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Methacrylate monolithic columns functionalized with epinephrine for capillary electrochromatography applications☆

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ABSTRACT

Epinephrine-bonded polymeric monoliths for capillary electrochromatography (CEC) were developed by nucleophilic substitution reaction of epoxide groups of poly(glycidyl-methacrylate-co-ethylenedimethacrylate) (poly(GMA-*co*-EDMA)) monoliths using epinephrine as nucleophilic reagent. The ring opening reaction under dynamic conditions was optimized. Successful chemical modification of the monolith surface was ascertained by *in situ* Raman spectroscopy characterization. In addition, the amount of epinephrine groups that was bound to the monolith surface was evaluated by oxidation of the catechol groups with Ce(IV), followed by spectrophotometric measurement of unreacted Ce(IV). About 9% of all theoretical epoxide groups of the parent monolith were bonded to epinephrine. The chromatographic behavior of the epinephrine-bonded monolith in CEC conditions was assessed with test mixtures of alkyl benzenes, aniline derivatives and substituted phenols. In comparison to the poly(GMA-*co*-EDMA) monoliths, the epinephrine-bonded monoliths exhibited a much higher retention and slight differences in selectivity. The epinephrine-bonded monolith was further modified by oxidation with a Ce(IV) solution and compared with the epinephrine-bonded monoliths. The resulting monolithic stationary phases were evaluated in terms of reproducibility, giving RSD values below 9% in the parameters investigated.

1. Introduction

The development of monolithic separation media in capillary electrochromatography (CEC) and related techniques has generated considerable interest in the last years [1–3]. Within the two main types of monolithic materials, the advantages of polymer-based over silica-based monoliths are a simpler and faster preparation, greater choices of surface functionalities, wider pH stability, and better biocompatibility. A variety of organic polymer-based monoliths based on polymethacrylate [4,5], or polyacrylate [6,7], polyacrylamide [8,9] and polystyrene [10,11] have been extensively investigated. Among them, poly(GMA-*co*-EDMA) monoliths enable straightforward and efficient derivatization at the epoxy group with using nucleophilic substitution reactions (SN_2) [12–24]. In contrast to the single step copolymerization, post-modifications of the monolith allow the independent tuning of mechanical and flow-through porous properties and surface chemistry of the parent monolith. Functionalization at the epoxy groups of GMA-based monoliths with amines [12–14], amino acids [15], poly(ethylene imine) [16], sodium sulfite [17], sodium hydrogen sulfide [18], sulfuric acid [19] and a variety of chiral reagents [20–24], to provide monoliths with different chromatographic properties (ion-exchange, hydrophobic/hydrophilic, chiral, *etc.*), have been described. Also, an important limitation in the development of hydrophobic monoliths has been the difficulty to copolymerize hydrophobic monomers with the hydrophilic ionizable monomers required for the generation of EOF in CEC [25]. Within this concern, the multi-step post-polymerization approach provides an excellent way of harmonizing incompatible reagents, including hydrophobic and hydrophilic substances, low solubility ion-pairing agents as well as oxidizing and reducing agents.

An important aspect of post-modification of epoxy groups is the evaluation of the yield of the functionalization reaction on the monolith surface, which should provide quantitative information about the amount of ligand bonded to the monolith surface; however, in most cases, this information is not available. To our knowledge, few reports [18,26] have afforded procedures to determine the yield of the functionalization reaction of the monolith surface. Thus, the content of thiol groups bonded to a GMA-based monolith was evaluated by using a disulfide exchange reaction with excess dipyridyldisulfide. Bonding of thiol containing reagents

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to monoliths has been of utmost interest to further attach other chromatographic ligands as gold nanoparticles to the reactive thiol-substrates [27].

In this work, parent monolithic stationary phases based on GMA as reactive monomer and ethylene dimethacrylate (EDMA) as crosslinker were functionalized with epinephrine, and the resulting monoliths were characterized for CEC applications. As shown in Fig. 1, functionalization with epinephrine adds to the monolith surface an aromatic ionizable catechol group, as well as an amino nitrogen, a chiral carbon atom and an extra hydroxyl. Thus, hydrophobic/hydrophilic interactions, permanent and π–π induced dipole attraction and hydrogen bonding between this ligand and the analytes could be expected. In addition, bonded epinephrine can be easily oxidized to bonded epinephrine-quinone, which constitutes a simple way of modifying the properties of the stationary phase, including the chromatographic selectivity. The experimental conditions of the functionalization reaction of poly(GMA-*co*-EDMA) with epinephrine (involving epoxide groups opening) were optimized. The epinephrine-bonded monolith was morphologically characterized by SEM and Raman spectroscopy. Also, the concentration of the epinephrine bound to the monolith surface was determined by redox reaction between the catechol groups and Ce(IV), followed by spectrophotometric monitoring of the remaining Ce(IV). A Ce(IV) solution was also used to convert the bonded epinephrine to bonded epinephrine-quinone, giving rise to a monolith with different retention properties. The CEC performance of the epinephrine-bonded and epinephrine-quinone-bonded monolithic columns was evaluated by studying the separation of uncharged (alkyl benzenes) and ionizable (anilines and phenols) solutes. The modified monoliths were compared to the parent monolith. The run-to-run and column-to-column reproducibilities of both columns were also evaluated.

