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Preparation and characterization of magnetic polymer nanospheres with high protein binding capacity

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Abstract

A novel magnetic support with high protein binding capacity was prepared by mini-emulsion polymerization. The magnetic poly(methacrylate-divinylbenzene) nanospheres prepared are 390 nm in diameter with narrow size distribution and star-like external morphology which leads to a large increase in specific surface area. Experimental results indicate that the maximum protein binding capacity is 316 mg bovine hemoglobin (BHb)/g support.

Keywords: Nanospheres; Synthesis; Surface modification; Mini-emulsion polymerization; Bioseparation; Divinylbenzene; Methacrylate; Protein binding; Hemoglobin

1. Introduction

Magnetic supports have been of interest since they exhibit wide applications in the fields of biotechnology and biomedical engineering, such as cell isolation, enzyme immobilization, immunoassay, protein separation, and drug targeting [1–5]. Magnetic separation is relatively rapid and easy, low cost and highly efficient. To make full use of the magnetic separation technology, magnetic supports with good properties are very necessary. For the present available magnetic supports used in bioseparation, low binding capacity and slow mass transfer kinetics are two limiting factors that restrict the applications of the supports to laboratory scale only [6].

One method to eliminate both limitations at a time is to produce nano-sized, non-porous magnetic particles, so as to obtain both a relatively large surface area and fast adsorption kinetics. However, there are two principal difficulties in preparing high-capacity magnetic supports for protein separation. First, a significant reduction in particle size must be achieved to provide the large surface area required, but too small a particle

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may not carry enough magnetite and, in practice, would cease to be magnetic. Second, an appropriate surface functionality should be introduced, with high enough density of functional groups on supports for efficient coupling of affinity ligands [7]. One possible solution to both binding capacity and mass transfer problems of magnetic supports is to develop high binding capacity magnetic particles with an appropriate size, which could be separated under the conventional magnetic field.

In this study, the novel magnetic nanospheres were synthesized by the mini-emulsion polymerization of methacrylate (MA) and cross-linker divinylbenzene (DVB) in the presence of oleic acid-coated magnetite nanoparticles. The most attractive feature of these nanospheres is that they have star-like external morphology, which leads to a large increase in specific surface area. Then they were highly functionalized with ethylenediamine via ammonolysis reaction. The morphology, magnetic property and surface functionality of these magnetic nanospheres were examined by TEM, VSM and FT-IR. It shows that these magnetic nanoparticles are of 390 nm in diameter with narrow size distribution, star-like external morphology and relatively high saturation magnetization (7.0 emu/g), which can be separated from the solution within 1 min with a permanent magnet (2000 Oersteds). For further protein adsorption experiment, the amino groups on the magnetic nanospheres were transferred to the imminodiacetic acid (IDA) by the subsequent carboxymethylation with sodium chloroacetate. After chelating copper ions, the magnetic supports were applied to protein adsorption using bovine hemoglobin (BHb) as a model protein. The result shows that the maximum protein binding capacity is up to 316 mg BHb/g of magnetic supports owing to their large specific surface area, which is apparently much higher than those reported in literature.

2. Experimental

2.1. Materials

Chemicals used were generally of reagent grade from commercial sources. MA and DVB were distilled under reduced pressure to remove the inhibitor prior to use. BHb was purchased from Sigma. All other materials were of analytical grade and used without any further purification including ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), sodium dodecylbenzene-sulfonate (SDS), cetyl alcohol (CA), aqueous ammonia (25%[w/w]), oleic acid, benzoyl peroxide (BPO), ethylenediamine, chloroacetic acid, *N,N*-dimethylformamide (DMF), ethylenedinitrilotetraacetic acid (EDTA) and $CuSO_4 \cdot 5H_2O$.

2.2. Preparation of oleic acid-coated magnetic Fe_3O_4 nanoparticles

The oleic acid-coated magnetic Fe_3O_4 nanoparticles were prepared by a co-precipitation method with some modifications [8]. Under nitrogen gas, 23.5 g of $FeCl_3 \cdot 6H_2O$ and 8.6 g of $FeCl_2 \cdot 4H_2O$ were dissolved in 500 ml deionized water. When the solution was heated to 85 °C, 35 ml $NH_3 \cdot H_2O$ were added. Then, 15 ml oleic acid were added dropwise within 20 min. The reaction was kept at 85 °C for 30 min. The black lump-like Fe_3O_4 gel was cooled to room temperature and washed several times with deionized water.

