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Synthesis of amino-silane modified superparamagnetic silica supports and their use for protein immobilization

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Abstract

A novel method to synthesize amino-silane modified magnetic silica supports was reported for affinity separation. The magnetite (Fe_3O_4) nanoparticles were prepared by the coprecipitation of ferrous and ferric salts with NH_4OH, and then their surface was coated with silica in sodium silicate solution by acidifying technology to form well-dispersed magnetic silica nanospheres. These magnetic silica nanospheres are of about 50–80 nm in diameter and exhibit superparamagnetic characteristics. The surface of these nanospheres was with amino-silane coupling agent for their attachment to affinity ligands. In this work, bovine serum albumin (BSA) was covalently immobilized onto the amino-silane modified magnetic silica supports by the glutaraldehyde method. The influence of pH, ionic strength as well as the initial protein concentration on BSA immobilization has been studied. The result shows that such amino-silane modified magnetic silica is a well-dispersed and effective superparamagnetic support for bioseparation and the maximum BSA immobilization capacity (up to 86 mg/g) was obtained in 0.1 M phosphate buffer at pH 5.0.

Keywords: Magnetic supports; Amino-silane; Protein immobilization; Bioseparation

1. Introduction

Magnetic supports have been shown wide interest since they have applied in the fields of bioseparation and biomedicine including protein and enzyme immobilization, immunoassay, RNA and DNA purification, cell isolation and target drug [1–6]. This technology is based on the immobilization of an affinity ligand on the surface of prefabricated magnetic support and the use of the resulting conjugates for the separation and concentration of biomacromolecules and cells. These magnetic supports can be covalently connected with affinity ligands such as proteins, enzymes and antibodies [7–9]. However, the interactions between affinity ligands and magnetic supports are not quite thoroughly investigated in the literature and the study of protein immobilization onto magnetic supports is important for optimizing conditions for preparation of immuno-magnetic supports with affinity ligands as well as enzyme immobilization.

Many types of organic and inorganic materials have been employed to prepare magnetic supports including natural macromolecules, synthetic polymers and some inorganic materials. Polystyrene (PSt) [1], polymethyl methacrylate (PMMA) [10], and chitosan [11] are the most frequently used materials for magnetic supports. However, for the current available magnetic supports used in separations of biological macromolecules, insufficient capacity and slow mass transfer kinetics are two limiting factors that restrict the applications of the magnetic supports to laboratory scales [12]. An effective way of eliminating both the limitations at one time is to produce nano-sized non-porous magnetic spheres, so as to obtain both relatively large surface area and fast adsorption kinetics [13]. High mechanical rigidity, resistance to solvents, and long shelf life make inorganic materials ideal supports. Among them, magnetic silica, which is based on the formation of magnetic iron oxide nanoparticles embedded in silica matrix, has been widely reported in the literature due to their diverse utility in bioseparation. Magnetic silicas with covalently immobilized affinity ligands have proved to be effective for affinity separation. However, the magnetic silica reported are either too small (less

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than 30 nm) [14,15] or too large (20–45 μm) [16]. They have some limitations [17] such as: in former cases, these nanoparticles, in practical terms, tend to form large aggregates and a part of magnetite would be exposed resulting in damage the activity of bioactive substances. It is often difficult to handle ultrafine particles like colloids. While for latter cases, complex operation and high cost are needed and a broad size distribution is observed. Furthermore, the prepared magnetic silica is not superparamagnetic, so that their applications are limited. Therefore, the development of particles of appropriate size, high magnetite content and well-dispersed magnetic supports are of significance for applications.

In this study, we described an easy and inexpensive method to prepare the highly functionalized, well-dispersed nano-sized magnetic supports and their use for protein immobilization. First, magnetite nanoparticles were prepared by the coprecipitation reaction of ferrous and ferric salts with NH_4OH, and then coated with silica, which was produced from sodium silicate solution directly. This coating prevents the core magnetite nanoparticles from possible degradation influenced by the outside environment and the formation of large aggregates. These magnetic silica can easily be separated with conventional permanent magnets. The outer silica surface is biocompatible and can be functionalized using a variety of known surface chemistry [18]. Herein, the surface of the magnetic silica was functionalized with amino-silane coupling agent. The morphology and properties of magnetic silica supports were examined by TEM and VSM. A series of experiments were carried out to investigate the protein immobilization onto these magnetic supports. Bovine serum albumin (BSA) was selected as a model protein and covalently immobilized onto these supports by the glutaraldehyde method. The influence of pH, ionic strength as well as initial protein concentration on BSA immobilization was studied. The condition of BSA immobilization is of significance for the preparation of magnetic supports with affinity ligands such as protein A and G.

