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Core/shell nanoparticles consisting of a Fe_3O_4 nanoparticle core and a mesoporous silica shell (Fe_3O_4/m-silica) were used as a matrix for immobilization of horseradish peroxidase (HRP) enzyme and subsequent design of an amperometric hydrogen peroxide biosensor. HRP enzyme was immobilized on the core/shell nanoparticles through the electrostatic interaction between oppositely charged HRP enzyme and the silica shell at neutral pH. The enzyme–core/shell nanoparticle hybrid material was deposited on screen printed electrodes and further characterized by ultraviolet–visible (UV-vis) spectroscopy and scanning electron microscopy (SEM). This set up was used as a biosensor to detect hydrogen peroxide. The hydrogen peroxide biosensor showed a detection limit of 4.3×10^{-7} M, at a signal-to-noise ratio of 3, and a sensitivity of 84.4 μA mM^{-1} cm^2.

Introduction

Hydrogen peroxide (H_2O_2) has practical applications in various fields such as food, clinical, pharmaceutical, industrial, biological, or environmental.[1–5] In addition, high concentrations of H_2O_2 can have a negative effect on human health, provoking eye and skin irritations.[5] Various H_2O_2 detection methods have been investigated, such as chromatography, photometry, fluorescence or electrochemical methods.[1–5] Among these methods, electrochemical detection has attracted extensive research interests due to rapid response, high sensitivity, simplicity, and relatively low cost.[5,6] Horseradish peroxidase (HRP) is usually used for the determination of H_2O_2 since HRP is a member of the superfamily of peroxidases and a heme containing glycoprotein.[7]

Effective enzyme immobilization is very important for the stabilization of the enzyme in biosensors. There are several enzyme immobilization methods such as physical adsorption,[8] sol–gel,[7,9] cross-linking,[10,11] or covalent bonding.[12] However, there are several drawbacks for each of these immobilization techniques, such as an analyte diffusion barrier for solid porous matrices, and enzyme leaching or denaturation due to synthesis conditions.[10] Nanoparticles, which can be functionalized by various materials have attracted research interest in the biosensors field due to their versatile physical and chemical properties.[1] The high surface area of nanoparticles presents the opportunity of higher enzyme loading. This is also combined with minimal analyte diffusion limitations when compared with planar porous immobilization hosts[13,14] Therefore, using nanoparticle platforms with lower diffusion limitations and higher enzyme loading, for biosensor design, holds promise for an improved sensitivity of analyte detection compared with their planar counterparts. For example, Luo *et al.* used Au nanoparticles to immobilize HRP,[15] Wang *et al.* reported a H_2O_2 biosensor using quantum dots (CdSe/ZnS),[16] Jia *et al.* demonstrated SnO_2 nanoparticles as a matrix for HRP enzyme immobilization,[7] and Zhang *et al.* reported Fe_3O_4–SiO_2 core/shell nanoparticles for a hydroquinone biosensor.[11] However, for most of these studies, the nanoparticles were agglomerated and therefore the advantage of a high surface area was largely lost.

In this study, core/shell nanoparticles consisting of a Fe_3O_4 nanoparticle core and a mesoporous silica shell (Fe_3O_4/m-silica), highly uniform in size, and well dispersed in aqueous solution were prepared as an effective support for HRP enzyme immobilization. The mesoporous surface of the silica shell brings advantages in terms of possible enzyme entrapment into the pores and biocompatibility. The core/shell nanoparticles were used as high surface area supports for HRP enzyme immobilization and the H_2O_2 biosensor was designed based on this hybrid platform.

Experimental

Materials

Horseradish peroxidase (HRP, 300 units mg^{-1}), hydrogen peroxide (H_2O_2, 30% w/v solution), KH_2PO_4, K_2HPO_4, iron chloride ($FeCl_3 \cdot 6H_2O$), sodium oleate, oleic acid, 1-octadecene, tetraethyl orthosilicate (TEOS), ammonium hydroxide, IGEPAL CO-520, methanol, ethanol and hexane (all reagent grade) were obtained from Sigma Aldrich for synthesis of Fe_3O_4/m-silica and subsequent biosensor testing. All chemicals were used without any additional purification and all experimental solutions were prepared with deionized (DI) water.