2.3. Synthesis of magnetic PMA-DVB nanospheres

To form the oil phase 10.0 g MA, 0.5 g DVB, and 2.0 g magnetic gel prepared above were mixed, while a certain amount of SDS and CA with molar ratio of 1:3 were dissolved in 100 ml deionized water to form the water phase. They were mixed together with ultrasonic treatment to form O/W mini-emulsion and then transferred to a 250 ml four-neck flask equipped with a condenser, a nitrogen inlet, and a stirrer. The mixture was agitated for 30 min. When the temperature increased to 80 °C, 1.0 g BPO was added to initiate the polymerization. The polymerization proceeded for 8 h at 80 °C. The magnetic nanospheres were separated by magnetic decantation and washed three times with deionized water and acetone. The resulted magnetic nanospheres are brown in color and exhibit magnetic properties.

2.4. Surface treatment

The above-prepared 5.0 g magnetic PMA-DVB nanospheres were washed twice with DMF and

redispersed in 100 ml DMF. Then 100 ml ethylenediamine were added. The mixture was agitated gently at 110 °C for 12 h. After washed with deionized water and ethanol for two times, the magnetic nanospheres with functional amino groups were obtained. To obtain the attachment of chelate ligands, the amino groups on the magnetic nanospheres were transferred to IDA groups by the subsequent carboxymethylation reaction. First, 10.0 g chloroacetic acid were dissolved in 60 ml H_2O, and 40 ml NaOH (10%) were added dropwise to adjust the pH to 7.0 with mild stirring. Then, the above amino-group-functionalized magnetic nanospheres were reacted with 1.0 g of Na_2CO_3 and 60 ml sodium chloroacetate at 70 °C with mild stirring for 12 h. After cooled and washed with deionized water, the magnetic PMA-IDA nanospheres were obtained with the IDA groups acting as chelating ligands.

To chelate copper ions, 3.0 g PMA-IDA nanospheres were added into 30 ml $CuSO_4 \cdot 5H_2O$ solution (50 mg/ml). The mixture was shaking at room temperature for 2 h. The resulted magnetic PMA-IDA-Cu^{2+} nanospheres were thoroughly washed with deionized water to remove the excess unbound Cu^{2+} and then stored in saline phosphate buffer (PBS) for further use.

2.5. Protein adsorption

BHb of different concentrations was dissolved in 1.5 ml adsorption buffer (0.1 M PBS, pH 8.1) and 1.0 mg magnetic PMA-IDA-Cu^{2+} supports were added, respectively. The experiments were conducted batchwise with continuous shaking at 30 °C for 30 min. The supports were separated by magnetic sedimentation and washed with adsorption buffer for three times. The supernatant was assayed for residual protein concentration by UV-Vis spectrophotometer at 406 nm [9]. The protein binding capacity of magnetic supports was calculated by mass balance.

2.6. Analysis and measurements

The size and morphology of magnetic nanospheres were observed by transmission electron microscopy (TEM, Hitachi 8100). The magnetic properties were analyzed with a vibrating sample magnetometer (VSM, model-155, Digital Measurement System, Inc.). Diffusive reflectance infrared spectroscopy (DR-IR) spectra of the magnetic PMA-DVB nanospheres both before and after ammonolysis were obtained by using FTIR spectrophotometer (FTIR, Bruker, Vector 22).

3. Results and discussion

3.1. Synthesis of magnetic PMA-DVB nanospheres

Nano-sized magnetic PMA-DVB spheres were prepared via the mini-emulsion polymerization method in this study. The morphology and size of magnetic nanospheres are shown in Figs. 1 and 2. The particle size distributions were calculated by statistical method to 300 particles in different regions of several TEM photos. It indicates that these nanospheres are of 390 nm in average diameter with narrow size distribution and star-like external morphology.

