Biosensors and Bioelectronics 41 (2013) 815–819

Contents lists available at SciVerse ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

An amperometric biosensor for the detection of hydrogen peroxide released from human breast cancer cells

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ARTICLE INFO

Article history:
Received 23 July 2012
Received in revised form
18 September 2012
Accepted 5 October 2012
Available online 12 October 2012

Keywords:
Electrochemical biosensor
Hydrogen peroxide
Horseradish peroxidase
Peptide
Tumor cells

ABSTRACT

The rapid, accurate and sensitive determination of hydrogen peroxide (H_2O_2) is of great significance in the physiological, pathological and environmental fields. In this work, we have proposed a highly sensitive and selective amperometric biosensor for the detection of extracellular H_2O_2 released from human breast cancer cells with the help of a sequence-specific peptide. Since the peptide immobilized on the electrode surface can specifically bind with horseradish peroxidase (HRP) in a favorable orientation, which then well promotes the catalytic activities of the immobilized enzyme toward the reaction of *o*-phenylenediamine and H_2O_2, the proposed biosensor can detect H_2O_2 in a wide linear range from 1.0×10^{-7} M to 1.0×10^{-4} M with a low detection limit of 3.0×10^{-8} M. It can be also directly used to efficiently trace extracellular H_2O_2 released from human breast cancer cells MCF-7. Furthermore, the reproducibility, stability and selectivity of the biosensor are also quite well compared with the previous report, so our biosensor might be potentially useful in physiological and pathological detection of H_2O_2 in the future.

1. Introduction

Hydrogen peroxide (H_2O_2) is often involved in food, biological, chemical and pharmaceutical procedures (Salimi et al., 2007; Sang et al., 2010). As a by-product of oxidative metabolism in vivo, H_2O_2 is also known as the most stable reactive oxygen species (ROS) implicated in cell proliferation, aging, death and signal transduction (Henzler and Steudle, 2000; Lindahl, 1993). Nevertheless, excessive amount of H_2O_2 has been found to have a detrimental impact on central nervous system that may cause various human central nervous system diseases, including Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (Luo et al., 2009; Mao et al., 2002). Besides, H_2O_2 is harmful to the atmosphere that may contribute to the formation of acid rain. Therefore, rapid, accurate and sensitive determination of H_2O_2 is of great importance in physiological, pathological and environmental fields.

Up to now, numerous techniques such as titrimetry, spectrophotometry, chemiluminescence and electrochemistry have been used for the detection of H_2O_2 (Chen et al., 2007; Lin et al., 2007; Lobnik and Čajlaković, 2001; Klassen et al., 1994). Among them, electrochemical techniques coupled with the use of redox proteins modified electrodes are most promising (Lu et al., 2010; Wang and Wang, 2004; Zhou et al., 2005; Zhu et al., 2007), benefiting from simple operation, low cost, high selectivity and sensitivity, while horseradish peroxidase (HRP), which may efficiently catalyze H_2O_2-dependent one-electron oxidation, is the most commonly used enzyme for the construction of electrochemical H_2O_2 biosensor (Zhu et al., 2011).

Since the immobilization of HRP on an electrode surface is essential to the performance of electrochemical biosensors, many approaches have been developed for HRP immobilization, including polymer entrapment, electropolymerization, sol–gels, layer-by-layer technique and covalent linking, etc. (Kafi et al., 2008; Liu et al., 2012; Mathew and Sandhyarani, 2011; Peng et al., 2009; Xi et al., 2009; Yemini et al., 2006; Zhang et al., 2008). Nonetheless, the previous approaches usually have a serious deficiency in common that they fail to control the orientation of the immobilized enzyme on the electrode surface, although favorable orientation is believed to play a crucial role in enhancing the electron transfer reactivity and biocatalytic activity of the immobilized enzyme. Therefore, many of the proposed biosensors cannot be used for the detection of H_2O_2 in complicated samples, not to mention tracing the extracellular H_2O_2 released from cells.

