MACROPOROUS CRYOGEL BASED SPIN COLUMN WITH IMMOBILIZED CONCANAVALIN A FOR ISOLATION OF GLYCOPROTEINS

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Abbreviations: BSA, bovine serum albumin; HEMA, hydroxyethyl methacrylate; PEGDA, poly(ethyleneglycol) diacrylate; APS, ammonium persulfate; TEMED, \(N,N,N',N''\)-tetramethylethylenediamine

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Abstract

In this work we have developed a hydrophilic poly(hydroxyethyl methacrylate-co-poly(ethylene glycol) diacrylate) cryogel placed in the centrifugal filter device. The composition of the polymerization mixture as well as the polymerization conditions were optimized in order to prepare a material with bimodal pore size distribution with 20-50 μm flow throw macropores and submicrometer pores in the polymer walls.

The optimized, mechanically stable, highly porous, material was used for spin column lectin chromatography. The surface of the monolithic scaffold was activated by epichlorohydrin and used for immobilization of concanavalin A to provide the affinity supports for selective isolation of glycoproteins containing high mannose glycan structures. The performance of the developed lectin modified cryogels was evaluated by analyses of glycoprotein mixtures. The efficiency and selectivity of the affinity supports were confirmed by MALDI-MS analysis.
1 Introduction

Glycosylation is one of the most important post-translational modifications of proteins [1]. Besides the vital role of glycosylation in protein folding, stability and recognition, changes in protein glycosylation have been associated with several diseases including cancer [2, 3] or allergies [4, 5].

One of the most useful techniques used for glycoprotein isolation/separation is lectin affinity chromatography [6]. Lectins are carbohydrate binding proteins, which are found in plants, animals and microorganisms. Concanavalin A is one of the most well characterized and widely used lectins. Concanavalin A is originally extracted from Canavalia ensiformis (Jack-bean) seeds and it specifically binds to internal and nonreducing terminal α-D-mannosyl and α-D-glucosyl groups found in various sugars, glycoproteins and glycolipids.

The biocompatibility of chromatographic stationary phases as well as supports for affinity ligands immobilization plays a crucial role in analyses of proteins and peptides. Macroporous cryogels have been introduced as a unique hydrophilic monolithic material for a wide range of applications including separation, cell cultivation and tissue engineering [7-9]. Cryogels represent the monolithic materials with the macroporous structure containing flow-through pores in the range of 10-100 µm [10]. The macroporous materials are formed by freezing the initial solution containing monomers, crosslinkers, free radical initiators and porogens. Since water is the most widely used porogen, the polymerization is usually initiated using a N,N,N',N'-tetramethylethylenediamine/ammonium persulfate (TEMED/APS) initiating system.

In this work we have focused on the development of a highly hydrophilic (meth)acrylate based cryogel. The main objective was the optimization of the reaction mixtures and polymerization conditions in order to prepare a monolithic cryogel with structured pore walls and increased surface area. The cryogels were synthesized using
hydroxyethyl methacrylate (HEMA) as a monomer and polyethyleneglycol diacrylate (PEGDA) as a crosslinker. The rate of the polymerization was controlled by varying the concentration of TEMED as an accelerator of the polymerization process. The optimized monolithic matrix was modified by immobilized concanavalin A and placed in the centrifugal filter device. The developed lectin modified spin column was used for selective isolation of glycoproteins containing high mannose glycan structures.

2 Materials and methods
2.1 Materials and reagents
Sodium carbonate, sodium acetate, calcium chloride, manganese chloride, sodium chloride, sulfuric acid, sodium periodate, epichlorohydrin, sodium cyanoborohydride, methyl-α-D-mannopyranoside, trifluoroacetic acid, methanol, LC-MS acetonitrile, and LC-MS water were purchased from Sigma-Aldrich (Prague, Czech Republic).

Hydroxyethyl methacrylate (HEMA), poly(ethyleneglycol) diacrylate (PEGDA, 3 ethylene oxide units), ammonium persulfate (APS), and N,N,N′,N′-tetramethylethylenediamine (TEMED) were purchased from Sigma-Aldrich. HEMA and PEGDA were purified by passing them through a column containing inhibitor remover (Sigma-Aldrich).

