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Short communication

Synthesis and performance of megaporous immobilized metal-ion affinity cryogels for recombinant protein capture and purification

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ABSTRACT

Megaporous cryogels with metal-ion affinity functionality, which possess enhanced protein-binding ability, were synthesized and their properties were investigated. These highly porous materials (pore sizes up to 100 μm) allowed the direct capture of a recombinant His_6-tagged protein from a partially clarified extract. The total ligand density of the material was found to be 770 μmol/g. Application of a partially clarified cell extract in order to recover a His_6-tagged protein (NAD(P)H-dependent 2-cyclohexen-1-one-reductase) yielded 12 mg of highly purified recombinant product *per* gram of adsorbent. Increased dynamic binding capacities were observed upon larger degrees of grafting, although some reduction in the quality of the system hydrodynamics was also observed. Nevertheless, these immobilized metal-ion affinity cryogels show potential for a convenient single-step purification of recombinant proteins from raw cell extracts without the need for laborious pre-chromatographic sample clean-up procedures.

1. Introduction

Several resource-intensive steps are required for the isolation and purification of a biologically or pharmaceutically relevant target protein or enzyme. In the case of recombinant proteins, such purification processes can be greatly facilitated by the introduction of protein affinity tags, which can then be suitably coupled to appropriate chromatographic adsorbents. However, adsorption in packed-beds normally requires extensive sample preparation, including a high degree of clarification in order to avoid column blockage and adsorbent fouling [1,2]. In the last decade, polymeric (macro- to mega-) porous materials have been introduced for protein capture, which, in some cases, avoid the extensive removal of biomass from fermentation broths [3,4]. These high-porosity materials, commonly known as cryogels, have been synthesized by polymerization under sub-zero temperatures [5–8]. However, one of the drawbacks of such a material is its limited surface area, which results in a low binding capacity for bioproducts [4,9–11]. However, polymer grafting can be utilized as a strategy to overcome such limitations and increase binding capacities by the introduction of moieties that enhance specific interactions between bioproducts and the adsorbent [12,13].

Affinity chromatography is a widely used technique for the purification and polishing of bioproducts largely due to its high specificity [14,15]. Immobilized metal-ion affinity chromatography (IMAC) is an affinity technique that takes advantage of strong coordinate–covalent interactions of certain biomolecular motifs with chelated metal-ions [16]. For example, imidazole groups in poly(His) residues form very strong complexes with Cu(II) ions [17]. Consequently, by immobilizing metal ions on the surface of chromatographic adsorbents, affinity-tagged biomolecules can be conveniently separated from crude feedstocks in a highly specific manner and in a single step. The most common IMAC systems are based on the immobilization of Ni(II) or Cu(II) ions on adsorbents *via* grafted chelating groups like iminodiacetic acid (IDA) or nitrilotriacetic acid (NTA) [18]. In this work, megaporous cryogels were synthesized, grafted with terminal epoxide moieties, and later functionalized with IDA. Cu(II) ions were then used to finally convey IMAC functionality to the cryogel. Furthermore, the applications of such highly porous adsorbents in the purification of a poly(His)-tagged recombinant protein were investigated.

2. Materials and methods

2.1. Chemicals and reagents

Methacrylic acid (MAA, 99% purity), ethylene glycol dimethacrylate (EGDMA, 98% purity), poly(ethylene glycol) diacrylate (PEGDA), glycidyl methacrylate (GMA), ammonium persulfate

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(APS), sodium carbonate, ethylenediaminetetraacetic acid (EDTA), imidazole, and the SDS-PAGE preparation kit were purchased from AppliChem GmbH (Darmstadt, Germany). *N,N,N′,N′*-tetramethylethylenediamine (TEMED) was purchased from SERVA Electrophoresis GmbH (Heidelberg, Germany). Ceric ammonium nitrate (CAN) and iminodiacetic acid (IDA) were purchased from Sigma Aldrich, Germany.

2.2. Physical characterization of IMAC-cryogels

The percentage of grafting (*G*%) was evaluated by the elementary weight gain method [19]. To avoid any traces of homo-polymer inside the cryogel, it was washed with hydrochloric acid (0.2 M) and water. Grafting percentage was calculated according to:

$$G\% = \frac{W_2 - W_1}{W_1} \times 100 \quad (1)$$

where the W_1 is the initial dry weight of the backbone, W_2 is the dry weight after grafting.

Scanning electron microscopy (SEM) was carried out as previously reported [3]. The samples were dried, gold sputtered and examined at different magnification using a JEOL JSM 5900 scanning electron microscope (Peabody Inc., USA).

2.3. Chemical characterization of IMAC-cryogels

FTIR spectra of dried material samples that were included in potassium bromide pellets were obtained using an FTIR-8300 spectrophotometer (Shimadzu). The concentration of the sample in KBr was approximately 0.2% by weight.