Recently, some peptide ligands were found to have a strong binding intensity to immobilize HRP onto the surface of a solid support with a favorable orientation, which can greatly promote the maintanence of the enzyme activities and facilitate the accessibility of the substrate to the immobilized enzyme

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http://dx.doi.org/10.1016/j.bios.2012.10.019

(Fu et al., 2010). Therefore, the use of peptide ligands for oriented immobilization of HRP onto an electrode surface with the enhanced enzymatic activity might provide an attractive alternative for fabricating HRP-based electrochemical biosensors. In this work, we have proposed an amperometric H_2O_2 biosensor with the selective immobilization of HRP onto the electrode surface in a favorable orientation by making use a sequence-specific peptide. The proposed biosensor has been proven to sensitively detect H_2O_2 in a wide linear range with low detection limit. It can be also used for the detection of H_2O_2 released from human breast cancer cells, so the biosensor might be potentially useful in physiological and pathological application in the future.

2. Experimental

2.1. Reagents

The sequence of the HRP-binding peptide (AHKVVPQRQIRHAYNRYGSC) was kindly provided by Dr. Jinglin Fu at Arizona State University. It was synthesized by China Peptides Co., Ltd. (Shanghai, China). HRP, *o*-phenylenediamine (*o*-PD), tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 6-mercapto-1-hexanol (MCH), phobol 12-myristate 13-acetate (PMA) were purchased from Sigma. H_2O_2 was purchased from Sinopharm Chemical Reagent Co., Ltd. For all the experiments, Milli-Q water (18.0 MΩ) purified by a Milli-Q Plus 185 ultrapure water system (Millipore Purification Pack) was used.

2.2. Preparation of HRP/peptide modified electrode

Firstly, the substrate gold electrode was polished on fine sand papers and alumina (particle size of about 0.05 μm)/water slurry on silk. Then, it was ultrasonicated in ethanol and water for 5 min, respectively. Finally, the electrode was electrochemically cleaned in 0.5 M H_2SO_4 to remove any remaining impurities. After being dried with nitrogen, the pre-treated electrode was immersed into a solution containing 10 μM peptide, 10 mM TCEP, 20 mM HEPES (pH 6.0) for 16 h at room temperature, followed by the treatment with 1 mM MCH for 0.5 h to remove the non-covalent binding peptide. After being thoroughly rinsed by water, the prepared peptide modified electrode was immersed into 10 mM sodium acetate (pH 6.0) containing 10 mg mL^{-1} HRP for 4 h at room temperature. Afterward, the prepared HRP/peptide modified electrode was rinsed with water, which was then ready for further electrochemical measurements. For the control experiment, bovine serum albumin (BSA) was used instead of HRP.

2.3. Detection of extracellular H_2O_2 released from MCF-7

Human breast cancer cells MCF-7 were obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science. The cells were maintained in RPMI-1640 supplemented with 10% FBS and incubated at 37 °C in a humidified incubator (5% CO_2–95% air). At the logarithmic growth phase, the cells were separated from the medium by centrifugation at 500 rpm for 5 min. After being washed twice with sterilized PBS (0.1 M, pH 7.2), the cells were re-suspended in PBS (0.1 M, pH 7.2) with the final concentration of 2×10^6 cell mL^{-1}. For the detection of extracellular H_2O_2 released from MCF-7, 1.7 μg mL^{-1} PMA was added into the cell suspension. For control experiments, 1.7 μg mL^{-1} PMA was added into a PBS buffer without MCF-7 cells.

2.4. Electrochemical measurements

Electrochemical impedance spectroscopy (EIS), cyclic voltammetry (CV) and chronoamperometry were performed on a CHI 660C electrochemical analyzer (CH Instruments) with a three-electrode system at room temperature. The three-electrode system consists of the modified gold electrode, a saturated calomel electrode (SCE) and a platinum counter electrode. The electrolyte solutions for CV and chronoamperometry were 100 mM PBS containing 10 mM *o*-PD (pH 7.0), while 100 mM PBS (pH7.0) containing 5 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl was used for EIS. Before the electrochemical measurements, all test solutions were thoroughly deoxygenated by bubbling high-purity nitrogen through the solution for at least 10 min. Then, a stream of nitrogen was blown gently across the surface of the solution in order to maintain the solution anaerobic throughout the experiments.

