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Magnetic single-enzyme nanoparticles with high activity and stability

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Abstract

Magnetic single-enzyme nanoparticles (SENs) encapsulated within a composite inorganic/organic polymer network were fabricated via the surface modification and in situ aqueous polymerization of separate enzyme molecule. The resultant nanoparticles were characterized by transmission electron microscope (TEM), Fourier transform infrared (FTIR) spectrometer and X-ray diffraction (XRD). These particles are almost spherical in shape and have a unique size of about 50 nm in diameter. Electrical and magnetic measurements reveal that the magnetic SENs have a conductivity of 2.7×10^{-3} S cm^{-1}, and are superparamagnetic with a saturation magnetization of 14.5 emu g^{-1} and a coercive force of 60 Oe. Compared with free enzyme, encapsulated enzyme exhibits a strong tolerance to the variation of solution pH, high temperature, organic solvent and long-term storage, thus showing significantly enhanced enzyme performance and stability.

Keywords: SENs; Enzyme; Magnetization; Activity; Stability

With the rapid development of nanostructured materials and nanotechnology, magnetic nanoparticles have received considerable attention [1,2]. Compared with the corresponding bulk material, magnetic nanoparticles possess unique property, namely superparamagnetism, in addition to their low toxicity and biocompatibility. This means that these particles are attracted to a magnetic field but retain no residual magnetism after the field is removed [3–5]. Thus, suspended superparamagnetic particles in solution can be removed from a reaction mixture using an external magnet, but they do not agglomerate after removal of the external magnetic field. The physiochemical properties of magnetic nanoparticles enable them to have a great potential for many applications in biomedical and catalysis fields [6–8]. In the past years, magnetic nanoparticles have been widely used as biomolecule carriers for immobilization of various enzymes [9–13]. However, the activity and stability of enzyme immobilized on these magnetic nanoparticles will greatly depend on the environmental factors, such as pH, temperature, organic solvent, immunological response, etc. Enzyme encapsulation provides an efficient route to improve resistance of enzyme to harsh conditions. Enzymes have been successfully encapsulated within polymer [14,15], silica [16,17], and gold [18] nanoparticles. These encapsulated enzymes exhibit enhanced activity and stability. Recently, a new class of enzyme catalysts has been created by surrounding single-enzyme molecules with a porous structure. The approach represents a novel way to modify and stabilize enzymes, and the new type of nanostructure is called "single-enzyme nanoparticles (SENs)". Kim and Grate have fabricated SENs via a multistep procedure involving enzyme surface modification, vinyl polymerization and shell condensation [19]. Thereafter, SENs were also synthesized by Yan et al. via a two-step procedure including enzyme surface acryloylation and in situ aqueous polymerization [20]. These caged SENs maintain good activity and stability, and thus hold potential for protein therapeutics and industrial biocatalysis. Nevertheless, until now, to the best of our knowledge, few works have been reported on the synthesis of magnetic SENs.

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In the present study, glucose oxidase (GOD), an enzyme that is widely used in clinical and chemical analyses, as well as in food industries [21], has been chosen as a model enzyme to explore the fabrication of magnetic SENs. The SENs encapsulated by a composite Fe_3O_4/conducting polymer shell were synthesized via the surface modification and in situ aqueous polymerization of separate enzyme molecule. The morphology, size, structure, and electrical and magnetic properties of the resulting magnetic SENs were characterized by TEM, FTIR, XRD, standard four-probe method and vibrating magnetometer. The activity and stability of GOD encapsulated within magnetic SENs were also examined in detail.

Experimental

Materials. Pyrrole monomer was obtained from Merck and distilled before use. Glucose oxidase (EC 1.1.3.4, Type II, 200 U mg^{-1}, from *Aspergillus niger*), *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethyl amino propyl) carbodiimide hydrochloride (EDC) were purchased from Sigma Chem. Co. and used as received. Crylic acid and acryloyl chloride were purchased from Aldrich. *N*-(3-Aminopropyl)-pyrrole and pyrrole-*N*-propylsulfonic sodium were prepared as described previously [22,23]. All other chemicals were of analytical grade and used without further purification. Deionized (DI) water (resistivity of 18 MΩ cm) was obtained from a Millipore Milli-Q Water System (Millipore Inc.), and was used for rinsing and for makeup of all aqueous solutions.

