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Calixarene-immobilized monolithic cryogels for preparative protein chromatography

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ABSTRACT

New cation exchanger monolithic stationary phases were prepared by immobilization of three different calixarene derivatives (i.e. tetracarboxylate calix[4]arene, *CLX-COO*, tetrasulfonate calix[4]arene, *CLX-SO*$_3$, and tetraphosphonate calix[4]arene, *CLX-PO*$_4$) onto a monolithic cryogel support (i.e. poly(2-hydroksyethylmethacrilate-*co*-glycidyl methacrylate, *P*) and investigated with respect to preparative protein chromatography. The obtained monoliths were characterized through various techniques such as FTIR spectroscopy, isoelectric point measurements, titrimetric analyses, and mercury intrusion porosimetry. Protein retention was investigated using some model proteins (i.e. lysozyme, cytochrome *c*, and α-chymotrypsinogen A, human serum albumin, and myoglobin), and the role of modifier (i.e. NaCl) concentration and pH was thoroughly analyzed under isocratic and gradient elution conditions. Overloading experiments were also conducted to study dynamic adsorption capacity and the obtained values were found to be ranging between 3 and 8 mg/mL depending on the type of calixarene molecule. Hence, higher or comparable protein adsorption capacities were seen to be applicable on calixarene-immobilized cryogels when compared to any other functionalized cryogels in the literature. Combined with the favorable properties of these monoliths, with respect to mass transport of large molecules, these results qualify calixarene functionalized monolithic cryogels as promising stationary phases for protein preparative purification.

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1. Introduction

After their first development, calixarenes have received great attention in chemistry, pharmaceutical and material sciences [1–5]. These cyclic oligomers are made up of a conical structure with defined upper and lower rims and a central annulus. Each rim can be independently functionalized and therefore numerous calixarene molecules exhibiting different physicochemical behavior can be synthesized. Moreover, calixarenes of varying cavity size (i.e. calix[4]arene, calix[6]arene, and calix[8]arene) can be prepared [6]. Their molecular structure allows an exceptional flexibility to interact with chemical species through diverse mechanisms. Such a flexible chemistry was found to be applicable in separation and purification processes [7,8].

Immobilization of calixarenes to solid supports is an indispensable process for their application in separation and purification processes. The concept has been successfully implemented in the preparation of novel calixarene-immobilized chromatography stationary phases [4] that have been successfully applied for the separation of some aromatic compounds, nucleosides and nucleobases [9], polycyclic aromatic hydrocarbons [10,11], antihypertensive drugs [12], steroids [13], and vitamins [14]. Hence, the use of calixarene-immobilized stationary phases in HPLC is a well-established concept for the chromatography of small molecules [4,8,9,11,12,15–17]. However, little or no attention has been paid to the chromatography of proteins; although the studies performed in solutions have revealed that calixarenes accommodating ionic functional groups could attract proteins through electrostatic forces [18]. This motivated us to investigate some calixarene derivatives containing different cation exchanger groups like carboxylate, sulfonate, and phosphonate for protein chromatography.

As it is well known, silica is the most widely used support material in calixarene immobilization for HPLC applications [4]. However, in chromatography of proteins, silica cannot meet all the requirements as a support material due to its poor chemical stability and inappropriate morphology. To prepare a calixarene-immobilized material that can be used as a stationary phase in protein chromatography, monolithic cryogels were thought to be

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beneficial owing to their supermacroporous morphology, high chemical stability, and suitability to *post*-surface modifications [19]. Cryogels are hydrogels prepared at sub-zero temperatures, and these class of polymeric materials have been extensively used to prepare monolithic support materials for adsorption and chromatography [19–22].

In previous studies, monolithic cryogels were found to be used as separation media in different chromatography applications like cation-exchange chromatography [19,23], anion-exchange adsorption [24], steric exclusion chromatography [25,26], affinity purification of protein G [27], chromatography applications based on hydrophobic affinity [28], immobilized metal affinity chromatography [29]. In these applications, cryogel supports were functionalized with various compounds and macromolecules like 3-Mercaptopropane sulfonic acid [19], humic acid [23], diethylaminoethanol [24], poly(carboxybetaine methacrylate) [26] and iminodiacetic acid [29]. Moreover, protein G-imprinting has been successfully applied on some cryogels [27]. The mentioned applications prove the adaptability of cryogels to different surface modification processes and to various modes of chromatography. Cryogels owe this flexibility to their chemistry that one can tailor the network of cryogels (*i*) by using functional monomers during the cryogellation process or (*ii*) by applying suitable *post-surface modifications*. Former has limited applications due to limited number of functional monomers that can be used in cryogellation, while latter is advantageous because many different low-molecular weight compounds and macromolecules are available for immobilization to suitable cryogel supports [19,23]. This concept can also be applied to immobilization of supramolecules like calixarenes.

Surprisingly, an approach that connects the chemistries of calixarenes and cryogels is lacking in the literature, although this might be beneficial in, for example, separation and purification of proteins. Hence, in this work, (*i*) chemical immobilization of three calixarene derivatives (i.e. tetracarboxylate calix[4]arene, *CLX-COO*, tetrasulfonate calix[4]arene, *CLX-SO$_3$*, and tetraphosphonate calix[4]arene, *CLX-PO$_4$*) onto poly(2-hydroxyethyl methacrylate-*co*-glycidyl methacrylate, *P*, and (*ii*) application of the prepared new monolithic stationary phases in cation-exchange chromatography have been investigated.