3. Results and discussion

3.1. Characterization of stepwise modification of the electrode surface

EIS has been used to characterize the stepwise modification of the gold electrode surface. As shown in Fig. 1, no impedance can be observed on the bare gold electrode, indicating the close approach of the electrochemical active molecules $[Fe(CN)_6]^{3-/4-}$ to the electrode surface. However, an increase of interfacial electron resistance can be observed after a monolayer of peptide has been self-assembled onto the electrode surface through the free thiol group of cysteine in C-terminal of the peptides, due to the steric hindrance resulted from the peptide that can prohibit $[Fe(CN)_6]^{3-/4-}$ reaching to the electrode surface. Furthermore, when HRP has been selectively captured by the peptide immobilized on the electrode surface, a much bigger interfacial electron resistance can be obtained, as the immobilized proteins have largely increased the steric hindrance on the electrode surface. In contrast, if BSA instead of HRP has been incubated with the peptide modified electrode, only a little increase of the resistance can be observed, which may be ascribed to the non-specific adsorption of BSA onto the electrode surface. Therefore, EIS results have not only revealed the stepwise assembling processes on the electrode surface, but also preliminarily proven the high

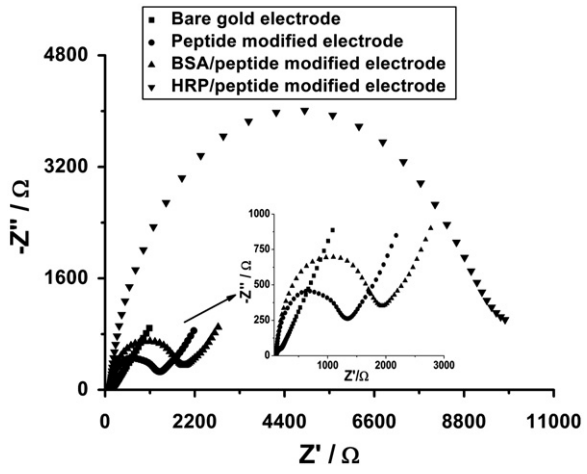

Fig. 1. Electrochemical impedance spectra (Nyquist plots) obtained at the bare electrode, and the electrode modified with peptide, BSA/peptide or HRP/peptide, in a 100 mM PBS buffer (pH 7.0) containing 5 mM $[Fe(CN)_6]^{3-/4-}$ and 100 mM KCl. Bias potential: 0.224 V. Frequency range: 0.1 Hz–100 kHz. Amplitude: 5 mV.

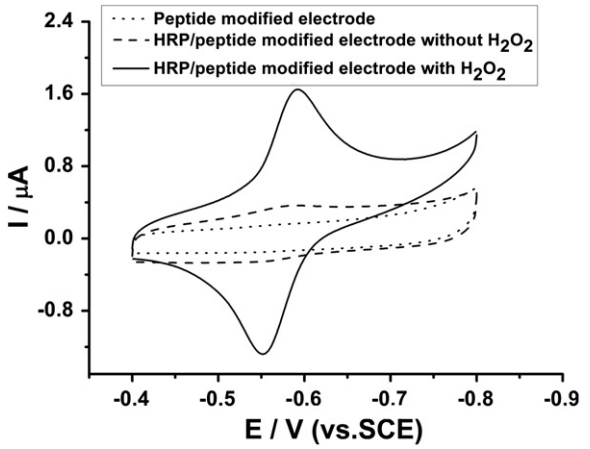

Fig. 2. Cyclic voltammograms obtained at the peptide and HRP/peptide modified electrode in the presence and absence of H_2O_2 in 100 mM PBS (pH 7.0) containing 10 mM *o*-PD. Scan rate: 100 mV/s. The concentration of H_2O_2 is 1 mM.

affinity and selectivity of HRP–peptide binding for HRP immobilization.