Ligand density was measured by quantification of the bound copper. The modified cryogel (MP-MA) was first saturated with copper and thoroughly washed with distilled water. Bound copper ions were released from the porous material employing a 0.1 M EDTA solution. The Cu(II)–EDTA complex was quantified by UV/Vis spectrophotometry at 730 nm employing a published value for the extinction coefficient of this complex (46.8 M^{-1} cm^{-1}) [20]. Control experiments to check Cu(II) loading on the base cryogel (MP-CM) were factored into the calculations for ligand density.

2.4. Adsorption system performance

The column pressure drop was measured as a function of flow rate using distilled water as mobile phase. Recorded pressure values were corrected for extra-column effects by measuring the pressure drop (Δp) across the column in the absence and presence of the cryogel. The hydraulic permeability was calculated using Darcy's law (see Eq. (2)) [21].

$$\frac{\Delta p}{L} = \frac{u\mu}{B_0} \quad (2)$$

where Δp stands for pressure drop (Pa), L is the column length (m), u is superficial velocity (m s^{-1}), μ stands for viscosity (Pa s) and B_0 is the hydraulic permeability (m^2).

System hydrodynamics were evaluated by residence time distribution (RTD) experiments using acetone as a tracer. The total number of theoretical plates (N) was calculated considering the residence time (t_R) and the peak width at half height (w_h) obtained from the tracer impulse. The number of plates (N) and the plate height (H) were calculated according to Eqs. (3) and (4), respectively [22,23].

$$N = 5.45\left(\frac{t_R}{w_h}\right)^2 \quad (3)$$

$$H = \frac{L}{N} \quad (4)$$

Dynamic binding capacity (DBC) was determined utilizing lysozyme as a model protein for breakthrough analysis [24]. The metal-ion affinity cryogel was equilibrated with 10 column volumes (CVs) of 20 mM phosphate buffer at pH 7 having 0.2 M sodium chloride and 0.02 M imidazole (buffer A) and saturated with the protein solution (2 mg/mL) in the same buffer. Protein concentration was recorded by monitoring the absorbance of the eluent at 280 nm. The DBC was calculated at 10% breakthrough using Eq. (5) [25].

$$q = \frac{C_0 \times (V - V_0)}{V_c} \quad (5)$$

where q is the dynamic binding capacity at 10% breakthrough (mg/mL), C_0 is the initial concentration of the sample (mg/mL), V is the volume at 10% breakthrough (mL), V_0 represents the dead volume of the system (mL), and V_c is the column volume (mL).

2.5. Tagged protein recovery and purification

The packed material was equilibrated with 10 CVs of a 20 mM phosphate buffer (pH 7), containing 0.2 M sodium chloride and 0.02 M imidazole (buffer A). 10 mL of the undiluted clarified homogenate (total protein content of almost 10 mg/mL) was applied to the material in the equilibration buffer (see Supporting information for preparation of homogenate). The flow rate of the process was 0.5 mL/min. For protein elution, 20 mM phosphate buffer (pH 7) containing 0.2 M sodium chloride and 0.2 M imidazole (buffer B) was employed (15 CVs). Fractions of the flow through and elution were collected and stored at 4 °C.

2.6. Protein purity and yield

Chromatographic fractions were analyzed by SDS-PAGE [26], employing a 12% tris–bisacrylamide gel. Protein bands were stained with Coomassie Blue as *per* the manufacturer's instructions (AppliChem GmbH, Darmstadt, Germany). Total protein content was measured spectrophotometrically by the Bradford protein assay [26]. The standard protein assay was performed using a range of BSA concentrations (200–1500 μg/mL). The concentration of unknown sample was calculated from the standard curve.

3. Results and discussion

3.1. Preparation of megaporous cryogels with IMAC functionality

The cryogel backbone was synthesized at −20 °C utilizing chemical monomers (MAA) in combination with a highly soluble cross-linking agent (*e.g.*, PEGDA) to give the required physical stability to the material (see Supporting information). This method has been known to generate typical pore sizes of approximately 100 μm [3]. Besides porosity, other beneficial properties of the synthesized structures, *e.g.*, a rapid re-swelling in water and remarkable elasticity, were also observed. Furthermore, the cryogels could be easily fitted into columns without cracking and were subsequently rehydrated *in situ* with an aqueous mobile phase.

Due to the presence of carboxylic acid groups on the backbone (MP-CM), the cryogel displayed weak cation-exchange characteristics. However, the binding capacity of MP-CM for macromolecules is quite low (2.5 mg *per* gram of dry adsorbent) due to the restricted internal surface area *per* unit volume, which is characteristic of highly megaporous structures [3].

In order to increase protein binding capacity values, GMA was employed as monomer for grafting due to its dual functionality (see Fig. 1). On one hand, the polymerization of vinyl groups leads to chain elongation with possible formation of polymer tentacles [19,27]. On the other hand, the epoxide group can be further