2. Experimental

2.1. Chemicals and aqueous solutions

Analytical reagent grade chemicals were used throughout the study. 2-hydroxyethylmethacrylate (HEMA; Aldrich), glycidyl methacrylate (GMA; Aldrich), *N,N,N′,N′*-tetramethylethylenediamine (TEMED; Aldrich), ammonium persulfate (APS; Sigma–Aldrich), *N,N′*-methylene-bis(acrylamide) (MBAAm; Fluka) were the chemicals used in the synthesis of the monolithic support. Phosphate buffers (20 mM) were used during the chromatographic runs, and for this purpose sodium salts of mono- and di-basic phosphates were used (Sigma). Dilute solutions prepared from stock HCl (Merck; 37%) and NaOH (Sigma) were used in pH adjustment of the aqueous solutions. 1.0 M NaCl solution was prepared from stock NaCl (Sigma) and used as a modifier in chromatographic runs. The modifier solutions were prepared in 20 mM phosphate buffer at various pH values (e.g. 5.0; 6.0 and 7.0). The solutions of model proteins (i.e. α-chymotrypsinogen (Chy; bovine pancreas; Sigma; p*I* ~9), cytochrome c (Cyt; equine heart; Sigma; p*I*~10) and lysozyme (Lys; hen egg white; Fluka; p*I*~11) were prepared in the same buffer used as running buffer. Human serum albumin (HSA; Sigma; p*I*~5) and Myoglobin (Mb; equine skeletal muscle; Sigma; p*I*~7) were also used in some of the chromatographic runs. All the solutions were prepared by using fresh ultrapure water, and filtered through membrane filters (0.45 μm; cellulose acetate) before use.

Solutions of ethylenediaminetetraacetic acid disodium salt (EDTA, Merck) and Eriochrome Black T (ECBT, Merck) were used as titrants and indicators, respectively, in EDTA titrations. All the chemicals used in the synthesis of calixarene derivatives were from Merck, and used as received.

2.2. Instruments

^{1}H NMR, ^{13}C NMR, and ^{31}P NMR spectra were recorded on an Agilent® 600 MHz instrument. FTIR spectroscopy analyses were carried out on a Bruker® Vertex model ATR-FTIR instrument. The spectra were recorded directly on the dried materials within 4000–400 cm^{-1} frequency range with 4 cm^{-1} resolution. Isoelectric point measurements were performed on a Malvern® Nano ZS90 model zetasizer instrument. Scanning electron microscopy (SEM) images were recorded on a Zeiss® Evo Basic model instrument. Mercury intrusion porosimetry measurements were done by using a Quantachrome® model Poremaster 60 instrument. Chromatographic runs were conducted on a modular HPLC system comprised of a degasser, a quaternary pump, a thermostatted column compartment, a 6-port valve, an automatic temperature controlled injection system, and a variable wavelength detector (Agilent® 1260 series). pH measurements were done by using a combined pH measurement system (Mettler-Toledo® Seven Easy). A Human® water purification system was utilized to produce ultrapure water. An external HPLC pump (Agilent®) was implemented to perform in-situ surface modification processes (i.e. calixarene immobilization).

2.3. Synthesis and characterization of calixarene derivatives

Calixarene derivatives [i.e. *CLX-COO* (600.53 g/mol); *CLX-SO$_3$* (823.67 g/mol); and *CLX-PO$_4$* (832.33 g/mol); the given molar masses are for the sodium salts of the calixarenes] used in immobilization process were synthesized according to the methods described in the literature [30–34]. Very detailed information about the synthesis procedures can be found in Supplementary Material. The purity and identity of the derivatives were confirmed by ^{1}H NMR, ^{13}C NMR, ^{31}P NMR, and FTIR spectroscopy techniques.

2.4. Preparation of monolithic support, and calixarene immobilization

To prepare the monolithic support (i.e. *P*), 1.6 mL HEMA and 0.1 mL GMA were dissolved in 5 mL degassed water. In another beaker, 0.28 g MBAAm was dissolved in 10 mL degassed water. Afterwards, both solutions were combined and mixed for 10 min in an ice bath. 0.02 g radical initiator (i.e. APS) was added, and finally, 25 μL TEMED was poured into the mixture. The final solution was rapidly (and carefully) poured into empty chromatographic columns (5.0 × 120 mm) previously placed in an ice-bath. The columns were put into a freezer and kept at −20 °C for 24 h [19]. Afterwards, the columns containing the support material, *P*, were put out of the freezer for thawing at room temperature for about 2 h. The monolith was, successively, rinsed with water, ethyl alcohol solution (10%, v/v), and water again before calixarene immobilization.

The procedure of calixarene immobilization on *P* was based on nucleophilic attack of the deprotonated hydroxyl groups of calixarene to the epoxide rings of *P*. A representative immobilization route is shown in Fig. 1. Briefly, aqueous solutions of the studied calixarene derivatives (5 × 10^{-3} M; 40 mL) were prepared, and pH of the solutions was set at 11–12 by titration with NaOH solution. In order to ensure complete deprotonation of the phenolic hydroxyl

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