In conventional emulsion polymerization, the smaller droplets have higher vapor pressure than larger droplets. The larger droplets are usually in the size range from 10 to 20 μm after they are growing at the expense of the smaller droplets. Fortunately, it is reported recently that oil droplets can be stabilized as minidroplets with submicron size (< 500 nm) by an emulsification process using a combination of an ionic emulsifier and a co-surfactant—a water-insoluble and monomer-soluble compound (dodecyl alcohol, CA, etc.) [10]. The minidroplets are very small and stable because of the combined effects of emulsifier and co-surfactant, which lowers the Gibbs free energy of droplets and thereby acts to decrease substantially the rate of oil diffusion from the smaller droplet to the larger one. As a result, the diffusion and solubilization of monomer phase into the aqueous phase can be suppressed and there is no micelle present in the aqueous phase since a much larger amount of emulsifier will be adsorbed on the surface of monomer minidroplets owing to a large increase in the specific surface area. Moreover, some researchers noted that the long-chain alcohol formed a liquid-crystal-like and electrically

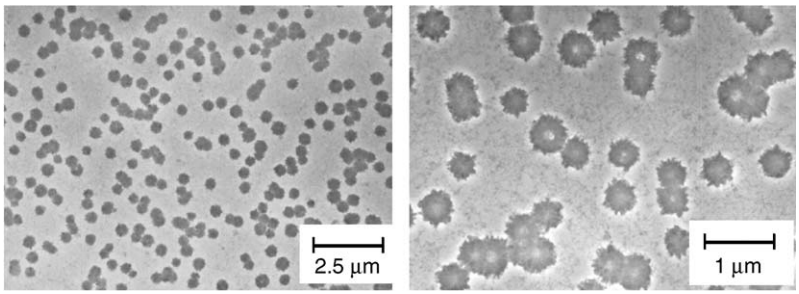


Fig. 1. FEM of magnetic PMA-DVB nanospheres with star-like external morphology.

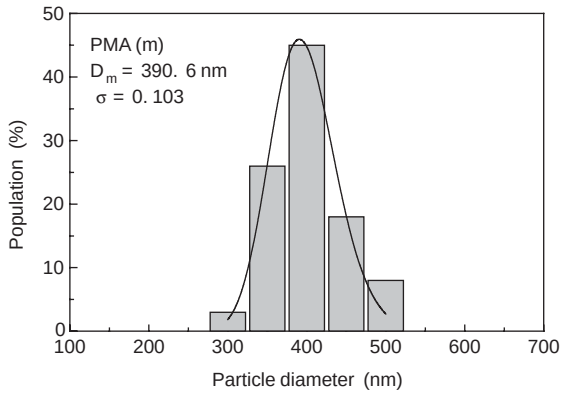


Fig. 2. Size distribution of magnetic PMA-DVB microspheres.

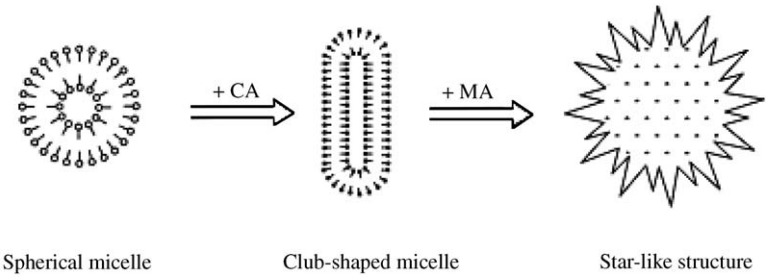
charged barrier with emulsifier molecules on the surface of the minidroplets to prevent them from coalescing [11]. Therefore, the possibility of nucleation in the aqueous phase could be avoided. This strategy was used to prepare submicron-sized magnetic PMA-DVB spheres.

To obtain a successful encapsulation, the magnetite nanoparticles were first made hydrophobic with oleic acid coating and became dispersible in hydrophobic monomers of MA and cross-linker DVB. Then the mixture of oil phase was added into the aqueous phase containing SDS and CA and minidroplets were formed by ultrasonic treatment. The hydrophobic initiator BPO used here is present predominantly inside the monomer minidroplet, so that the polymerization proceeds similar to the bulk polymerization inside each individual monomer minidroplet, but the escape and reentry of radicals also take place during polymerization.

