Enzyme nanoparticles-based electronic biosensor

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A simple and effective method to prepare an enzyme electronic biosensor by immobilizing enzyme nanoparticles directly onto the gold electrode surface is described; prepared horseradish peroxidase nanoparticles have been successfully used to develop reagentless electronic biosensors for H_2O_2 detection without promoters and mediators and offer great potential to develop enzyme-based electronic biosensors.

There has been considerable interest in recent years in synthesizing nanostructured materials because of their unique optical, electronic, chemical, and mechanical properties. Different materials, such as metal (gold, silver), carbon, and polymers (polypyrrole and polyaniline), have been used to prepare nanomaterials such as nanoparticles,[1,2] nanotubes,[3,4] and nanowires.[5–7] Special attention has been given to bioassay applications using such nanomaterials to develop biosensors, biomedical devices, biofuel cells, and bioreactors.[8–10] DNA, antibodies, and enzymes have been immobilized to nanostructure materials by covalent binding, adsorption, and entrapment to develop sensitive biosensors and bioassay methods. Recently, a large number of enzyme biosensors based on nanoparticles[11–13] or nanotubes[14–19] have been developed. The ability to tailor the properties of nanomaterials offers excellent prospects for enhancing the performance of enzyme-based biocatalytic sensors. Flavin adenine dinucleotide functionalized gold nanoparticles were used as current collecting and electron-relaying units to reconstitute apo-glucose oxidase, which exceeded the electron-transfer features of the native enzyme.[13] Template-synthesized polymeric microcapsule arrays were employed to immobilize enzyme molecules as bioreactors.[18] Gooding's group[19] investigated the electron-transfer properties of microperoxidase MP-11 using arrays of aligned carbon nanotubes, while Kim *et al.*[20] developed enzyme–polymer nanoparticles that surround a single enzyme molecule with a porous composite organic/inorganic network of less than a few nanometers thick. Direct adsorption of proteins, such as enzymes, onto bulk metal surfaces frequently results in denaturation of the protein and loss of bioactivity.[21] In contrast, when such enzymes are first adsorbed onto metal nanoparticles, and the enzyme-covered nanoparticles are then electrodeposited onto bulk electrode surfaces to make enzyme biosensors, the bioactivity of the enzymes is often retained.[21–23]

This communication reports on electronic biosensors based on surface-confined enzyme nanoparticles and on the direct electron-transfer characteristics of such biocatalytic particles on gold electrodes. Protein nanoparticles, such as human serum albumin[24] and insulin,[25] have been developed and used in drug delivery systems owing to their potential to improve protein stability, prolonged therapeutic effect, and promise for administration through non-parenteral routes. As far as we know, there is no report on enzyme nanoparticles.

Here we report the preparation of horseradish peroxidase (HRP) nanoparticles and their use for bioelectronic sensing applications for the first time. Briefly, HRP nanoparticles were prepared by desolvation with ethanol and subsequent crosslinking with glutaraldehyde (Fig. 1). Briefly, 2 mg of HRP in 1 ml purified water titrated to pH 7–10 were transformed into nanoparticles by continuously adding 4 ml of desolvating agent ethanol under stirring at 500 rpm at room temperature. The addition speed was 0.5 ml/minute by manual control. After the desolvation process, 8% glutaraldehyde in water (0.47 μl) was added to induce particle crosslinking. The crosslinking process was performed by stirring the suspension over a time period of 24 hours in an ice bath. Then 0.1 g of L-cysteine was added, and the suspension was stirred for the next 4 hours. The resulting nanoparticles were purified by three cycles of differential centrifugation (14 000 × *g*, 10 min) and redispersion of the pellet to the original volume in water. Each redispersion step was performed in an ultrasonication bath over 5 min. Bovine serum albumin (BSA) nanoparticles were prepared using the same procedure. The resulting enzyme nanoparticles were dispersed in 0.1 M phosphate buffer (pH 7.4) and stored at 4 °C.

