitterionic structure with an equal amount of positive and negative charges. The polymers containing PC groups were coated or grafted to prepared biomimetic materials for exploitation in a broader range of technologies like physiological environments [23–25]. This kind of material has been proven to have the potential of nonspecific selectivity and application in protein separation by RPLC media containing PC polymer and zwitterion exchange chromatography media [26–29]. The earliest synthetic polymeric monomer containing a PC group was 2-methacryloyloxy ethylphosphorylcholine (MPC) [30]. Regarding its molecular structure, MPC has a hydrophilic polar dipole ion and hydrophobic non-polar long-chain alkyl, and thus, it can be modified on the surface of silica to prepare a HILIC/HIC stationary phase with a biomimic structure [31,32].

On the one hand, the PC zwitterionic functionality has both a positively charged quaternary ammonium group and a negatively charged phosphoric group, but research has showed that materials with a MPC group reveal electronegativity over a wide pH range [33]. Therefore, the MPC could be used as a ligand to prepare a cation exchange chromatography (WCX) mode stationary phase. Previous experiments have found that MPC was grafted onto silica to obtain a zwitterionic chromatography (ZIC) stationary phase containing PC groups, and we also synthesized a ZIC mode stationary phase by modifying phosphorylcholine dichloride containing PC groups onto silica, which can rapidly separate the acidic and alkaline proteins from egg white [29]. The above results show that the stationary phase containing PC groups has good separation properties and selectivity.

MPC has a hydrophilic cation/anion group, which is highly hydrated. A zwitterionic stationary phase was synthesized by the graft polymerization of MPC onto the surface of porous silica particles for performing the ZIC/HILIC mode [31]. It was shown that small peptides can be separated in HILIC mode. After that, Jiang [34] prepared porous zwitterionic monolith by the thermal copolymerisation of MPC and ethylene glycol dimethacrylate (EDMA) within capillaries. This stationary phase showed very good selectivity for a range of polar test analytes, especially small peptides, and the reason might be the good biocompatibility of the PC functionality. Additionally, as a supplement for RPLC and substitution for traditional chromatography, HILIC showed superiority and potential for analysing polar and hydrophilic compounds. There are many polar components contained in Chinese medicine that cannot be retained in RPLC. Jin [35] constructed a chromatographic fingerprint of Ligusticum chuanxiong with HILIC and RPLC to gain more chemical information about the polar compounds and weakly polar compounds contained in Chinese medicine. Flavonoids in traditional Chinese medicine were well-preserved but could not be separated on RPLC, while Zhang [36] successfully separated the flavonoids from the same fractions on RPLC using HILIC.

In this work, we selected MPC as the ligand to synthesize a MMC stationary phase based on silica by the "thiol-ene" click reaction. This ligand could provide electrostatic adsorption, electrostatic repulsion and hydrophobic interaction. The separation properties and retention characteristics in HILIC and WCX modes as well as refolding with simultaneous purification of reduced/denatured lysozyme and inclusion body of recombinant human Delta-like1-RGD (rhDll1-RGD) were investigated. A novel high performance MMC, with the stationary phase having HILIC/WCX MMC functionality that was prepared, could be used as effective strategy for polar compounds, protein separation, and refolding with simultaneous purification of denatured proteins.

2. Materials and methods

2.1. Chemicals and materials

Full porous spherical silica (5 μm diameter, 180 Å pore size for protein separation; 100 Å pore size for nucleoside separation) were purchased from Lanzhou Institute of Chemical Physics (Lanzhou, China). Sodium dodecyl sulfate (SDS), acrylamide,lysozyme(Lys), cytochrome (Cyt-c), myoglobin(Myo), α-Chymotrypsinogen A(α-ChyA), Ribonuclease A(RNase A), Ribonuclease B(RNaseB),thymidine, uridine, adenosine, cytidine, guanosine,Glutathione oxidized (GSSG),Glutathione(GSH) and isopropylthio-β-D-galactoside (IPTG) were purchased from Sigma-Aldrich(St Louis,MO, USA). 2-methacryloyloxyethyl phosphorylcholine (MPC) was derived from Joy Natural Institute of Technology (Nanjing, China). γ-mercaptopropyltrimethoxysilane was obtained from Aladdin (Shanghai, China). Azodiisobutyronitrile (AIBN) was obtained from Xi'an Wolsen Bio-technology Co., Ltd (Shanxi, China). All other chemical reagent is of analysis grade.

2.2. Instrumentation

All chromatographic tests were conducted using an LC-20A chromatographic system (Shimadzu, Japan), including two LC-20ATvp pumps, an SCL-10Avp system controller, an SPD-20Avp UV–vis detector, and a N-2000 chromatography workstation. Samples were injected through a Rheodyne 7725i valve. The stationary phase was characterized by elemental analyzer (Varios EL III, Elementar Co., Ltd, Germany) and X-ray photoelectron spectroscopy (XPS) (K-Alpha Thermo Electron Corporation, USA). A Mini-Protean II electrophoresis system (Bio-Rad, Hercules, CA, USA) was used for SDS–polyacrylamide gel electrophoresis assays.A Dual wavelength thin layer scanner Cs-9301PC (Shimadzu, Japan), and a MALDI-TOF-MS (Axima CFR plus, Kratos,Shimadzu, Japan).

2.3. Preparation of MPC-modified silica stationary phase

2.3.1. Preparation of mercaptopropyl silica

2.0 g of silica were dispersed in 50 mL of dried toluene in a 250 mL three-necked flask. After the mixture was degassed by purging it with nitrogen for 30 min with stirring, 1.0 mL of γ-mercaptopropyltrimethoxysilane was added slowly. Under nitrogen atmosphere,the reaction mixture was stirred at 110 °C for 24 h. The obtained mercaptopropyl silica was filtered, washed with toluene, acetone, methanol in sequence,and dried under vacuum at 60 °C for 12 h.

2.3.2. Grafting of MPC-modified silica and packing

The prepared mercaptopropyl silica (2.0 g), 0.65 g MPC and 0.04 g AIBN were dispersed into 50 mL absolute ethanol in a three-necked flask. The reaction system was purged with nitrogen for 30 min to remove the air and performed for 24 h at 60 °C with stirring. Then the product was filtered, washed with ethanol, water and methanol in sequence, and dried under vacuum for 12 h at 60 °C. The obtained white spherical solid is the MPC-silica.

The MPC-modified silica was slurry-packed into the stainless-steel column (100 mm × 4.6 mm i.d.) by using methanol as the slurry solvent and propulsion solvent under 30–40 Mpa for 30 min.

Please cite this article in press as: C. Xiong, et al., Preparation and evaluation of a hydrophilic interaction and cation-exchange chromatography stationary phase modified with 2-methacryloyloxyethyl phosphorylcholine, J. Chromatogr. A (2018), https://doi.org/10.1016/j.chroma.2018.02.059