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Conjugation of α-chymotrypsin on a polymeric hydrophilic nanolayer covering magnetic nanoparticles

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Abstract

Magnetic nanoparticles, covered by a polymeric hydrophilic nanolayer containing reactive amino groups, were obtained *via* Hoffman degradation of the polyacrylamide-coated Fe_3O_4 nanoparticles synthesized by photochemical in situ polymerization, and then conjugated the model enzyme—α-chymotrypsin (CT) by use of EDC· HCl and NHS at room temperatures. The mechanism of photochemical in situ polymerization was briefly proposed in this paper. Superparamagnetic properties were retained for Fe_3O_4 after enzyme immobilization while slightly reducing the value of saturation magnetization. Crystalline structure of Fe_3O_4 after CT immobilization was consistent with that of the freshly prepared Fe_3O_4 by X-ray diffraction (XRD) analysis. The binding capacity was 69 and 61 mg enzyme/g nanogel determined by thermogravimetric (TG) analysis and by standard BCA protein assay, respectively. Specific activity of the immobilized CT was 0.93 U/(mg min), only 59.3% as that of free CT. Thermal stability of CT was improved after being bound to the amine-functionalized magnetic nanogel.

Keywords: Immobilized enzyme; Photochemical in situ polymerization; α-Chymotrypsin; Magnetic nanogel

1. Introduction

Nowadays, superparamagnetic Fe_3O_4 nanoparticles have become a subject of considerable research interests due to their inherent merits of strong magnetization and low toxicity to human bodies [5]. Fe_3O_4 functionalized with biodegradable and biocompatible materials such as enzyme, protein and hydrophilic polymers has been widely applied in the biochemical and biomedicine fields for cell separation [1], nucleic acids detection and separation [9], targeted drug systems [10,19–21] and MRI contrast agent [6].

Recently, a number of methods such as microemulsion polymerization, emulsion polymerization [15], and in situ polymerization [18] have been introduced to prepare functional core–shell composites. Photochemical in situ polymerization, which was proposed by our group, was a novel route to synthesize core–shell composites in a wide variety of compositions. Up to now, magnetic nanogels with hydroxyl groups or amino groups have been synthesized [14], and successfully applied in biosensor, targeted drug delivery systems and chiral resolution. Photochemical in situ polymerization was endowed with the unique advantages as follows:

(i) There is only one Fe_3O_4 nanoparticle core in the core–shell magnetic nanogel, which is desirable for MRI contrast agent.
(ii) High magnetic content and strong magnetization of Fe_3O_4 guaranteed that magnetic nanogels can be easily separated from the reaction medium by a magnet.
(iii) Polymeric content on the Fe_3O_4 as well as zeta potential can be manipulated by adjusting irradiation intensity, irradiation time and monomer concentration, etc.

In the present study, magnetic nanogel with reactive amino group was obtained *via* Hoffman degradation of the polyacrylamide-coated Fe_3O_4 nanoparticles prepared by photochemical in situ polymerization. CT was conjugated to the magnetic nanoparticles covered by polymeric hydrophilic nanolayer by use of EDC·HCl and NHS at room temperatures. The immobilized CT was characterized by FT-IR, transmission electronic

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microscopy (TEM), photo correlation spectroscopy (PCS), X-ray diffraction (XRD) analysis, thermogravimetric (TG) analysis and vibrating sample magnetometer (VSM) measurement. Amount of the enzyme bound to the amine-functionalized magnetic nanogel was determined by both TG analysis and standard BCA assay. Furthermore, thermal stabilities of the bound CT were investigated.

2. Experimental

2.1. Materials

N-Benzoyl-L-tyrosine ethyl ester (BTEE), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), sodium sulfite, ammonia (25 wt.%), acrylamide (AM), *N*,*N*′-methylene-bis-(acrylamide) (MBA), 1-ethyl-3-(3-dimethylamine propyl) carbodiimide hydrochloride (EDC·HCl), *N*-hydroxysuccinimide (NHS), and α-chymotrypsin (CT) were all of analytic grade and commercially available from Shanghai Chemical Reagents Corp. Of all the reagents above, only AM and MBA were recrystallized from acetone prior to use. The water was doubly distilled after deionization. Five hundred watts xenon lamp was purchased as irradiation source.