A typical high resolution transmission electron microscopy (TEM) image of the free-standing HRP nanoparticles is shown in Fig. 2. The seemingly white nanoparticles, which result from the transparency of the protein to the electron beam, have a diameter around 100 nm, indicating that a single HRP nanoparticle is composed of over 1000 cross-linked enzyme molecules (diameter around 6 nm). The presence of thiol groups was confirmed by energy-dispersive X-ray analysis in the TEM instruments.

Ultraviolet–visible spectroscopy was used to verify whether the enzyme molecules in HRP nanoparticles maintain protein structure characteristics after the cross-linking and functionalization process. A well-defined characteristic absorption peak of protein (at 280 nm) is observed with 0.1 M phosphate buffer containing 0.1 mg ml^{-1} of HRP nanoparticles (Fig. 3). These profiles are similar to those observed for HRP dissolved in the buffer solution. Such an absorption band is not observed in

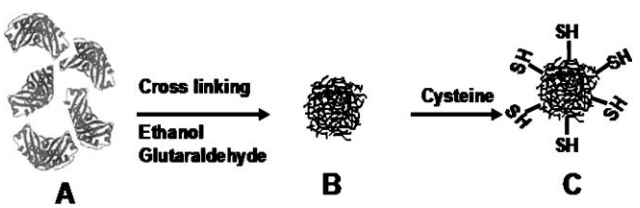

Fig. 1 Scheme of enzyme nanoparticle preparation, A: free enzyme molecules; B: cross linked enzyme nanoparticles; C: thiotinated enzyme nanoparticles.

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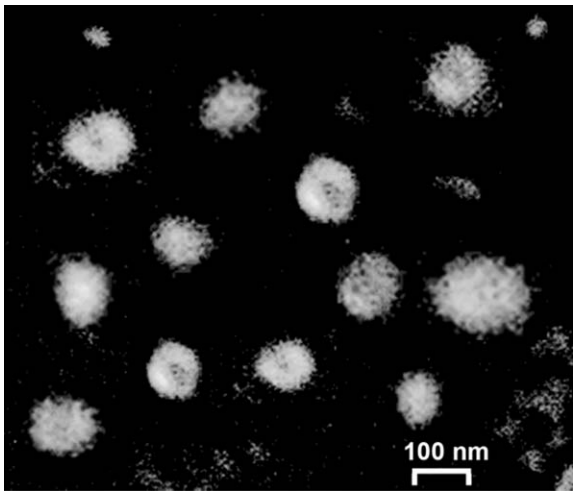

Fig. 2 TEM image of HRP nanoparticles. The micrograph was taken with a Hitachi H7000 instrument operated at 75 kV.

control experiments involving a glutaraldehyde and cysteine solution (not shown), indicating that most of the enzyme molecules preserve their native protein structure characteristics after the crosslinking and functionalization steps.

Thiol groups on the surface of HRP nanoparticles provide a facile method for attaching the particles to the gold electrode surface to develop an enzyme nanoparticle-based electronic biosensor and thus circumventing complications associated with solution systems. Before the experiment, we scanned the potential of the cleaned bare gold electrode over the 0.5 to 1.5 V range in freshly prepared 0.2 M H_2SO_4 until the voltammogram characteristic of the clean polycrystalline gold was established. The polycrystalline gold electrode was placed in the HRP nanoparticle solution under mild stirring at 4 °C for 12 h to give a nanoparticle self-assembled HRP nanoparticle layer. The HRP nanoparticle/gold (HRP NP/Au) was rinsed with a 0.1 M of phosphate buffer (PB, pH 7.4) carefully and stored in a PB buffer at 4 °C when not in use.