Fabrication of magnetic single-enzyme nanoparticles (SENs). The synthesis of magnetic SENs containing GOD consists of the following processes. According to the protocol of Wang et al. [24], the surface amino groups of GOD were first reacted with acryloyl chloride to yield surface vinyl groups. Subsequently, 20 mg of GOD, acryloylated and solubilized in 10.0 mL hexane, was mixed with 3 mL crylic acid. UV light (365 nm) initiated vinyl polymerization between acryloylated GOD and crylic acid in the presence of the free radical initiator 2,2′-azobis-(2,4-dimethylvaleronitrile) with vigorous stirring. After polymerization for 2 h at 30 °C, the resultant carboxyl-terminated GOD was purified by gel filtration, and then reacted with *N*-(3-aminopropyl)-pyrrole dissolved in a phosphate buffer solution (0.05 M, pH 6.0) containing 0.01 M EDC and 0.02 M NHS under constant stirring for 1 h. Thereafter, the as-formed pyrrole-terminated GOD was purified again by gel filtration, and then mixed with pyrrole-*N*-propylsulfonic sodium aqueous solution (0.5 M) containing abundant $FeCl_3{\cdot}6H_2O$ (0.5 M) and $FeCl_2{\cdot}4H_2O$ (0.3 M), followed by ultrasonic irradiation for 3 h at nitrogen atmosphere. The solution was filtered through a syringe filter, and the product was dispersed in 0.2 M NH_4OH aqueous solution for 2 min with vigorous stirring, immediately followed by dilution with deionized water (enzyme activity assays revealed a slight loss of enzymatic activity (less than 5%) before and after reacting with NH_4OH solution). Finally, the resulting magnetic SENs were accumulated by a permanent magnet, washed several times with deionized water to remove unreacted reagents, and then stored in the refrigerator at 4 °C.

Characterization. The morphology and size of the resulting SENs were examined on a Hitachi model H-800 transmission electron microscope (TEM) at an accelerating voltage of 120 kV. Infrared (IR) spectra were recorded on Nicolet 200SXV Fourier transform infrared (FTIR) spectrometer using a KBr wafer. XRD was performed on a Rigaku D/MAX-RC X-ray diffractometer using Cu Kα radiation. Iron content in SENs was determined using an ICA9000 (N+M) plasma spectrophotometer. The conductivity of SENs powder at room temperature was measured by the conventional four-point probe technique with a digital multimeter and a programmable dc voltage/current source on compressed pellets of dried powders. The magnetization measurements were performed at room temperature using model 155 vibrating magnetometer.

Enzyme activity assays. The activity of free and encapsulated GOD were determined spectrometrically at 505 nm based on the change of solution color resulting from the production of quinoneimine dye in reaction solution. In detail, the chemical equations of the aforementioned reactions are described as follows:

$$\text{glucose} + H_2O + O_2 \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{phenol} + \text{4-AAP} \xrightarrow{\text{peroxidase}} \text{quinoneimine} + H_2O$$

Generally, 0.10 mL suspension of the SENs or free GOD solution was added into the 5.0 mL of substrate solution (pH 5.8) containing 0.1 M phosphate buffer, 10.0 mM glucose, 0.5 mM 4-aminoantipyrene (4-AAP), 0.05 M phenol, and 0.01 mg peroxidase from horseradish (HRP). After mixing at 30 °C for 5 min by vortex, the light absorption of the resulting mixture was immediately recorded on a spectrophotometer. Based on the theoretical analysis, the recorded absorbance should be proportional to enzyme activity in the same mass of enzyme. The equivalent volume of free or encapsulated GOD was used in every group of experiments to get comparable result. The stability of free and encapsulated GOD was investigated by assaying their residual activity. The pH-dependence measurements of enzyme activity were performed in buffer solution with pH range of 5.5–9 at 30 °C, the thermal stability and stability in organic solvent were assessed after the enzyme solutions were incubated at a certain temperature or in a certain organic solvent for 20 min, and the storage stability was carried out by measuring successively activity of GOD stored in a phosphate buffer solution (pH 5.8) at 4 °C in the dark for 1 month.