2.2. Preparation of superparamagnetic Fe_3O_4 nanoparticles

Superparamagnetic Fe_3O_4 was synthesized in partial reduction method. The preparation was illustrated as follows: $FeCl_3 \cdot 6H_2O$ (6.5 g) was dissolved in 100 ml of water, and charged it into a 500 ml of three-necked flask. With an injector, 50 ml of 0.16 M sodium sulfite solution, which was freshly prepared, was added slowly into the flask. After the red solution changed its color to yellow, 40 ml of diluted ammonia (24 ml of concentrated ammonia was diluted with 16 ml water) was rapidly injected into the flask, while stirring and bubbling intensively with nitrogen gas as protective gas. The reaction was kept at 60 °C for 30 min, before being maturated for about 2 h at room temperatures. After completion of the reaction, the black precipitate was collected by an external magnetic field and washed twice with sodium chloride solution followed by distilled water.

2.3. Synthesis of magnetic nanogel with reactive amino groups

Polyacrylamide (PAM)-coated Fe_3O_4 was synthesized by photochemical in situ polymerization. The typical procedures was as follows: 0.5 g of AM monomer was dissolved in 120 ml of water, and mixed with 4 ml of 1% MBA solution and 20 mg of Fe_3O_4 nanoparticles, and then irradiated by 500 W xenon lamp for 1.5 h. N_2 was bubbled as protective gas throughout the experiment.

Magnetic nanoparticles, covered by polymeric hydrophilic nanolayer containing amino groups, were obtained *via* Hoffman degradation of the PAM-coated Fe_3O_4 nanoparticles. After completion of Hoffman degradation, the amine-functionalized magnetic nanogel was immediately gathered by a magnet and lyophilized.

2.4. Conjugation of CT on the amine-functionalized superparamagnetic nanogel

EDC·HCl (5 mg) and NHS (6 mg) were dissolved in 3 ml of phosphate buffer solution (50 mM, pH 7.4). Twenty milligrams of the lyophilized amine-functionalized powder was added into the mixture. The mixture was well mixed by ultrasonic treatment for 10 min at 0 °C. Subsequently, 5 mg of CT was added into the above mixture, and then shaken for 24 h at room temperature. The immobilized enzyme was collected by an external magnetic field and washed several times with PBS (pH 7.4, 50 mM) until no free enzyme was detected in the washing solution. Finally, the immobilized enzyme was dispersed in aqueous medium and adjusted the solution pH to 3 by 0.1 M HCl for further measurements.

2.5. Characterization

Morphology of the immobilized CT was investigated with a PHILIPS CM120 transmission electron microscopy (TEM) with an accelerated voltage of 80 kV. Size distribution of the samples was determined by a Zetasizer 3000HS photo-correlation spectroscope (PCS, Malvern Instruments Ltd.). The proof conjugation of CT on magnetic nanogel with reactive amino group was confirmed by a Nicolet FT-IR spectrophotometer in a wave number range of 4000–400 cm^{-1} with a resolution accuracy of 4 cm^{-1}. Powder X-ray diffractions were recorded on a Philips X-ray diffractometer (Cu Kα radiation, λ = 1.5418 Å). Thermogravimetric analysis was estimated by a simultaneous DTA-TG and DSC apparatus (Shimadzu, Japan) by heating the samples from room temperature to 700 °C under N_2 atmosphere at a heating rate of 10 K min^{-1}. Magnetic properties of the samples, which were vacuum dried, were obtained with a Princeton Applied Research vibrating sample magnetometer model 155 (VSM) and a Quantum Design SQUID MPMS-XL (ac and dc modes and maximum static field 5 T).

2.6. Assay of the amount of CT immobilized on the magnetic nanogel with amino groups

Amount of CT immobilized on the magnetic nanogel was determined by standard BCA protein assays, with BSA as a standard, of the original enzyme solution and the washing solution after immobilization, respectively. The amount of the immobilized enzyme on the magnetic nanogel with amino groups was calculated as

$$M\ (\text{mg enzyme/g nanogel}) = \frac{C_1 V_1 - C_2 V_2}{W}$$

where M is the amount of CT immobilized onto the magnetic nanogel, C_1 and C_2 the concentrations of the enzyme initial and washing solution after immobilization, respectively, V_1 and V_2 the volumes of the enzyme solution and the washing solution after immobilization, W the weight of the magnetic car-

rier. All data used in this formula are averages of duplicated experiments.