Concanavalin A (Canavalia ensiformis), cytochrome c (bovine), ribonuclease B (bovine), serum albumin (bovine, BSA), α-lactalbumin (bovine), peroxidase (horseradish), insulin (bovine), myoglobin (horse), super-dihydroxybenzoic acid (9:1 mixture of dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid) were purchased from Sigma-Aldrich.

2.2 Instrumentation
A MALDI-TOF/TOF instrument (model 5800, AB Sciex, Framingham, MA) was used for MALDI spectrometric detection and the samples were applied using the “dried-droplet” technique. The 20 mg/mL super-dihydroxybenzoic acid was prepared in 50% aqueous acetonitrile containing 0.1% trifluoroacetic acid. Before analysis, the samples and matrix were mixed in a 1:1 ratio. Mass spectrometric measurements were carried out in the positive ion linear mode with a scan range of 5000-90 000 m/z.

Scanning electron micrographs were obtained using the MIRA3 microscope (TESCAN, Brno, Czech Republic). The samples were sputtered with a 10 nm thick layer of gold before SEM imaging.

2.3 Preparation and activation of the macroporous cryogel

The cryogels were prepared in the low-density polyethylene (LDPE) tube (7 mm id, 15 cm long). A mixture consisting of 15% (v/v) HEMA, 5% (v/v) PEGDA, 80% (v/v) water, and 1% (w/v, with respect to monomers) APS were stirred (1400 rpm) in the ice bath for 5 min. Then, 0.25-1% (v/v, with respect to monomers) TEMED was added and the mixture was stirred (1400 rpm) for 1 min. The LDPE tube was filled with the mixture, sealed at both ends, and frozen at –20°C in an ethanol cooled cryostat (Huber CC505, Offenburg, Germany). After being kept frozen for 24 h, cryogels were cut into small pieces (7 mm in diameter, 10 mm long), defrosted at room temperature and washed with water.

The cryogels were immersed in a solution consisting of 10% (v/v) epichlorohydrin, 45% (v/v) methanol and 45% (v/v) 50 mmol/L sodium carbonate, and stirred (250 rpm) for 3 h at room temperature. After incubation, the cryogels were washed with methanol and water.

2.4 Immobilization of concanavalin A
The epichlorohydrin activated cryogels were immersed in a solution of 0.5 mol/L sulfuric acid and stirred (250 rpm) at room temperature for 18 h. After hydrolysis of epoxide groups, the cryogels were washed with water, stirred in 0.1 mol/L sodium periodate (250 rpm for 90 min in dark), washed again with water and equilibrated with a binding buffer. A solution of 50 mmol/L sodium acetate pH 6.5 containing 1 mmol/L calcium chloride, 1 mmol/L manganese chloride and 150 mmol/L sodium chloride was used as the binding buffer. Concanavalin A (2.5 mg/mL) was dissolved in the binding buffer containing 5 mg/ml sodium cyanoborohydride. Cryogels were immersed in the lectin solution for 5 h at room temperature, washed with the binding buffer and stored in the buffer at 4°C before further use.

2.5 Isolation of glycosylated proteins

The protein mixture consisting of insulin, cytochrome, ribonuclease B, myoglobin, α-lactalbumin, peroxidase and BSA was prepared in the binding buffer containing 10% acetonitrile. The concentration and masses of individual proteins are shown in Table 1.

A piece of the cryogel was placed in the Millipore Ultrafree-MC centrifugal filter unit with the microporous membrane (5-μm pore size, Millipore, Billerica, MA) and equilibrated with the binding buffer containing 10% acetonitrile (500 μL). The sample (150 μL) was applied on the cryogel and after 10 min incubation centrifuged using the Eppendorf MiniSpin Plus centrifuge (800 rpm) for 30 s. Finally, the cryogel was washed five times with the binding buffer containing 10% acetonitrile (500 μL) and eluted by the eluting buffer (150 μL). A solution of 50 mmol/L sodium acetate pH 6.5 containing 1 mmol/L calcium chloride, 1 mmol/L manganese chloride, 150 mmol/L sodium chloride and 200 mmol/L methyl-α-D-mannopyranoside was used as the eluting buffer.
The cryogel was washed five times with the binding buffer containing 10% acetonitrile (500 µL) before application of another sample. The collected fractions were desalted using a C18 ZipTip (Millipore) following the protocol suggested by the manufacturer before MALDI-MS analysis.

