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Composite imprinted macroporous hydrogels for haemoglobin purification from cell homogenate

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ABSTRACT

Purification of haemoglobin (Hb) has been studied for many years due to its ability to act as an oxygen carrier and its possible use in urgent clinical treatment. In this study, different types of chromatography columns were developed for Hb purification. Two of them showed satisfactory results as affinity chromatography columns and were thus studied more extensively. The affinity adsorbents were prepared by molecular imprinting techniques. In the first case, Pickering emulsion polymerization was used to prepare affinity adsorbents based on molecular imprinting technology. The imprinted particles were immobilized via covalent bonds on the surface of cryogel, a macroporous hydrogel produced by free radical polymerization under sub-zero temperature. In the second case, the affinity sites for Hb were formed directly on an acrylamide cryogel by protein imprinting during the cryogelation. The dynamic binding capacity of the composite cryogel with the immobilized particles and the directly imprinted acrylamide cryogel was found to be 5.2 mg/g and 3.6 mg/g, respectively. The affinity columns showed high selectivity towards Hb in spite of the presence of serum albumin as well as other interfering substances in non-clarified cell homogenates. The maximum capacity in batch mode, the fluid flow and other physical and chemical properties of these columns were investigated.

1. Introduction

Blood transfusion is a routine medical treatment in cases of severe blood loss (≥30% of the blood volume). Failing to balance the blood pressure and maintain oxygen delivery will cause serious damage to patients [1]. However, there are some challenges when using human's blood for transfusion e.g. lack of healthy blood donors, short shelf-life of blood, increasingly ageing world population and risk of transmitting pathogens such as HIV. These challenges can be addressed by developing blood substitutes [2]. Haemoglobin-based oxygen carriers (HBOCs) are a class of blood substitutes which have proved useful for delivering oxygen. These carriers can be produced from different sources such as human and bovine [3]. Regardless of the original source of Hb, the protein must be purified and separated from the by-products generated during the upstream process.

Affinity chromatography columns have attracted much interest due to their high efficiency and separation resolution in downstream processing [4]. Affinity ligands based on biological molecules such as antibodies have been used in these columns for many years due to their high selectivity [5–7]. However, their high cost, sensitivity to harsh conditions, tendency of denaturation, low capacity and short shelf life, demands of correct orientation and risk of leakage are some of the shortcomings. Consequently, it becomes attractive to develop alternative synthetic ligands which are potentially inexpensive, chemically well-defined and stable [8–10].

One of the techniques for synthesizing affinity ligands can be based on molecular imprinting. Molecularly imprinted polymers (MIPs) are often synthesized as rationally designed ligands with high selectivity while the extent of non-specific interactions can be reduced. MIP ligands are prepared by polymerization of cross-linkers and functional monomers in the presence of target (bio)molecules which act as templates. After polymerization, the target molecules are removed to leave behind cavities with specific shape, size and functional groups in the crosslinked polymers to enable highly selective molecular recognition towards the target molecules [11–13]. MIPs have shown promising results for capturing small molecules at low concentrations in the last few decades [14]. Recently, utilizing MIPs for recognition of also large biomolecules such as proteins, DNA, etc., has drawn attention [11,15]. The use of conventional MIP approaches for capturing biomolecules may not be suitable due to the harsh synthetic conditions such as the frequent use of organic solvent, which can easily denature biomacromolecular templates. In addition, the issues of

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the large size, complex structure and sensitivity to pH, temperature or high salt concentration, as well as low solubility in nonpolar solvent demand water-compatible polymerization techniques to be adopted in order to overcome these obstacles [16]. Pickering emulsion polymerization was first introduced by our group for synthesizing water-compatible MIP particles [17,18]. Positioning of silica nanoparticles on water/oil interfaces to form stable emulsions is the key for successful molecular imprinting by Pickering emulsion polymerization. The binding capacity and water compatibility of MIP particles are strongly dependent on monomer selection and the amount of porogen used in the polymerization process [19]. By fixing a template on the surface of the stabilizing silica nanoparticles, it has been proven possible to generate surface-imprinted MIP beads in an oil-in-water Pickering emulsion. This approach for synthesizing MIP particles towards Hb proteins was used in this study.

Packed-bed affinity columns have been well studied in downstream processing for purification of different molecules. However, the conventional column configuration is not a suitable option when dealing with particulate-containing fluids such as urine, blood and crude cell extracts. The molecular size of many biologics is in the range of 20–300 nm while packed-bed columns often only can operate with compounds smaller than 10 nm [20]. A macroporous hydrogel, cryogel, is a fairly recent class of monolithic column which is an end-product of cryogelation polymerization. The polymerization takes place at sub-zero temperature while water acts as a porogen [21,22]. Cryogel has a spongy texture and harbours pores in the range 1–100 μm. The macroporous structure allows excellent mass transfer and high flow rates through the column [23–26]. Since most of the cryogel structure is composed of pores, its surface area is only limited to thin walls and, as a consequence, its binding capacity may be low especially when treating large molecules [27]. To address the issue of low capacity, composite cryogels have been designed with different approaches such as embedding or immobilizing particles/adsorbents inside or on the surface of the wall of cryogels [28–31].

