Uniform Superparamagnetic Fe₃O₄/CMCS Composite Nanospheres for Lysozyme Adsorption

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In this study, uniform superparamagnetic Fe₃O₄/carboxymethyl chitosan composite nanospheres with high saturation magnetization were successfully synthesized via a modified inverse emulsion crosslinking approach, using genipin as a cross-linking agent. These nanospheres were then characterized, and their protein adsorption capacity was further investigated under various conditions. The implementation of a sonication treatment of a mixture containing Fe₃O₄ nanoparticles and carboxymethyl chitosan before the emulsion process significantly promoted the homogeneity of Fe₃O₄ nanoparticles in an aqueous phase system. The Fe₃O₄/carboxymethyl chitosan composite nanospheres were of uniform spherical structure, were approximately 230 nm in size, and possessed superparamagnetic characteristics with a mean saturation magnetization as high as 35 emu g⁻¹, corresponding to a magnetite content of 43%. Lysozyme was then employed as a model protein to investigate the effects of pH, incubation time and ion strength on the protein adsorption capacity of the as-synthesized composite nanospheres. The as-obtained composite nanospheres could serve as a promising candidate for fast and efficient protein adsorption.

Keywords: Superparamagnetic, Composite Nanospheres, Lysozyme Adsorption, Genipin.

1. INTRODUCTION

In recent years, nano materials have attracted much attention due to their promising potential in biomedical applications including drug delivery, bioseparation, magnetic resonance imaging, and disease diagnosis. In particular, magnetic polymer composite spheres were of great interest because of their unique superparamagnetic nature. Chitosan, a biocompatible, biodegradable, bioactive, and nontoxic polymer derived from the partial deacetylation of the long chain polymer, chitin, is frequently chosen as a component for the synthesis of magnetic polymer composite spheres. To date, several methods have been applied for the preparation of the magnetic chitosan composite spheres, such as suspension cross-linking techniques, other crosslinking methods, two-step emulsion polymerization, solvothermal reduction approaches, reduction–precipitation methods, and inverse emulsion crosslinking approaches.

Among these methods, the inverse emulsion crosslinking technique was especially appealing due to the advantage that is conferred in the large-scale production of spheres with uniform structure and shape. The two-step preparation combined with the Triton X-100 reversed-phase microemulsion system has been used to fabricate magnetic chitosan nanoparticles with a narrow size distribution. However, these nanoparticles showed relatively low saturation magnetization. Dense and monodispersed magnetic fluorescent chitosan nanoparticles have been prepared in water-in-oil (W/O) microemulsion, however, only a few Fe₃O₄ nanoparticles were encapsulated into the nanoparticles and were heterogeneous. In many reports, a plethora of synthetic cross-linking agents, such as glutaraldehyde, have been studied. However, findings regarding their toxicity and irritation have limited any further biomedical application. Therefore, to prepare uniform magnetic chitosan spheres with high saturation magnetization and excellent morphology using a facile and environmentally friendly method would be an attractive work.

In this work, a modified inverse emulsion crosslinking approach was proposed, with genipin as a cross-linking agent, to prepare uniform Fe₃O₄/carboxymethyl chitosan (CMCS) composite nanospheres with high saturation magnetization (Scheme 1). Firstly, the chitosan was carboxymethylated to improve its solubility in water, which would promote the dispersion of Fe₃O₄/CMCS composite...
nanospheres in aqueous media. After that, the sonication treatment of the aqeous phase containing Fe₃O₄ nanoparticles and CMCS was introduced to obtain the Fe₃O₄/CMCS composite nanospheres in which the Fe₃O₄ nanoparticles were homogeneously dispersed. And then, under high stirring speed, the aqeous phase was added into the organic phase to form the homogeneous hybrid droplets due to van der Waals forces. Finally, in the presence of genipin, the uniform composite nanospheres were formed gradually. Genipin, an iridoid glucoside extracted from the fruits of Gardenia jasminoides, exhibits much lower cytotoxicity and better biocompatibility than conventional glutaraldehyde. Genipin has also been shown to be a promising crosslinking agent for the preparation of biomedical materials, as it can spontaneously and effectively react with primary amino groups of CMCS to stabilize the emulsion system. In our research, the excellent morphology and a series of excellent properties of the as-synthesized Fe₃O₄/CMCS composite nanospheres were characterized. Furthermore, the protein adsorption capacity of the synthesized nanospheres was characterized in order to investigate their potential for use. The results of the research indicated both that the modified technique was successful and that the morphology and the properties of the resulting nanospheres were highly promising for extremely efficient protein adsorption.