2. Experimental

2.1. Materials

Chemicals were generally of reagent grade from commercial sources. Amino-silane coupling agent, *N*-(2-aminoethyl)-3-aminopropyltrimethoxysilane (AEAPS), was obtained from Sigma–Aldrich Chemical Co. BSA was purchased from Beijing Chemical Reagent Company. All other materials were of analytical grade and used without any further purification, including ferrous chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$), ferric chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), ammonium hydroxide (25% (w/w)), sodium silicate, glycerol, hydrochloric acid, glutaraldehyde, and methanol. Water was deionized and deoxygenated prior to use.

2.2. Synthesis of magnetic silica nanospheres

The magnetite nanoparticles were prepared by a conventional co-precipitation method with some modifications [19]. 23.35 g $FeCl_3{\cdot}6H_2O$ and 8.60 g $FeCl_2{\cdot}4H_2O$ were dissolved in 800 ml deionized water under nitrogen with vigorous stirring at 90 °C. A 30-ml of 25% $NH_3{\cdot}H_2O$ was added to the solution. The color of bulk turned from orange to black immediately. The magnetite precipitates were washed twice with deionized water by magnetic decantation.

Ninety-five grams of sodium silicate was dissolved in deionized water and the pH value of the solution was adjusted to 12–13 by hydrochloric acid. The sodium silicate solution and Fe_3O_4 nanoparticles prepared as described above were poured into a 2-l beaker equipped with a mechanical stirrer. The mixture was sonicated for 30 min. Then, the temperature of the mixture was increased to 85 °C. Hydrochloric acid was added dropwise to adjust pH value to 6.0. The precipitates were washed several times with deionized water by magnetic decantation. This step could be repeated several times to ensure that the desired amount of silica has been coated on the magnetite Fe_3O_4. Finally, the resulted precipitate was dried into powders at room temperature under vacuum. The particles obtained are brown yellow in color.

2.3. Surface treatment by amino-silane

Two grams of magnetic silica nanospheres prepared above, 2.5 ml H_2O and 10 ml AEAPS were added into 250 ml methanol. The mixture was treated by ultrasound for 30 min, mixed with 150 ml glycerol and then transferred to a 500 ml three-necked flask equipped with a mechanical stirrer. The temperature was kept at 85–90 °C with rapid stirring for 3 h. The resulting nanospheres were washed with deionized water and methanol for several times. These nanospheres were immersed in 5% (v/v) glutaraldehyde solution with 0.1 M phosphate buffer, pH 7.4 for 6 h at room temperature and then washed with deionized water.

2.4. Protein immobilization

All BSA immobilization experiments were conducted batchwise at continuous shaking at 30 °C for 4 h, which proved to be a sufficient period for any run. In a typical experiment, an appropriate amount of BSA was dissolved in 5 ml of buffer solution of specified pH and 25 mg of glutaraldehyde activated magnetic supports was added. The mixture was incubated at 30 °C for 4 h. Then the magnetic supports were separated by permanent magnet (2000 Oe). The amount of BSA immobilized onto magnetic supports was determined by measuring the initial and final concentrations of BSA from the absorbance at 280 nm of the supernatant using a calibration curve prepared previously.

To remove the physically adsorbed BSA, the sediments of the BSA-immobilized magnetic supports were resuspended in glycine buffer at pH 11.0 for 2 h. The desorbed amounts

of BSA were determined from the absorbance at 280 nm of supernatants. In order to remove perfectly a very small amount of wafting particles, all the separated supernatants were centrifuged twice at 50,000 × g before measuring.

2.5. Analysis and measurements

The size and morphology of the magnetic silica supports were observed by transmission electron micros (TEM, Hitachi 8100, 200 kV). The magnetic properties were analyzed with a vibrating sample magnetometer (VSM) (Model-155, Digital Measurement System, Inc.). Protein concentrations were measured from the absorbance at 280 nm by Lambda Bio-40 spectrophotometer (Perkin–Elmer, Norwalk, CT, USA).

