Journal of Chromatography A, 1386 (2015) 13–21

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Strong cation-exchange chromatography of proteins on a sulfoalkylated monolithic cryogel

Işık Perçin[a,c], Rushd Khalaf[a], Bastian Brand[a], Massimo Morbidelli[a], Orhan Gezici[a,b,*]

[a] ETH Zurich, Institute for Chemical and Bioengineering, Vladimir-Prelog-Weg 1, CH-8093 Zurich, Switzerland
[b] Nigde University, Faculty of Science and Arts, Chemistry Department, 51200, Nigde, Turkey
[c] Hacettepe University, Department of Biology, Molecular Biology Division, Beytepe, 06800 Ankara, Turkey

ARTICLE INFO

Article history:
Received 9 December 2014
Received in revised form 15 January 2015
Accepted 25 January 2015
Available online 2 February 2015

Keywords:
Gradient elution
HPLC
Ion-exchange
Protein
Separation
Yamamoto Model

ABSTRACT

A new strong cation exchanger (SCX) monolithic column was synthesized by at-line surface modification of a cryogel prepared by copolymerization of 2-hydroxyethylmethacrylate (HEMA) and glycidylmethacrylate (GMA). Sodium salt of 3-Mercaptopropane sulfonic acid (3-MPS) was used as the ligand to transform the surface of the monolith into a strong cation exchanger. The obtained material was characterized in terms of elemental analysis, infrared spectroscopy (FTIR), Scanning Electron Microscopy (SEM), Brunauer–Emmett–Teller (BET) N_2 adsorption, and used as a stationary phase for strong-cation exchange chromatography of some proteins, such as α-chymotrypsinogen, cytochrome c and lysozyme. Water permeability of the column was calculated according to Darcy's law (2.66×10^{-13} m^2). The performance of the monolithic cryogel column was evaluated on the basis of Height Equivalent to a Theoretical Plate (*HETP*). Retention behavior of the studied proteins was modeled on the basis of Yamamoto model to understand the role of the ion-exchange mechanism in retention behaviors. The considered proteins were successfully separated, and the obtained chromatogram was compared with that obtained with a non-functionalized cryogel column.

1. Introduction

Ion-exchange chromatography (IEC) is one of the most widely used modes of High Performance Liquid Chromatography (HPLC) for separation and purification of proteins, because it offers an efficient, non-denaturing and inexpensive separation. As in all modes of HPLC, the stationary phase is a key factor in IEC of proteins and therefore the development of efficient stationary phases is essential. High capacity, selectivity, chemical and mechanical stability and reversible protein retention are some important features being sought for in an IEC stationary phase [1–4].

Silica-based materials are popular in HPLC because of the mechanical stability and suitability of silica to various types of surface modifications. However, silica-based materials tend to dissolve at pH values above 8 and below 2. This inherent poor chemical stability is a serious drawback in protein purification processes where cleaning in place techniques are often implemented with 1 M NaOH [1]. For this reason, polymeric supports have taken considerable attention in IEC of proteins, and are often preferred to silica derived materials.

In IEC of proteins, mass-transfer resistance and high backpressure represent the most prominent drawbacks. The first results in poor efficiency while the latter may cause support degradation. These limitations can typically be overcome when using monolithic columns [5,6]. A monolithic material comprises of an interconnected channel network through which the mobile phase can easily flow [6,7]. Since their development in 1970s, cryogels have gained considerable attention because of their good mechanical properties and high biocompatibility. Cryogels can be described as polymeric hydrogels prepared at sub-zero temperatures. A continuous network that comprises of interconnected macropores (ranging in size from 10 to 100 μm) makes cryogels a suitable medium for separation and purification of high-molecular weight species. Little or no mass-transfer resistance, applicability of high flow rates [8,9], ease of preparation and suitability to further modifications are among the most important characteristics of monolithic cryogels. The mentioned features can help to design new support materials and stationary phases for different types of chromatographic applications [10].