3 Results and discussion

3.1 Optimization of polymerization mixture

The macroporous cryogel was produced by radical polymerization in the frozen state of HEMA as a monomer, PEGDA as a crosslinker and APS/TEMED as an initiating system in water. HEMA and PEGDA were chosen in order to prepare a hydrophilic sorbent with minimized non-specific protein interactions. The cryogels were prepared in the tube (7 mm in diameter, 15 cm long) at –20°C, cut into 10 mm long pieces and thawed at room temperature.

The prepared material was characterized by scanning electron microscopy (SEM) after sputtering with a 10 nm thick layer of gold. Figure 1 shows the comparison of monolithic matrices prepared from the same mixture at 25°C and –20°C. Although the monolithic matrix prepared at room temperature shows the typical globular structure with the small flow-through pore (the size of ~1 µm), the polymerization at –20°C resulted in the macroporous structure with interconnected pores (the size up to 50 µm) formed by the ice crystals.

The appropriate porosity and mechanical stability are the main requirements during the preparation of cryogels used as centrifugal devices for affinity chromatography. It is well known that the pore morphology as well as mechanical properties of prepared cryogels is controlled by several parameters such as the freezing rate and starting concentrations of monomer, crosslinker and initiator. Therefore, all the solutions and LDPE tubes were kept in the ice bath and the time was accurately controlled in order to obtain reproducible conditions.
for cryogel preparation. In the first experiments, the amount of monomers (HEMA and PEGDA) in the polymerization mixture was optimized. Concentrations of monomers in the range of 5-30% (v/v) were tested. Based on SEM micrographs, the use of lower concentration of monomers in the polymerization mixture resulted in preparation of a highly porous material with very thin polymer walls. On the other hand, an increase in initial concentration of monomers resulted in increase in the thickness of the polymer walls and in decrease of the pore volume. As a compromise, a polymerization mixture consisting of 20% (v/v) monomers and 80% (v/v) water was used for preparation of a stable rigid matrix containing macropores with the size of 10-50 µm. The prepared cryogels are highly elastic and have a sponge-like morphology withstanding deformation during centrifugation steps.

Although most of the prepared macroporous cryogels had smooth, non-porous polymer walls (based on the SEM micrographs reported) [11-16], we have observed that the concentration of the APS/TEMED initiating system in the polymerization mixture influenced the polymerization rate, significantly affecting the structure of the cryogel walls. Therefore, various concentrations of TEMED (0.25-1% (v/v)) were added to the polymerization mixture. The SEM micrographs of the resulting macroporous monolithic matrices are shown in Figure 2. While smooth non-porous polymer walls of the cryogels were prepared in the presence of 0.25% (v/v) TEMED (Figure 2A, 2D), the cryogels prepared in the presence of 1% (v/v) TEMED were characterized by the porous globular wall structure caused by the reaction induced phase separation (Figure 2C, 2F). The microporosity of the polymer walls significantly increased the surface of the developed material having the macropores in the size range of 20-50 µm. Similar combination of cryostructuration and the polymerization induced phase separation has been shown recently [17]. In this case of polyacrylamide-based cryogel synthesis the phase separation was induced by the addition of inert solvents such as methanol or acetone to the polymerization mixture.
3.2 Immobilization of concanavalin A

Prior to lectin immobilization, the pore surface of the poly(HEMA-co-PEGDA) macroporous cryogel was activated in order to allow the lectin immobilization under mild conditions. The simple way to introduce reactive functionalities on the surface of the poly(HEMA-co-PEGDA) cryogel is O-alkylation with epichlorohydrin under alkaline conditions as described in the section 2.4. The immobilization of concanavalin A was performed using the reductive amination technique. The epoxide functionalities of the epichlorohydrin activated cryogel were hydrolyzed by sulfuric acid followed by oxidation of diols to aldehyde functionalities using sodium periodate. Then, the cryogels were placed in the centrifugal filter unit and used for lectin immobilization. Concanavalin A was covalently coupled to the surface of the cryogel via primary amine functionalities in the presence of sodium cyanoborohydride.