Apparatus and measurements

The electrochemical measurements of H_2O_2 concentration were performed with a BASi epsilon C3 cell stand. Ultraviolet–visible

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(UV-vis) spectroscopy measurements were carried out with a SpectraMax M5 spectrometer (Molecular Devices). The microstructure of the Fe_3O_4/m-silica nanoparticles was evaluated by transmission electron microscopy (TEM) with a Titan 80–300 (FEI Company, USA) operated at 300 eV. Scanning electron microscopy (SEM) images of the Fe_3O_4/m-silica and the surface of the screen printed electrodes (SPE) were obtained using a FEI Nova NanoSEM 200 operating at 10 kV.

Synthesis of Fe_3O_4/m-silica core/shell nanoparticles

In order to prepare the Fe_3O_4/m-silica, Fe_3O_4 nanoparticles were synthesized by a thermal decomposition method.[17] In the first step, iron oleate was prepared as a precursor of Fe_3O_4 from iron chloride (0.54 g) and sodium oleate (1.83 g). The mixture including iron chloride, sodium oleate, hexane, ethanol and DI water was heated to 65 °C for 4 h. Then, the solution was washed by DI water and dried overnight. Next, iron oleate (1.8 g) was added into the flask including oleic acid (0.29 g) and 1-octadecene (10 g). Then, the flask was heated to 320 °C for 30 min to obtain the Fe_3O_4 nanoparticles of 13 nm in diameter. The resulting nanoparticles were washed with ethanol and dispersed in hexane.

The silica shell was formed around Fe_3O_4 nanoparticles by a reverse microemulsion method.[18] IGEPAL-CO 520 (0.6 ml) which is a surfactant used to modify the surface property was added in hexane (12 ml). Then the as-synthesized Fe_3O_4 nanoparticles (1 ml) were added in the solution. Aqueous ammonia (0.1 ml) and TEOS (0.3 ml) were added to the mixture as a reaction catalyst and silica precursor, respectively. The solution was stirred for 24 h at room temperature and the resulting product was washed with ethanol. Finally, Fe_3O_4/m-silica was dispersed in DI water.

Preparation of the H_2O_2 biosensors

Phosphate buffer saline (PBS) solution (0.05 M, pH 7.0) containing KH_2PO_4 and K_2HPO_4 was prepared and the pH value was adjusted from 4.0 to 9.0 using HCl or NaOH. Next, HRP was added to the PBS solution (100 μl). The solution containing HRP enzyme (typically 1.36 U) was incubated for 2 h with Fe_3O_4/m-silica (50 μl) solution to immobilize the enzyme on the surface of the core/shell nanoparticles. Screen printed electrodes (SPE; area = 5 × 4 mm^2, Pine Research Instrumentation) were used as working electrodes. In order to change the surface properties to hydrophilic, the SPE was pretreated by direct current (DC) potential amperometry (1750 mV *vs.* Ag/AgCl) for 5 min. HRP–Fe_3O_4/m-silica (3 μl) was then deposited on the pretreated SPEs. The SPEs were dried overnight and stored in 0.05 M PBS solution at 4 °C before biosensor testing for determination of H_2O_2.

Characterization of H_2O_2 biosensors

A conventional three-electrode system was used for the biosensor testing with SPE as the working electrode, a platinum wire as an auxiliary electrode, and a Ag/AgCl electrode as a reference electrode. Cyclic voltammetric (CV) curves and amperometric response of SPEs were obtained in 3 ml PBS (0.05 M, pH 7.0) containing 1.0 mM $K_4Fe(CN)_6$ as an electron mediator, under magnetic stirring (150 rpm).