One way to properly functionalize cryogels would be to prepare them by the co-polymerization of suitable monomers. However, it is often difficult to find a functional monomer which is suitable

* Corresponding author at: Nigde University, Faculty of Science and Arts, Chemistry Department, 51200, Nigde/Turkey. Tel.: +90 388 2254030; fax: +90 388 2250180.
E-mail addresses: ogezici@gmail.com, ogezici@nigde.edu.tr (O. Gezici).

http://dx.doi.org/10.1016/j.chroma.2015.01.075
0021-9673/

for a specific application. On the other hand, surface modification of a cryogel with a target ligand easily provides a full variety of functional cryogels. The main drawback with poly(acrylamide) cryogels, which are among the most widely studied cryogels, is the absence of reactive functional groups for surface modifications. Some protocols based on transamidination introduce surface modifications with amine compounds. However, in this type of reactions a high amount of amine-compound and (usually) elevated temperatures are needed to obtain sufficiently high modification yields [10,11]. Compared to poly(acrylamide) cryogels, poly(vinyl alcohol) cryogels are more suitable for surface modifications. However, in poly(vinyl alcohol) cryogels, a supplementary activation step is needed to convert alcoholic —OH groups into more reactive groups before attachment of targeted ligands. Reactions with dialdehydes and epichlorohydrin have been used for surface activation of poly(vinyl alcohol) cryogels. It should be noticed that harsher conditions may be necessary to obtain sufficiently high degree of surface activation for poly(vinyl alcohol) cryogels [12]. Graft polymerization has also been used as an alternative way to obtain cryogels with ion exchanger groups [13–16]. For example, grafting 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) onto cryogel matrix has been successfully applied on polyacrylamide-based cryogel matrix. In such kind of grafting processes, the cryogel matrix is usually dried before grafting. Moreover, in-situ graft polymerization (without drying the cryogel matrix) of the above mentioned monomer has also been reported [8].

Cryogels prepared by co-polymerization of 2-hydroxyethylmethacrylate (HEMA) and glycidylmethacrylate (GMA) offer various alternatives for functionalization. On the contrary of poly(acrylamide) and poly(vinyl alcohol) cryogels, poly(HEMA-*co*-GMA) cryogels do not require a supplementary surface activation step (and the associated harsh conditions) for the attachment of a targeted ligand due to the good reactivity of the epoxide group. This group in fact offers easy one-step surface functionalization under mild conditions with relatively little use of chemicals. Through ring-opening reactions of oxirane (epoxide) groups in poly(HEMA-*co*-GMA), a wide range of ligands with specific functionalities can be attached to a cryogel matrix. As reported by Lozinsky et al. [12], immobilization of ion-exchange, hydrophobic or affinity ligands can be directly performed in a cryogel-filled column. Such an at-line ligand-attachment procedure for poly(HEMA-*co*-GMA) cryogels seems to be useful for the development of cryogel-based stationary phases through one-step attachment of targetted ligand. This methodology was succesfully applied to attach iminodiacetic acid to poly(HEMA-*co*-GMA) [17], and the resulting material was used to bind urease after loading Ni(II) to the functionalized cryogel. The attachment of targetted ligand to poly(HEMA-*co*-GMA) matrix seems to be advantageous over surface functionalization of poly(acrylamide)- and poly(vinyl alcohol)-based cryogels as well as graft polymerization, because it allows for a wide variety of functionalization with less use of chemicals.

In this work a new procedure is presented for incorporating a strong-cation exchange functionality into poly(HEMA-*co*-GMA) cryogels. The methodology is based on the formation of a thioether bond between the cryogel matrix and the sodium salt of 3-Mercaptopropane sulfonic acid (3-MPS) under alkaline conditions. Attachment of 3-MPS is done "*in-situ*" by pumping the ligand solution through the monolithic cryogel column. According to our knowledge, this is the first study that reports attachment of sulfopropyl groups to cryogel matrix through such a sulfoalkylation process. We believe that this represents an easy, one step and non-destructive functionalization process for cryogel monoliths. The ion-exchange character of the monolithic cryogel was investigated by using three model proteins, and the obtained chromatographic data were modelled on the basis of Yamamoto Model [18,19].