Results and discussion

Preparation and structural characterization

Single-enzyme nanoparticles encapsulated by a composite inorganic/organic polymer network were synthesized via the surface modification and in situ aqueous polymerization of separate enzyme molecule. As shown in Fig. 1, the vinyl groups were grafted onto the surface of enzyme molecule by acryloylation. Subsequently, the carboxyl-terminated SENs were obtained via vinyl polymerization. The peripheral groups of modified enzymes were then activated through peptide linkage with a pyrrole ring using the linkage reagents NHS and EDC. As the resultant pyrrole-terminated SENs were mixed with the solution containing pyrrole-*N*-propylsulfonic sodium, $FeCl_3$ and $FeCl_2$ under sonication, the poly(pyrrole-*N*-propylsulfonic acid) incorporating iron ions would be generated on the surface of enzyme molecule, followed by treatment with NH_4OH aqueous solution, the iron ions in polymer were changed to iron oxide (Fe_3O_4), that is, the SENs with conductivity and magnetism were obtained. Fig. 2A shows the morphology of the resulting encapsulated enzyme molecule, TEM observation indicates the as-prepared magnetic SENs are almost spherical in shape, quite polydisperse and have an unique size of about 50 nm in diameter. The uniform characteristics of the SENs would provide them with rapid response toward magnetic field, which is of interest to their application.

FTIR spectroscopy proved to be useful to characterize the SENs. Poly(pyrrole-*N*-propylsulfonic acid) and SENs were analyzed by FTIR (Fig. 2B). It was obvious that all characteristic bands of poly(pyrrole-*N*-propylsulfonic acid)

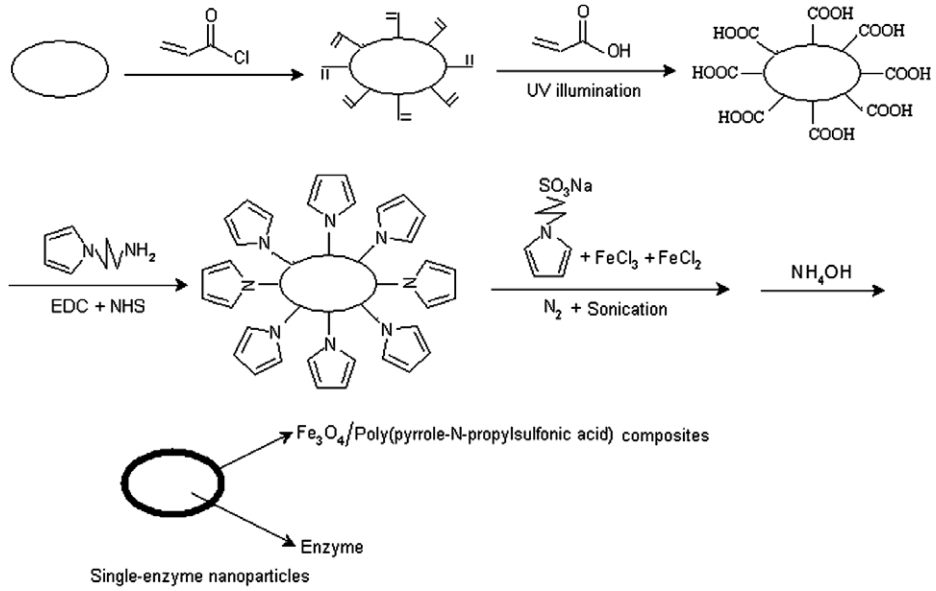

Fig. 1. Schematic for the synthesis of single-enzyme nanoparticles (SENs).