2. MATERIALS AND METHODS

2.1. Materials

Iron(III) chloride hexahydrate (FeCl₃·6H₂O), iron(II) chloride tetrahydrate (FeCl₂·4H₂O) and sodium monochloroacetate (C₃H₅ClNaO₂) were purchased from Sigma. Isopropanol, sodium citrate, span 80 and liquid paraffin were purchased from Forest Science and Technology Development Co. Ltd. (Chengdu, China). Lysozyme was purchased from Solarbio Science and Technology Co. Ltd. (Beijing, China). The Pierce® BCA Protein Assay Kit was purchased from Wandoa Biotech Development Co. Ltd. (Chengdu, China). Chitosan (CS, Mw = 20 KDa) was obtained from College of Marine Life Sciences, Ocean University of China (Qingdao, China). Other chemicals were analytical grade, pure reagents. Deionized water was used in this work.

2.2. Synthesis of CMCS

Chitosan was carboxymethylated according to the previously reported method. Briefly, 3 g chitosan was added into 100 mL of a isopropanol/water (80/20) mixture with 15 g sodium hydroxide at 60 °C to swell and alkalize for 1 h. Then, 20 mL of C₆H₅CINaO₂ solution (0.75 g mL⁻¹ in isopropanol) was added dropwise into the mixture under mechanical stirring for 4 h at 60 °C. Then, 200 mL of ethyl alcohol (70%) was added to stop the reaction. Finally, the solid was obtained by suction filtration, and then washed several times with 70% and 99% ethyl alcohol alternatingly for desalting and dewatering, followed by drying in vacuum condition at room temperature.

2.3. Synthesis of Fe₃O₄ Nanoparticles

The Fe₃O₄ nanoparticles were synthesized by the co-precipitation of Fe²⁺ and Fe³⁺ using NH₄OH. 100 mL of deionized water was heated to 50 °C in a three-neck flask, with deaeration of O₂ by bubbling N₂ through the liquid. Next, 1000 mg FeCl₃·6H₂O, 2448 mg FeCl₂·6H₂O, and 76 mg sodium citrate were added to the water, with the addition of 35 mL of NH₄OH into the reaction mixture under vigorous stirring with nitrogen gas protection. After allowing the reaction to proceed for 1 h at 80 °C, the black suspension was added into 100 mL of water in a conical flask, to stop the reaction, and then the black sediment was collected under an external magnetic field, following washed several times with water until the pH = 7. Finally, Fe₃O₄ nanoparticles were re-suspended well in water to form stable ferrofluid.

2.4. Synthesis of Fe₃O₄/CMCS Composite Nanospheres

The Fe₃O₄/CMCS composite nanospheres were prepared by a modified inverse emulsion crosslinking method. Firstly, 50 mg CMCS was dissolved in 250 μL of water, and then 500 μL of Fe₃O₄ suspension was added, following the mixture was homogeneously blended by sonication at a power level of 50% for 5 minutes (Sonic and Materials Inc. VCX 130PB, 130 W). The black mixture was added into 10 mL of liquid paraffin containing 3 mL of span 80 at 2000 rpm for 20 min. Then, 200 μL of genipin aqueous solution (125 mg mL⁻¹) was added in order to begin the cross-linking reaction by mechanical stirring at 1000 rpm. After allowing the reaction to proceed for 4 h at 60 °C, the product was collected by an external magnetic field, and then washed successively several times with petroleum ether, ethyl alcohol, and water. Finally, the obtained product was re-dispersed in distilled water.

2.5. Adsorption Studies of Lysozyme

Lysozyme was dissolved in 10 mM Britton-Robison buffer solution at pH 4–12. Then, 1 mg of Fe₃O₄/CMCS composite nanospheres was added into 1 mL of 0.5 mg mL⁻¹ lysozyme solution. After stirring for 0.08–36 h at 30 °C,
the Fe₃O₄/CMCS composite nanospheres were removed from the lysozyme solution by an external magnetic field. The concentration of redundant lysozyme in the samples was estimated with the Pierce® BCA Protein Assay Kit according to manufacturer’s instructions, measuring the absorbance at 562 nm on a plate reader. The amount of absorbed lysozyme were estimated from the following equation:26

\[ q = \frac{V_o(C_o - C)}{W} \]

where \( q \) is the adsorbed amount at equilibrium on the magnetic composite nanospheres, that is, adsorption capacity at equilibrium (mg g⁻¹); \( C_o \) and \( C \) are the protein concentrations of the initial and equilibrium solution, respectively (mg mL⁻¹); \( V_o \) is the volume of the aqueous phase (mL) and \( W \) is the weight of the magnetic composite nanospheres (g).

