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Direct electrochemistry of horseradish peroxidase immobilized on a monolayer modified nanowire array electrode

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ABSTRACT

Vertically aligned nanowire array electrodes (NAEs) were prepared by electrodeposition of gold into an anodic aluminium oxide membrane (AAM), providing an ordered three-dimensional (3D) matrix for immobilization of redox proteins. Third-generation H_2O_2 biosensors were prepared by covalent immobilization of horseradish peroxidase (HRP) on the self-assembled monolayer modified NAEs. Direct electron transfer and electrocatalytic performances of the HRP/NAEs with different nanowire lengths (deposition time of 2, 4 and 5 h) were investigated. Results showed that with longer nanowires, better performances were achieved. The HRP/NAE_{5h} (5 h deposition time) exhibited remarkable sensitivity ($45.86 \mu\text{A mM}^{-1} \text{cm}^{-2}$) towards H_2O_2 with a detection limit of $0.42 \mu\text{M}$ ($S/N=3$), linearity up to 15 mM and a response time of 4 s. The ordered 3D gold nanowire array with high conductivity, excellent electron transfer capability and good biocompatibility proved promising for fabricating sensitive, selective, stable and mediator-free enzymatic biosensors.

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1. Introduction

Direct electron transfer between redox proteins and electrodes has attracted considerable attention due to its significance in both theoretical (Leger and Bertrand, 2008) and practical applications (Freire et al., 2003; Wu and Hu, 2007) in biosensors, bioelectronics and energy systems (Guo et al., 2008). Studies based on direct electrochemistry of redox proteins have been employed to investigate the three-dimensional (3D) structures of enzymes and the relations between the structures and the catalytic properties, for a better understanding of the kinetics and thermodynamics of the biological redox process (Leger and Bertrand, 2008). Direct electro-communication of biomolecules and electrodes established a foundation for fabricating mediator-free and sensitive biosensors (Kang et al., 2009). Third-generation biosensors usually offer better selectivity by operating in a potential range close to the redox potential of the enzyme itself, thus being less exposed to interfering reactions (Freire et al., 2003). These biosensors are also advantageous for in vivo detection due to its simplicity and harmlessness (Wang, 2008; Wang et al., 2008).

To achieve direct electron transfer, enzyme immobilization is crucial as the enzymatic redox centres are usually embedded

deeply into the low conductive amino acid chains, resulting in a slow electron transfer rate in most orientations (Kang et al., 2009). Enzymes denature easily or become inactive when directly adsorbed on a conventional electrode. To overcome this drawback, different approaches such as covalent, cross-linking attachment or affinity interaction (Chaki and Vijayamohan, 2002; Zheng et al., 2006), entrapment or encapsulation in a polymer or a sol-gel/inorganic matrix (Gupta and Chaudhury, 2007) and a nanostructure combined method (Xiao et al., 2000; Zhao et al., 2002) have been utilized for enzyme immobilization (Wang et al., 2008). The 2D immobilization methods (Wu and Hu, 2007), such as self-assembled monolayer (SAM) (Chaki and Vijayamohan, 2002) and layer-by-layer (LBL) (Zheng et al., 2006), allow the enzyme to anchor orientally on the electrode surface, thus achieving direct electron transfer by reducing the distance between the active site and electrode surface (Freire et al., 2003; Zimmermann et al., 2000). Entrapment/encapsulation immobilization (Gupta and Chaudhury, 2007) is widely applied owing to its ability to immobilize more electroactive enzymes on the electrode (Jia et al., 2002). In comparison to the 2D approach, entrapment/encapsulation provides a 3D porous structure which drastically increases the active sites for enzyme immobilization. Further improvements have been reported by combining nanostructures with SAM and/or entrapment (Kang et al., 2009; Yin et al., 2009). However, the distribution of enzyme in the gel or the polymer 3D matrix is usually not uniform, sometimes spatially hindered, resulting in relatively low electron transfer efficiency because only a very

