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Phosphatidylcholine covalently linked to a methacrylate-based monolith as a biomimetic stationary phase for capillary liquid chromatography

Dana Moravcová[a,*,1], Enrique Javier Carrasco-Correa[b,1], Josef Planeta[a], Michael Lämmerhofer[c], Susanne K. Wiedmer[d,**]

[a] Institute of Analytical Chemistry of the ASCR, v. v. i., Veveří 97, 60200 Brno, Czech Republic
[b] Department of Analytical Chemistry, University of Valencia, Dr. Moliner 50, 46100 Burjassot, Valencia, Spain
[c] Institute of Pharmaceutical Sciences, University of Tübingen, Auf der Morgenstelle 8, 72076 Tübingen, Germany
[d] Department of Chemistry, P.O. Box 55, 00014 University of Helsinki, Finland

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ABSTRACT

In this study a strategy to immobilize phospholipids onto a polymer-based stationary phase is described. Methacrylate-based monoliths in capillary format (150 × 0.1 mm) were modified by soybean phosphatidylcholine through 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide coupling to obtain stationary phases suitable to mimic cell surface membranes. The covalent coupling reaction involves the phosphate group in phospholipids; therefore, the described methodology is suitable for all types of phospholipids.

Immobilization of soy bean phosphatidylcholine on the monolith was confirmed by attenuated total reflectance Fourier transform infrared spectroscopy and gas chromatography-mass spectrometry of the fatty alcohol profile, generated upon reductive cleavage of the fatty acyl side chains of the phospholipid on the monolith surface with lithium aluminium hydride.

The prepared stationary phases were evaluated through studies on the retention of low-molar mass model analytes including neutral, acidic, and basic compounds. Liquid chromatographic studies confirmed predominant hydrophobic interactions between the analytes and the synthesized stationary phase; however, electrostatic interactions contributed to the retention as well. The synthesized columns showed high stability even with fully aqueous mobile phases such as Dulbecco's phosphate-buffered saline solution.

1. Introduction

Predictive models for drug absorption, distribution, excretion, and also metabolic pathways in living organisms are important in the field of drug discovery in pharmaceutical industry and medicine (drug screening). Although the octanol–water partition coefficient ($P_{o/w}$) proposed by Fujita et al. [1] as a model for biological partitioning is widely used and well accepted for evaluating the lipophilicity/hydrophobicity of compounds, there are still efforts to develop other systems providing better insight into biological partitioning processes. For example, the human intestinal Caco-2 cell line is often used as a simple reference model in prediction of cellular permeation of drug candidates [2]. Physico-chemical methods employing analytical separation methodologies based on immobilized (phospho)lipids or (phospho)lipid aggregates, especially capillary electromigration and liquid chromatographic (LC) techniques, are also indispensable for rapid screening of drug permeability. Comprehensive outlines of application of these techniques to analyte–membrane interactions studies are included, e.g., in reviews from Wiedmer et al. [3] or from Cserháti et al. [4].

Retention of analytes under reversed-phase LC conditions similarly as octanol–water partitioning do not consider the ionization of analytes and merely hydrophobicity alone is used for prediction of drug absorption [5]. However, since most drugs are charged at physiological pH values, the use of biomimetic stationary phases may provide better insight into their biological partitioning processes. These biomimetic stationary phases are prepared by modification of silica or soft gel particles such as sepharose. Surface modification can be based on covalent or

* Corresponding author. Tel.: +420 532290218.
** Corresponding author. Tel.: +358 294150183.
E-mail addresses: moravcova@iach.cz (D. Moravcová), susanne.wiedmer@helsinki.fi (S.K. Wiedmer).
[1] The authors share first authorship.

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non-covalent bonding of lipids. Non-covalent bonding methods include coating [6,7] and hydrophobic binding [8–12]. The covalently bonded stationary phases possess improved stability over non-covalently bonded ones and cover sorbents such as cholesterol stationary phases [13–15], alkyl-phosphate stationary phases [16], phosphatidylcholine (PC) stationary phases [17], and sphingomyelin stationary phases [18]. Another approach to the synthesis of phospholipid-modified stationary phases was introduced by Gallagher et al. [19]. They stabilized a phospholipid bilayer containing small unilamellar vesicles (SUVs) on non-porous silica particles by UV- and redox polymerization. SUVs were prepared from a mixture of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine and 1,2-bis[10-(2′,4′-hexadieoyloxy)decanoyl]-*sn*-glycero-2-phosphocholine (a phospholipid containing double bonds in its alkyl chains).

Despite a tremendous progress in synthesis of new LC chromatographic stationary phases, only the ones based on monolayers of phospholipids covalently bonded to aminopropyl silica particles (cf. immobilized artificial membranes, IAM) developed by Ch. Pidgeon [20–22] are commercially available.

Monolithic stationary phases have gained increased popularity during the last years due to their unique hydrodynamic properties and a wide variety of chemistries available for their synthesis. At present, monolithic polymer-based [23,24], inorganic oxide-based [25], or hybrid organic–inorganic oxide-based [26] materials are synthesized and evaluated.

There are two fundamentally different ways to manufacture polymer-based monolithic columns with dedicated surface chemistry, namely, one-step in-situ polymerization and post-polymerization surface functionalization. A variety of in-situ prepared monoliths have been described for reversed-phase LC [27], ion-exchange LC [28], and advanced chromatographic modes such as enantioselective [29] and affinity-type [30] separations. Concerning the chromatographic properties, the success of one-step in-situ polymerization strongly depends on a proper choice of both the monomers and the porogenic solvents in the polymerization mixture. Attempts to modify well-known polymerization mixtures by incorporating new monomers require careful re-optimization of the whole polymerization mixture to preserve the pore structure [31].