### 2.6. Characterization

The morphology of the magnetic composite nanospheres was observed by scanning electron microscopy (SEM, Hitachi S-4800, Japan) and transmission electron microscopy (TEM, JEOL, JEM-2010, Japan). The samples were deposited on silicon wafer for SEM and on copper grid coated carbon for TEM, and then dried at room temperature. The zeta potential and size distribution of the grid coated carbon for TEM, and then dried at room temperature. The morphology of the magnetic composite nanospheres was observed by SEM and TEM (Fig. 1). The composite nanospheres were entirely spherical and uniform in size, at ~500–4000 cm⁻¹ related to the vibration of the Fe–O bond and the characteristic peaks related to CMCS (CH, CO, CN, NH, and OH stretching and deformation vibrations)²⁷,²⁸ were shown in the spectra of the Fe₃O₄/CMCS composite nanospheres. These results confirmed the existence of Fe₃O₄ and CMCS in the products.

XRD patterns revealed the crystal structures of CMCS, Fe₃O₄ nanoparticles, and Fe₃O₄/CMCS composite nanospheres. As shown in Figure 2, both the strong characteristic absorption band at 580 cm⁻¹ related to the vibration of the Fe–O bond and the characteristic peaks related to CMCS (CH, CO, CN, NH, and OH stretching and deformation vibrations)²⁷,²⁸ were shown in the spectra of the Fe₃O₄/CMCS composite nanospheres. These results confirmed the existence of Fe₃O₄ and CMCS in the products.

The two peaks at 2θ of 12.52° and 27.82° for CMCS in the Fe₃O₄/CMCS composite nanospheres were diminished compared with that of free CMCS, which might indicate that the crystallinity of CMCS was decreased. However, the Fe₃O₄ nanoparticles encapsulated into the Fe₃O₄/CMCS composite nanospheres might have been oxidized to maghemite more or less over time, because the maghemite also had the same spinel-type structure with the

### 3. RESULTS AND DISCUSSION

#### 3.1. Morphology and Size Distribution

The size and morphology of the Fe₃O₄/CMCS composite nanospheres and Fe₃O₄ nanoparticles were directly observed by SEM and TEM (Fig. 1). The composite nanospheres were entirely spherical and uniform in size, at ~230 ± 50 nm in diameter. The Fe₃O₄ nanoparticles with an initial size of ~10 nm (inset of Fig. 1(b)) were evenly dispersed in the Fe₃O₄/CMCS composite nanospheres (Fig. 1(b)), which was attributed to the sonication treatment of the aqueous phase containing Fe₃O₄ nanoparticles and CMCS before emulsification.

![Figure 1](image1.png)  
**Figure 1.** SEM micrograph (A) and the size histogram by DLS (the inset of A) of Fe₃O₄/CMCS composite nanospheres. TEM micrographs of Fe₃O₄/CMCS composite nanospheres (B) and Fe₃O₄ nanoparticles (the inset of B).

#### 3.2. Micro-Structural Study

FTIR were measured in order to characterize the chemical composition of CMCS, Fe₃O₄ nanoparticles, and Fe₃O₄/CMCS composite nanospheres. As shown in Figure 2, both the strong characteristic absorption band at 580 cm⁻¹ related to the vibration of the Fe–O bond and the characteristic peaks related to CMCS (CH, CO, CN, NH, and OH stretching and deformation vibrations)²⁷,²⁸ were shown in the spectra of the Fe₃O₄/CMCS composite nanospheres. These results confirmed the existence of Fe₃O₄ and CMCS in the products.

![Figure 2](image2.png)  
**Figure 2.** FTIR spectra of CMCS (a), Fe₃O₄ nanoparticles (b) and Fe₃O₄/CMCS composite nanospheres (c).
same XRD pattern. These results demonstrated that the Fe$_3$O$_4$/CMCS composite nanospheres retained the spinel structure of magnetite crystal and that the crystallinity of encapsulated CMCS was lower than that of free CMCS.

To obtain quantitative information, TGA was performed on CMCS, Fe$_3$O$_4$ nanoparticles, and Fe$_3$O$_4$/CMCS composite nanospheres (Fig. 4). The mass of the Fe$_3$O$_4$ nanoparticles decreased only 12%, due to a low amount of sodium citrate on their surface. The TGA curve of CMCS showed a two-step process of degradation. The first stage (below 200 °C) was associated with the loss of water, while the second (200–450 °C) was attributed to the degradation and deacetylation of CMCS; after this step, ~62% solid residue was left, which may have been due to the good crystallinity of CMCS. These results regarding the microstructure of the components and nanostructures investigated were consistent with previous reports. For the Fe$_3$O$_4$/CMCS composite nanospheres, a multiple-step degradation process was also observed. The first stage (below 200 °C) could be ascribed to the evaporation of water, and the second mass-loss-step between 200 and 450 °C was attributed to the decomposition of the free outer layer CMCS and sodium citrate. In the third stage, the TGA curve suddenly plateaued from 600 to 700 °C, due to the desorption and decomposition of the inner layer CMCS and sodium citrate that was bound to the Fe$_3$O$_4$ nanoparticles. The CMCS in the Fe$_3$O$_4$/CMCS composite nanospheres was more easily decomposed than pure, free CMCS because of its decreased crystallinity, which was deduced according to the XRD results. Therefore, the magnetite content of the Fe$_3$O$_4$/CMCS composite nanospheres was at least 43%.