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small percentage of the enzyme immobilized is electroactive. Thus, ordered 3D matrices, such as nanotube arrays (Cui et al., 2007; Lan et al., 2008), ordered porous materials (Zhu et al., 2009) and nanowire/nanorod/nanopillar electrode ensembles (Anandan et al., 2006; Gu et al., 2009; Yu et al., 2003), have been used for increasing the active surface area for enzyme immobilization. Nanowire electrode ensembles (NEEs) prepared from a polycarbonate (PC) membrane (Roberts and Kelley, 2007; Yang et al., 2007) and nanowire array electrodes (NAEs) prepared from an anodic alumina membrane (AAM) (Lee et al., 2008) have been reported for various enzyme immobilizations. The ordered 3D nanowires with high aspect ratios improve the signal-to-noise ratio significantly, resulting in significantly higher sensitivity and a lower detection limit (De Leo et al., 2007). Direct electrochemistry of a H_2O_2 biosensor on NEEs fabricated from a PC membrane based on direct adsorption immobilization of haemoglobin (Hb) has been reported (Yang et al., 2007). The results showed that the quantity of electroactive Hb varied with the changing of the electrode morphology and increased with the nanowire length.

Hydrogen peroxide (H_2O_2) is necessary for the metabolism of proteins, carbohydrates, fats, vitamins, and minerals. Besides its essential role for the production of estrogens, progesterone, and thyroxine in the body, H_2O_2 helps regulate blood sugar and cellular energy production. Oxidative damages in the body are caused by cellular H_2O_2 imbalance as this chemical plays an important role in cell signalling and communication (Dröge, 2002). Cisplatin used in testicular cancer treatment, affects an increase in the H_2O_2 production to kill the cells. Cancer cells that are resistant to cisplatin or other cancer therapies are capable of synthesizing larger amounts of peroxiredoxin to degrade H_2O_2 (Wood et al., 2003). A significant amount of work in enzyme based biosensors is related to the detection of H_2O_2 , a co-product of enzymatic oxidation using oxidases (Bakker, 2004). Therefore, the detection of H_2O_2 is of great interest and importance in analytical, environmental and biomedical chemistry as well as food and environmental monitoring. Horseradish peroxidase (HRP) is one of the most important peroxidase enzymes for H_2O_2 or other peroxides (Freire et al., 2003). The enzyme has a heme catalytic centre and the surrounding protein. The electrochemically active centre in HRP is normally buried deeply in its extended 3D structure, which makes direct electron transfer between HRP and the electrode surface prohibitively slow (Wu and Hu, 2007). Direct physical adsorption of HRP on a bare electrode, often with denaturation because of multiple contacts and interaction with the surface, can create an insulating layer that blocks electron transfer to the electrode (Freire et al., 2003; Wu and Hu, 2007).

In this paper, NAEs with different nanowire lengths were prepared using the template of AAM. By combining the advantages of SAM modification for oriented attachment of enzymes, biocompatibility and electron transfer properties of the gold nanowires and the ordered 3D nanowire matrix having a large active surface area, HRP was covalently immobilized on these self-assembled monolayer modified gold NAEs. The direct electrochemistry and biosensing performance of HRP/NAEs were presented and discussed in detail. To our knowledge, this is the first third-generation H_2O_2 biosensor based on the direct electrochemistry of HRP immobilized on a SAM-modified gold nanowire array electrode.

2. Experimental

2.1. Reagents and materials

Hydrogen peroxide (H_2O_2 , 30 wt.%), horseradish peroxidase (HRP, EC 1.11.1.7, Type II, 150–250 units/mg), L-cysteine, and glu-

taraldehyde (GA, Grade I, 25%) were purchased from Sigma–Aldrich (Dublin, Ireland). Deionised water ($R = 18.2 \text{ M}\Omega$) obtained from a Milli-Q (Millipore, Bedford, MA) water purification system was used. All reagents were of analytical grade with highest purity. The Au electrode bath (PURAMET 402) was purchased from AMI DODUCO. The AAM template has a nominal pore size of 200 nm (Whatman Inc).