2.7. Enzymatic activity measurement

Enzymatic activity of the immobilized CT was determined by continuous spectrophotometric rate determination:

$$\text{BTEE} + \text{H}_2\text{O} \xrightarrow{\text{CT}} \text{benzoyl} - l - \text{tyrosine} + \text{ethanol}$$

The assay mixture was consisted of 1.42 ml of Tris–HCl buffer (80 mM, pH 7.8), 1.4 ml of 1.18 mM BTEE and 0.08 ml of 2 M $CaCl_2$). After addition of 0.1 ml of enzyme solution, the reaction was carried out at 25 °C for 3 min. The suspension was immediately separated by an external magnetic field of 0.5 T and measured the absorbance of the solution at 256 nm in a UV/vis spectrophotometer (Shimadzu, Model 1601; Tokyo, Japan).

2.8. Thermal stability measurement

Thermal stabilities of free and bound CT were investigated by measuring their residual activities after being incubated for 30 min in the temperature range of 35–85 °C. Thermal stabilities of free and bound CT were also examined by assaying their residual activities after being incubated at 45 °C for a required period.

3. Results and discussion

3.1. Synthesis of superparamagnetic Fe_3O_4 nanoparticles

Superparamagnetic Fe_3O_4 nanoparticles were synthesized in partial reduction method according to Refs. [3,4]. They were about 20 nm in diameter (hydrodynamic diameter), with a polydispersity index of 0.184 (Fig. 1). Saturation magnetization (66.3 emu/g), which was in agreement with 67.8 emu/g of 12.4 nm in size reported [12], and immeasurable coercive force (H_c) and remanence could be obviously obtained from Fig. 8, which demonstrated that Fe_3O_4 were of high magnetization and superparamagnetic [16], respectively.

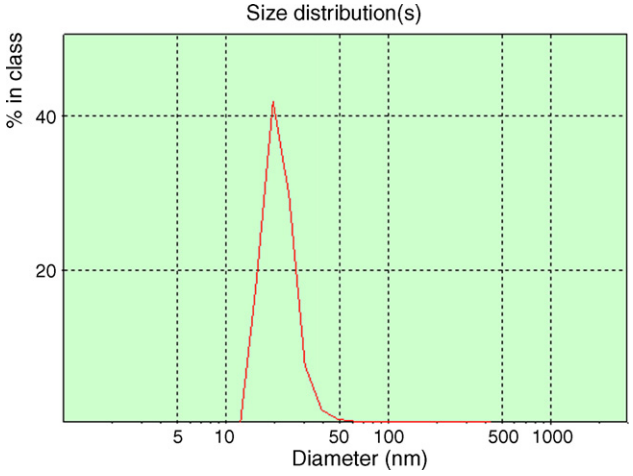

Fig. 1. Particle size distribution of Fe_3O_4 nanoparticles measured by PCS.

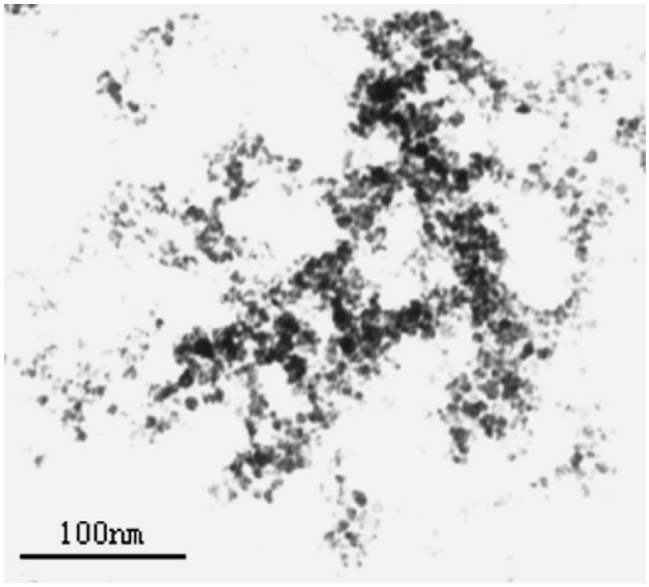

Fig. 2. TEM image of Fe_3O_4 nanoparticles.

