A Highly Efficient Nano-Cluster Artificial Peroxidase and Its Direct Electrochemistry on a Nano Complex Modified Glassy Carbon Electrode

Jun HONG,^{*†} Wei WANG,^{*} Kun HUANG,^{*} Wei-Yun YANG,^{*} Ying-Xue ZHAO,^{*} Bao-Lin XIAO,^{*} Yun-Fei GAO,^{*} Zainab MOOSAVI-MOVAHEDI,^{**} Hedayatollah GHOURCHIAN,^{***} and Ali Akbar MOOSAVI-MOVAHEDI^{***†}

^{*}College of Life Science, Henan University, Kaifeng 475000, China

^{**}Chemistry & Chemical Engineering Research Center of Iran (CCERC), Tehran, Iran

^{***}Institute of Biochemistry and Biophysics, University of Tehran, Tehran, Iran

A nano-cluster with highly efficient peroxide activity was constructed based on nafen (NF) and cytochrome *c* (Cyt *c*). UV-Vis spectrometry and transmission electron microscopy (TEM) methods were utilized for characterization of the nano-structured enzyme or artificial peroxidase (AP). The nano-cluster was composed of a Chain-Ball structure, with an average ball size of about 40 nm. The Michaelis-Menten (K_m) and catalytic rate (k_{cat}) constants of the AP were determined to be $2.5 \pm 0.4 \mu\text{M}$ and $0.069 \pm 0.001 \text{ s}^{-1}$, respectively, in 50 mM PBS at pH 7.0. The catalytic efficiency of the AP was evaluated to be $0.028 \pm 0.005 \mu\text{M}^{-1} \text{ s}^{-1}$, which was $39 \pm 5\%$ as efficient as the native horseradish peroxidase (HRP). The AP was also immobilized on a functional multi-wall carbon nanotube (MWCNTs)-gold colloid nanoparticles (AuNPs) nano-complex modified glassy carbon (GC) electrode. The cyclic voltammetry of AP on the nano complex modified GC electrode showed a pair of well-defined redox peaks with a formal potential ($E^{\circ'}$) of $-45 \pm 2 \text{ mV}$ (vs. Ag/AgCl) at a scan rate of 0.05 V/s. The heterogeneous electron transfer rate constant (k_s) was evaluated to be 0.65 s^{-1} . The surface concentration of electroactive AP on GC electrode (Γ) was $7 \times 10^{-10} \text{ mol cm}^{-2}$. The apparent Michaelis-Menten constant (K_m^{app}) was 0.23 nM.

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Introduction

Direct electron transfer between a redox protein and an electrode can provide a suitable model to understand the electron-transfer mechanism in biological systems, and to establish a foundation for fabricating electrochemistry biosensors and electrochemical devices without mediators.¹⁻⁴ Researchers have studied the carbon nano-tube and its application, because it is special structure and properties (e.g. high surface area), which make it easier to transfer electrons.⁵⁻⁷ Gold nanoparticles (AuNPs) are among the most stable metal nanoparticles. Because of its novel optical, electrical, catalytic properties and favorable biocompatibility, AuNPs have been widely applied to analytical chemistry and electrochemistry.^{8,9} Each type of nano-material has its own physical and chemical characteristics, making it difficult to design and prepare a biosensor based on only one type of nano-material. Thus, using a complex of several kinds of material will be more superior.¹⁰ Recently, several nano-materials, including carbon nanotube (CNT), and nanoparticles of Au, Ag, TiO₂, Fe₃O₄ or MnO₂, have been applied to electrochemical studies of redox protein.¹¹⁻¹⁴

Native proteins or enzymes are usually not suitable to be used directly in industrial, agricultural, medical and other fields because of their expensive price, difficulty to gain, instability or

incapacity to work under different conditions. The designs of heme proteins have recently become an active area of research. Among these, artificial peroxidases are designed not only for practical applications as artificial enzymes, but also for understanding the enzymatic mechanisms. The most successful peroxidase-like enzyme mimics have put emphasis on the heme structure and the surroundings of the natural heme enzyme.¹⁵⁻¹⁷

Cytochrome *c* (Cyt *c*) is a small and very stable globular protein with 104 amino acids.¹⁸ The catalytic activity of native Cyt *c* is low.¹⁹ Nafen (NF) is a polymer material, composed of a fluorocarbon backbone and the top of sulfonic acid groups in the side chain. At a certain pH, NF turns to aggregation in solution, because of electrostatic interactions of the sulfonate anion.²⁰ It could be firmly fixed by the number of electroactive material through ion exchange.^{21,22} NF has a high chemical stability, and good biocompatibility, and is a biological macromolecule.²³⁻²⁵ NF have been used to help immobilize redox proteins onto electrode surfaces.²⁶⁻²⁸

In the present study, a new nano-cluster complex with highly efficient peroxidase activity was constructed based on Cyt *c* and NF. UV-Vis spectrometry and transmission electron microscopy (TEM) methods were utilized for additional characterization of the nanostructured enzyme or artificial peroxidase (AP). The AP was also immobilized on a nano-composite consisting of functionalized multi-wall carbon nanotubes (MWCNTs) and AuNPs. Then the nano-composite was placed on a glassy carbon (GC) electrode and covered with a NF membrane. Using the thus-prepared electrode, direct electron transfer of AP was

[†] To whom correspondence should be addressed.

E-mail: hongjun@henu.edu.cn; moosavi@ibb.ut.ac.ir

realized, so that it could be applied as a highly sensitive biosensor for the detection of H_2O_2 .

