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Novel humic acid-bonded magnetite nanoparticles for protein immobilization

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ABSTRACT

The present paper is the first report that introduces (i) a useful methodology for chemical immobilization of humic acid (HA) to aminopropyltriethoxysilane-functionalized magnetite iron oxide nanoparticles (APS-MNPs) and (ii) human serum albumin (HSA) binding to the obtained material (HA-APS-MNPs). The newly prepared magnetite nanoparticle was characterized by using Fourier transform infrared spectroscopy (FTIR), transmission electron microscopy (TEM), scanning electron microscopy (SEM), thermogravimetric analysis (TGA), and elemental analysis. Results indicated that surface modification of the bare magnetite nanoparticles (MNPs) with aminopropyltriethoxysilane (APS) and HA was successfully performed. The protein binding studies that were evaluated in batch mode exhibited that HA-APS-MNPs could be efficiently used as a substrate for the binding of HSA from aqueous solutions. Usually, recovery values higher than 90% were found to be feasible by HA-APS-MNPs, while that value was around 2% and 70% in the cases of MNPs and APS-MNPs, respectively. Hence, the capacity of MNPs was found to be significantly improved by immobilization of HA. Furthermore, thermal degradation of HA-APS-MNPs and HSA bonded HA-APS-MNPs was evaluated in terms of the Horowitz–Metzger equation in order to determine kinetic parameters for thermal decomposition. Activation energies calculated for HA-APS-MNPs (20.74 kJ mol^{-1}) and HSA bonded HA-APS-MNPs (33.42 kJ mol^{-1}) implied chemical immobilization of HA to APS-MNPs, and tight interactions between HA and HA-APS-MNPs.

1. Introduction

Interactions between solid substrates and proteins (and other biomolecules) are important in various applications such as protein purification and separation, peptide synthesis, and diagnostic assays [1,2]. Despite the fact that interaction of proteins with material surfaces is a complicated phenomenon, protein binding to a solid surface leads to the development and design of different types of tools and techniques such as biochips, biosensors, bioreactors and diagnostic techniques [3]. In a number of disciplines such as biology, biotechnology, biochemical engineering and biomedicine, interaction between proteins and surfaces plays an important role [4]. For instance in biomedicine, protein binding on polymer particles includes the following: artificial tissues and organs, drug delivery systems, biosensors, solid-phase immunoassays, immunomagnetic cell separations, and immobilized enzymes [5–8]. In recent years, separation and purification of target protein and elucidation of protein function are some of the major tasks facing researchers. Thus, development of tools to enhance protein studies is critical. Many tools have been developed to immobilize, purify or separate individual proteins from biological matrices. With this respect, application of magnetite nanoparticles, MNPs (microspheres, nanospheres and ferrofluids), in the mentioned process purification, separation and immobilization of protein and enzymes is straightforward [9–11].

Iron oxide magnetite particles are a group of the paramagnetic nanoparticles which are usually modified with various functional groups such as epoxy, amine and aldehyde in order to improve their efficiency [12–14]. Immobilization of biomolecules to a material possessing magnetic feature leads to a quick, easy and gentle separation of biological molecules and macromolecules by using an external magnetic field gradient [15–17]. Although some nanomaterials have excellent physical and chemical bulk properties, they do not possess suitable surface properties for specific applications. Consequently, it may be necessary to modify the surface of such materials properly [18,19]. Modification of the surface of magnetite nanoparticles with functional groups enhances recognition properties, and affinity of MNP toward target biomolecule. The most common way is to attach suitable organic groups to the surface.

Humic acid (HA), which occurs naturally by decomposition of mainly plant residues, is a widespread natural macromolecular organic matter on Earth [20]. HA has a high interaction capability with different types of species owing to its multifunctional macromolecular structure

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containing carboxyl, phenolic hydroxyl, carbonyl, methoxyl, alcoholic hydroxyl, ether and amino groups covalently bonded to a hydrophobic framework [21–23]. Although HA has a multifunctional character and thus constitutes a suitable environment for various species, some drawbacks, such as its high solubility and difficulties encountered to separate it from its suspensions, restrict utilization of solid HA as a substrate [24]. For this reason, immobilization of HA to a suitable solid support with good mechanical properties is deemed important in order to take the benefit of its multifunctional character [25]. In the past three decades, different types of inorganic and organic backbones, such as anion-exchange resins [26], hematite particles [27], alginate gels [28], synthetic polymers [29] and silica particles [30–34] were used as solid support for immobilization of HA. At this context, it is a good tentative idea to combine the multifunctional character of HA and magnetic properties of iron oxide particles through careful immobilization of HA [35]. Despite the fact that there is a growing interest on HA-based magnetite nanoparticles, the concept of preparation of HA-based magnetite nanoparticles is usually based on co-precipitation and/or physical interactions between magnetite nanoparticle and HA. This leads to materials with poor mechanical strength and thus makes it difficult to utilize the obtained materials over a wide range of pH. Although the environmental applications of magnetite nanoparticle based HA have been extensively studied [36–38], little or no literature is available on HA–protein interaction. So, HA-bonded magnetite nanoparticles are expected to be a suitable linker system for protein immobilization. Therefore, the aim of this study is to explore the applicability of a new methodology for immobilization of HA to aminopropyl triethoxysilane modified superparamagnetite iron oxide nanoparticle via covalent bond formation and to explore protein binding efficiency of the obtained material toward human serum albumin (HSA).