3.3 Extraction of glycoproteins

The piece of the cryogel modified by concanavalin A was placed in the centrifugal filter unit and used for glycoprotein isolation. The selectivity of the developed spin column was investigated by the analysis of the model mixture containing 7 proteins in the concentration range of 0.6-0.8 µmol/L (Table 1). Only ribonuclease B and horseradish peroxidase are glycoproteins containing high-mannose type glycans with high affinity towards concanavalin A binding sites.

The protein mixture was applied on the spin column followed by washing using the binding buffer. The retained glycoproteins were eluted with methyl-α-D-mannopyranoside and the collected fractions were desalted using the C18 ZipTips following the protocol suggested by the manufacturer and analysed by MALDI-MS (Figure 3). The spectrum of the
sample applied on the concanavalin A contains all 7 proteins (Figure 3A); however, only ribonuclease B and horseradish peroxidase were detected in the elution fraction (Figure 3B) confirming the selectivity of the cryogel spin column. On the other hand, no glycoproteins were detected in the “flow-through” fractions by MALDI-MS. We can assume the binding capacity of the cryogel column is approximately 2-3 orders of magnitude lower in comparison to the conventional agarose-based beads with immobilized concanavalin A (e.g., 25 mg horseradish peroxidase/mL Con A Sepharose 4B resin).

After the elution step, the spin columns were washed five times using the binding buffer before application of the next sample. No non-specific interactions were observed since hydrophobic BSA was not detected in the elution fraction. The spin columns were reused five times with no observable loss of affinity.

4 Concluding remarks

Macroporous cryogels with immobilized affinity ligands placed in the centrifugal filter device represent a simple tool enabling rapid as well as efficient isolation of targeted analytes in glycoproteomic analysis. Although the cryogel was only modified by concanavalin A, other lectins can be immobilized on the surface as well and used for isolation of specific glycoproteins.

In principle, the binding capacity of the cryogels is not comparable with the conventional monolithic or even packed column due to the smaller surface area. However, the highly permeable macroporous matrices with immobilized affinity ligands such as lectins or antibodies might find a useful application in the isolation of whole cells or membrane domains based on the specific recognition.

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Conflict of interest

The authors declare no financial/commercial conflicts of interest.

References


Table 1. Composition of protein mixture

<table>
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<th>protein</th>
<th>high mannose glycan structure in the molecule</th>
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<th>m/z</th>
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<tbody>
<tr>
<td>1</td>
<td>bovine insulin</td>
<td>no</td>
<td>1</td>
<td>5.7 kDa</td>
</tr>
<tr>
<td>2</td>
<td>bovine cytochrome c</td>
<td>no</td>
<td>10</td>
<td>12.2 kDa</td>
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<tr>
<td>3</td>
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<td>no</td>
<td>10</td>
<td>14.1 kDa</td>
</tr>
<tr>
<td>4</td>
<td>bovine ribonuclease B</td>
<td>yes</td>
<td>10</td>
<td>14.8 kDa</td>
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<tr>
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<td>bovine serum albumin</td>
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<td>50</td>
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</table>
Figure 1. SEM micrographs of the poly(HEMA-co-PEGDA) monoliths prepared at (A) 25°C and (B) –20°C. Magnification: 1000x.
Figure 2. SEM micrographs of the macroporous monolithic poly(HEMA-co-PEGDA) cryogel prepared in the presence of various TEMED concentrations: (A, D) 0.25% (v/v), (B, E) 0.5% (v/v), (C, F) 1% (v/v). Magnification: (A, B, C) 250x, (D, E, F) 1000x.
Figure 3. MALDI/MS spectrum of (A) the protein mixture and (B) the glycoprotein fraction extracted using the concanavalin A modified cryogel. Proteins: 1, insulin; 2, cytochrome c; 3, α-lactalbumin; 4, ribonuclease B; 5, myoglobin; 6, peroxidase; 7, albumin.