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Immobilization of cholesterol oxidase on magnetic fluorescent core-shell-structured nanoparticles

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ABSTRACT

The magnetic fluorescent core-shell structured nanoparticles, Fe_3O_4@SiO_2(F)@meso–SiO_2 nanoparticles, were prepared. Cholesterol oxidase (COD) was immobilized on their surface to form Fe_3O_4@SiO_2(F)@meso–SiO_2@COD nanoparticles. Optimal immobilization was achieved with 2.5% (v/v) APTES, 2.0% (v/v) GA, 10 mg COD (in 15 mg carrier) and solution pH of 7.0. Fe_3O_4@SiO_2(F)@meso–SiO_2@COD nanoparticles showed maximal catalytic activity at pH 7.0 and 50 °C. The thermal, storage and operational stabilities of COD were improved greatly after its immobilization. After the incubation at 50 °C for 5 h, the nanoparticles and free COD retained 80% and 46% of its initial activity, respectively. After kept at 4 °C for 30 days, the nanoparticles and free COD maintained 86% and 65% of initial activity, respectively. The nanoparticles retained 71% of its initial activity after 7 consecutive operations. Since Fe_3O_4@SiO_2(F)@meso–SiO_2@COD nanoparticles contained tris(2,2-bipyridyl)dichloro-ruthenium(II) hexahydrate ($Ru(bpy)_3Cl_2$) and were optical sensitive to oxygen in solution, it might be used as the sensing material and has the application potential in multi parameter fiber optic biosensor based on enzyme catalysis and oxygen consumption.

1. Introduction

It is well known that high cholesterol level in blood is associated with the clinical disorders such as hypertension, arteriosclerosis, myocardial infarction, nephrosis and diabetes mellitus. Therefore the determination of cholesterol using rapid, cheap and reliable methods is of great importance in clinical diagnosis. Fiber optic biosensors have many advantages including high sensitivity, fast response, immunity from electrical interference and low cost, and could be the effective way to determine cholesterol concentration. Cholesterol oxidase (COD) catalyzes the oxidation of cholest-5-en-3-one and then the isomerization to cholest-4-en-3-one [1]. For the fiber optic biosensor based on cholesterol oxidase catalysis, the sensing performance depends mainly on the enzyme properties. As a native enzyme, COD has the drawbacks of high cost, poor stability and resource shortage. However, the properties of COD can be greatly improved after its immobilization on suitable carriers due to the fact that the immobilized enzyme offers several advantages such as its reuse, improved stability and ease removal from the reaction medium.

A certain amount of work has been done on COD immobilization. Chen et al. used functionalized sepharose particles as the carrier to immobilize COD [2]. The thermal, pH and storage stabilities of COD were increased by immobilization. Yapar et al. immobilized COD in conducting network via complexation of chitosan with alginic acid [3]. The immobilized COD still maintained 63% of its initial activity after 44 measurements, showing an excellent operational stability. Gilles et al. immobilized COD on to Fe_3O_4 magnetic nanoparticles [4]. The immobilized enzyme exhibited a better tolerance to pH and temperature, and it also had an improved stability. In addition, COD has been immobilized on perlite [5], polyaniline films [6], hollow fiber [7], silk mat [8], functional polymetric supports [9], hydrogel [10], Alkylamine glass beads [11], mesoporous silica materials [12] and sol-gel film [13]

SiO_2 nanoparticles have the advantages of ease to prepare, good biocompatibility and large specific surface area [14,15]. Fluorescent silica nanoparticles offer significant advantages compared to free dyes due to the fact that the encapsulation of luminescent molecules in silica nanoparticles often increases their photostability and emission quantum yield [16–19]. The reported sensing materials for fiber optic biosensor based on enzyme catalysis usually consist of two parts [20–22], the optical sensing part and catalytic enzyme part. If the enzyme is immobilized on the fluorescent SiO_2 nanoparticles containing oxygen sensing photosensitizer and these complex nanoparticles are used as the sensing materials for fiber optic biosensor, the catalysis effect of enzyme can arrive at the photosensitizer directly and the performance of fiber optic biosensor will be improved. The complex nanoparticles with the magnetic core will be beneficial to the isolation of the immobilized enzyme from the reaction mixture. If different enzymes were immobilized on magnetic luminescent silica nanoparticles and

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the enzyme performance was controlled, it will be possible to develop the multi parameter fiber optic biosensor based on enzyme catalysis and oxygen consumption.

