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Monolithic capillary columns synthesized from a single phosphate-containing dimethacrylate monomer for cation-exchange chromatography of peptides and proteins

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ABSTRACT

Monoliths containing phosphoric acid functional groups were synthesized from only one monomer, bis[2-(methacryloyloxy)ethyl] phosphate (BMEP), in 75-μm i.d. UV transparent fused-silica capillaries by photo-initiated polymerization for cation exchange chromatography of peptides and proteins. Various synthetic conditions, including porogen solvents, monomer concentration, and polymerization time, were studied. The hydrophobicities of the resulting monoliths were evaluated using propyl paraben under reversed-phase conditions and synthetic peptides under ion-exchange conditions. These monoliths exhibited low hydrophobicities and relatively low porosities due to their highly cross-linked structures. A dynamic binding capacity (lysozyme) of 73 mg/mL of column volume was measured using the best performing monolith. Synthetic peptides were eluted in approximately 30 min without addition of acetonitrile to the mobile phase, yielding a peak capacity of 28. Efficiencies of 52,900 plates/m for peptides and 71,000 plates/m for proteins were obtained under isocratic conditions. The effects of separation conditions, i.e., mobile phase pH and salt gradient rate, were studied. Good run-to-run reproducibility was achieved with a relative standard deviation (RSD) less than 1.5% for retention times of proteins. The column-to-column retention time reproducibility for peptides was less than 3.5% RSD. A monolithic column was used to follow the deamidation of ribonuclease A. The kinetics of deamidation were founded to be first order with a half life of 195 h. A cytochrome C digest was also separated using a linear gradient of sodium chloride.

1. Introduction

Ion-exchange chromatography (IEC) is an important chromatographic separation mode for analytes that can participate in ionic interactions, such as peptides, proteins, oligonucleotides, and viruses [1–4]. Compared to other chromatographic separation modes, such as reversed-phase chromatography (RPC) and affinity chromatography, IEC employs mild separation conditions that avoid denaturing of the proteins. IEC is often used in two-dimensional separations for peptide mapping of protein digests [5,6], where it is followed by RPC, which is an orthogonal separation mechanism.

Monoliths are considered to be emerging chromatographic stationary phases, which provide an alternative to particle packed columns. They provide lower back pressure in liquid chromatography (LC) and are easy to prepare. Several reviews have described the synthetic methods and applications of monoliths in LC [7–14]. Polymeric monolithic columns are typically used to separate biomolecules due to their pH stability and biocompatibility. Several approaches, including adsorption of surfactants [15,16], post-modification [17–20], and copolymerization [21–25], have been utilized to introduce functional groups into monolith backbones to prepare capillary cation-exchange columns. Among these approaches, copolymerization is the simplest, since it only requires one step, which leads to easy control of the total concentration of functional groups in the monolith. However, monoliths synthesized according to this approach suffer from low density of functional groups on the surface. Most of the functional groups are buried in the body of the monolith. Fortunately, the porogen solvents can be carefully optimized to produce more functional groups on the surface, thereby increasing the dynamic binding capacity (DBC) of the monolith [23–25].

Recently, several ion-exchange monoliths were prepared by various methods. Krenkova et al. [26] grafted 2-acrylamido-2-methyl-1-propanesulfonic acid and acrylic acid on a poly(2,3-dihydroxypropyl methacrylate-co-ethylene methacrylate) mono-

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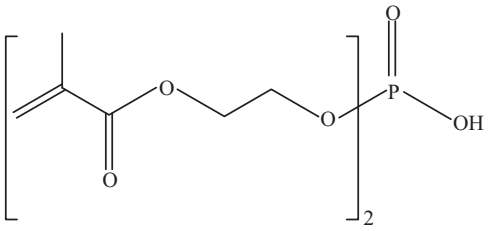

BMEP

Fig. 1. Chemical structure of BMEP.

lith to prepare strong and weak cation-exchange monoliths for separation of peptides and proteins. Good separations were obtained. DBC values of 29 and 21 mg/mL for strong and weak cation-exchange monoliths were measured. However, no peak capacities were reported for separations of peptides and proteins. Past work in our laboratory has been based on *in situ* copolymerization to prepare several cation-exchange monolithic columns [23,25,27]. Excellent separations of peptides and proteins were achieved in 15 min with peak capacities over 25.

Normally, a monomer and a cross-linker are the major components used to prepare a monolith. In this work, only a cross-linker was used to prepare a cation-exchange polymeric monolith. With only one monomer, the reproducibility of synthesized monoliths should improve, and the resulting highly cross-linked structure should ensure long-term stability. However, only a few studies of monoliths synthesized from one monomer have been reported. Lubbad and Buchmeiser [28] used tetrakis(4-vinylbenzyl)silane (TVBS) to prepare a highly cross-linked polymer to separate both low, medium and high-molecular-weight analytes. The monolith showed low swelling propensity due to the highly cross-linked structure. Greiderer et al. [29] used 1,2-bis(p-vinylphenyl)ethane (BVPE) to obtain a monolith for simultaneous separation of low and high-molecular-weight compounds. The resulting monolith showed a broad bimodal pore size distribution from flow-through channels in the μm range, and mesopores to small macropores in the range of 4–500 nm. Tremendous enhancement of surface area (101 m^2/g) was obtained compared to typical organic monoliths (20 m^2/g). Good reproducibility and low swelling propensity were also achieved due to highly cross-linked structures. Recently, a series of monoliths prepared for hydrophobic interaction chromatography was reported by our group using either polyethylene glycol diacrylate or dimethacrylate as single monomer [30]. Excellent separations of proteins with good peak capacities were obtained. The limited number of reports on monoliths synthesized from one monomer primarily results from the limited availability of suitable monomers.