3.2. Studies of the catalytic activity of the immobilized HRP

We have utilized CV to examine the catalytic activity of the immobilized HRP by using *o*-PD as electron mediator (Fornera and Walde, 2010). As shown in Fig. 2, no electrochemical response can be observed on the peptide modified electrode, while a pair of small redox peaks can be obtained after HRP is immobilized onto the peptide modified electrode. Furthermore, when 1 mM H_2O_2 is added into the test solution, a pair of very big and well defined redox peaks can be observed with the formal potential at -0.572 V (vs. SCE), owing to the production of electroactive 2, 3-diaminophenazine (DAP) from HRP-catalyzed oxidation of *o*-PD by H_2O_2 (Niu et al., 2004). On the other hand, in the absence of the peptide, comparatively very small CV peak can be observed since HRP is immobilized on the electrode surface through non-specific adsorption, thus the fixed HRP can only show quite low catalytic ability. Therefore, the strong binding of HRP with the peptides as well as the favorable orientation of HRP on the peptide modified electrode may provide the opportunity to sensitively detect H_2O_2. Meanwhile, since the immobilized HRP on the electrode surface with the help of the peptides can exhibit a nice catalytic activity toward the oxidization of *o*-PD with H_2O_2, the CV results have also demonstrated the well biocompatibility of the peptide and its ability to maintain the enzymatic activity of the immobilized enzyme.

3.3. Quantitive detection of H_2O_2

Previous reports have pointed out that production of the electroactive DAP as well as its electrochemical response may only depend on H_2O_2 concentration when *o*-PD and HRP are excessive (Cui et al., 2008). So, it might be possible for us to use the HRP/peptide modified electrode to quantitively detect H_2O_2. In order to obtain a higher sensitivity, a much more sensitive electrochemical technique, chronoamperometry, has been employed in this work (Zhao et al., 2012). Fig. 3a shows a typical current-time curve obtained at the HRP/peptide modified electrode with successive injection of H_2O_2. As is shown (Fig. 3a), a gradual increase of amperometric current can be observed with the successive addition of H_2O_2, and the current can attain to a stable steady within 5 s by each addition, suggesting a fast response of our biosensor. Moreover, the increase of the peak

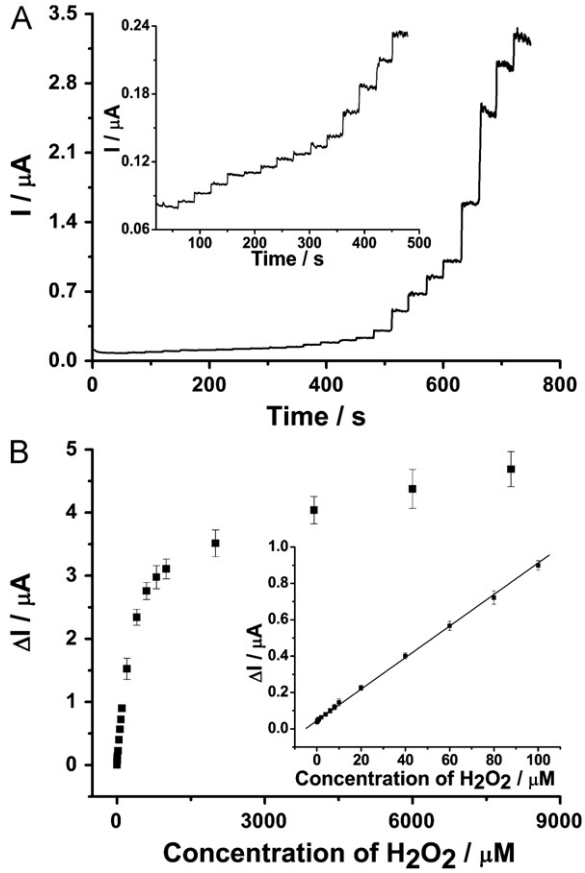

Fig. 3. (A) Amperometric response curves obtained at HRP/peptide modified electrode for successive additions of different concentrations of H_2O_2 (from 1.0×10^{-9} M to 8.0×10^{-4} M) in a stirring 100 mM PBS (pH 7.0) containing 10 mM *o*-PD at the applied potential of -590 mV. The inset shows the amperometric response obtained with the concentration of H_2O_2 ranged from 1.0×10^{-9} M–1.0×10^{-5} M. (B) Calibration plots of the increase of peak current (ΔI) with the concentration of H_2O_2. Inset is the linear relationship between ΔI and the concentrations of H_2O_2 from 1.0×10^{-7} M to 1.0×10^{-4} M. Error bars represent the standard deviations of three independent measurements.