In this work, the magnetic fluorescent core-shell structured nanoparticles, Fe_3O_4@SiO_2(F)@meso–SiO_2 nanoparticles, were prepared. The core of nanoparticles consisted of a single Fe_3O_4 nanoparticle encapsulated in fluorescent dye $Ru(bpy)_3Cl_2$ co-doped nonporous silica, their shell was made from ordered mesoporous silica. COD was immobilized on their surface to form Fe_3O_4@SiO_2(F)@meso–SiO_2@COD nanoparticles. Immobilization conditions were investigated and optimized. The properties of Fe_3O_4@SiO_2(F)@meso–SiO_2@COD nanoparticles such as catalytic activity as a function of pH and temperature, thermal, storage and operational stabilities were studied. Since Fe_3O_4@SiO_2(F)@meso–SiO_2@COD nanoparticles have improved catalytic performance and oxygen sensing property, they will have the application potential in multi parameter fiber optic biosensor based on enzyme catalysis and oxygen consumption. To the best of our knowledge, there has been no report on this kind of magnetic materials with the properties of enzymatic catalysis and optical oxygen sensing.

2. Experimental

2.1. Chemicals

COD (E.C. 1.1.3.6, 20 U/mg), Cholesterol, $Ru(bpy)_3Cl_2$ (99.0%), hexadecyltrimethyl ammonium bromide (CTAB) were purchased from Aldrich-Sigma. 3-aminopropiltrietoxysilane (APTES) was purchased from Alfa Aesar. Tetraethoxysilane (TEOS) and glutaraldehyde (GA) (25% aqueous solution) were purchased from Sinopharm Chemical Reagent Co.. All reagents were with analytical grade and used without further purification. Double-distilled water was used throughout the experiments.

2.2. Preparation of Fe_3O_4@SiO_2(F) nanoparticles

The magnetic Fe_3O_4 nanoparticles were prepared using the method of Massart [23]. 4 mg of $Ru(bpy)_3Cl_2.6H_2O$ was reacted with 30 μL of APTES in 5 mL of ethanol under dark condition for 24 h. The prepared fluorescent agent precursor solution was kept at 4 °C. 155 mL of ethanol, 27 mL of H_2O, 8 mL of ammonia, 5 mL of fluorescent agent precursor solution, and 4 mL of Fe_3O_4 nanoparticles solution were added in a 250 mL conical flask consecutively. Finally, 0.44 mL of TEOS was added slowly. After ultrasonic treatment for 10 min, the mixture was stirred at 25°C for 6 h. The product was washed with H_2O to neutral and dry in vacuum at 60°C

2.3. Preparation of Fe_3O_4@SiO_2(F)@meso–SiO_2 nanoparticles

The Fe_3O_4@SiO_2(F)@meso–SiO_2 nanoparticles were prepared by a modified stöber sol-gel method [24]. 0.20 g of Fe_3O_4@SiO_2(F) nanoparticles, 0.17 g of CTAB, 0.5 mL of ammonia, 25 mL of ethanol were added in 54 mL of H_2O consecutively. 0.3 mL of TEOS was added slowly. After ultrasonic treatment for 10 min, the mixture was stirred at 25°C for 8 h. After the particles were magnetically separated and washed with ethanol twice, they were refluxed in acidic ethanol at 100°C under N_2 for 24 h to remove CTAB. The product was washed with ethanol to neutral and dry in vacuum at 60°C.