2.2. Apparatus

Amperometric measurements (I/t) and cyclic voltammetry (CV) were performed using a CHI 1040A electrochemical workstation (CH Instruments, Austin, TX) at room temperature. The three-electrode system consists of a gold nanowire array electrode ($\Phi = 5 \text{ mm}$), an Ag/AgCl (3 M NaCl) reference electrode (BAS, West Lafayette, IN) and a Pt wire counter electrode. The morphology of NAEs was determined by a JEOL JSM 6100 scanning electron microscopy (SEM). The transmission electron microscope (TEM) images were obtained with a JEOL 2010 TEM (JEOL, Ltd., Japan) operating at 200 kV.

2.3. Synthesis of gold nanowire array

Vertically standing gold nanowire arrays with different lengths were fabricated using a template electrodeposition technique. Typically, a thin layer of Au (thickness of 300 nm, 5 mm in diameter) was first sputtered onto one side of the 3 M transparent polymer film. The AAM template was attached on the gold-coated film by a specially designed sample holder. Electrodeposition of Au was performed at a constant current density at 1.0 mA cm^{-2} for 2–5 h in the Au bath (PURAMET 402). After deposition, the AAM template was etched out by submerging the sample in 6.0 M NaOH solution for 1 h. The resulting NAEs were rinsed and kept in deionised water.

2.4. Electrode preparation

The gold nanowire array electrode was cleaned with Piranha (3:1 (v/v) mixture of concentrated H_2SO_4 and H_2O_2), followed by rinsing with ethanol and deionised water. The electrode was attached to an electrochemical cell containing 0.5 M H_2SO_4 for cleaning by cyclic voltammetry between -0.2 and $+1.5 \text{ V}$ at 0.1 V s^{-1} until a stable CV profile was obtained.

The resulting electrode was immersed in a freshly prepared 10 mM cysteine (saturated with N_2) for 30 min. After rising with water, the cysteine-modified gold nanowire array electrode was incubated into 5 mg HRP in 1.0 mL of 50 mM pH 7.0 phosphate buffer (containing 2% glutaraldehyde) for 12 h at 4°C to effect enzyme immobilization via glutaraldehyde cross-linking.

3. Results and discussion

3.1. Morphology of the gold nanowire array electrode

SEM images of the gold nanowire modified electrodes made from different deposition times (2, 4 and 5 h) are shown in Fig. 1A–C. The average lengths of the nanowires were ~ 5 , 15 and $20 \mu\text{m}$ for $\text{NAE}_{2\text{h}}$, $\text{NAE}_{4\text{h}}$ and $\text{NAE}_{5\text{h}}$ respectively. The diameters of the nanowires were $\sim 200 \text{ nm}$, consistent with the pore size of the AAM template. Nanowires of NAEs were bended or bundled together on the top part due to the surface tension force exerted on them during evaporation of the washing solvent. The TEM image in Fig. 1D showed that the Au nanowires have rough surfaces with small bumps of $\sim 10 \text{ nm}$, possibly owing to the casting of the rough surface of the template pore wall. A further HRTEM micrograph in Fig. 1E showed that these bumps exhibit single crystalline struc-

Fig. 1. (A–C) SEM image of the NAE_{2h}, NAE_{4h} and NAE_{5h}; (D) TEM image of gold nanowires after AAM removed, most nanowires have rough surfaces; (E) HRTEM image of a single Au nanowire, bump on nanowire has a single crystalline nature.

ture nature with clearly visible lattice fringes, a useful feature for the biosensing behaviour.

3.2. Electrochemical characterization of the gold nanowire array electrode

Cyclic voltammetry of the flat gold electrode (circular Au coated polymer with same diameter of 5 mm) and the NAEs were performed in 0.5 M H₂SO₄ at a scan rate of 0.1 V s^{−1} (Fig. 2A). The supplementary information of Fig. S1 shows the expanded cyclic voltammogram of the flat gold electrode. Roughness ratios of the NAEs are presented in Table 1, calculated as the ratio of the reduction peak area (integrating the voltammogram from +0.7 to +1.2 V) of each NAE relative to the flat electrode. The roughness ratios of the NAEs enlarged several tens of times compared to the flat gold electrode, thus, providing more active sites for enzyme immobilization. Of interest was the comparison of the nanowire electrodeposition time (nanowire length) and the roughness data. The roughness ratio of the NAE_{4h} was about twice of the NAE_{2h}, while the roughness ratio increased 1.3-fold from the NAE_{4h} to the NAE_{5h}.