3. Results and discussion

3.1. Properties of magnetic silica supports

Magnetite (Fe_3O_4) nanoparticles were prepared by the co-precipitation method from ferrous and ferric ion solutions at the exact 1:2 ratio. It was reported that magnetite nanoparticles prepared by the coprecipitation method have extensive hydroxyl groups on the surface by contact with aqueous phase [20]. Silicic acid is formed gradually from sodium silicate solution during the addition of dropped hydrochloric acid, which undergoes the polymerization reaction. The silanol groups (Si–OH) are transformed to siloxane bonds (Si–O–Si) via the condensation reaction and the silica coating forms around [21]. It is assumed that these silicic acid molecules associated with Fe_3O_4 particles by forming covalent bonds through water condensation. The subsequent silicic acid polymerizes likewise and finally the compacted silica layers are formed around the particles. Consequently, it is likely that each of the magnetic particles is far apart and they will not aggregate. Compared with the microemulsion method [14] and "sol–gel" technique [15] for the preparation of magnetic silica, this approach is easy to manipulate and the size of particles obtained can be controlled from tens of nanometers to several hundred nanometers by changing the ratio of SiO_2 to Fe_3O_4 or by repeating the coating procedure. Many factors affect the particle size and size distribution including pH, temperature, reagent concentration and Si/Fe ratio. The pH affects the surface charge of Fe_3O_4 and their dispersity in sodium silicate solution. The temperature greatly influences the condensation speed from silicic acid dimer to polymer. The two factors are critical to the formation of well-dispersed magnetic silica nanospheres. The magnetite nanoparticles were coated with silica for the purpose of preventing the magnetite aggregation and avoiding the rapid biodegradation when they are directly exposed to biological system. The naked magnetite will damage the activity of biological substances. The magnetic silica nanospheres were well-dispersed as shown in Fig. 1 with the average size of about 50–80 nm. The surface of magnetic silica is biocompatible and can be functionalized with the silane coupling agent. Herein, AEAPS is a commercially available amino-functional silane coupling agent. They were covalently bound to the surface of magnetic silica nanospheres through condensation reaction as shown in Fig. 2. The terminal amino groups are reactive and can be transferred to aldehyde groups by a glutaraldehyde method to facilitate the attachment of affinity ligands.

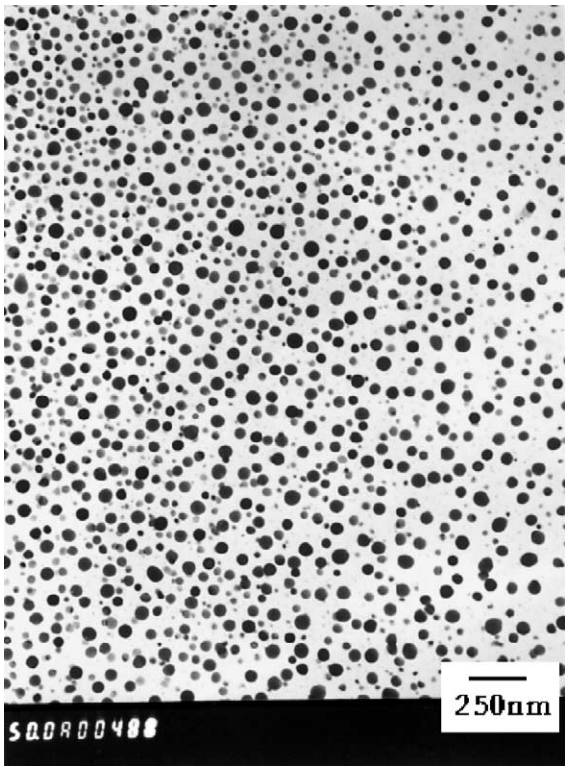

Fig. 1. TEM of magnetic silica nanospheres.

The magnetic properties of magnetic silica nanospheres were analyzed by VSM. Fig. 3 shows the magnetization curve of the Fe_3O_4 nano-particles coated with silica. The magnetite content is 33%. These particles exhibit superparamagnetic characteristics with zero remanence and coercivity. Because the diameter of prepared magnetic Fe_3O_4 particles is about 15 nm [19], which is smaller than the D_P (superparamagnetic critical size) of Fe_3O_4 particles ($D_P = 30$ nm), so they behave superparamagnetic. Fig. 3 shows that their specific saturation magnetization (σ_s) is 19.8 emu/g. These magnetic silica nanospheres can be easily separated within 1 min by using a conventional permanent magnet (2000 Oe).