2. Materials and methods

2.1. Chemicals and reagents

All chemicals were of analytical reagent grade and used without further purification. 2-hydroxyethylmethacrylate (HEMA; Aldrich), glycidylmethacrylate (GMA; TCI), N,N,N′,N′-tetramethylenediamine (TEMED; Aldrich), ammonium persulfate (APS; Sigma–Aldrich), and N,N′-methylene-bis(acrylamide) (MBAAm; Fluka) were used to prepare the monolithic cryogel. The prepared cryogel was modified by using 3-MPS (Aldrich). Sodium salts of mono- and di-basic phosphates were from Fluka and used to prepare phosphate buffers (20 mM; at pH 5.0, 6.4 and 7.0). 1 M NaCl solutions at pH 5.0 and 6.4 (in 20 mM buffer as described above) were prepared from stock NaCl (Merck), and used as modifier in chromatographic experiments. 5 mg/mL samples of α-chymotrypsinogen (*Bovine pancreas*; Sigma), cytochrome c (*Horse heart*; Across Organics) and lysozyme (*Hen egg white*; Fluka) were prepared in the same buffer used as mobile phase. A mixture of the studied proteins (2 mg/mL for each) was prepared in 20 mM buffer at pH 6.4. An aqueous dextran (80 kDa; Sigma–Aldrich) solution was prepared in phosphate buffer (20 mM; pH 7.0) and used as sample to test performance of the column. Ultrapure water obtained by a Millipore water purification system was used throughout the experiments. All the solutions and samples were filtered through 0.2 μm membrane filters before use.

2.2. Apparatus and instruments

An Agilent 1100 Series HPLC system with a degasser, quaternary pump, thermostatted column compartment, auto injector, and variable wavelength detector was used in chromatographic experiments. A conductivity detector with a flow-through cell was also used to measure the mobile phase conductivity. A combination pH electrode and a pH meter system (Mettler-Toledo) were used to measure pH of the buffers. Elemental analyses were performed by using LECO elemental analyzer. FTIR measurements were done on a Bruker Vertex70 model ATR-FTIR (Attenuated Total Reflectance-Fourier Transformed Infrared Spectroscopy) instrument. Scanning electron microscopy (SEM) images of lyophilized material were recorded on a Zeiss Gemini 1530 FEG model instrument. The samples were prepared by platinum sputtering by using Balzers MED 010, and coating thickness (5 nm) was determined by a quartz microbalance after coating the samples with platinum. BET surface area was determined on the same samples by using a Micromeritics Tristar model instrument. A Gel-Permeation Chromatography system (Merck LaChrom) with a refractive index detector was used to study the performance of the column through some experiments conducted at different flow rates (an aqueous solution of 80 kDa dextran was used as sample).

The monolith was prepared in a chromatographic glass column (GE Healthcare) of 130 × 5.0 mm ID with filters. An HPLC pump was used to pass 3-MPS solution through the column, and thus to modify the monolith into a strong cation exchanger form. Ultrapure water was freshly produced and used to prepare aqueous solutions.

2.3. Methods

2.3.1. Preparation of monolithic cryogel column

Monolithic cryogel was prepared by co-polymerization of HEMA and GMA, and MBAAm was used as cross-linker [17]. Briefly, 1.6 mL HEMA and 0.1 mL GMA were dissolved in 5 mL of degassed water, and 0.28 g MBAAm were dissolved in another 10 mL of degassed water. Both solutions were mixed and the mixture was stirred in an ice-bath for 10 min. 0.02 g APS (radical initiator) was added to the reaction mixture. Finally, 25 μL of TEMED was added to the