Results and discussion

Microstructural characterization of Fe_3O_4/m-silica core/shell nanoparticles

Fig. 1 shows the TEM and SEM images of Fe_3O_4/m-silica, indicating the spherical nanoparticles have uniform size and shape and are well dispersed in DI water. Fig. 1a and b show TEM images of Fe_3O_4/m-silica with a 13 nm Fe_3O_4 core and a 20 nm mesoporous silica shell. The diameter of the core/shell nanoparticles was determined to be about 50 nm. The uniform size and absence of agglomeration of the Fe_3O_4/m-silica were supported by the SEM images in Fig. 1c and d. In order to confirm the composition of Fe_3O_4/m-silica, electron energy loss spectra (EELS) of the core/shell nanoparticles were obtained. Fig. 2a and b show the zero loss and Fe mapping images of Fe_3O_4/m-silica, respectively. The white areas in Fig. 2b indicate the iron atoms. The iron mapping results are consistent with the Fe_3O_4 core area from the zero loss image.

Immobilization of HRP into Fe_3O_4/m-silica core/shell nanoparticles

The HRP enzyme in PBS was added into a solution containing Fe_3O_4/m-silica to immobilize the enzyme. After incubation for 2 h, HRP immobilization was evaluated by UV-vis spectroscopy. Fig. 3a shows the UV-vis absorption spectra of 100 μl free HRP enzyme and 100 μl HRP–Fe_3O_4/m-silica. HRP enzyme has a characteristic absorption band at around 404 nm.[7,19] The UV-vis results show the same absorption peak positions for both HRP enzyme and HRP–Fe_3O_4/m-silica composite (Fig. 3a). This shows that HRP enzyme was immobilized successfully onto the surface of Fe_3O_4/m-silica without a significant alteration of

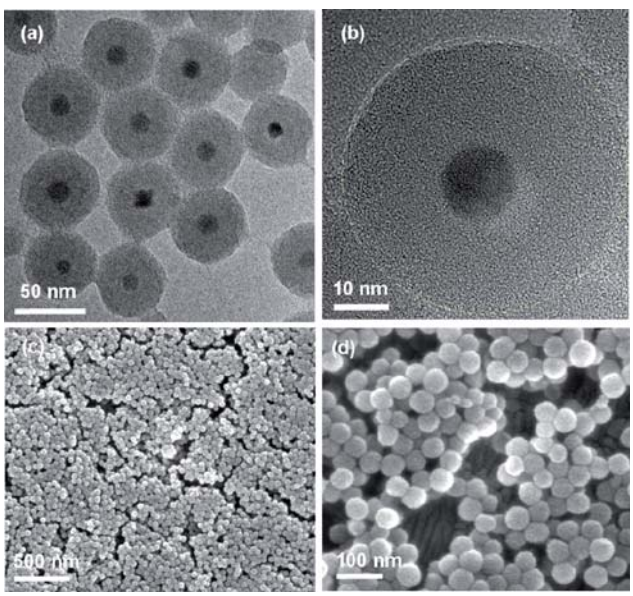

Fig. 1 (a), (b) TEM images and (c), (d) SEM images of Fe_3O_4/m-silica dispersed in DI water.

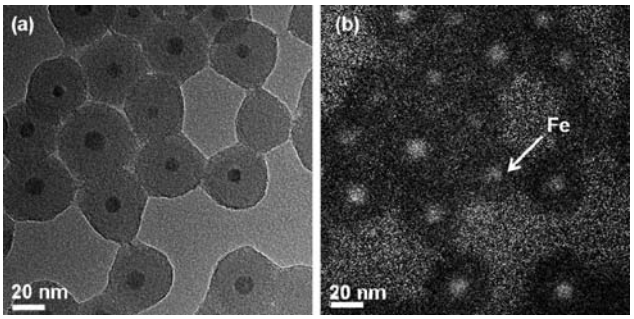

Fig. 2 EELS mapping images of Fe_3O_4/m-silica: (a) zero loss image and (b) Fe mapping images.

