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Synthesis of tentacle type magnetic beads as immobilized metal chelate affinity support for cytochrome *c* adsorption

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Abstract

Magnetic poly(2-hydroxyethylmethacrylate) (mPHEMA) beads with an average diameter of 100–140 μm were produced by suspension polymerization in the presence of magnetite particles (i.e. Fe_3O_4). Specific surface area and average pore size of the magnetic beads was found to be 50 m^2/g and 819 nm, respectively. Ester groups in the mPHEMA structure were converted to imine groups by reacting with poly(ethyleneimine) (PEI) in the presence of NaH. Amino ($–NH_2$) content of PEI-attached mPHEMA beads was determined as 102 mg PEI/g. Then, Cu^{2+} ions were chelated on the magnetic beads in the range of 20–793 μmol Cu^{2+}/g. Cytochrome *c* (cyt *c*) adsorption was performed on the metal chelating beads from aqueous solutions containing different amounts of cyt *c* at different pHs, Cu^{2+} loadings and temperatures. Cyt *c* adsorption on the mPHEMA/PEI beads was 4.6 mg/g. Cu^{2+} chelation increased the cyt *c* adsorption significantly (40.1 mg/g). Adsorption capacity increased with Cu^{2+} loading and then reached a saturation value. Cyt *c* adsorption decreased with increasing temperature. Cyt *c* molecules could be reversibly adsorbed and eluted ten times with the magnetic adsorbents without noticeable loss in their cyt *c* adsorption capacity. The applicability of two kinetic models including pseudo-first order and pseudo-second order model was estimated on the basis of comparative analysis of the corresponding rate parameters, equilibrium capacity and correlation coefficients. Results suggest that chemisorption processes could be the rate-limiting step in the adsorption process. In the last part of this article, cyt *c* adsorption experiments were performed in a magnetically stabilized fluidized bed (MSFB) system at optimum conditions determined from the batch experiments. The adsorption capacity decreased significantly from 46.8 to 15.4 mg/g polymer with the increase of the flow-rate from 0.5 to 4.0 ml/min. The resulting magnetic chelator beads possessed excellent long-term storage stability.

Keywords: Affinity chromatography; Metal-chelate affinity beads; Polyethyleneimine; Cytochrome *c*; Magnetic beads

1. Introduction

Immobilized metal affinity chromatography (IMAC) has been used extensively for purification of a variety of proteins [1–3]. Many transition metals can form stable complexes with electron-rich compounds and may coordinate molecules containing O, N and S by ion dipole interactions. Metal ion ligands are first-row transition metal ions (Zn^{2+}, Ni^{2+}, Cu^{2+} and Fe^{2+}) chelated by iminodiacetic acid, nitrilotriacetic acid, tris(carboxymethyl)ethylene-diamine. IMAC introduces a new approach for selectively interacting materials on the basis of their affinities for chelated metal ions. The separation is based on the interaction of a Lewis acid (electron pair acceptor), i.e., a chelated metal ion, with an electron donor atoms (N, O and S) on the surface of the protein [4,5]. Proteins are assumed to interact mainly through the imidazole group of histidine and, to a lesser extent, the indoyl group of tryptophan and the thiol group of cysteine. Cooperation between neighboring amino acid side chains and local conformations play important roles in protein binding. Aromatic amino acids and the amino-terminal of the peptides also have some contributions. The benefits of IMAC – ligand stability, relatively high selectivity, high protein loading, mild elution conditions, simple regeneration, low cost of metal ions, and reuse of adsorbents for hundred of times without and detecable loss of metal-chelating properties – are decisive when developing large-scale purification processes for industrial applications [6].

There have been several separation approaches performed under magnetic field [7]. The most well known technique is the magnetically stabilized fluidized bed. Magnetically stabilized

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fluidized bed exhibits combination of the best characteristics of both packed and fluidized bed. These include the efficient fluid–solid mass transfer properties, elimination of particle mixing, low pressure drop, high feed-stream solid tolerances, good fluid–solid contact, elimination of fouling and continuous countercurrent operation [8]. Magnetically stabilized fluidized bed is desirable because of high convective transport rates. Magnetic adsorbents can be efficiently used for the purification of proteins and nucleic acids [9]. Magnetic adsorbents can be manufactured from polymers since they have a variety of surface functional groups, which can be tailored to use in different applications [10–16].

It was demonstrated that the attachment of polymer chains onto the surface of porous polymer beads to form tentacle-type supports could sufficiently increase the adsorption capacity of proteins [17]. The enhanced adsorption capacity of these modified polymer adsorbents would apparently arise from tentacles extending away from the support surface a sufficient distance to allow proteins to penetrate the polymer layer and bind in multiple layers [18]. Also with tentacle type chains the contact between the protein and the base support surface is markedly reduced which minimize undesirable hydrophobic interactions between the protein and the base support surface. Enlightened by this idea, we assumed that this attachment technique could be an efficient way to enhance the adsorption capacity of proteins on magnetic beads.