appeared in the IR spectrum of SENs, as expected. For instance, pyrrole rings vibration at 1540 and 1456 cm^{-1}, =C—H in-plane vibration at 1300 and 1066 cm^{-1}, and =C—H out-of-plane vibration at 780 and 902 cm^{-1}. In addition, it was noted that two new bands at 570 and 1652 cm^{-1} appeared in SENs, which were attributed to Fe_3O_4 (Fe—O stretching) and amide I (C=O/C—N stretching), respectively. These results strongly confirmed the formation of SENs encapsulated by Fe_3O_4/poly(pyrrole-*N*-propylsulfonic acid) composites. Moreover, the data also indicated that no obvious interaction existed between magnetic particles and conducting polymers, namely Fe_3O_4 particles were embedded into conducting polymers shell by a blend method. In order to further verify the presence of magnetic particles/conducting polymer composites on enzyme molecule, XRD was employed to gain evidence for the SENs. Fig. 2C shows the XRD patterns of the SENs, as well as the Fe_3O_4 nanoparticles. The main peaks at $2\theta = 30.1°$, 35.4°, 43.1°, 56.9°, and 62.5°, which were in agreement with the XRD peaks of pure Fe_3O_4 nanoparticles, were observed in the SENs. Additionally, a broad peak at about $2\theta = 25°$ was a characteristic peak of poly(pyrrole-*N*-propylsulfonic acid). Thus, XRD further confirmed that Fe_3O_4 magnetic particles and conducting polymer existed in the SENs. Furthermore, this also revealed that the phase change of Fe_3O_4 did not take place in SENs, that is, Fe_3O_4 particles were blended with conducting polymers, this was consistent with the result by FTIR analysis.

Electrical and magnetic properties

FTIR and XRD proved that the obtained SENs contained Fe_3O_4 magnetic particles and conducting polymers, which would provide the SENs with a doubly functionalized property, namely conductivity and magnetization. As we all know, the Fe_3O_4 content in composites affects significantly electrical and magnetic properties. It has been widely reported that the increasing Fe_3O_4 content will result in reduction of the conductivity, whereas increase of the magnetization [25,26]. The decrease in conductivity may be attributed to the insulting behavior of Fe_3O_4 particles embedded in composites, which inhibits electron translation in conducting polymers. In the present study, we mainly focus on the synthesis of SENs with high magnetism. The Fe content in SENs obtained in our experiments is the maximum content, exhibiting the maximal saturation magnetization. Elemental analysis results show that the Fe content in SENs reaches nearly 23 wt.%. The conductivity of SENs measured by standard four-probe method is 2.7×10^{-3} S cm^{-1}.

The magnetic property of as-synthesized SENs was investigated using a magnetometer. Fig. 3 presents the dependence of magnetization on the applied magnetic field for SENs at 30 °C. A weak hysteresis loop was observed, indicating that the resultant SENs were nearly superparamagnetic. From the plot of *magnetization* vs H, the saturation magnetization (M_s) and coercive force (H_c) were estimated to be 14.5 emu g^{-1} and 60 Oe, respectively. These magnetic properties are quite different from bulk Fe_3O_4 particles ($M_s = 84$ emu g^{-1} and $H_c = 500$–800 Oe [27]). The reduced M_s may be a result of the presence of conducting polymers on the surface of Fe_3O_4 particles, which may quench the magnetic moment, whereas the low H_c may be resulted from the size of Fe_3O_4 particles blended with conducting polymers. In addition, it was worth noting that the remanence of the SENs was zero once the applied magnetic field was removed, which further proved the superparamagnetic behavior of the SENs. Since conducting polymer was not magnetic, the ferromagnetic properties of SENs should derive from the magnetic Fe_3O_4 particles in SENs. The superparamagnetic property of the SENs is critical