2. Materials and methods

2.1. Chemicals and other materials

Glycidyl methacrylate (GMA), ethylene dimethacrylate (EDMA), [2-(methacryloyloxy)ethyl]trimethyl ammonium chloride (75% in water, META), 1,4-butanediol, 3-(trimethoxysilyl)propyl methacrylate, epinephrine, sodium tetraborate decahydrate and cerium(IV) ammonium nitrate were from Aldrich (Milwaukee, WI, USA). Cyclohexanol, 1-dodecanol, 1-propanol, HPLC-grade acetonitrile (ACN) and methanol (MeOH) were from Scharlau (Barcelona, Spain). Azobisisobutyronitrile (AIBN) and tris(hydroxymethyl)aminoethane (Tris) were from Fluka (Buchs, Switzerland). Thiourea as EOF marker, and several alkyl benzenes, anilines and phenols from Riedel-de Haën (Seelze, Germany), were used. Hydrochloric acid (37%) was supplied by Panreac (Barcelona). Unless otherwise stated, other chemicals used were of analytical grade. Deionized water was obtained with a Barnstead deionizer (Sybron, Boston, MA, USA). Stock solutions of alkyl benzenes, anilines, phenols and thiourea prepared in ACN at 1 mg mL^{-1} each were kept at 4 °C until use. Test mixtures of these solutions (100 μg mL^{-1} each analyte and thiourea) were prepared daily by dilution with the mobile phase.

Uncoated fused-silica capillaries of 33.5 cm total capillary length and 375 μm O.D. × 100 μm I.D. with UV-transparent external coating (Polymicro Technologies, Phoenix, AZ, USA) were used. The effective monolithic bed length was 8.5 cm.

2.2. Instrumentation

UV crosslinker (model CL1000) from UVP (Upland, CA, USA), equipped with UV lamps (5 × 8 W, 254 nm), was used to photo-initiate the polymerization. Conditioning steps and functionalization of the monolithic columns were achieved with an HPLC pump (1100 series, Agilent Technologies, Waldbronn, Germany). SEM images were taken with a scanning electron microscope (S-4100, Hitachi, Ibaraki, Japan) provided with a field emission gun, a back secondary electron detector and an EMIP 3.0 image data acquisition system (Rontec, Normanton, UK). Raman spectra of monolithic materials (confined within capillary support) were obtained with an "in via" Renishaw spectrometer (Renishaw, Gloucestershire, UK) equipped with a Renishaw HPNIR laser (15 mW at 785 nm) and a microscope (Olympus, Hamburg, Germany). Analyses on different sites in unfunctionalized and functionalized monoliths were recorded. The absorbance of Ce(IV) solutions was measured with a model 8453 UV-VIS spectrophotometer (Agilent Technologies) provided with a 1 cm optical-path quartz cell (Hellma, Müllheim, Germany). CEC experiments were performed on a HP^{3D}CE instrument (Agilent) equipped with a diode array UV detector, and pressurized at both capillary ends with an external nitrogen supply. Data acquisition was performed with the ChemStation Software (Rev.A.10.01, Agilent).

2.3. Preparation of poly(GMA-co-EDMA) monolithic columns

Prior to the preparation of the columns, and in order to ensure covalent attachment of the monolith to the inner wall of the fused-silica capillaries, surface modification with 3-(trimethoxysilyl)propyl methacrylate was performed as described [28,29]. Monoliths were prepared from polymerization mixtures obtained by weighing GMA (20 wt%), EDMA (5 wt%), and a binary pore-forming solvent constituted by cyclohexanol (70 wt%) and dodecanol (5 wt%). AIBN (1 wt% with respect to the monomers) was added as polymerization initiator. After mixing and to obtain a clear solution, sonication for 10 min followed by purging with nitrogen for 10 more min was applied. The preconditioned capillary was filled with the mixture up to a length of 8.5 cm. Photopolymerization was accomplished by irradiation of the capillaries within the UV irradiation chamber at 0.9 J/cm^2 for 30 min. After UV polymerization, an HPLC pump was used to flush the columns for 30 min with MeOH to remove the pore-forming solvents and possible unreacted monomers.

2.4. Functionalization of poly(GMA-co-EDMA) with epinephrine

The reaction scheme of poly(GMA-*co*-EDMA) functionalization with epinephrine is given in Fig. 1. Chemical modification of the poly(GMA-*co*-EDMA) surface was performed under dynamic conditions, by flushing the capillary with a continuous stream of derivatizing solution. For this purpose, the capillaries, placed in a thermostated water bath were allowed to react with a solution of epinephrine (10 mM in 100 mM sodium tetraborate at pH 8.0 at 60 °C) for 2 h at a flow-rate of 4.0 μL min^{-1}. Then, the functionalized monoliths were flushed with MeOH for 30 min in order to remove the unreacted epinephrine, followed by mobile phase for 60 more min. A poly(GMA-*co*-EDMA) column (parent monolith) was also flushed with MeOH for 30 min followed by mobile phase for 60 min before injection of the test solutes.

2.5. Evaluation of the bonding of epinephrine on the monolith surface

For the determination of the amount of bonded epinephrine molecules bound to the monolith surface, the capillaries were flushed with a 2 mM Ce(IV) solution using a HPLC pump at flow rate of 4 μL min^{-1}. Several fractions (1 mL each) were collected at increasing time values along 12 h, and the unreacted Ce(IV) present in each fraction was spectrophotometrically measured at 320 nm. The scheme of the redox reaction, where bonded epinephrine is