The effective surface areas of the NAEs were estimated using ferrocyanide as a redox probe. Fig. 2B shows the cyclic voltam-

mograms of the flat gold electrode and different NAEs in 0.1 M KCl containing 5 mM K₃Fe(CN)₆ at a scan rate of 0.1 V s^{−1}. The well-defined redox peaks were observed, with $\Delta E_p = 155, 137, 107$ and 66 mV for the flat gold electrode, NAE_{2h}, NAE_{4h} and NAE_{5h}, respectively, indicating a quasi-reversible reaction at each electrode (Wang, 2006). The CV features of the high-density NAEs were similar to the flat gold electrode rather than a sigmoidal steady-state curve, due to the overlapping of the diffusion layers from individual nanowires (Li et al., 2003). The electroactive surface areas (A_{eff}) of the flat electrode, NAE_{2h}, NAE_{4h} and NAE_{5h} were calculated as 0.22, 0.28, 0.40 and 0.44 cm², respectively (Table 1), according to the Randles–Sevcik equation (Cui et al., 2007):

$$I_p = 2.69 \times 10^5 A n^{3/2} D_0^{1/2} v^{1/2} C_0$$

where I_p , A , n , D_0 , v and C_0 represent the redox peak current (A), the effective surface area of the electrode (cm²), electrons per molecule oxidized or reduced ($n = 1$), the diffusion coefficient of K₃Fe(CN)₆ in 0.1 M KCl (0.673×10^{-5} cm² s^{−1}) (Chailapakul et al., 2000), the scan rate (V s^{−1}) and the concentration of redox species (mol cm^{−3}). Similar to the roughness ratios, the NAE with longer deposition time showed larger electroactive surface area although the enlarge-

Fig. 2. (A) Cyclic voltammograms of the flat gold electrode (a), NAE_{2h} (b), NAE_{4h} (c) and NAE_{5h} (d) in 0.5 M H₂SO₄ at 0.1 V s^{−1}. (B) Cyclic voltammograms of the flat gold electrode (a), NAE_{2h} (b), NAE_{4h} (c) and NAE_{5h} (d) in 0.1 M KCl containing 5 mM K₃Fe(CN)₆ at 0.1 V s^{−1}.

Table 1

Key parameters of the HRP modified flat gold and gold nanowire array electrodes.

	Roughness ratio ^a	A_{eff} (cm ²)	$\Gamma^* \times 10^{11}$ (mol cm ⁻²)	k_s (s ⁻¹)	Linearity (mM)	Detection limit ^b (μM)	Sensitivity ^b (μA mM ⁻¹ cm ⁻²)	K_M^{app} (mM)
Flat AuE	1	0.22	4.2	0.62	1.8	3.90	0.07	2.06
NAE _{2h}	43.3	0.28	14.2	2.03	15	0.97	10.78	1.87
NAE _{4h}	91.6	0.40	19.8	2.14	15	0.64	26.65	1.54
NAE _{5h}	125.1	0.44	24.8	2.22	15	0.42	45.86	0.64

^a Calculated as the ratio of the reduction peak area (integrating the voltammogram from +0.7 to +1.2 V) of each NAE relative to the flat electrode.^b Detection potential: -0.1 V.

ment of the effective surface area was moderate compared to the increase of the roughness ratio (e.g. A_{eff} of NAE_{5h} was only slightly larger than that of NAE_{4h}, whereas the roughness ratio of NAE_{5h} and NAE_{4h} was ~1.3). This may be attributed to the fast reaction rate of K₃Fe(CN)₆, resulting in only the top part of the nanowires contributing to the increase in the electroactive surface for electron transfer (Anandan et al., 2007; De Leo et al., 2007).