3.2. Protein immobilization studies

3.2.1. The effect of pH on protein immobilization

The influence of different factors on the immobilization capacity (mg/g) of BSA onto the glutaraldehyde activated magnetic supports was studied. Fig. 4 presents the change of immobilized BSA at equilibrium against the pH with the

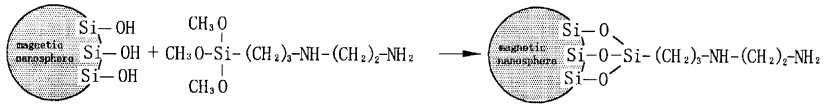

Fig. 2. Process of the silanization reaction of AEAPS with magnetic SiO_2.

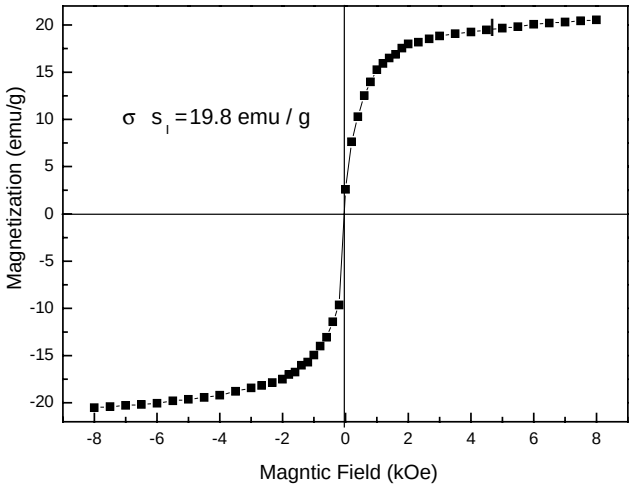

Fig. 3. VSM magnetzation curve of the magnetic silica nanospheres.

initial BSA concentration of 3 mg/ml. In order to minimize the influence of the buffer, all experiments were carried out in the 0.1 M phosphate buffer. The pH value was varied from 3 to 9. Like other studies in the literature [22], maximum value of immobilization capacity was realized at pH 5.0, which was at around the pI of BSA (pI = 4.7).

This result is not unusual, because maximum adsorption of a protein can be accomplished when it has a neutral charge, i.e. at the isoelectric point. At pH different from the pI, the protein molecules are charged and repel from each other. The neutral original magnetic support surface acquires a positive or negative charge, which prevents the further protein immobilization. As a certain amount of protein molecules on the surface, the process is terminated. Consequently, lower and higher pH values resulted in decreased immobilization capacity for BSA.

3.2.2. *The effect of ionic strength on protein immobilization*

In order to find out the effect of the ionic strength on the protein immobilization, the BSA immobilization in sodium chloride solutions was studied with the initial BSA concentration of 3 mg/ml. The NaCl concentration was varied from 0 to 1 M. All experiments were carried out in 0.1 M phosphate buffer with pH 5.0 and with pH 9.0, respectively. The results are presented in Fig. 5. It was found that the presence of NaCl did not affect the BSA immobilization obviously in 0.1 M buffer with pH 5.0. However, the amount of BSA immobilized substantially increases with the increase of the salt concentration at pH 9.0. NaCl concentration increases from 0 to 0.6 M caused a distinct enhancement in the amount of the BSA immobilized. The further increase in the ionic strength above 0.6 M up to 1 M NaCl did not affect the immobilization of BSA greatly.

Maximum immobilization capacity is realized at the pH = pI, where the protein has a neutral charge and minimum solubility. The change of NaCl concentration has no influence on the protein charge and solubility. On the other hand, at pH 9.0, the basic media caused the protein molecules negatively charged and repel from each other, increasing the solubility of protein. Certainly, the high salt concentration can shield the charge of protein molecules. This eliminates or, at least, reduces substantially the electrostatic repulsion of the molecules [23]. It leads to a decrease in protein solubility and ensures that more protein molecules bind to magnetic supports. This is the reason why the BSA immobilization capacity increases with the increase of NaCl concentration at pH 9.0.

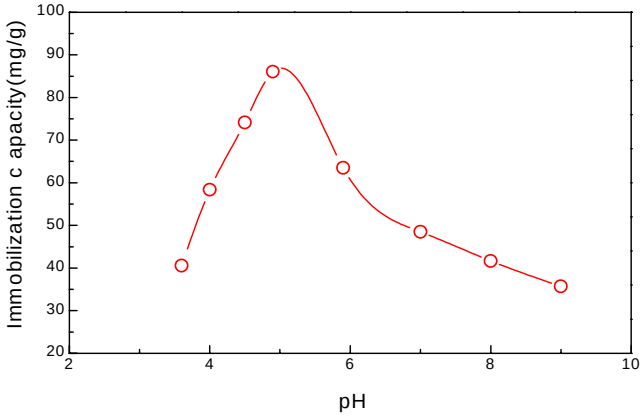

Fig. 4. Effect of pH on BSA immobilization (initial BSA concentration 3 mg/ml, 30 °C).