Direct electrochemical reactions of redox protein and enzyme at solid electrodes may bring new insights into biological electron transfer processes as well as enable new classes of reagentless biosensors. The resulting HRP NP/Au interface is characterized by

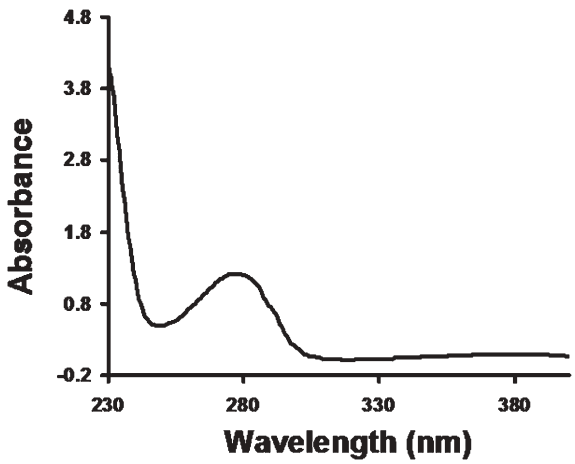

Fig. 3 Ultraviolet invisible spectrum of 0.1 mg ml^{-1} HRP nanoparticles/0.1 M phosphate buffer (pH 7.4).

cyclic voltammetry (CV) to observe whether the enzyme retains its redox activity after crosslinking and self-assembly on a gold surface. Cyclic voltammetric measurements were performed with a three-electrode system comprising a platinum wire as auxiliary electrode, a Ag/AgCl as reference, against which all potentials were measured, and HRP nanoparticle–gold (HRP NP/Au), BSA nanoparticle–gold or gold as a working electrode was used for all electrochemical experiments. Cyclic voltammetric measurements were done in an unstirred electrochemical cell. The voltammogram of the surface-confined enzyme nanoparticles in 0.1 M phosphate buffer (pH 7.4) is shown in Fig. 4a. A pair of stable and well-defined redox peaks with regard to Fe(III) to Fe(II) conversion of the immobilized HRP nanoparticles were observed, with a cathodic peak at −0.08 V and the corresponding anodic peak at 0.15 V. The reduction potential is close to the standard-potential value (−0.22 V) of native HRP in solution,[26] suggesting that most of the enzyme molecules preserve their native characteristics after the crosslinking and self-assembly steps. Control experiments, performed with a gold electrode modified with non-redox protein (BSA) nanoparticles (Fig. 4b) and a polycrystalline gold electrode (Fig. 4c), display no redox activity at both electrodes. Both the anodic and cathodic peak currents of HRP were increasing with the increase of the scan rate in the range from 10 to 100 mV s^{-1} (Fig. 5). The ratio of the cathodic to the anodic peak current is near unity. Peak currents vary linearly with the scan rate, as shown in the inset to Fig. 5, indicating that the electrode reaction is a typical surface-controlled quasi-reversible process.

The HRP-NP/Au association does not seem to impair the redox and electrocatalytic properties of HRP. Such behavior has also been demonstrated for the redox processes of hydrogen peroxide that are of considerable interest to the operation of oxidase-based amperometric biosensors. Amperometric experiments were carried out in the stirred system by applying a potential −300 mV on steady cell. The stirring rate was 800 rpm, and current–time curves were recorded after a constant background had been established. Fig. 6 displays a typical current–time plot of the HRP NP/Au electrode for successive additions of H_2O_2. A fast electrocatalytic

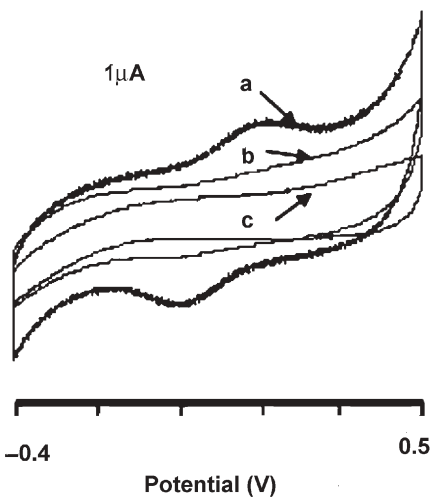

Fig. 4 Cyclic voltammograms of the HRP NP/Au electrode (a), gold electrode modified by bovine serum albumin nanoparticles (b), and polycrystalline gold electrode in 0.1 M phosphate buffer, scan rate: 100 mV/s.