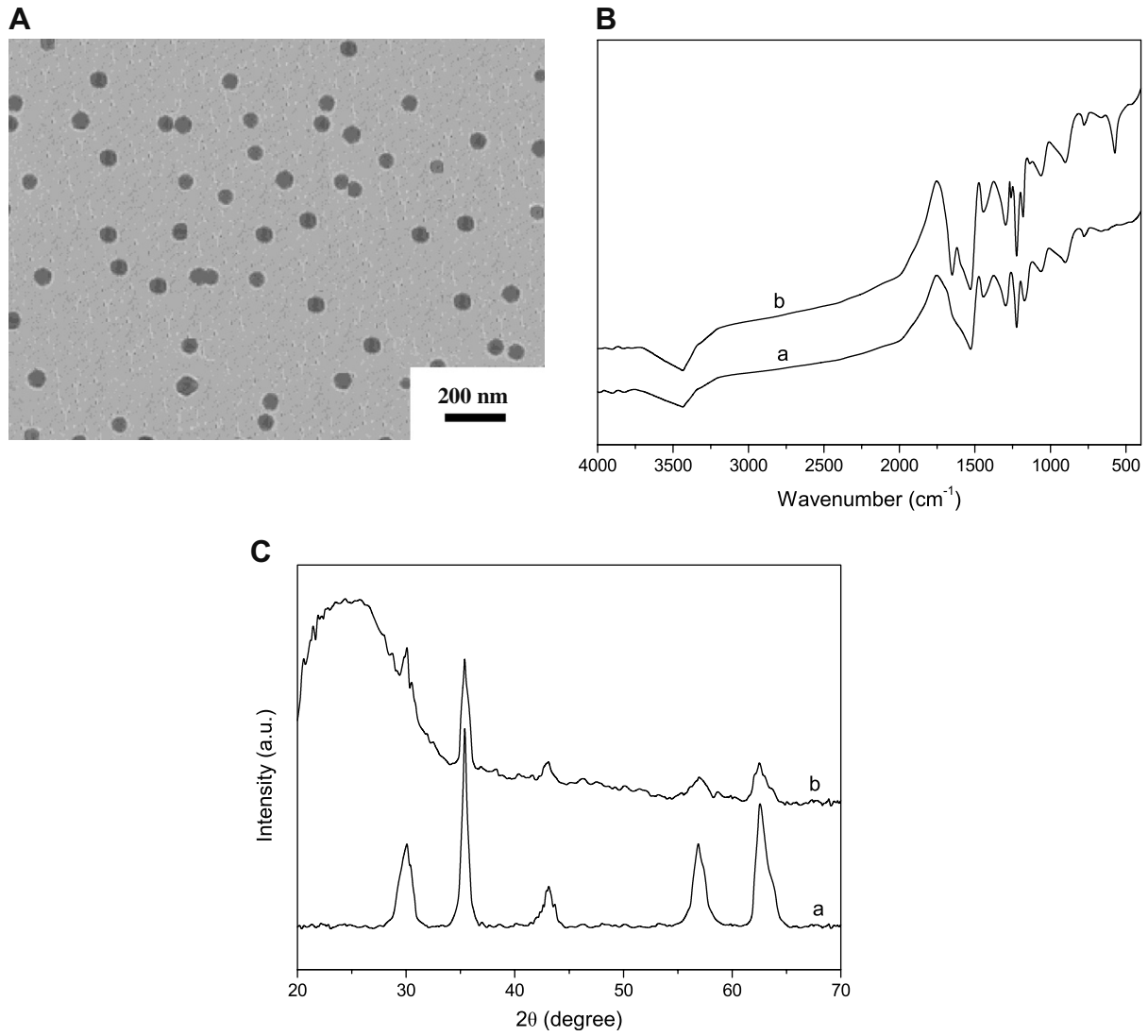

Fig. 2. TEM image (A), FTIR spectra (B), and XRD patterns (C) of synthesized SENs.

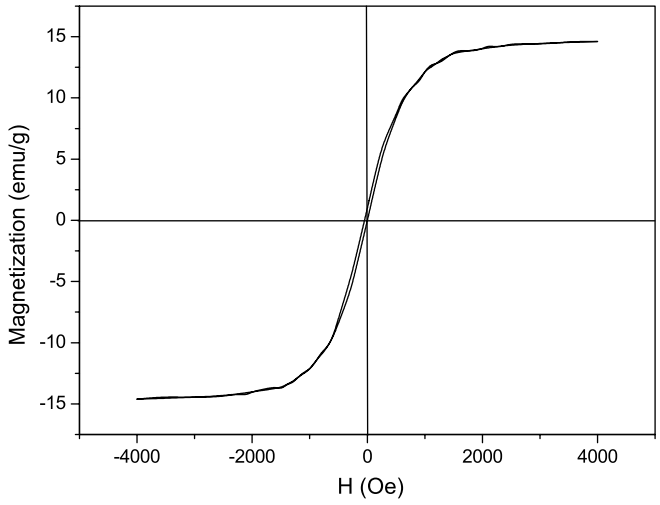

Fig. 3. Dependence of the applied magnetic field on the saturated magnetization of SENs.

for their application in industrial biocatalysis, biomedical, and bioengineering field, which prevents them from aggregation and enables them to redisperse rapidly when the magnetic field is removed [28].