3.3. Direct electron transfer of HRP on the gold nanowire array electrode

The performance of electron transfer depends strongly on the immobilization procedure. Self-assembled monolayer (SAM) exhibits high organisation and homogeneity, making it very attractive for surfaces tailored with desired properties (Freire et al., 2003). Fig. 3A illustrates the stepwise fabrication process of the HRP/NAEs. L-cysteine was self-assembled onto the gold nanowires, leaving the amine groups for oriented enzyme attachment (Zhang et al., 2005). Covalent immobilization of the redox protein on the SAM avoided the influence of the mass transfer and enabled a diffusion-less electron transfer process (Wu and Hu, 2007). As L-cysteine and glutaraldehyde are both small molecules, the distance between the electroactive group of HRP and the electrode was close to facilitate direct electron transfer.

Fig. 3B compares the cyclic voltammograms of different HRP/NAEs and the HRP/flat Au electrode in 50 mM deoxygenated phosphate buffer (pH 7.0). The expanded CV profile of the HRP/flat Au electrode and the associated redox process are explained in the supplementary information (Fig. S2). Well-defined redox peaks were observed on the HRP/NAEs, with the peak-to-peak separation of 72 mV and the formal potential ($E^{\circ'} = (E_{\text{pa}} + E_{\text{pc}})/2$) of 18 mV at 0.1 V s⁻¹ vs. 3.0 M Ag/AgCl. The value of $E^{\circ'}$ was smaller than the HRP/Nafion-cysteine/Au electrode (60 mV vs. Ag/AgCl or 257 mV vs. NHE) (Hong et al., 2007), suggesting better electron transfer efficiency.

The scan rate effect on the response of the HRP/NAE_{2h} was investigated to determine kinetic parameters of HRP (Fig. 3C). As shown in Fig. 3C inset, the anodic or cathodic peak current was proportional to the scan rate, as $I_{\text{pa}}(\mu\text{A}) = 1.0488 + 113.7v(\text{V s}^{-1})$ ($R = 0.9987$) and $I_{\text{pc}}(\mu\text{A}) = -1.0063 - 113.84v(\text{V s}^{-1})$ ($R = 0.9984$). This further suggested the electrochemical behaviour of HRP on the NAEs was typical of a surface-controlled quasi-reversible process (Yin et al., 2009), verifying that immobilized HRP was stable on the NAEs.

The relationship between the peak potential (E_p) and the natural logarithm of scan rate ($\ln v$) for HRP/NAEs in 50 mM PB, pH 7.0 was studied. The cathodic peak potential (E_{pc}) changed proportionally to $\ln v$ with a linear regression equation of $E_{\text{pc}}(\text{V}) = -0.0494 \ln v(\text{V s}^{-1}) - 0.2802$ ($R = 0.9958$) in the range from 0.35 to 0.5 V s⁻¹. According to the Laviron equation (Laviron, 1979):

$$E_p = E^{\circ'} + \frac{RT}{\alpha nF} - \frac{RT}{\alpha nF} \ln v$$

where α is the cathodic electron transfer coefficient, n is the electron transfer number, R is the gas constant ($R = 8.314 \text{ J mol}^{-1} \text{ K}^{-1}$), T is the temperature in Kelvin ($T = 298 \text{ K}$) and F is the Faraday con-

stant ($F = 96493 \text{ C mol}^{-1}$). The αn was calculated to be 0.52. Given $0.3 < \alpha < 0.7$ in general (Ma et al., 2000), it could be concluded that $n = 1$ and $\alpha = 0.52$. So, the redox reaction between HRP and the NAE was a single electron transfer process. When $n\Delta E_p < 200 \text{ mV}$, the electron transfer rate k_s can be estimated with the Laviron's equation $k_s = \alpha nFv/RT$ (Laviron, 1979). The heterogeneous electron transfer rate of each NAE was shown in Table 1. The HRP/NAEs exhibited much higher k_s values compared to the HRP/flat Au electrode, due to the high electrocatalytic reaction with elevated amounts of HRP on the NAEs and the excellent electron transfer accelerating property of the well-aligned gold nanowires. The electron transfer rate of HRP/NAEs was also much higher than the k_s values of HRP immobilized on zinc oxide nanorods (1.15 s^{-1}) (Gu et al., 2009) and a Nafion-cysteine/Au electrode (1.13 s^{-1}) (Hong et al., 2007), suggesting superior electron transfer kinetics of HRP on the NAEs.