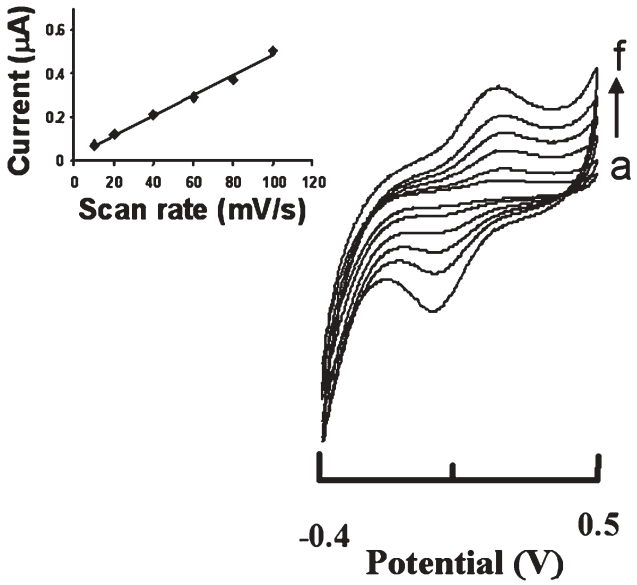

Fig. 5 Cyclic voltammograms of gold electrode modified by HRP nanoparticles in 0.1 M phosphate buffer (pH 7.4) at 10, 20, 40, 60, 80, 100 mV/s (from a to f). Inset: plots of peak currents *versus* scan rate.

response was obtained with 95% of the steady-state current being achieved in less than 5 s. The corresponding calibration plot is linear over the entire 1 μM to 9 μM range (see inset). No response was obtained with the control electrodes above the concentration range (not shown). The detection limit was estimated to be 100 nM at a signal-to-noise ratio of 3. The HRP NP/Au electrode showed good reproducibility, with a variation coefficient of 2.54% for 10 successive assays at the 2 μM H_2O_2 level.

In conclusion, we have demonstrated a simple and effective method to prepare an enzyme electronic biosensor by immobilising enzyme nanoparticles directly onto a gold electrode surface. Immobilized enzyme nanoparticles on the gold electrode retain their redox and electrocatalytic activities and were used to develop reagentless biosensors for H_2O_2 detection without promoters and

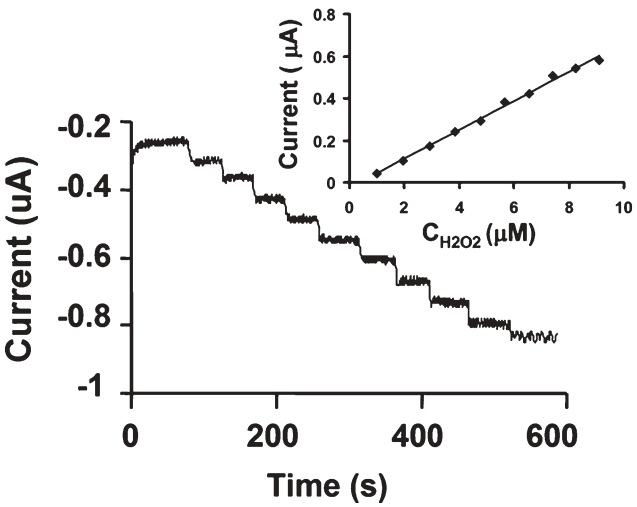

Fig. 6 Typical steady-state response of the biosensor on successive injections of 0.1 mmol l^{-1} H_2O_2 into 1 ml of stirring PB. Applied potential, −300 mV, supporting electrolyte, 0.1 mol l^{-1} pH 7.4 PB. Inset: the corresponding calibration plot.

mediators. The new immobilization method represents an attractive route for designing biosensors, biomedical devices, biofuel cells, and enzymatic bioreactors. Functionalized enzyme nanoparticles could also be used as novel tags for bioaffinity assays of protein and DNA. The new biosensor technology is thus expected to open new opportunities for biosensors, clinical diagnostics, and bioanalysis in general.

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