Activity and stability of enzyme encapsulated within SENs

The activity of free and encapsulated GOD as a function of pH were determined in the pH range of 5.5–9 at 30 °C (Fig. 4A). The maximum activity of both free and encapsulated GOD was found to be at pH 5.8, implying that the solution pH around the SENs might be similar to that around the free GOD. With the increase of pH to 9.0, the encapsulated GOD displayed a significantly improved stability compared with that of free GOD. At pH 9.0, the encapsulated GOD retained about 85% of its maximum activity, which was approximately four times higher than that of free GOD. Thus, it is suggested that the activity of enzyme is less susceptible to solution pH after being

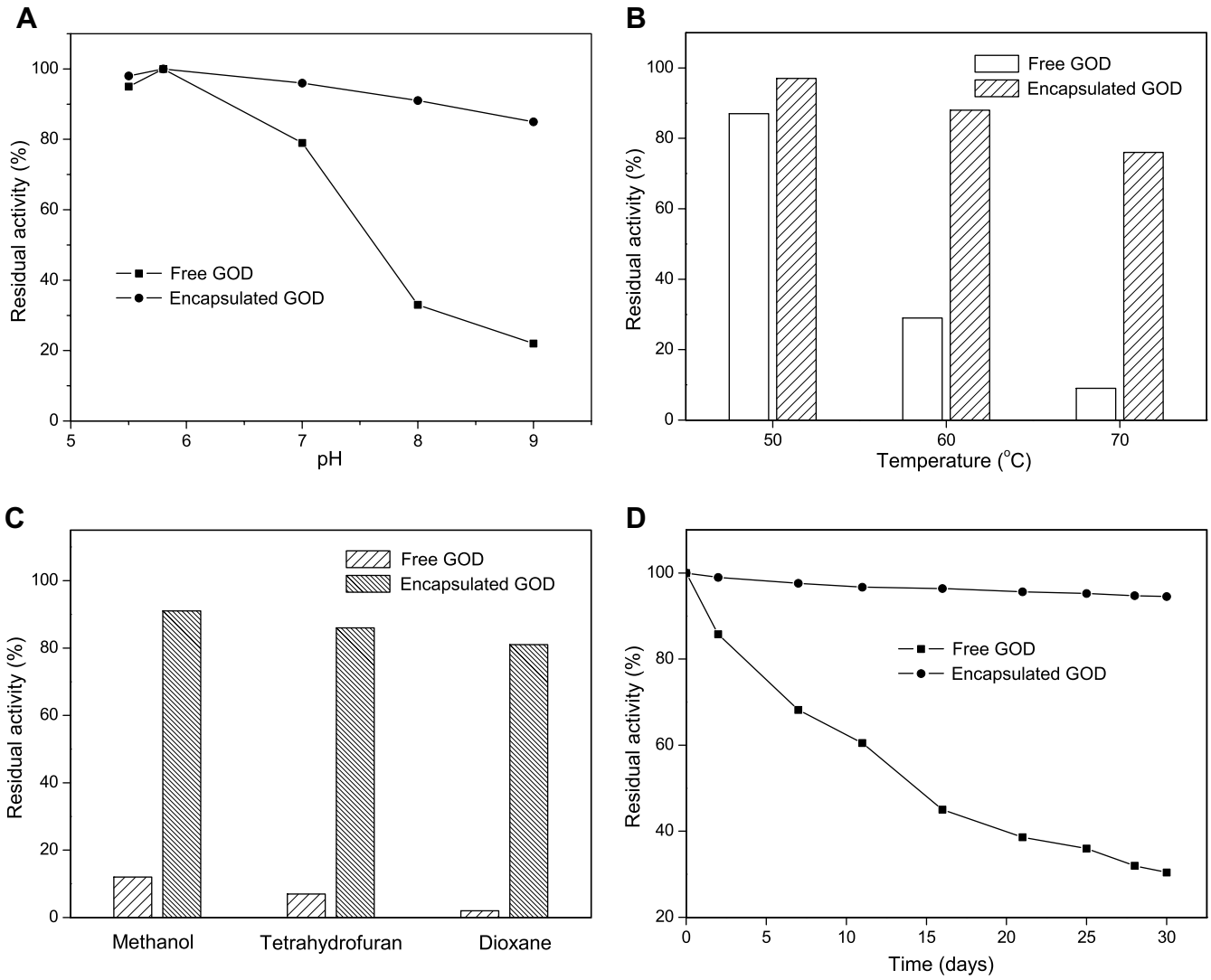

Fig. 4. Activity and stability of GOD encapsulated within SENs at different conditions. (A) Residual activity of GOD as a function of pH. (B) Thermal stability of the free and encapsulated GOD after incubation for 20 min at various temperatures. (C) Residual activity of GOD after exposing to an aqueous solution containing 10% (v/v) organic solvent for 20 min at 30 °C. (D) Storage stability of the free and encapsulated GOD in the phosphate buffer solution (pH 5.8) at 4 °C.

