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Preparation and characterization of pH-responsive polyacrylamide molecularly imprinted polymer: Application to isolation of recombinant and wild type human serum albumin from biological sources

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ABSTRACT

In this work pH-responsive neutral and cationic polyacrylamide molecularly imprinted polymers (nMIP and cMIP, respectively) were prepared for separation of recombinant and wild type human serum albumin (HSA, pI 4.7) using mixture of polymerization initiators. The effect of pH during preparation and adsorption stages at $pI_{(HSA)} \pm 2.0$ on binding capacity and selectivity; imprinting factor (IF) was thoroughly investigated. SE-HPLC and RP-HPLC were employed for thorough evaluation of the stability of HSA at the studied experimental conditions and for simultaneous determination of HSA and erythropoietin (EPO) in their mixtures, respectively. Results showed that nMIP were generally superior to cMIP, where nMIP prepared at pH 2.7 and tested at pH 6.7 showed superior binding characteristics (IF 42.91). The pH at the preparation stage imposed minimal effect on the stability of HSA owing to entrapment of HSA within the polymer network. Adsorption experiments carried out at pH 2.7, regardless of polymer type and pH of preparation revealed poor selectivity. Adsorption of HSA onto MIP followed Sips model with pseudo second-order kinetics. Scanning electron microscopy (SEM) revealed a rough surface for MIP and a smooth one with wider pore diameter for non-imprinted polymer (NIP). Successful separation of recombinant HSA from its binary mixture with EPO and wild type HSA from crude plasma was demonstrated using RP-HPLC. This suggested that MIP should be applicable for downstream purification of therapeutic grade HSA at scale either from plasma or recombinant sources and isolation of HSA from plasma for diagnostic purposes.

1. Introduction

Biopharmaceuticals present a breakthrough in biomedical research since they enable the treatment of a wide range of life-threatening incurable diseases [1]. They differ from low molecular weight conventional pharmaceuticals in being more complex in structure, relatively less stable and more difficult to purify, characterize and control [2–4].

Human serum albumin (HSA) is a globular protein composed of 585 amino acids (66.5 kDa, pI 4.7) and has a rigid, heart-shaped conformational structure [5]. It contains 35 Cys residues forming 17 intra-chain disulfide bonds and one free sulfhydryl group at Cys34. The concentration of HSA in human plasma ranges from 35 to 45 mg mL^{-1} representing the main determinant of plasma oncotic pressure and exhibiting an antioxidant function [5–7]. HSA is used typically, at dosage more than 10 g per dose for the treatment of hemorrhagic shock, surgical blood loss, serious burn injuries, liver diseases and other conditions that cause hypoalbuminemia. Its main therapeutic role is to restore normal colloid oncotic pressure and to increase circulating plasma volume [8]. HSA is also a diagnostic biomarker for numerous pathological conditions such as oxidative stress, cancer and rheumatoid arthritis [5,7]. Moreover, recombinant HSA is used as an excipient in biopharmaceutical formulations and supplement in cell culture media used for production of vaccines and biopharmaceuticals [9].

Most of the commercial HSA preparations are obtained through fractionation of human plasma which entails possible contamina-

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tion by viruses or prions [8]. Recently, recombinant technology has been employed for production of HSA using several hosts [5,10]. The recombinant versions were found bioequivalent to plasma-derived HSA with respect to safety and therapeutic characteristics [9,11]. Structural differences have been noted which was attributed to differences in posttranslational modifications of recombinant HSA version that is largely depends on the host employed for production [12,13].

Since HSA is administrated at high doses, purity has always been a critical quality attribute for HSA in order to avoid any undesirable immunogenic responses [9]. A combinations of several fractionation and/or chromatographic steps are generally required for purification of HSA [14]. However, direct product capture at high purity and low cost has always been the main challenge. Owing to the low cost, stability and ease of production, approaches using molecularly imprinted polymers (MIP) appear attractive in this context [15–17]. MIP involves preparation of robust artificial affinity adsorbents in other words "synthetic receptors". MIP of proteins is still an emerging bioanalytical technique that should ultimately replace expensive bio-reagents; such as antibodies and aptamers required for affinity chromatography applications [17–19].

Despite the numerous reports, advances towards generic and robust MIP have been slow. Most of these reports covered the separation of low molecular weight compounds [20]. When it comes to protein MIP, no universal approach has been reported and general recommendations based on the structure, hydrophobicity and surface charge distribution were reported [21–23]. MIP are generally prepared using a reaction mixture of functional monomer(s), cross linker(s), polymerization initiator(s) and most importantly the template; either a whole protein or an exposed small molecular weight epitope [24]. Complementary binding sites that recognize structural features of the target protein are then formed [17].