In contrast to single step copolymerization, post-modifications of the monolith allow independent tuning of mechanical, flow-through porous properties, and also surface chemistry of the parent monolith. The use of glycidyl methacrylate (GMA) containing an epoxy group is particularly useful for preparing monoliths which can be easily functionalized. Using ethylene dimethacrylate (EDMA) as a crosslinker, poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) (poly(GMA-*co*-EDMA)) monoliths with a highly reactive surface are obtained. Monoliths with a variety of chromatographic properties (ion-exchange, hydrophobic/hydrophilic, chiral, etc.) have been obtained by bonding amines [32], amino acids [29], polymers [33], thiol derivatives [34], and chiral reagents [35] to the parent monolith.

The use of monolithic supports for preparation of chromatographic sorbents suitable for mimicking biological cell membranes is not common. Kuroda et al. [36] modified a silica-based monolithic column by capturing phospholipids (egg phosphatidylcholine and a mixture of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoserine) through hydrophobic interactions with octadecylsilane (C18) alkyl chains. Zhao et al. [37] prepared phospholipid functionalized capillary monolithic columns by in-situ copolymerization of 12-methacryloyl dodecylphosphatidylcholine, 12-methacryloyl dodecylphosphatidylserine, and ethylene dimethacrylate. The same approach for synthesis of a cholesterol-based polymeric monolithic column was reported by Szumski et al. [38], who used cholesteryl methacrylate as a functional monomer and trimethylolpropane trimethacrylate as a crosslinker in the polymerization mixture.

In our earlier paper we described a procedure for preparation of silica-based monolithic capillary columns covered by spherical unilamellar liposomes, in which immobilization was performed through amino groups via Schiff's base formation [39]. Due to the fact that, in general, secondary and tertiary amino groups do not undergo a Schiff's base reaction, only liposomes with primary amino group were able to react.

In this work we suggest another approach to synthesize biomembrane mimicking stationary phase using soy bean phosphatidylcholine as a model phospholipid. Immobilization was done onto a poly(GMA-*co*-EDMA) monolith. The reaction takes place via 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) coupling (a reaction commonly used for protein-conjugation to carboxylic acid linkers, e.g., in the preparation of enzyme reactors [40]), whereby a covalent phosphoramidate bond is created between amino groups present on the stationary phase and the phosphate group of a phospholipid. The developed stationary phases were characterized by attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR), gas chromatography-mass spectrometry (GC-MS), and capillary LC, confirming the presence of immobilized phospholipids onto the monolith.

2. Experimental part

2.1. Chemicals and reagents

Acetonitrile (ACN, LC/MS-grade), alkylbenzenes (C_0–C_6), formic acid, oxalic acid, citric acid, drugs (Table 1), lidocaine, imidazole, glutaraldehyde (GA), sodium cyanoborohydride (SCBH), ammonium bifluoride (NH_4HF_2), glycidyl methacrylate (GMA), ethylene dimethacrylate (EDMA), 3-(trimethoxysilyl)propyl methacrylate (γ-MAPS), lithium aluminium hydride ($LiAlH_4$), and 1-octadecanol were purchased from Sigma-Aldrich (Vienna, Austria). HPLC-grade methanol (MeOH) and isopropanol (IPA) were from Scharlab (Barcelona, Spain). Azobisisobutyronitrile (AIBN) and *N*-(3-dimethylaminopropyl)-*N'*-ethyl-carbodiimide hydrochloride (EDC) were from Fluka (Buchs, Switzerland). Hexamethylene-1,6-diamine (HMD) was from BASF (Ludwigshafen, Germany). L-α-Phosphatidylcholine (PC) extracted from soy bean (hereafter called soyPC) was from Avanti Polar Lipids (Alabaster, AL, USA) and it has the following fatty acid distribution: 14.9 wt% of 16:0; 3.7 wt% of 18:0; 11.4 wt% of 18:1; 63.0 wt% of 18:2; 5.7 wt% of 18:3; 1.2 wt% of unknown lipids. Uncoated fused-silica capillaries 375 μm O.D. × 100 μm I.D. (Polymicro Technologies, Phoenix, AZ, USA) were used for preparation of monolithic columns.

2.2. Instrumentation

A syringe pump (Model 100, KD Scientific, New Hope, PA, USA) was employed to introduce the derivatization reagents into the monolithic capillary column. An HPLC pump (1100 series, Agilent Technologies, Waldbronn, Germany) was used for washing the prepared monolithic columns. FTIR was performed with a Jasco 4100 type A spectrophotometer (Jasco, Easton, MD) fitted with a single reflection ATR accessory. Spectra were accomplished from 4000 to 500 cm^{-1} in the absorbance mode at a 2 cm^{-1} resolution with 100 scans.

GC-MS analysis was done on a Focus DSQ II gas chromatograph provided with an AI 3000 autosampler and single quadrupole MS detector from Thermo Fisher Scientific. The analytical fused-capillary column was a TR-5MS (30 m, 0.25 mm I.D., 0.25 μm film thickness) from Thermo Fisher Scientific. The GC oven single ramp temperature program was operated as follows: the initial oven temperature of 50 °C was kept for 2 min, the temperature was