In this work, bis[2-(methacryloyloxy)ethyl] phosphate (BMEP) (chemical structure shown in Fig. 1) was used as a single monomer to prepare polymeric cation exchange monolithic columns for LC by *in situ* photo-initiated copolymerization. The performance of these columns for separation of peptides and proteins under both isocratic and gradient ion-exchange conditions are presented.

2. Experimental

2.1. Materials and chemicals

Uracil, 2,2-dimethoxy-2-phenylacetophenone (DMPA, 99%), 3-(trimethoxysilyl)propyl methacrylate (TPM, 98%), BMEP, a natural peptide mixture (H2016), peptides (i.e., D-Leu-Gly, Gly-Gly-Tyr-Arg, Gly-Tyr, angiotensin II, and leucine enkephalin), proteins (i.e., trypsinogen from bovine pancrease, ribonuclease A from bovine pancrease, cytochrome C from bovine heart, α-chymotrypsinogen A from bovine pancreas, trypsin from bovine pancreas, and lysozyme from chicken egg white) were purchased from Sigma–Aldrich (Milwaukee, WI) and used without further purification. Propyl paraben was purchased from Spectrum (Gardena, CA). A synthetic peptide standard (CES-P0050) was obtained from Alberta Peptides Institute (Edmonton, Alberta, Canada). Solvents for monolith synthesis and chemicals for mobile phase preparation were HPLC or analytical reagent grade. Fused-silica capillaries (75-μm i.d. × 360-μm o.d.) were purchased from Polymicro Technologies (Phoenix, AZ, USA).

2.2. Preparation of polymeric monolithic columns

UV-transparent fused-silica capillaries were first silanized with TPM to introduce pendant vinyl groups to anchor the polymer monolith to the capillary wall [24,31]. Polymeric monoliths were prepared as previously described [23]. The polymerization mixture was prepared in a 4 mL glass vial by mixing initiator, monomers, and porogens. The mixture was vortexed and ultrasonicated for 1 min to help form a homogeneous solution and eliminate oxygen. The monomer solution was introduced into the capillary by capillary action. The capillary was placed directly under a PRX 1000-20 Exposure Unit UV lamp (TAMARACK Scientific, Corona, CA) for various times (2–10 min). The resulting monolith was then flushed with methanol and water sequentially for 30 min each to remove porogens and unreacted monomers using an LC pump. The capillaries were stored in 10% methanol aqueous solutions to prevent the monoliths from drying. Scanning electron microscopy (SEM) images of the monoliths were obtained as previously described [23].

2.3. Capillary LC

Capillary LC of peptides and proteins was performed using an LC system comprised of two ISCO 100 DM syringe pumps and a flow controller. A Valco splitting tee (Houston, TX) was positioned between the static mixer of the syringe pumps and the 60-nL Valco internal loop sample injector. A 40-cm-long capillary (30-μm i.d.) was used as a capillary splitter and a 10-cm-long capillary (30-μm i.d.) was connected between the splitting tee and the injector. The mobile phase flow rate was set at 40 μL/min, and the actual flow rate in the monolithic capillary column was approximately 0.32 μL/min. The mobile phase was 5 mmol/L aqueous phosphate buffer at various pH values. All mobile phases were filtered through a 0.2 μm Nylon membrane filter (Supelco, Bellefonte, PA). A Model UV3000 detector from Thermo Separations (San Jose, CA) was used at a wavelength of 214 nm. Data were acquired with ChromQuest 2.5.1 software (ThermoQuest, San Jose, CA). Capillary LC of a cytochrome C digest was performed using an Ultimate 3000 high pressure gradient LC system (Dionex, Sunnyvale, CA) equipped with an FLM-3300 nano flow manager [30]. A section of 50 μm i.d. poly(vinyl alcohol)-coated fused silica capillary was used as the sample loop, and the loop volume was calculated to be 200 nL. Detailed chromatographic conditions are given in the figure captions.

For evaluation of the relative hydrophobicities of the monoliths, reversed-phase capillary LC elution measurements of propyl paraben and uracil were performed. The mobile phase was 20% (v/v) acetonitrile in water. The pump flow rate was 40 μL/min, and the detection wavelength was 254 nm. Uracil was used as an unretained marker. The retention factor for propyl paraben was obtained from the equation, $k = (t_p - t_u)/t_u$, where k is the retention factor, and t_p and t_u are the retention times of propyl paraben and uracil, respectively.