As mentioned earlier in this study, MIP particles for capturing haemoglobin (Hb) were prepared first by using Pickering emulsion polymerization. An additional functional group was introduced onto the surface of the MIP particles during the synthesis. Hb-selective cryogels were formed in different processes: 1) by immobilizing MIP particles onto a pre-made cryogel via covalent conjugation; 2) by embedding MIP particles inside the cryogel's walls during the polymerization process; and 3) cryogel was formed in the presence of Hb molecules during the cryogelation process, where the protein acted as template to create specific binding sites directly in the cryogel. The physical properties and selectivity of all the macroporous columns were studied. The binding capacity of the affinity hydrogels was evaluated and compared with each other in both batch mode operation and in chromatographic systems.

2. Materials and methods

2.1. Materials

Acrylamide (Am), *N,N,N′,N′*-tetramethylethylenediamine (TEMED) and silica dioxide nano powder (spherical, porous, 5–15 nm, (Si) were purchased from Sigma-Aldrich, China. *N,N′*-Methylenebis(acrylamide) (MBAm), human Hb (lyophilized powder, acetic acid, bovine serum albumin (BSA), ethanolamine and ethylene glycol dimethylacrylate (EGDMA) were from Sigma-Aldrich, USA. Hydrofluoric acid (HF), sodium phosphate, sodium carbonate, ammonium persulfate (APS), ethylenediamine (99%, sulphuric acid (95–97%) and nitric acid were from Sigma-Aldrich, Germany. Toluene and methanol were purchased from Thermo-Fisher, Germany. Sodium hydrogen carbonate, Triton X-100 (99.5%), azobisisobutyronitrile (AIBN, 98%) and methacrylic acid (MAA) were from Merck-Schuchardt, Germany. Sodium hydrogen phosphate and sodium dodecyl sulphate (SDS) were purchased from VWR, France and BDH Chemicals, England, respectively. Allyl glycidyl ether (AGE) was from EGA-Chemi, Germany. All the chemicals and reagents were of analytical grade and used without any purification or pre-treatment step.

Adult human Hb (HbA) was kindly provided by Department of Clinical Sciences, Lund University. *E. coli* cells were cultured following a previously published protocol [32,33]. The cells were cultured overnight at 30 °C in Terrific broth media with a shaking speed of 150 rpm. After harvesting, the cells were washed twice in 10 mM phosphate buffer (0.1 M, pH 6.0) and then pelleted by centrifugation and snap-frozen in liquid nitrogen. The cell pellets were stored at −80 °C until further use.

2.2. Synthesis of MIP/NIP particles containing epoxy groups by Pickering emulsion polymerization

Haemoglobin-imprinted polymer particles were synthesized by Pickering emulsion polymerization following a previously published procedure [17,18] with a few modifications. Briefly, 50 mg silica nanoparticles and 50 mg Hb were suspended in 15 mL distilled water for over an hour on a rocking table. Due to the electrostatic interactions between the Hb and silica nanoparticles, the template non-covalently binds to the particles. Then the suspension was centrifuged and the excess Hb in the supernatant was discarded. The particles were re-suspended in 15 mL distilled water. A Pickering emulsion was prepared by mixing the particle suspension with 0.5 g Am, 0.7 g MAA, 0.38 g AGE and 50 mg AIBN in a mixture of 0.5 mL toluene and 2 mL EGDMA. The obtained mixture was sonicated for a minimum of 5 min in an ice bath. For the ultrasound sonication, a power intensity of 65% was used, with a 2 s interval between sonication pulses. The obtained emulsion was left to stand on a bench at room temperature for 1–2 h until it stabilized. After this step, the polymerization was induced and continued in an oven for 24 h at 70 °C. The upper phase was discarded and the particles formed were collected by centrifugation, and washed with 20 mL methanol on a rocking table for 1 h. The silica nanoparticles were removed by treatment with a 30 mL mixture of methanol and hydrofluoric acid (30: 1 v/v%) for 1 h at room temperature while stirring. The template (Hb molecules) was removed by washing with 10 mL mixture of acetic acid (10% v/v) and SDS (5% w/w) solution. The final polymer beads were washed with copious water, then dried under vacuum at room temperature.

The same procedure was followed to prepare the non-imprinted polymer (NIP) particles, except that the template was replaced by 50 mg Triton X-100. The MIP and NIP particles containing epoxy groups on their surface are denoted as MIP_{ep} and NIP_{ep}, respectively.

2.3. Synthesis of MIP particles containing amino groups

MIPs containing amino groups on their surface were prepared by suspending 4 g of MIP_{ep} particles in a 50 mL mixture of water and ethylenediamine (1: 1 volume ratio) for 4 h at 80 °C while stirring. The beads were dialysed against water for 48 h and freeze-dried. The obtained particles were denoted as MIP_{am}. For synthesizing NIP_{am}, NIP_{ep} were modified following the same procedure.

2.4. Preparation of cryogels

Preparation of acrylamide cryogel using radical polymerization has been reported elsewhere [29]. In this work the total monomer

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