### 3.3. Magnetic Properties

The magnetic properties of the Fe$_3$O$_4$ nanoparticles and Fe$_3$O$_4$/CMCS composite nanospheres were investigated by VSM at 300 K (Fig. 5). As expected, the hysteresis loops of all samples showed negligible coercivity and remanence at 300 K, indicating the superparamagnetic nature of the Fe$_3$O$_4$ nanoparticles and Fe$_3$O$_4$/CMCS composite nanospheres. The mean saturation magnetization values of the Fe$_3$O$_4$ nanoparticles and the Fe$_3$O$_4$/CMCS composite nanospheres were ~64 emu g$^{-1}$ and ~35 emu g$^{-1}$, respectively. These results of VSM were validated by TGA via the following formula:

$$M_s = M_{Fe_3O_4} (1 - \omega)$$

where $M_{Fe_3O_4}$ is the saturation magnetization of the synthesized pure Fe$_3$O$_4$ nanoparticles without sodium citrate and $\omega$ is the mass loss (%). The $M_s$ value, calculated with the corresponding formula, of the Fe$_3$O$_4$/CMCS composite nanospheres was ~31 emu g$^{-1}$, which was close to that obtained using the VSM, further strengthening these results.
3.4. Lysozyme Adsorption Studies

The protein adsorption capacity of the Fe₃O₄/CMCS composite nanospheres was studied. Lysozyme adsorption experiments were carried out on three parallel samples at different media pH, incubation time and ion strength.

Figure 6(A) shows the effect of pH on the adsorption amount of lysozyme at 35 °C. As the pH increased from 4 to 10, both the electrostatic repulsion between lysozyme molecules and the electrostatic attraction between lysozyme and Fe₃O₄/CMCS composite nanospheres were weakened. The latter had the largest effect, resulting in the increased adsorption capacity of lysozyme. In explanation of these results, when pH increased from 4 to 7, the adsorbed amount of lysozyme slightly decreased due to a decrease in the net charge number of the Fe₃O₄/CMCS composite nanospheres within the pH range (Fig. 6(B)). When pH increased from 8 to 10, close to the isoelectric point of lysozyme (pI = 10.8), the amount of lysozyme adsorption increased to a maximum value of up to 361 mg g⁻¹, representing an optimum pH for adsorption, and plateaued owing to the relatively weaker electrostatic repulsion interaction between lysozyme molecules near the isoelectric point. As pH further increased above the isoelectric point, the electrostatic repulsive forces increased, resulting in a decrease in the amount of lysozyme adsorption.

The effect of incubation time on the adsorbed amount of lysozyme has been represented in Figure 7. When incubation time increased from 5 min to 20 min, the amount of adsorbed lysozyme also increased gradually, becoming saturated over 20 min due to the abundant charges of the composite nanospheres. Even if the incubation time had been prolonged, the amount of adsorbed lysozyme would not remarkably increase, since the surface of the composite nanospheres had been coated with enough adsorbed lysozyme to impede further adsorption. These results revealed that the Fe₃O₄/CMCS composite nanospheres could quickly and effectively accomplish saturated levels of lysozyme adsorption within 20 min.

The effect of ionic strength on the lysozyme adsorption at 35 °C was investigated by the addition of the NaCl solution, as shown in Figure 8. The adsorption capacity of lysozyme decreased with an increase of ionic strength. When the concentration of NaCl solution increased to
pared Fe$_3$O$_4$/CMCS composite nanospheres could serve as point of lysozyme. These features indicated that the prepared Fe$_3$O$_4$/CMCS composite nanospheres became less effective for lysozyme adsorption under the condition of high ion strength.

4. CONCLUSIONS

We have successfully synthesized uniform superparamagnetic Fe$_3$O$_4$/CMCS composite nanospheres with high saturation magnetization via a modified inverse emulsion crosslinking method using genipin as a crosslinker reagent. The as-synthesized composite nanospheres showed uniform spherical structure, and the encapsulated Fe$_3$O$_4$ nanoparticles were dispersed homogeneously in the composite nanospheres. The magnetic composite nanospheres exhibited superparamagnetism with mean saturation magnetization of up to 35 emu g$^{-1}$ and at least 43% magnetite content. In particular, the composite nanospheres exhibited fast and effective adsorption of lysozyme with the maximum amount of adsorption as high as 361 mg g$^{-1}$ within 20 min, near the isoelectric point of lysozyme. These features indicated that the prepared Fe$_3$O$_4$/CMCS composite nanospheres could serve as a promising candidate for fast and efficient protein adsorption in biomedical field.

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References and Notes

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