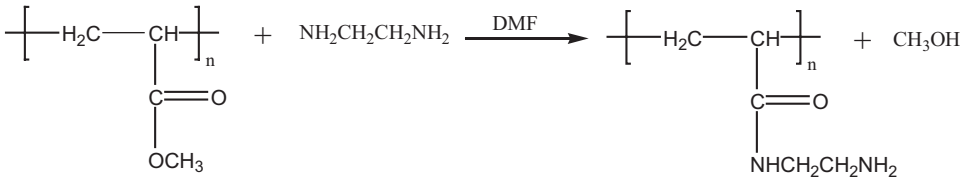
The morphology of the complex of SDS–CA is the combination of spherical SDS micelle and amorphous CA. The molar ratio of SDS to CA determines the shape of the complex SDS–CA. With the increase of the amount of CA, the compound becomes more club-shaped rather than spherical-oriented. At the exact 1:3 molar ratio of SDS to CA, the SDS–CA complex becomes regular club-shaped structure. After the monomer MA added, the club-shaped crystal is gathering to form star-like structure with diameter approximated to the length of club-shaped crystal. As a result, the magnetic PMA-DVB nanospheres with star-like external morphology were obtained, the average diameter being 390 nm. However, as more monomer MA is added, the complex will become more spherical-oriented as shown in scheme 1.

3.2. Surface modification of magnetic PMA-DVB nanospheres

The surface chemical reaction was employed to introduce functional groups ($-NH_2$) and it is different from the conventional co-polymerization method. In monomer co-polymerization (one of them is the functional monomer), most of functional groups were buried in the polymer with only a small part left on the surface [7,12]. Herein, the amino-group-functionalized magnetic nanospheres were prepared by ammonolysis reaction that performed between the methoxyl ($-OCH_3$) groups on the polymer surface and



Scheme 1. Process of the formation of magnetic PMA-DVB nanospheres with star-like structure.



Scheme 2.

ethylenediamine in the presence of DMF as shown in scheme 2.

The result was proven by the comparison of DR-IR spectra of magnetic PMA-DVB before ammonolysis (A) and after (B) as shown in Fig. 3. After ammonolysis, the intensity of the carboxyl band decreased due to the ammonolysis that took place between CH_3O–C═O groups and amino groups. The above –OCH_3 characteristic band at 1373 cm^{-1} disappeared. Meanwhile, the intensity of C–O–C characteristic bands at 1160 and 1270 cm^{-1} decreased obviously. It can be seen that, compared with the unmodified magnetic nanospheres, the amino-modified nanospheres possess absorption bands in 1650 and 1545 cm^{-1} due to the stretching vibration of amide I (mainly due to C═O stretching) and amide II (mainly due to N–H bending), respectively.

To facilitate the attachment of chelating ligands, the amino groups on the magnetic PMA-DVB-NH_2 nanospheres were transferred to IDA through carboxymethylation by using sodium chloroacetate as shown in scheme 3. To obtain high conversion, it is more preferable to use the sodium chloroacetate rather than chloroacetic acid. Meanwhile, due to the hydrolysis of sodium chloroacetate, more sodium chloroacetate solution was added during the reaction process. The reaction resulted in the transformation of amino groups to IDA groups, which are ready for chelating metal ions such as Cu^{2+}, Ni^{2+}, Co^{2+}, and Zn^{2+}.

In this work, Cu^{2+} was selected as the metal chelating ligand because of its high chelation efficiency with IDA groups and high binding capacity with proteins having exposed histidine. After the completion of chelation (scheme 4), the resulted supports were thoroughly washed with deionized water to remove any unbound Cu^{2+} while the bound Cu^{2+} could be replaced with EDTA solution. When the Cu^{2+} immobilized magnetic nanospheres were treated with 0.1 M EDTA, the EDTA solution turned the light blue, indicating that Cu^{2+} was already chelated. The amount of Cu^{2+} chelated to magnetic PMA-IDA nanospheres was qualitatively measured by atomic absorption spectrometry (AAS). The Cu^{2+} chelated was up to 1.83 mmol/g magnetic supports.