In view of the fact that Fe^{2+} tended to be oxidized in air, nitrogen gas was bubbled to prevent the oxidation throughout the experiment. Besides, reaction temperature, maturation time and molar ratio of ammonia to ferric ions were the key factors to synthesis of the Fe_3O_4 nanoparticles. Crystallinity formed at lower temperature (such as room temperature) could enhance saturation magnetization of Fe_3O_4 nanoparticles [13] and the particles' crystalline structure was confirmed to be consistent with the JCPDS No.19-0629 magnetite by XRD measurement (Fig. 9). The six characteristic peaks of the particle crystals occurred at 2θ of 30.1, 35.5, 43.2, 53.5, 57.0, and 62.8, represent corresponding indices (2 2 0), (3 1 1), (4 0 0), (4 2 2), (5 1 1), and (4 4 0), respectively, which revealed that the magnetic particles were pure Fe_3O_4 with spinel structure. Mean particle size calculated using the Debye–Scherrer formula from the reflection of (3 1 1) was 12.4 nm, which was in accordance with the TEM result (10 ± 2 nm, in Fig. 2) within error range of the measurements.

3.2. Preparation of magnetic nanogel with amino groups for enzyme immobilization

Photochemical in situ polymerization was initially practiced in our group [14]. The possible mechanism and preparation route could be briefly expressed as shown in Fig. 3. In the experiment, polyacrylamide-coated Fe_3O_4 nanoparticles were synthesized by xenon lamp irradiation for 1.5 h in the surfactant and initiator free aqueous medium. As known to us, photon section of nano-scaled Fe_3O_4 was much larger than that of AM monomer due to large surface-to-volume ratio, therefore, a majority of photons were adsorbed by Fe_3O_4 nanoparticles. Active holes [2], which could initiate AM monomer polymerizing on the surface of Fe_3O_4 in the presence of MBA, were generated on the surface of nano-scaled Fe_3O_4 while exposed to UV light. Chain length of the polymer and amount of the monomer polymerized on

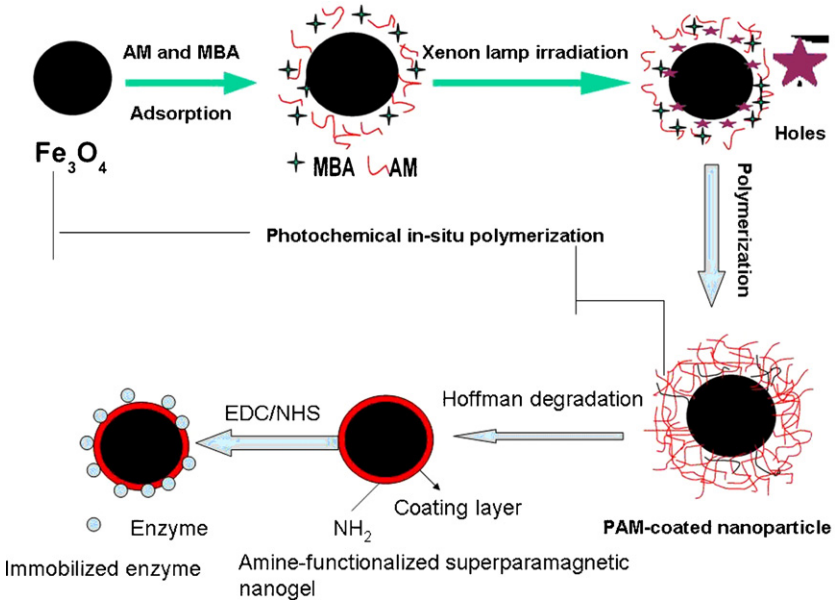

Fig. 3. Schematic view of the preparation of the amine-functionalized nanogel and immobilized CT.

the surface of Fe_3O_4 could be manipulated by changing irradiation time, intensity and monomer concentration, etc. There was only one nanoparticle core (Fe_3O_4) contained in each magnetic nanogel, which was suitable for MRI contrast agent to solve the aggregation [7]. Certain amount of chain transfer agent (usually ethanol or isopropyl alcohol) was added to inhibit the homopolymerization of AM in the solution.