According to Faraday's law:

$$I_p = \frac{nFQv}{4RT} = \frac{n^2F^2A\Gamma v}{4RT}$$

the surface average concentration of electroactive HRP (Γ^* , mol cm⁻²) on the SAM-modified NAEs was estimated as follows

$$\Gamma^* = \frac{Q}{nFA}$$

where I_p is the reduction current (A), v is scan rate (V s⁻¹), Q is the charge (C) by integrating of the anodic peak of the HRP/NAE, n is the electron transfer number, F is the Faraday constant and A is the electrode area (cm²). The surface average concentration of active HRP on the NAE_{2h}, NAE_{4h} and NAE_{5h} were 1.42×10^{-10} , 1.94×10^{-10} and $2.48 \times 10^{-10} \text{ mol cm}^{-2}$, respectively (Table 1). The value of Γ^* increased with the length of the nanowires, which was the reason that the direct electron transfer peak current increased towards the enhancement of the roughness ratio. The surface concentration of electroactive HRP was larger than the saturated monolayer concentration of HRP ($5.0 \times 10^{-11} \text{ mol cm}^{-2}$) (Xiao et al., 2000) because HRP was coated over the entire surface topology of the nanowire electrode. Comparing to those reported for HRP surface concentration in a multilayer modified ZnO nanorod electrode (Gu et al., 2009), cysteine/AuNWs are more efficient for HRP immobilization.

3.4. Electrocatalytic reduction of H₂O₂

Electrocatalytic reduction of hydrogen peroxide by the HRP/NAEs was a single electron transfer process, which could be expressed as (Hong et al., 2007):

Fig. 4A shows the cyclic voltammograms of the HRP/NAE_{2h} in 50 mM PB, pH 7.0 in the absence and presence of 100 μM H₂O₂. The cathodic peak increased greatly upon the addition of H₂O₂, indicating an enzymatic catalytic reaction on the HRP/NAE_{2h}. The reduction peak observed at -0.1 V was selected as the detection potential throughout this study.

Fig. 3. (A) Schematic illustration of stepwise fabrication of the HRP/NAEs electrodes. (B) Cyclic voltammograms of the HRP/flat Au electrode (a), HRP/NAE_{2h} (b), HRP/NAE_{4h} (c) and HRP/NAE_{5h} (d) in 50 mM deoxygenated PB (pH 7.0) at 0.1 V s⁻¹. (C) Cyclic voltammograms of the HRP/NAE_{2h} in 50 mM deoxygenated PB (pH 7.0) at different scan rates (20–200 mV s⁻¹ with an increase of 20 mV s⁻¹ each step). Inset: anodic and cathodic current plotted against the scan rate.

Fig. 4. (A) Cyclic voltammograms of the HRP/NAE_{2h} in 50 mM PB, pH 7.0 in the absence (a) and presence (b) of 100 μM H₂O₂. (B) The typical current–time responses of the HRP/flat Au electrode (a), HRP/NAE_{2h} (b), HRP/NAE_{4h} (c) and HRP/NAE_{5h} (d) at -0.1 V with successive addition of 10 μM of H₂O₂.

The typical current–time responses of different HRP/NAEs and the HRP/Au electrode at -0.1 V with successive addition of 10 μM of H₂O₂ were illustrated in Fig. 4B. The signal response of H₂O₂ at the HRP/NAEs was large compared to that of the flat electrode, reflecting superior performances of the gold nanowire electrodes. Nevertheless, the amplified current response of the HRP/flat electrode towards H₂O₂ was shown in Fig. S3. The response of the HRP/NAEs towards H₂O₂ was fast (3–4 s), with linearity up to

15 mM, suggesting a fast catalytic process and high catalytic activity of the immobilized enzyme. The response time was faster than the reported results of HRP immobilized on different types of 3D electrodes as shown in Table 2. This could be attributed to three aspects: first, HRP was exposed to the surface of the gold nanowires, therefore, H₂O₂ could diffuse freely to the enzyme. Second, favourable orientation of HRP on cysteine/AuNWs enabled high biocatalytic efficiency. Third, nano-gold on the nanowire surface may acceler-

Table 2

Performance of the biosensors based on direct electron transfer of HRP in 3D matrices.