3.3. Magnetic properties of magnetic nanospheres

The magnetic properties of both amino-modified and unmodified magnetic PMA-DVB nanosphere were analyzed with VSM as shown in

Fig. 4. The saturation magnetization of amino-modified nanospheres, which was found to be 7.0 emu/g, is comparable to the unmodified nanospheres of 7.8 emu/g. The result shows that the surface modification reaction has little impact on the magnetism of nanospheres before and after modification. It has been clear that, for ultrafine magnetically ordered particles, it exists a critical size below which the granules can acquire only single magnetic domains even in zero magnetic fields. The critical size was estimated as 25 nm [13]. This suggests that the magnetic nanosphere prepared in this work are superparamagnetic because the Fe_3O_4 particles used herein are about 8 nm [14], smaller than the Dp (superparamagnetic critical size) of Fe_3O_4 particles (Dp = 25 nm). The remanence and coercivity measured by VSM

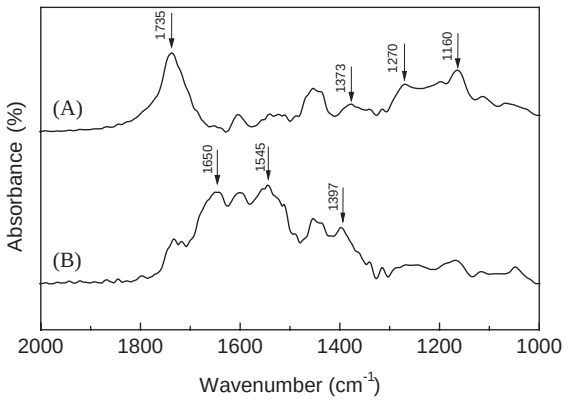


Fig. 3. Comparison of FT-IR spectra of magnetic PMA-DVB nanospheres before ammonolysis (A) and after (B).

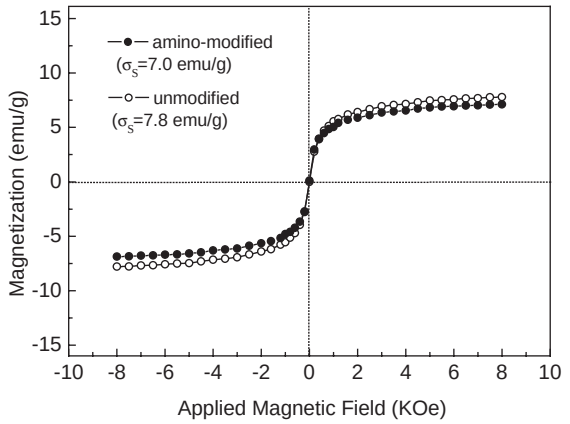
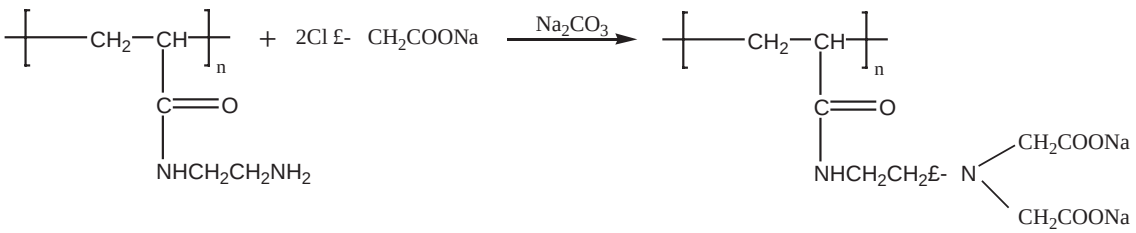
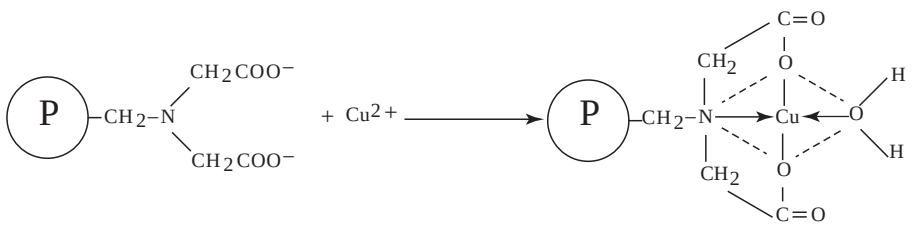