2.4. Preparation of Fe_3O_4@SiO_2(F)@meso–SiO_2@COD nanoparticles

15 mg Fe_3O_4@SiO_2(F)@meso–SiO_2 nanoparticles was added into 4 mL 2.5% (v/v) APTES solution and stirred at 25°C for 24 h. The modified nanoparticles were washed with H_2O and redispersed in 3.68 mL phosphate buffer solution (PBS, 0.01 mol/L, pH = 7.3). 0.32 mL of 25% (v/v) GA was added in the mixture and stirred for 1.5 h. The mixture was washed with neutral PBS for several times. The activated Fe_3O_4@SiO_2(F)@meso–SiO_2 nanoparticles were obtained after centrifugation. For the immobilization of COD, 2 mL of COD (5 mg/mL) was allowed to contact with the activated Fe_3O_4@SiO_2(F)@meso–SiO_2 nanoparticles in a tube at 4°C for 12 h with occasional shaking. Then the Fe_3O_4@SiO_2(F)@meso–SiO_2 nanoparticles were washed with PBS buffer (0.05 M, pH = 7.0) thoroughly to remove free COD and Fe_3O_4@SiO_2(F)@meso–SiO_2@COD nanoparticles were obtained and stored in PBS buffer (0.05 M, pH = 7.0).

Followed Bradford method [25], by subtracting the residual free GOD content in the solution from the initial free GOD content the amount of GOD protein loaded onto the porous fluorescent SiO_2 nanoparticles was measured.

2.5. Assay of COD activity

The activity of free COD and Fe_3O_4@SiO_2(F)@meso–SiO_2@COD nanoparticles were determined spectrophotometrically [9] 2.5 mL of cholesterol solution was contacted with free COD or Fe_3O_4@SiO_2(F)@COD nanoparticles. After incubation of 2 min, 2.5 mL of solution containing 4-aminoantipyrine, phenol, peroxidase were added and waited for 10 min to complete quinoneimine dye formation. The COD activity was measured, in triplicate, by recording the absorbance of the red dye at 500 nm at which the dye complex exhibited its maximal adsorption. One unit of COD activity converts 1.0 μmol of cholesterol to 4-cholesten-3-one per min at pH 7.0 and 25 °C.

2.6. Stability of COD

To study the thermal stability of free COD and Fe_3O_4@SiO_2(F)@meso–SiO_2@COD nanoparticles, enzyme activity was determined in different time of incubation at 60 °C. The storage stability of free COD and Fe_3O_4@SiO_2(F)@meso–SiO_2@COD nanoparticles was investigated by storing them at 4 °C for 30 days and determining their residual activity every five days.

To investigate the operation stability of Fe_3O_4@SiO_2(F)@meso–SiO_2@COD nanoparticles, several consecutive operation cycles were performed by oxidizing cholesterol. At the end of each oxidation cycle, PBS buffer (0.1 M, pH 7.0) was used to wash the nanoparticles for several times and the procedure was carried out repeatedly with a fresh aliquot of substrate.

2.7. Characterizations

The morphologies of Fe_3O_4@SiO_2(F)@meso–SiO_2 nanoparticles and Fe_3O_4@SiO_2(F)@meso–SiO_2@COD nanoparticles were observed using a transmission electron microscopy (JEM-100 CXII, JEOL, Japan). Pore size and porosity measurements were performed by the Brunauer–Emmett–Teller (BET) method on an ASAP 2020 instrument. The magnetic properties of nanoparticles were characterized using 4HF vibrating sample magnetometer (VSM). The fluorescence spectra were recorded using a fluorescence spectrophotometer (F-4500, HITACHI, Japan). FT–IR spectrum was recorded on a Thermo Nicolet Nexus FT–IR spectrometer with the standard KBr pellet method. The activities of Fe_3O_4@SiO_2(F)@meso–SiO_2@COD nanoparticles were measured by spectrophotometer (UV-2450, Shimadzu, Japan).

3. Results and discussion

3.1. Preparation and characterization of Fe_3O_4@SiO_2(F)@meso–SiO_2 nanoparticles and Fe_3O_4@SiO_2(F)@meso–SiO_2 @COD nanoparticles

Fe_3O_4@SiO_2(F)@meso–SiO_2 nanoparticles were prepared by a multistep process. Monodisperperse superparamagnetic Fe_3O_4 nanoparticles were first prepared using the method of Massart [23]. Then the prepared Fe_3O_4 nanoparticles were coated with a layer of silica. The formation of SiO_2 nanoparticles consisted of hydrolysis, nucleation and