	Response time (s)	Detection limit (μM)	Linear range	K_M^{app} (mM)	References
HRP-SAM-AuNAE _{5h}	<4	0.42	0.74 μM to 15 mM	0.64	This paper
HRP-AuNPs-SF/GCE	<8	5	10 μM to 1.8 mM	1.22	(Yin et al., 2009)
HRP-CNT-Chitsan-sol-gel/GCE	5	1.4	4.2 μM to 5 mM	6.51	(Kang et al., 2009)
HRP-LBL-ZnO nanorod	<5	1.9	5 μM to 1.7 mM	10.72	(Gu et al., 2009)
HRP-TiO ₂ nanotube arrays	NA	0.1	50 μM to 1 mM	1.9	(Wu et al., 2008)
HRP-AuNP-TiO ₂ nanotube arrays	<5	2.0	5 μM to 0.4 mM	NA	(Kafi et al., 2008)
HRP-flower ZnO-AuNP-Nafion/GCE	<5	9.0	15 μM to 1.1 mM	1.76	(Xiang et al., 2009)
HRP-MSHS-Nafion/GCE	<5	1.2	3.9 μM to 0.14 mM	0.22	(Cao et al., 2009)

ate electron transfer between the enzyme and the electrode (Jia et al., 2002). The HRP/NAE_{2h}, HRP/NAE_{4h} and HRP/NAE_{5h} exhibited high sensitivity of 10.78, 26.65 and 45.86 $\mu\text{A mM}^{-1} \text{cm}^{-2}$ and low detection limits of 0.97, 0.64 and 0.42 μM ($S/N=3$), respectively, compared to 0.07 $\mu\text{A mM}^{-1} \text{cm}^{-2}$ and 3.90 μM at the HRP immobilized on the flat gold electrode. The sensitivity of the HRP/NAEs was much greater than that of HRP entrapped in 3D sol-gel or immobilized on nanotube or nanorod electrodes (see Table 2), evincing that well-aligned nanowires provided larger and more uniform electroactive sites for immobilizing biomolecules and facilitated electron transfer.

The apparent Michaelis–Menten constant (K_M^{app}), a reflection of the enzymatic affinity and the ratio of microscopic kinetic constants (Hong et al., 2007), can be obtained from the electrochemical version of the Lineweaver–Burk equation (Xiao et al., 1999):

$$\frac{1}{I_{\text{ss}}} = \frac{1}{I_{\text{max}}} + \frac{K_M^{\text{app}}}{I_{\text{max}}C}$$

where I_{ss} is the steady-state current after the addition of substrate, I_{max} is the maximum steady-state current measured under saturated substrate condition and C is the bulk concentration of the substrate. The K_M^{app} values of HRP/NAE_{2h}, HRP/NAE_{4h} and HRP/NAE_{5h} were 1.87, 1.54 and 0.64 mM, respectively, significantly lower than reported values in Table 2. These results indicated that the immobilized HRP possessed high enzymatic activity and the HRP/NAEs exhibited high affinity for H_2O_2 (Yin et al., 2009).

When stored in pH 7 phosphate buffer at 4 °C, the biosensors based on HRP covalently immobilized on the NAEs were stable and retained 96% of its initial activity for H_2O_2 after four weeks. Among five electrodes prepared under the same condition, their response to hydrogen peroxide only varied 4.6% (RSD). Excellent stability and reproducibility could be attributed to the firm and consistent attachment of HRP on the SAM-modified NAEs and good biocompatibility of the gold nanowires.

4. Conclusion

In brief, the direct electron transfer between HRP and the monolayer modified NAEs was achieved by combining the superiorities of the ordered 3D nanowires and the self-assembled monolayer. The resulting HRP/NAEs displayed prominent improvement regarding electroactivity, sensitivity and stability, comparing with the HRP/flat gold electrodes. This attractive and versatile procedure, with great clinical and environmental significance, opens up new opportunities for the construction of various mediator-free biosensors with high sensitivity, low detection limit and rapid response.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2009.10.018.

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