Fig. 4. VSM magnetization curve of amino-modified and unmodified magnetic PMA-DVB nanospheres.



Scheme 3.



Scheme 4.

were so low that hysteresis could hardly be observed. This feature is also typical of superparamagnetism. Both the amino-modified and unmodified magnetic nanospheres can be separated within 1 min by a conventional permanent magnet (2000 Oersteds).

3.4. Adsorption of bovine hemoglobin

BHb with exposed histidines has been selected as a model protein to study the affinity protein binding capacity of magnetic PMA-IDA-Cu^{2+} supports. The affinity is mainly due to the chelating effect between the Cu^{2+} and the imidazole of histidines [15]. Cu^{2+} acts as an electron-accepter group and histidine as an electron-donor group. The adsorption experiment should be performed in a buffer solution with an appropriate pH value, so that the imidazole on the protein surface is at least partially unprotonated. The optimal pH value is determined by the nature of metal ion, the chelating ligand, and the structure of protein. For metal chelating ligand, the optimal adsorption often occurs in the pH range of 5–9 [16]. In the present work, the adsorption of BHb was carried out at pH 8.1. With the non-porous structure, the magnetic PMA-IDA-Cu^{2+} supports were not prone to fouling and the mass transfer efficiency was high. The adsorption equilibrium can be achieved within 30 min. Because of the strong affinity interaction between PMA-IDA-Cu^{2+} and BHb, little protein can be desorbed by changing the pH and the ion strength. Fig. 5 shows the isothermal adsorption curve of BHb onto magnetic supports. The experimental data are in agreement with the result fitted by Langmuir function. As the equilibrium BHb concentration is over 4 mg/ml, the saturated protein binding capacity is up to 316 mg/g of supports, which is much higher than those of similar works reported in Ref. [17]. This magnetic support with such high capacity is equal to the commercial porous resin supports, which has a specific surface area of 50–60 m^2/g. However, the calculated specific surface area of magnetic PMA-IDA-Cu^{2+} supports (average diameter 390 nm) is about 15 m^2/g. It can be explained that the star-like external morphology of supports leads to a large increase in specific surface area, which resulted in high binding capacity to a certain degree.

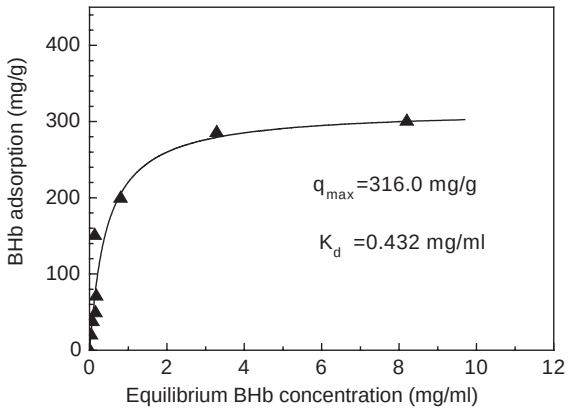


Fig. 5. Isotherms of BHb adsorption onto magnetic PMA-IDA-Cu^{2+} supports.

The preliminary experiment shows that the amino-group-functionalized magnetic poly(-MA–DVB) carriers were highly effective for antibody purification after being covalently coupled with protein A by the glutaraldehyde method as demonstrated by the direct purification of mouse IgG2a from ascites (the capacity was 22 mg IgG2a/g carriers). Further experimentation is now in progress to explore the potential of these magnetic nanospheres in the field of affinity separation, especially for antibody purification, immunoassay, and cell isolation.

Acknowledgements

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