It was proved that the magnetic nanogel with amino groups was obtained successfully through Hoffman degradation of the PAM-coated Fe_3O_4 nanoparticles in our former work [14]. As amino groups tended to be oxidized, therefore, nitrogen gas was bubbled as protective gas throughout the Hoffman degradation. Its mean particle size was about 25 nm measured by PCS. The amination degree of the amine-functionalized magnetic nanogel after Hoffman degradation, determined by conductometric titration, was about 74.8%. There were still some uneliminated carbonyl groups on the magnetic nanogel, otherwise further Hoffman elimination would destroy surface structure of the magnetic nanogel.

3.3. Enzyme immobilization

3.3.1. Immobilization protocol

CT, which was commonly employed as biocatalyzer for ester hydrolysis, had been studied intensively in the past decade. In this paper, CT as a model enzyme was successfully conjugated onto the magnetic nanogel with reactive amino groups by use of EDC·HCl and NHS at room temperatures [8]. The immobilization protocol was as illustrated in Fig. 4.

3.3.2. Confirmation of CT immobilized onto the amine-functionalized magnetic nanogel

In order to confirm the binding of CT onto the magnetic nanogel with amino groups, FT-IR spectrum measurement was performed. As shown in Fig. 5, the presence of Fe_3O_4 core could

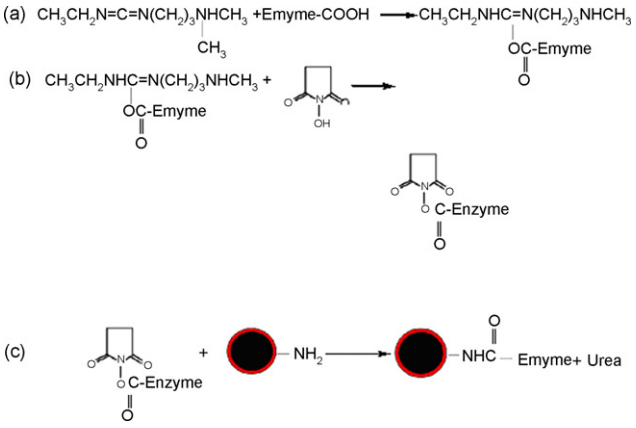

Fig. 4. Schematic presentation of CT immobilization on the magnetic nanogel with reactive amino groups.

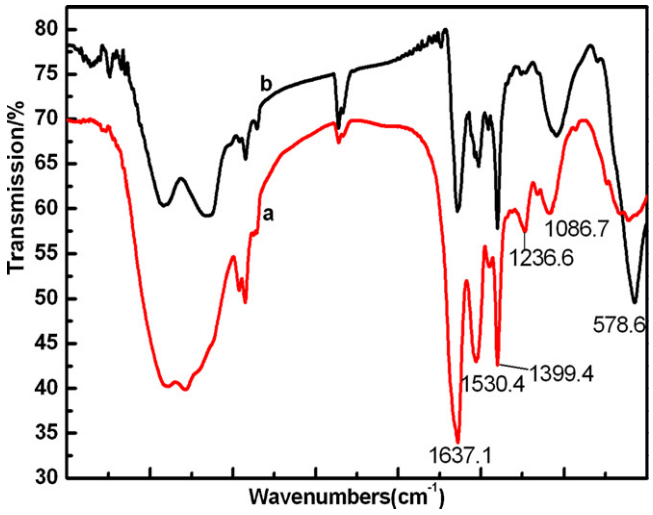

Fig. 5. FT-IR spectra of (a) free CT and (b) immobilized CT.

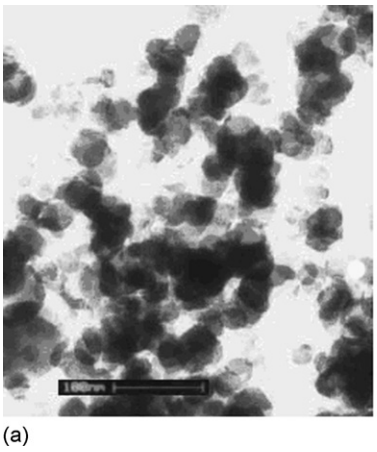

Fig. 6. TEM images of immobilized CT (untreated by ultrasonic treatment) (a) whole and (b) discrete immobilized enzyme recognized from the whole.

be seen by the strong absorption band at around 578.6 cm^{-1}, which corresponded to the Fe–O bond of naked Fe_3O_4. The peaks of 1637.1 cm^{-1}, 1530.4 and 1399.4 cm^{-1}, which also existed in the IR spectrum of the immobilized CT, were the characteristic peaks of CT. The results confirmed that CT was conjugated to the magnetic nanogel successfully.