current (ΔI) has been found to be linear with H_2O_2 concentration in the wide range from 1.0×10^{-7} M to 1.0×10^{-4} M (Fig. 3b). The detection limit can be as low as 3.0×10^{-8} M defined at a signal-to-noise ratio of 3. The apparent Michaelis–Menten constant calculated from the Lineweaver–Burk equation is 0.401 mM, which is also much lower than the previous reports. So, as shown in Table 1, compared with the previous reports, the performance of our biosensor can be satisfactorily good (Wang et al., 2010; Yang et al., 2009; Yuan et al., 2010; Zhou et al., 2010).

3.4. Reproducibility, stability and selectivity of our biosensor

The relative standard deviation (RSD) for series of repetitive measurements with different concentrations of H_2O_2 are all within 5%, and the RSD for five successive determination at 1.0×10^{-4} M H_2O_2 is 3.9%, so the reproducibility of this biosensor can be satisfactory. Meanwhile, stability of the biosensor has also been checked by recording the current response for 1.0×10^{-4} M H_2O_2 detection every week when stored at 4 °C in PBS buffer. Since no obvious decrease of the peak current can be observed in the first week and the current can retain about 90% of its initial value even after 4 weeks storage, stability of our biosensor can be satisfactorily good. Besides, we have also studied the selectivity of our biosensor under the applied potentials (Table 2). Since the presence of some substances interferences, like NO_3^-, glucose,

Table 1
Comparison of the proposed H_2O_2 biosensor with other HRP-based biosensors.

H_2O_2 biosensor	Linear range (μM)	Detection limit (μM)	K_M^{app} (mM)	Reference
Nafion/HRP/GNSs–TiO_2	41–630	5.9	0.63	Wang et al. (2010)
HRP/silica matrix	20–200	3	0.88	Yang et al. (2009)
HRP/SiO_2/BSA/AuNP/thionine–nafion	8–3720	2.0	2.3	Yuan et al. (2010)
Au/graphene/HRP/chitosan	5–5130	1.7	2.61	Zhou et al. (2010)
HRP/peptides	0.1–100	0.03	0.401	Our work

Table 2
Interference experiments with the proposed H_2O_2 biosensor.

Interfering substrate (IS)	$\Delta I_{H_2O_2+IS}/\Delta I_{H_2O_2}$
NO_3^-	0.97
Glucose	0.98
Dopamine	0.99
Acetic acid	0.96
Citric acid	1.00
Ethanol	1.02
Ascorbic acid	0.85