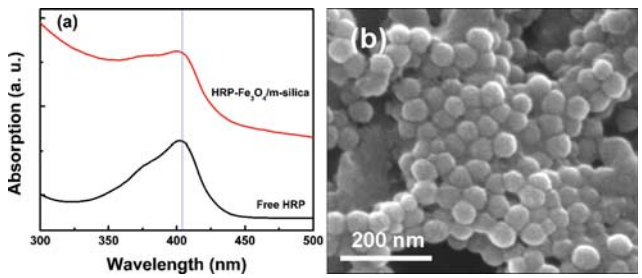

Fig. 3 (a) UV-vis absorption spectra of HRP–Fe_3O_4/m-silica and free HRP and (b) SEM image of the SPE with HRP–Fe_3O_4/m-silica.

properties. The mechanism of enzymatic immobilization is through electrostatic interaction between the HRP and the silica shell. This could be explained through the two components being oppositely charged between pH values of 3 and 7.2. This arises from the isoelectric points of HRP enzyme and silica being 7.2[20] and 3,[21] respectively. The surface morphology of the SPE coated HRP–Fe_3O_4/m-silica was checked by SEM (Fig. 3b). The particles are significantly more agglomerated after enzyme immobilization (Fig. 1d), which is attributed to electrostatic interactions between Fe_3O_4/m-silica and the HRP enzyme.[6]

Electrochemical characterization of the H_2O_2 biosensor

SPEs with HRP–Fe_3O_4/m-silica were prepared by the process described above. A solution of 1.0 mM $K_4Fe(CN)_6$ was used as an electron mediator.[6,22] The reaction sequence below shows a proposed reaction mechanism of the amperometric H_2O_2 biosensor and the effect of the electron mediator.[6,22]

$$HRP_{(red)} + H_2O_2 \rightarrow HRP_{(ox)} + H_2O$$

$$Fe(CN)_6]^{4-}{}_{(red)} + HRP_{(ox)} \rightarrow HRP_{(red)} + [Fe(CN)_6]^{3-}{}_{(ox)}$$

$$[Fe(CN)_6]^{3-}{}_{(ox)} + e^- \rightarrow Fe(CN)_6]^{4-}{}_{(red)}$$

HRP is oxidized by H_2O_2 in this reaction. The electron mediator, $K_4Fe(CN)_6$ is then oxidized to $K_3Fe(CN)_6$ and the resulting $[Fe(CN)_6]^{3-}$ is reduced to $[Fe(CN)_6]^{4-}$. The electrical current that is generated through these reactions is indicative of the presence of H_2O_2.

The CVs of SPEs fabricated with HRP–Fe_3O_4/m-silica were obtained with different scan rates (10–300 mV s^{-1}) in PBS solution (0.05 M, pH 7.0) containing 0.1 mM $K_4Fe(CN)_6$ as shown in Fig. 4a. The redox peaks corresponding to the HRP Fe^{III}/Fe^{II} redox couple occur at 280 and 180 mV at a scan rate of 50 mV s^{-1}, respectively, with a separation of peak potential of about 100 mV. Fig. 4b shows peak currents of oxidation and reduction with different scan rates. The peak currents show a linear relationship with an increase in the scan rate.[6,19] At the same time, the oxidation and reduction peak potentials of HRP shifted to positive and negative potential values, respectively. This indicates a surface-controlled and quasi-reversible process.[19] Fig. 5 shows a schematic representation of the hydrogen peroxide sensor constructed on the Fe_3O_4/SiO_2 core/shell particles.

Optimization of experimental parameters

The current sensitivity is influenced by the pH value of the test PBS solution, applied potential, and concentration of enzyme. These experimental variables were optimized to achieve a sensitive biosensor. Fig. 6a shows the current response with different pH values of test PBS solution (pH 4.0–9.0) at a constant concentration of H_2O_2 (18 μM). The three SPE working electrodes prepared under the same conditions from HRP–Fe_3O_4/m-silica were tested and the current response was averaged by repeating each measurement three times. At a pH value of 5.0 of