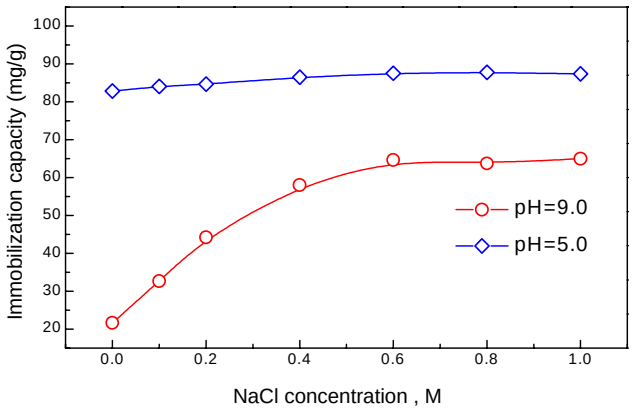

Fig. 5. Effect of NaCl concentration on BSA immobilization (initial BSA concentration 3 mg/ml, 30 °C).

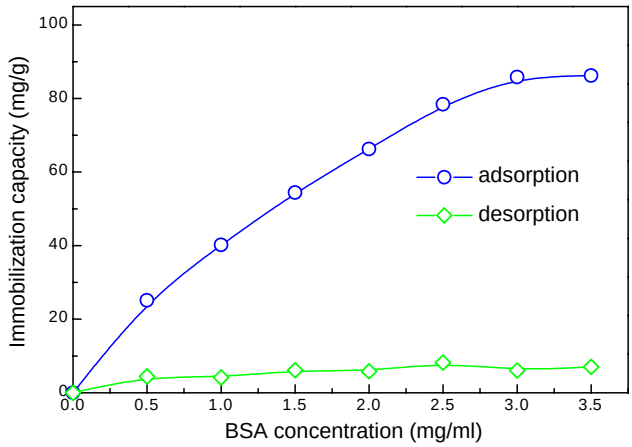

Fig. 6. Effect of initial BSA concentration on immobilization (pH 5.0, 30 °C).

3.2.3. The effect of BSA concentration on protein immobilization

Fig. 6 shows the change of the amount of BSA immobilized at equilibrium with different initial BSA concentration in 0.1 M phosphate buffer, pH 5.0. The amounts of BSA immobilized and desorbed are plotted against the BSA concentration. For assuring the elution of unattached BSA, desorption experiments were carried out in glycine buffer at pH 11.0 under ionic strength 0.01 as the literature reported [7].

In Fig. 6, the amount of BSA immobilized first increased with increasing BSA concentration, but reach a plateau value at 3 mg/ml BSA concentration. As it was expected, only small amount of BSA were detached from the magnetic supports and hence most of the BSA were covalently immobilized onto the glutaraldehyde activated magnetic supports. The maximum immobilization capacity is about 86 mg/g at an initial BSA concentration over 3 mg/ml.

4. Conclusion

A novel and facile method for the preparation of highly functionalized, nano-sized inorganic magnetic supports for bioaffinity separation has been synthesized based on the magnetite/silica combination. They have the average size of about 50–80 nm in diameter and exhibit superparamagnetic characteristics. These inorganic composite magnetic supports have the advantage of high mechanical rigidity, no aggregation and biocompatibility. They were activated by the glutaraldehyde method for attachment to biomolecules. Their use in protein and enzyme immobilization was investigated using BSA as a model protein. The maximum value of BSA immobilization capacity (up to 86 mg/g) was obtained in 0.1 M phosphate buffer at pH 5.0. This study of BSA immobilization is of significance for preparation of immuno-magnetic supports with affinity ligand such as protein A and G. Preliminary experiments showed that the amino-silane modified magnetic silica nanospheres are applicable to the purification of antibodies from ascites using protein A as ligands immobilized onto these magnetic supports. Further experimentation is now in progress to explore the potential of this technique in the field of affinity separation, especially for antibody purification, immunoassay, and cell isolation.

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