encapsulated, namely a strong tolerance of encapsulated enzyme to the variation of pH values. This might be due to the multipoint covalent attachments of enzyme molecules in the hybrid nanoparticles, which increases the stability of encapsulated enzyme.

The thermal stability of free and encapsulated GOD was assessed after an incubation period of 20 min at various temperatures (50, 60, and 70 °C). As shown in Fig. 4B, the activity of free GOD was strongly dependent on temperature, the residual activity of free GOD was 87% at 50 °C, 29% at 60 °C, and 9% at 70 °C, indicating a serious loss of enzymatic activity with the increasing temperature. However, the activity of encapsulated GOD appeared to be less sensitive to temperature changes, the residual activity was over 80% for all temperatures, revealing a strong resistance of encapsulated GOD to denaturation of enzyme caused by high temperature. The significantly enhanced thermal stability of encapsulated enzyme, we believe, is due to the fixation of enzyme in SENs that hinders the thermal inactivity of enzyme resulting from thermal fluctuation at high temperature.

Fig. 4C shows the residual activity of free and encapsulated GOD after exposing to an aqueous solution containing 10% (v/v) polar organic solvent (methanol, tetrahydrofuran, and dioxane) for 20 min at 30 °C. It was clear that the encapsulated GOD retained most portion of its activity (over 80%), whereas the free GOD lost almost all its activity. The result indicates that encapsulated enzyme is more stable against organic solvent in comparison with free enzyme in solution, which is consistent with the results of enzyme encapsulated in nanogel [20]. The considerably improved stability of encapsulated enzyme in organic solvent may be attributed to two factors, on one hand, multipoint attachments of enzyme molecules in

SENs strengthen the structure of enzyme molecules and avoid organism-induced conformational changes. On the other hand, the hydrophilic groups in SENs provide a hydrophilic environment similar to the ''essential water'', and the enzyme is able to display its biological function, while the stripping of the essential water by the polar organic solvents causes the denaturation of free enzyme [29].

The storage stability, or the ability of enzyme to maintain activity over a long period of time, is an important aspect to be considered in the practical application of enzyme. To evaluate the long-term storage stability, the free or encapsulated GOD was stored in a phosphate buffer solution (pH 5.8) at 4 °C in the dark. The activity of GOD was tested repeatedly nine times during 1 month's storage. Fig. 4D shows the residual activity of GOD as a function of the time elapsed. Under the same storage conditions, the activity of encapsulated GOD decreased slower than that of free GOD. After one month, the residual activity of encapsulated GOD was nearly 95% of its initial activity, whereas the free GOD could only retain less than 31% of its original activity. This obviously reveals that the stability of enzyme molecules and their bioactivity are preserved well after being encapsulated. The enhanced storage stability of encapsulated enzyme can be attributed to the improved resistance to thermal denaturation and conformational changes of enzyme in the buffer solution.

Conclusion

A novel and efficient method has been developed to fabricate magnetic SENs. The analyses of TEM, FTIR, and XRD indicate that the obtained nanoparticle size is about 50 nm, and the nanoshell entrapping GOD is composed of Fe_3O_4/conducting polymer composites. Magnetic measurement shows that the resultant SENs are superparamagnetic and their saturation magnetization is reduced after GOD entrapping. The superparamagnetic property of the SENs will prevent them from aggregation and enable them to redisperse rapidly when the external magnetic field is removed. Furthermore, experiments have proved that these SENs have a significantly improved activity and stability toward pH change, high temperature, organic solvent, and long-term storage, as compared to free GOD. The magnetic SENs with small diffusion limitation, high activity and stability, we believe, will find much potential for a large variety of applications.

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