Monomers of organic siloxanes [25], metal chelators [26–28], oligosaccharides [29] and polyacrylamide monomers have been reported for synthesis of protein MIP. Polyacrylamide-based polymers have been employed using a variety of monomers including neutral [30–35] and charged monomers [36–40]. This could be attributed to the biocompatibility of polyacrylamide polymers [41] and availability of large number of monomers of different chemical properties as outlined above. Moreover, it has been reported that polyacrylamide MIP showed high recognition specificity [35] and the most promising results in terms of imprinting efficiency [42]. The effect of pH during synthesis and adsorption stages has been considered in few reports [30,43,44]. However, to the best of our knowledge systemic evaluation of the effect of pH not only on MIP synthesis/application but also on the stability and conformational structure of template protein has not been reported. We believe that this could be one of the most important reasons behind the low reproducibility and lack of predictable response of MIP. This had largely hindered translation of MIP research from bench to biopharmaceutical industry.

In the present study, pH responsive polyacrylamide MIP for separation of HSA have been synthesized, characterized and tested at pH range $pI_{(HSA)} \pm 2$. The capacity and selectivity were evaluated relative to equivalent non-imprinted polymer (NIP) using a stability-indicating orthogonal testing protocol. The applicability of optimum MIP was demonstrated through separation of either recombinant or wild type HSA from its mixtures with EPO and human plasma proteins, respectively.

2. Materials and methods

2.1. Chemicals and reagents

Plasma-purified HSA (0.20 g mL^{-1}) and recombinant EPO (300.00 μg mL^{-1}) reference standards were kindly supplied by the National Organization for Research and Control of Biologicals (Egypt). Gerepo 4000 IU, Vials, North China Pharmaceuticals Group (China), labeled to contain 36 μg mL^{-1} recombinant EPO and 2.5 mg mL^{-1} recombinant HSA was obtained from local market. Human plasma was obtained from the Holding Company for Biological Products and Vaccines (VACSERA), Egypt and was used as a source for wild type HSA. Bradford total protein assay kit was obtained from Thermoscientific (USA). Acrylamide was purchased from HIMEDIA laboratories Pvt. Ltd. (India) while *N,N'* ethylenebis(acrylamide) (MBA), *N,N,N',N'*-tetramethylethylenediamine (TEMED) and ammonium persulfate (APS) were purchased from BioBasic INC (USA). 2-(dimethylamino)ethyl methacrylate (DMAEMA) was purchased from Sigma (USA). Q Sepharose Fast Flow anion exchange resin was obtained from GE Healthcare (USA). All other chemicals and solvents were of HPLC grade and were obtained from Sigma (USA). Ultrapure water was obtained using a MilliQ UF-Plus system (Millipore, Germany) with a resistivity of at least 18.2 MΩ cm at 25 °C and total organic carbon (TOC) value below 5 ppb.

2.2. Instruments

All chromatographic separations were carried out using an Agilent 1200 HPLC system (Agilent Technologies, Germany) equipped with PDA detector, temperature controlled autosampler and column compartment. System control and data analysis were achieved using Chemstation software (Agilent Technologies, Germany). Spectrophotometric analysis was carried out using a UV–vis spectrophotometer (Shimadzu Corporation, Japan) controlled with UVProbe Software (Shimadzu Corporation, Japan). Template extraction and adsorption experiments were carried out using vacuum manifold and Bond Elut solid phase extraction (SPE) columns, 6 mL with two Frits (Agilent Technologies, Germany) manually packed with the polymer under investigation and operated at mild vacuum.

2.3. Bioanalysis techniques

2.3.1. Size exclusion chromatography

A previously developed SE-HPLC method was used to assess the stability of HSA at the experimental conditions that might be encountered during this study [45]. Briefly, analysis was conducted using YMC Pack-Diol 200 column (500 × 8 mm), particle size 5 μm and pore size 200 Å, molecular weight range 10–100,000 kDa (YMC Corporation, Japan). The temperature of the column compartment and sample tray were maintained at 25 °C and 4–8 °C, respectively and the injection volume was 50 μL. A mobile phase composed of 0.1 M phosphate buffer (pH 7.0 ± 0.05) containing 0.1 M sodium sulfate was used. Isocratic elution was employed at a flow rate of 1.0 mL min^{-1} for 30 min and detection was achieved at 214.0 nm. Separation efficiency was demonstrated using HSA degraded samples and system suitability parameters were calculated [46]. A set of HSA standard solutions was prepared over a concentration range of 10.00–200.00 μg mL^{-1} and analyzed. Calibration curve was constructed and the regression equation was obtained. Method verification was performed according to ICH guidelines [47] and the following parameters were investigated: specificity, accuracy and precision.

2.3.2. Reversed phase chromatography

2.3.2.1. Method optimization and system suitability. A previously reported set of separation conditions [48] were optimized with respect to column type, gradient elution program and flow rate. All separations were carried out using Zorbax 300SB-C18 column (250 mm × 4.6 mm), particle size 5 μm and pore size 250 Å (Agilent technologies, USA). The HPLC system was operated in gra-