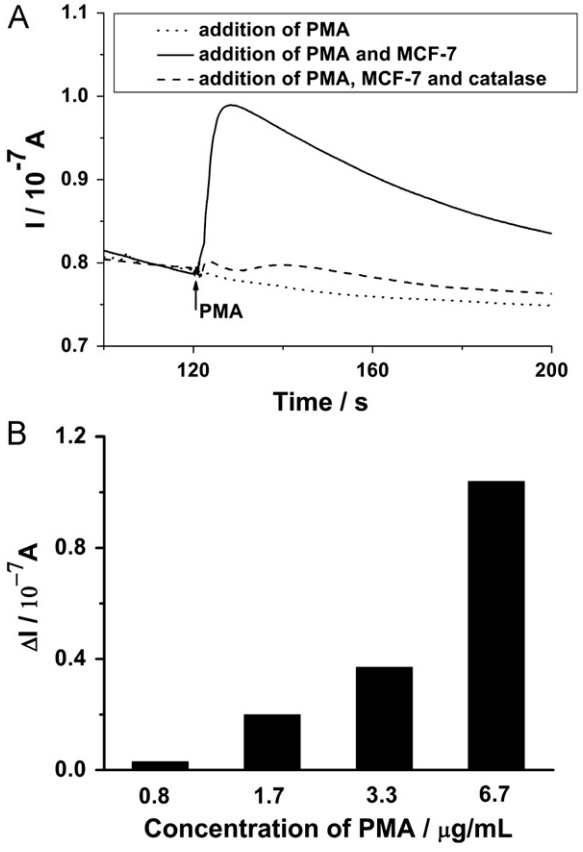

Fig. 4. (A) Typical amperometric response obtained at the HRP/peptide modified electrode after PMA (1.7 μg mL^{-1}) is added into the test solution in the presence and absence of MCF-7. The dash line shows the case that both PMA (1.7 μg mL^{-1}) and catalase (22.8 U mL^{-1}) are added into the MCF-7 cell suspension. (B) The increase of the peak current obtained at the HRP/peptide modified electrode when different concentration of PMA are added into the MCF-7 cell suspension. The concentration of MCF-7: 2×10^6 cell mL^{-1}. Others are as in Fig. 3.

dopamine, acetic acid, citric acid and ethanol with as high as 1 mM have nearly no inference with the electrochemical response for 1.0×10^{-4} M H_2O_2 detection, the selectivity of our biosensors is also quite well, although 1 mM ascorbic acid is found to cause about 15% decrease of the peak current, which is ascribed to the consumption of H_2O_2 for the oxidation of ascorbic acid (Yang et al., 2006).

3.5. Detection of extracellular H_2O_2 released from human breast cancer cells MCF-7

We have used our biosensor to detect extracellular H_2O_2 released from human breast cancer cells MCF-7. Before the measurements, PMA that can induce the generation of H_2O_2 by MCF-7 cells has been added to the test solution to stimulate the release of H_2O_2 from the cells (Wu et al., 2011). As shown in Fig. 4a, with the addition of PMA into the test solution containing MCF-7 cells, an obvious peak can be obtained at the HRP/peptide modified electrode, and a maximum current of 20 nA can be achieved after a quite short time, showing the release of H_2O_2 from MCF-7 induced by PMA. Afterward, with the consumption of H_2O_2 by the oxidation of *o*-PD, the peak current is found to decay gradually. As a comparison, an extremely low peak current that is almost down to the background can be observed if catalase is also added into the test solution, since catalase is the selective scavenger of H_2O_2. In the other words, the released H_2O_2 from MCF-7 cells will be consumed by catalase, thus there is no H_2O_2 that can be detected by our sensor. The effects of PMA dose on the release of H_2O_2 have been evaluated. As is shown in Fig. 4b, a higher dose of PMA can accelerate the release of more H_2O_2 from MCF-7, thus the peak current is found to increase in a PMA dose-dependent manner.

4. Conclusion

In conclusion, we have herein reported a highly sensitive and selective electrochemical biosensor for the detection of H_2O_2 with the help of a sequence-specific peptide. Since the peptide immobilized on the electrode surface may make the immobilization of HRP in a favorable orientation manner, the immobilized enzyme can show excellent catalytic activity toward the oxidation of *o*-PD with H_2O_2. The biosensor may have a wide detection range with a low detection limit for the detection of H_2O_2. Moreover, it can be also used to efficiently monitor the extracellular H_2O_2 released from human breast cancer cells MCF-7. Therefore, this biosensor might be potentially useful for the further physiological and pathological applications, like cancer. What is more, since diverse peptide ligands can be designed for binding different target proteins (enzymes) and modulate their functions, the peptide modified electrode proposed in this work may be extended to facilitate the development of more kinds of biosensors in the future.

Acknowledgments

This work is supported by the National Science Fund for Distinguished Young Scholars (Grant no. 20925520), and the Leading Academic Discipline Project of Shanghai Municipal Education

Commission (J50108). The authors also cordially thank Dr. Jinglin Fu for kindly providing the sequences of HRP-binding peptide.

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