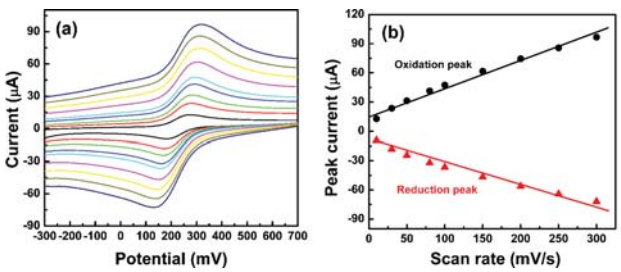

Fig. 4 (a) Cyclic voltammograms of the SPE with HRP–Fe_3O_4/m-silica in PBS solution containing 1.0 mM $K_4Fe(CN)_6$ under different scan rates, from 10 to 300 mV s^{-1} and (b) plot of oxidation (black line) and reduction (red line) peak current *vs.* the scan rate.

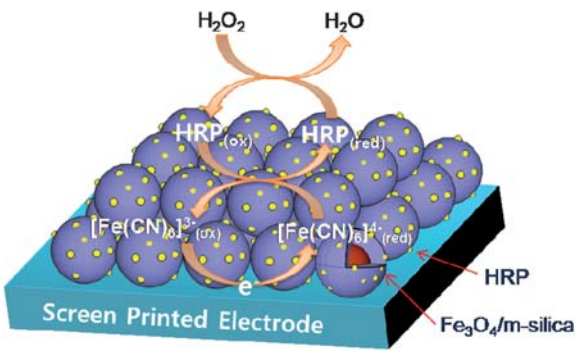

Fig. 5 Schematic representation of the hydrogen peroxide biosensor constructed on the Fe_3O_4/m-silica.

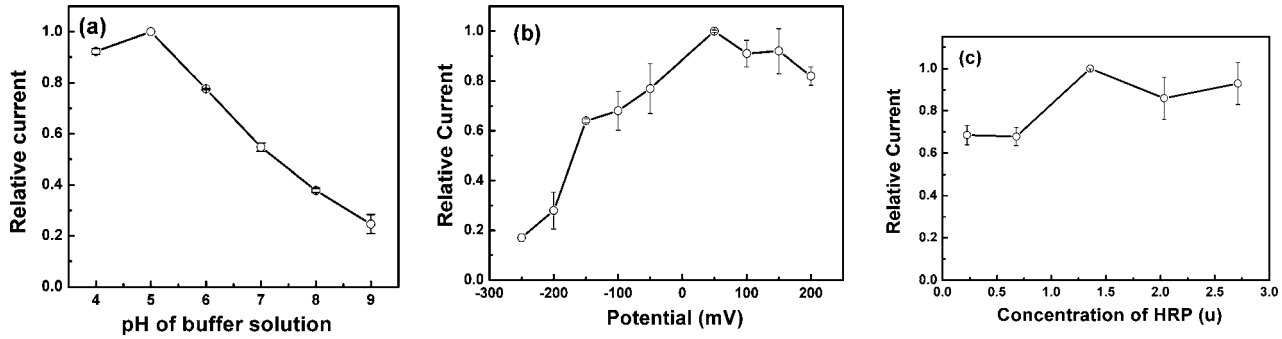

Fig. 6 (a) Effect of the pH of PBS solution, (b) effect of the applied potential, and (c) effect of the HRP concentration on the current response of the SPE with HRP–Fe_3O_4/m-silica to 18 μM H_2O_2.