In this study, we assessed the suitability of a magnetically stabilized fluidized bed (MSFB) utilising Cu^{2+}-chelated poly (hydroxyethyl methacrylate)/poly(ethyleneimine) [mPHEMA/PEI] beads for the adsorption of cyt *c*. Polyethyleneimine (PEI) is chosen to attach on magnetic beads, as the resultant polymeric magnetic materials are anticipated to expand the adsorbent based separation technology. We are interested in PEI, as it is known a branched chain polymer with a lot of amine groups, has extensively used to form complexes with metal ions [19]. The number of amine groups can easily exceed 1000 depending on the molecular weight of the PEI. The higher flexibility and durability of this complexing ligand as well as significantly lower material and manufacturing costs are also very important. PHEMA gels have been largely employed in biomedical applications and as separation or adsorption matrixes for various proteins [20–23]. For this reason, PHEMA beads were produced by suspension polymerization in the presence of magnetite particles. Ester groups in the PHEMA structure were converted to amine groups by reacting with poly(ethyleneimine) (PEI) in the presence of NaH. Then, cyt *c* adsorption onto the Cu^{2+}-chelated mPHEMA-PEI beads is discussed.

2. Experimental

2.1. Materials

Cytochrome *c* (from horse heart, Mr: 12.384, p*I*: 10.6), poly(ethyleneimine) (PEI; average molecular weight of 25,000, branched, ratio of primary amine, secondary amine and tertiary amine: 1/2/1), 50 wt.% aqueous solution were supplied by Sigma (St. Louis, USA). Hydroxyethyl methacrylate (HEMA) and ethylene glycol dimethacrylate (EGDMA) were purchased from Fluka A.G. (Buchs, Switzerland) filtered through an activated basic alumina column, distilled under reduced pressure and stored at 4 °C. Radical initiator azobisisobutyronitrile (AIBN) was obtained from Fluka (Switzerland) and crystallized from methanol. Poly(vinyl alcohol) (PVAL; MW: 100,000, 98% hydrolyzed) and magnetite (Fe_3O_4; <1 μm) were supplied from Aldrich Chem. Co. (Milawaukee, WI, USA). All other reagents, unless specified, were of analytical grade and were used without further purification. Laboratory glassware was kept overnight in a 5% nitric acid solution. Before use the glassware was rinsed with deionised water and dried in a dust-free environment. Buffer and sample solutions were prefiltered through a 0.2 μm membrane (Sartorius, Göttingen, Germany). All water used in the adsorption experiments was purified using a Barnstead (Dubuque, IA) ROpure LP® reverse osmosis unit with a high flow cellulose acetate membrane (Barnstead D2731) followed by a Barnstead D3804 NANOpure® organic/colloid removal and ion exchange packed-bed system.

2.2. Preparation of mPHEMA beads

mPHEMA beads were prepared as described in elsewhere [24]. Polymerization was carried out in an aqueous suspension medium containing polyvinyl alcohol (PVAL; 0.2 g), which was used as a stabilizer. Toluene and EGDMA was included in the recipe as a pore former and cross-linker, respectively. The monomer phase containing HEMA (4.0 ml), EGDMA (8.0 ml), toluene (12 ml), magnetite particles (0.5 g) and benzoyl peroxide (60 mg) was added to the dispersion medium (50 ml distilled water) within a laboratory type reactor (i.e., a two neck flask with a volume of 500 ml) provided with a blade type stirrer. The mixture was degassed by argon purging prior to the addition of benzoyl peroxide. The polymerization was then allowed to proceed at 70 °C for 4 h and 90 °C for 2 h. Final magnetic beads were extensively washed with ethanol and water to remove any unreacted monomer or diluent and then stored in distilled water at 4 °C. When not in use, the resulting adsorbents were kept under refrigeration in 0.02% NaN_3 solution for preventing of microbial contamination.

2.3. PEI attachment

Following procedure was applied for covalent attachment of PEI onto the mPHEMA beads, which was given elsewhere [25]. One hundred milliliters of PEI solution and 500 ml of benzene were boiled in Dean–Stark apparatus at the reflux temperature for azeotropic removal of water. After removal of water in the PEI solution with benzene, 10 g of PEI was dissolved in tetrahydrofuran (THF). NaH (3.0 g) was added into this solution. A 10 g of dry magnetic beads was weighed and transferred into this solution mixture. The solution was stirred in Dean–Stark apparatus at the reflux temperature for 24 h in an N_2 atmosphere. After completion of reaction, the mPHEMA/PEI beads were filtered and washed thoroughly with methanol several times and then dried in vacuo at room temperature for 24 h.