3.3.3. *Properties of the immobilized CT*

Mean particle size of the immobilized CT was measured to be about 31 nm in diameter. Considering particle size of single CT molecule was 4.6 nm [17], therefore, CT immobilized on the magnetic nanogel might be mono-molecular layer. Considerable dispersibility could be observed optically from its TEM image (Fig. 6a). Discrete CT-immobilized nanoparticle could be recognized from the TEM image (Fig. 6b) in comparison with the results reported [11].

TG–DSC analysis was carried out to estimate the amount of CT immobilized on the amine-functionalized magnetic nanogel, as well as thermal stability. It was performed under nitrogen atmosphere to avoid minimizing mass increase due to oxidation. Weight ratio of CT bound to the amine-functionalized magnetic nanogel was calculated to be 6.9%, and the binding capacity was 6.9 mg enzyme/ g nanogel. The polymeric shell was decomposed at about 180 °C and completed at about 460 °C (Fig. 7), which demonstrated that thermal stability of the immobilized CT was similar to that of the amine-functionalized magnetic nanogel.

Magnetic properties of the immobilized CT were also studied at room temperatures by VSM measurement (Fig. 8). The immobilized enzyme exhibited excellent susceptibility to applied magnetic field, and its saturation magnetization was determined to be 58.5 emu/g. Taking into account the weight of polymeric nanolayer (PAM and CT), saturation magnetization of Fe_3O_4 was slightly decreased in comparison with the naked Fe_3O_4 [6]. Furthermore, excellent magnetic response was guaranteed by the high magnetic content of Fe_3O_4 (88.1 wt.%), which was required for reuse of immobilized enzymes in the biotechnological applications.

Hitherto, there was lack of literature focusing on the effects of UV light irradiation on the crystalline structure of Fe_3O_4. In

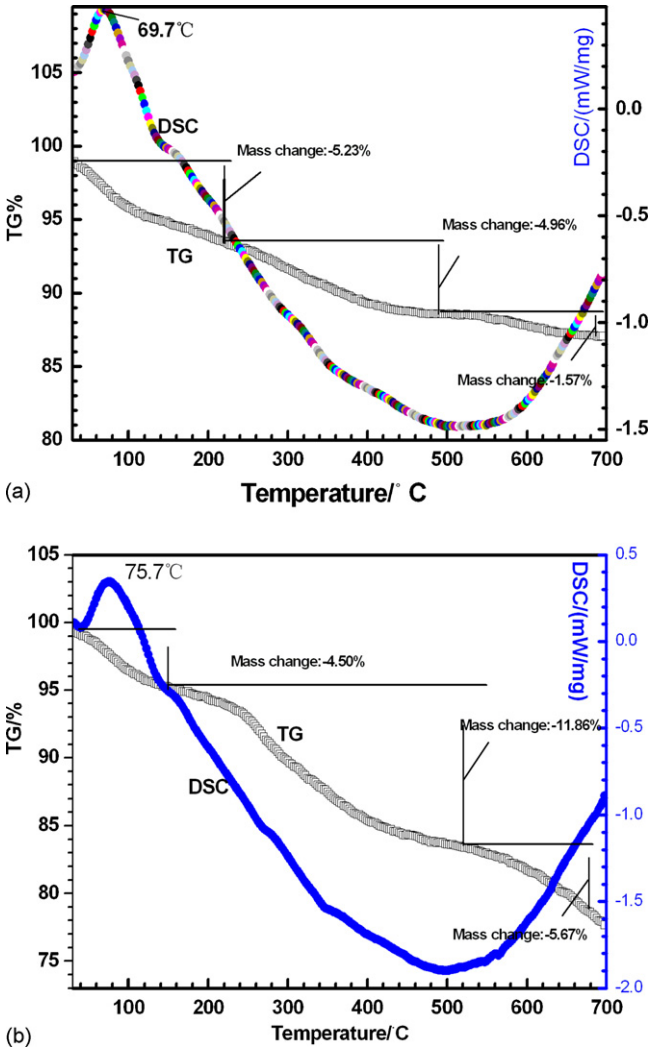

Fig. 7. TG–DSC curves of (a) amine-functionalized superparamagnetic nanogel and (b) immobilized CT.