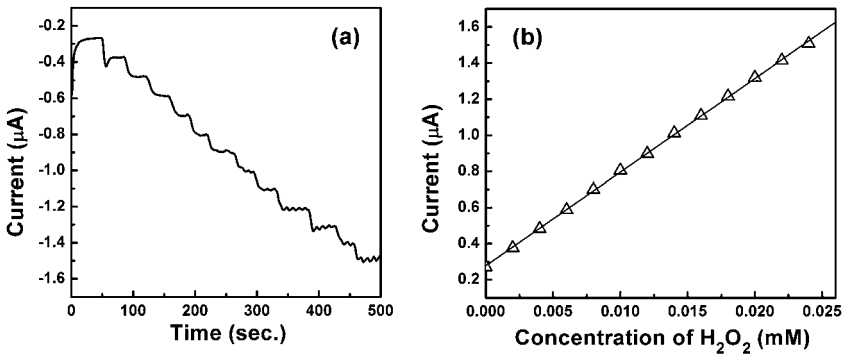

Fig. 7 (a) Amperometric response of the fabricated biosensor to successive addition of H_2O_2 into stirring PBS solution (0.05 M, pH 5) containing 1.0 mM $K_4Fe(CN)_6$; the applied potential was 50 mV *vs.* Ag/AgCl and (b) calibration curve and linear fitting curve between the current and the H_2O_2 concentration.

the PBS solution, the SPEs showed the highest current response. Thus, a PBS solution with a pH of 5.0 was used for subsequent biosensor tests. To find an optimum potential for the biosensor, various applied potential values, from −250 to 250 mV, were used as shown in Fig. 6b. The current response reached the maximum point at an applied potential of 50 mV. In addition, the influence of HRP enzyme concentration was investigated. As shown in Fig. 6c, the current was increased with increase of HRP enzyme amount until 1.36 U. When the amount of HRP enzyme was higher than 1.36 U, the saturation limit was reached. Thus a HRP concentration of 1.36 U was chosen for the preparation of the SPEs.

Amperometric response of the H_2O_2 biosensor

Fig. 7a shows the amperometric current *vs.* time curve of the SPE with HRP–Fe_3O_4/m-silica. H_2O_2 (2 μM) was added successively into the test PBS solution (0.05 M, pH 5.0) containing 1.0 mM $K_4Fe(CN)_6$. The current responses showed a linear relationship with the concentration of H_2O_2 (2–24 μM) as shown in Fig. 7b. The correlation coefficient was 0.999 ($n = 13$). The detection limit was 0.43 μM H_2O_2, determined from the linear graph (signal-to-noise ratio = 3). To determine the sensitivity of the biosensor, some assumptions for the calculation of the effective surface area were made. The HRP–Fe_3O_4/m-silica nanoparticles are considered to be uniformly coated on the SPE. The core/shell nanoparticles were assumed to be monodisperse, with a diameter of 50 nm, as actually confirmed by the TEM results. The sensitivity of the biosensor, which was calculated by taking into account the total surface area of enzyme modified core/shell nanoparticles was 84.4 μA mM^{-1} cm^2, which is a relatively high value compared to other similar published data.[3] The results show that HRP–Fe_3O_4/m-silica system can be used as a high surface area materials platform for the design of a sensitive amperometric H_2O_2 biosensor.

Stability of the H_2O_2 biosensor

The stability of the prepared biosensor was investigated by repeating the amperometric measurements over time. The current response of the biosensor was measured by adding 18 μM H_2O_2. After fabrication, the SPEs were stored in a PBS solution (0.05 M, pH 7.0) at 4 °C. After two months of storage, measurements of the current response of the biosensor to 18 μM H_2O_2 showed more than 90% retention of the biological activity.

Conclusion

Hybrid nanoparticles containing a Fe_3O_4 nanoparticle core, a mesoporous silica shell, and immobilized HRP enzyme were tested as material platforms for the design of an amperometric H_2O_2 biosensor. Uniform, non-agglomerated Fe_3O_4/m-silica nanoparticles of 50 nm diameter were synthesized by thermal decomposition and a reverse microemulsion process. The nanoparticles were well dispersed in DI water, which is an indication

of their biocompatibility. The HRP enzyme was immobilized onto the Fe_3O_4/m-silica particles through electrostatic interaction between HRP and Fe_3O_4/m-silica. This platform was subsequently used for the design of an amperometric biosensor for the detection of H_2O_2. The biosensor showed a detection limit of 0.43 μM of H_2O_2 and a sensitivity of 84.4 μA mM^{-1} cm^2. In addition, the biosensor displayed retention of 90% of activity after being stored at 4 °C for 2 months.

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