2. Experimental

2.1. Materials and methods

Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$, >99%), ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$, >99%), tris(hydroxymethyl)-aminomethane (99.8–100.1%), ammonium hydroxide (25%, w/w), sodium hydroxide (≥97%), hydrochloric acid (37%) and ethanol (>99.2%) were obtained from Merck (Darmstadt, Germany). (3-Aminopropyl)-triethoxysilane (APS, 99%), albumin from human serum (97–99%) and phosphate-buffered saline solution (PBS: pH 7.4, 0.1 M) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium form of Aldrich HA was used as the source of HA after a simple purification process [25,32] that involves dissolution under alkaline conditions, filtration of undissolved particles and re-precipitation of HA by addition of acid. In this way, the material was also turned into its protonated form. HA was turned into humyl chloride (HUM-Cl) by reaction of oxalyl chloride (Merck). Dimethylformamide (DMF, Merck), triethylamine (TEA, Merck), acetone (Merck) and dichloromethane (Merck) were the solvents and/or reagents used during the immobilization of HA to APS-MNPs and subsequent purification steps.

All aqueous solutions were prepared with deionized water that had been passed through a Millipore Milli-Q Plus water purification system. All chemicals were of analytical grade and used as received.

2.2. Instruments

Elemental analyses were performed by a Leco CHNS-932 analyzer, and changes in elemental composition were used to evaluate the efficiency of surface modifications. IR spectra were recorded on a Perkin-Elmer spectrum 100 FTIR spectrometer with an ATR compartment. The spectra were compared with each other to understand the mechanism of surface modifications and protein binding to HA-APS-MNPs. Thermogravimetric analysis (TGA) was carried out with a Setaram SETSYS thermal analyzer at a temperature range of 25–950 °C at a heating rate of 10 °C min^{-1} under argon atmosphere with a gas flow rate of 20 mL min^{-1}. The size and shape of the NPs were determined by transmission electron microscopy (TEM, FEI Company-Tecnai™ G2 Spirit/Biotwin). SEM images were obtained using a Zeiss LS-10 field emission SEM instrument equipped with an Inca Energy 350 X-Max (Oxford Instruments) spectrometer. Samples were sputter-coated with a Au (60%) and Pd (40%) alloy using a Q150R (Quorum Technologies) instrument. Images were obtained at 3×10^{-4} Pa working pressure and 15 keV accelerating voltage using InLens detection mode (2 mm of working distance). Fluorescence spectra of HSA were recorded on a Perkin-Elmer LS-55 fluorescence spectrometer, and the instrument was used to determine the concentration of HSA in solutions. Finally, a combination Orion 410 A^+ pH meter was used for the pH measurements.

2.3. Magnetite nanoparticles and surface modifications

Bare MNPs were prepared according to the method described by Can et al. [9]. Briefly, 3.1736 g of $FeCl_2 \cdot 4H_2O$ (0.016 mol) and 7.5684 g of $FeCl_3 \cdot 6H_2O$ (0.028 mol) were dissolved in 320 mL of deionized water, such that $Fe^{2+}/Fe^{3+} = 1/1.75$. The mixed solution was stirred under N_2 at 80 °C for 1 h. Then, 40 mL of $NH_3 \cdot H_2O$ was injected into the mixture rapidly, stirred under N_2 for another 1 h and then cooled to room temperature. The precipitated particles were washed five times with hot water until the supernatant reached pH 7 and separated by magnetic decantation. Finally, magnetic nanoparticles were dried under vacuum at 70 °C.

In order to improve the efficiency of surface modification, it is a very common way to attach proper linkers to the surface before immobilization of a target macromolecule [9]. Surface silanization with aminopropyltriethoxysilane (APS) is one of the useful methods used for this purpose. Aminopropyl groups on the surface of the obtained product (APS-MNPs) are good linkers for the attachment of HA via amide bond formation mechanism. The same mechanism was used for immobilization of HA to aminopropyl silica, and the obtained materials were found to be stable [25,32]. By turning carboxylic acid groups of HA into their respective acid chlorides (humyl chloride, HUM-Cl) [39], the efficiency of HA immobilization onto an aminopropyl-functionalized surface can be improved. Therefore, surface modification of MNPs with HA was performed on the basis of reaction between APS-MNPs and HUM-Cl.

APS-MNPs were prepared according to the methods described in the literature [40,41]. Briefly, 4.2252 g of MNPs was sonicated in a 150 mL ethanol/water (volume ratio, 1:1) solution for 30 min to get uniform dispersion. Then 16.1600 g of APS was added to solution under N_2 atmosphere at 40 °C for 2 h. After that the solution was cooled to room temperature. The prepared APS-MNPs were collected with a magnet, and washed with ethanol, followed by deionized water three times. Finally, APS-MNPs were dried under vacuum at 70 °C.

HUM-Cl was prepared by adding 25 mL of anhydrous oxalyl chloride to 1.50 g of dried HA in 50 mL of anhydrous DMF with stirring. The reaction mixture was refluxed for 72 h, and the resulting mixture was evaporated to dryness under reduced pressure. Finally, obtained crude product was used immediately for the next step without further purification. 1.5 g of APS-MNPs was interacted with the prepared HUM-Cl in the presence of 1.00 mL of TEA in 50 mL of anhydrous DMF for 72 h at 60 °C. After magnetic separation, obtained magnetite nanoparticles were washed in sequence three times with warm DMF, dichloromethane, acetone and distilled water. Acid chloride groups of immobilized HUM-Cl turn rapidly into their respective carboxylic acid (and/or carboxylate) groups through the final washing step, yielding HA-APS-MNPs (Scheme 1). The product was dried under vacuum at 100 °C for 3 h and kept in a desiccator before use. According to the results of thermal analyses, the amount of HA bonded onto APS-MNPs was found to be approximately 151 mg HA/g of magnetite nanoparticle. IRν_{max} (ATR)/cm^{-1}: 3110; 2933; 1714; 1505; 1204; 1137; and 549.