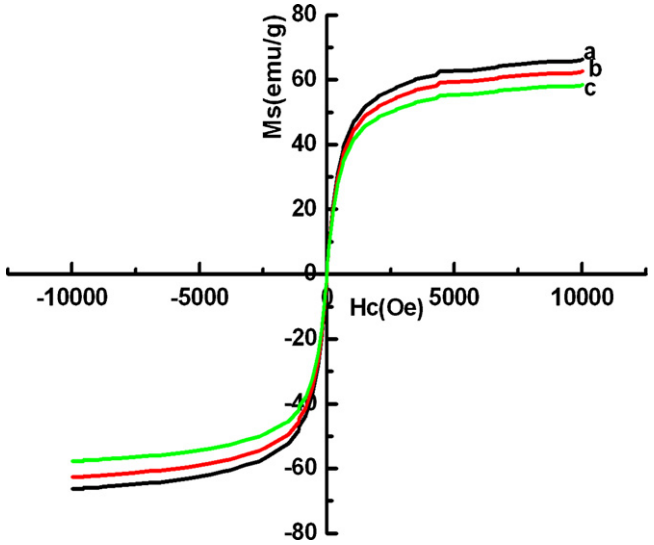

Fig. 8. Hysteresis loops of (a) Fe_3O_4 and (b) magnetic nanogel with amino groups, and (c) bound CT measured at room temperatures.

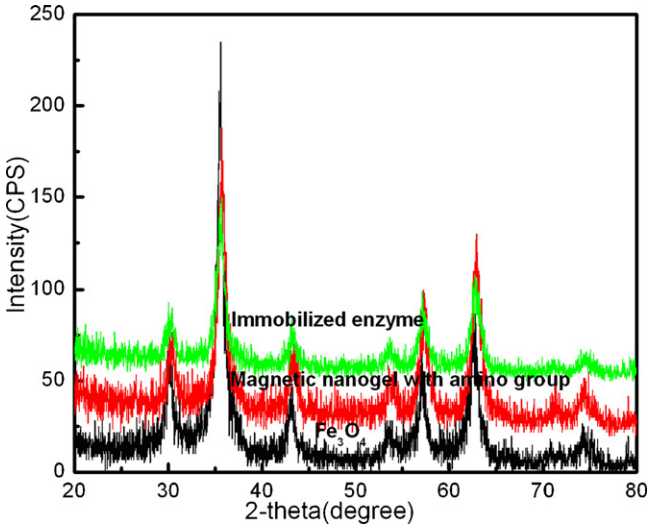

Fig. 9. XRD patterns of naked Fe_3O_4, amine-functionalized superparamagnetic nanogel and immobilized CT.

view of these, crystalline structures of the amine-functionalized superparamagnetic nanogel and the bound CT were checked by XRD analysis. Results showed that crystalline structure of Fe_3O_4 was not affected by both UV light irradiation and enzyme immobilization (Fig. 9).

Unit of enzyme activity (U) was defined as: 1 mg of protein will hydrolyze 1.0 μmol of BTEE per minute at pH 7.8 at 25 °C. Specific activity of the immobilized CT was determined to be 0.93 U/ (mg min), only 59.3% as that of free CT. It agreed well with the results reported [11]. Amount of the enzyme immobilized on the amine-functionalized magnetic nanogel was also determined by standard BCA protein assay, with BSA as a standard. The binding capacity was about 61 mg enzyme/g nanogel, in accordance with the result determined by TG analysis within error range of the measurements.

Thermal stability of an immobilized enzyme is one of the most important criteria of their application. In general, activity

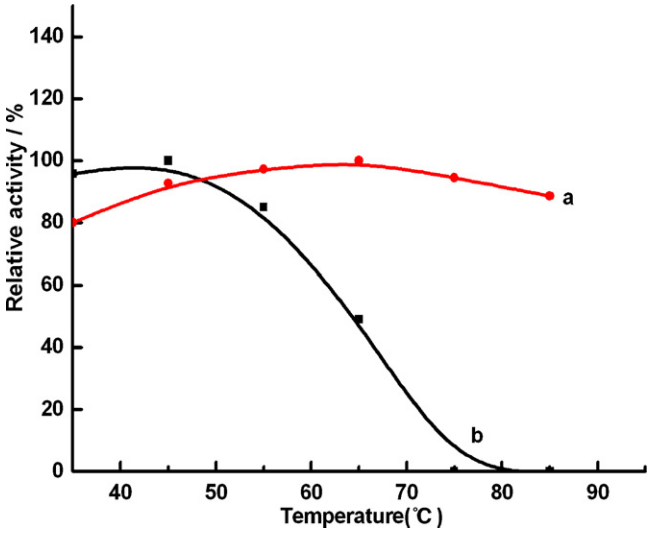

Fig. 10. Thermal stabilities of (a) immobilized CT and (b) free CT incubated in the temperature range of 35–85 °C for 30 min.

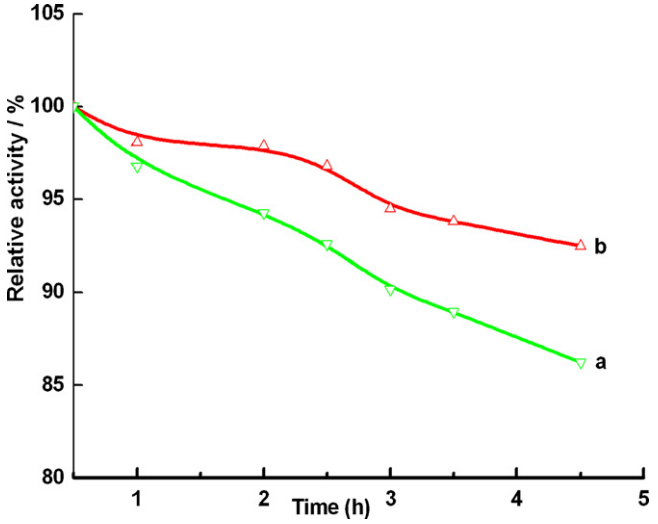

Fig. 11. Thermal stabilities of (a) free CT and (b) bound CT incubated at 45 °C.

of the immobilized enzyme, especially in a covalently bound system, is more resistant than that of the soluble form against heat and denaturing agents. Therefore, thermal stability experiment was carried out with free and immobilized CT. As presented in Fig. 10, almost no activity was retained for free enzyme when incubation temperature was above 75 °C. However, the activity of the bound CT still had a residual activity of 88.7% even at 85 °C. Furthermore, residual activity of the immobilized enzyme after an incubation period of 4.5 h at 45 °C was as high as 92.5% (Fig. 11), which was higher than that of the free enzyme (85.6%). The experiment clearly revealed that thermal stability of CT was improved after being conjugated to the amine-functionalized magnetic nanogel, in agreement with the results previously reported [22–24].

4. Conclusions

A novel route to synthesize magnetic nanoparticles, covered by a polymeric hydrophilic nanolayer for enzyme immobilization, has been reported in this paper. Uncoated superparamagnetic Fe_3O_4 with mean particle size about 10 nm (dried state) and narrow size distribution was prepared in partial reduction method. Magnetic nanogel with reactive amino groups was obtained *via* Hoffman degradation of the PAM-coated Fe_3O_4 nanoparticles. The amination degree of the sample after Hoffman degradation, determined by conductometric titration, was about 74.8%. CT was conjugated on the amine-functionalized magnetic nanogel by use of EDC·HCl and NHS at room temperatures. Mean particle size of the immobilized enzyme was 31 nm in diameter, and its discrete morphology could be recognized from its TEM image. Crystalline structure of Fe_3O_4 was not affected by both UV light irradiation and enzyme immobilization. Superparamagnetic properties were retained for Fe_3O_4 after enzyme immobilization while slightly reducing the value of saturation magnetization. The binding capacity was 61 mg and 69 enzyme/g Fe_3O_4 nanogel determined by standard BCA assay and TG analysis, respectively. Enzymatic activity of the immobilized CT was 0.93U/(mg min), only 59.3% as that of free CT.

Thermal stability of CT was improved after being immobilized on the magnetic nanogel with amino groups. It can be expected that the immobilized enzyme might make economically viable the use of expensive enzymes and hence opens a new horizon for enzymatic catalytic biotechnology.

Acknowledgements

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