Experimental

Reagents and chemicals

Horse heart Cyt *c*, guaiacol, NF (5% in ethanol), L-cysteine (Cys), HAuCl_4 and sodium citrate were purchased from Sigma and used without further purification. MWCNTs, prepared by chemical vapor deposition, were purchased from Shenzhen Nanotech Port Ltd. Co., China. Hydrogen peroxide, sodium dihydrogen phosphate (NaH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4) were obtained from Shanghai Chemicals Co., China. All solutions were prepared in double-distilled deionized water. Hydrogen peroxide (H_2O_2) stock solutions were prepared by appropriate dilutions of 30% (v/v) H_2O_2 in deionized water. All other chemicals were of analytical grade and used without further purification.

AP preparation

Typical AP could be constructed by injecting 7 μl NF into 3 ml of 50 mM phosphate buffer solution (PBS) containing 1 μM Cyt *c*, and then mixed to form a homogeneous colloidal solution.

UV-vis absorption spectra and steady state kinetics

UV-vis absorption spectra were collected using a TU-1901 spectrophotometer (Beijing Purkinje General Instrument Co., China) with a 1-cm path-length cell equipped with a thermostat holder and an external temperature controller (Shanghai Hengping Instrument Co., China) at $25 \pm 0.1^\circ\text{C}$. The steady-state kinetics of the guaiacol oxidation by H_2O_2 , catalyzed by the modeled catalyst, was monitored at 470 nm (colored product of the reaction is tetraguaiacol) (Eq. (1)).^{15,17} The extinction coefficient of $26.6 \text{ mM}^{-1} \text{ cm}^{-1}$ (470 nm) was used for tetraguaiacol.²⁹⁻³¹

The activity of the artificial enzyme was determined using the TU-1901 spectrophotometer. An amount of guaiacol was added to the Cyt *c*-NF nano-cluster solution, and reaction was initiated by the addition of H_2O_2 . Progress curves of the enzyme activity reactions were obtained at various guaiacol concentrations. The obtained initial rates were used to describe the Michaelis-Menten curves according to the rate of oxidation of guaiacol. The initial rate of guaiacol oxidation was measured by the rate of tetraguaiacol formation. The concentration of H_2O_2 was kept at 1.2 mM.¹⁵

TEM image of AP

TEM was performed using a Philips CM120 (Philips, Holland). TEM images of the NF-Cyt *c* nano-cluster were prepared by the following method: At first, a solution with the same volume of 0.2 mM Cyt *c* and 5% NF was prepared. Then, a drop of the solution was added to formvar/carbon coated grids (400 mesh),³² dried, and then viewed under a TEM operating at a voltage of 100 kV.

AuNPs preparation

AuNPs were prepared as previously reported in the literatures.³³⁻³⁵ Briefly, after a 0.01% HAuCl_4 solution was heated, a 20 mM sodium citrate solution was dropped quickly into the hot HAuCl_4 solution while it was vigorously agitated. At first, the color of the solution changed from light yellow to grey, then to black and gradually to wine red color. At this step the color did not change anymore, so the heating was stopped and the solution mixture was continuously agitated until it cooled down to room temperature. The prepared AuNPs were stored in the dark at 4°C . UV-Vis absorption spectra of the AuNPs were collected using a TU-1901 spectrophotometer (Beijing Purkinje General Instrument Co., China), using a 1-cm path-length cell equipped with a thermostat holder and an external temperature controller (Shanghai Hengping Instrument Co., China) at $25 \pm 0.1^\circ\text{C}$.

Functional MWCNTs preparation

MWCNTs were functionalized according to the literatures.³⁶ Briefly, MWCNTs were treated with a mixture of concentrated H_2SO_4 and HNO_3 (v/v = 1/3) under an ultrasonic bath for 4 h at 80°C . Then, by using NaOH, the pH of the solution was adjusted to 7.0. Thereafter the carboxylic acid functionalized MWCNTs (MWCNTs-COOH) were centrifuged and washed with water three times. The obtained MWCNTs-COOH was dried at room temperature, and then viewed under a TEM using a Hitachi HU-12A (Hitachi, Japan) operating at 75 kV.

Apparatus and measurements

Electrochemical studies were carried out in a conventional three-electrode cell powered by an electrochemical system comprising a CHI650C (CHI Instrument, USA). An Ag/AgCl-saturated KCl, a Pt wire and a GC electrode of 3 mm diameter (CHI Instrument, USA) were used as the reference, counter and working electrodes, respectively. Electrochemical measurements were carried out in a N_2 -saturated 0.05 M sodium phosphate buffer solution (PBS, pH 7.0) at $25 \pm 1^\circ\text{C}$.

Preparation of an AP modified electrode

The preparation of a GC electrode was similar to that given in previous articles.^{20,32,37-40} Prior to coating, the GC electrode was mechanically polished twice with alumina (particle sizes 1, 0.3 and 0.05 μm , respectively) to a mirror finish. Then, it was treated electrochemically in 200 mM sulfuric acid, cycling between -1.0 and $+0.5 \text{ V}$ (vs. Ag/AgCl) at a sweep rate of 0.1 V/s for approximately 10 min. Thereafter, the working electrode was placed in a 50 mM PBS (pH 7.0), and an anodic potential of 1.7 V (vs. Ag/AgCl) was applied for 3–5 min. After the electrode was washed, the AuNPs were electro-deposited in the range of 0–1.1 V (vs. Ag/AgCl) at a scan rate of 0.1 V/s. Then, the GC electrode was dipped in 1 mM Cys for 30 min. After washing with water, 3 μl of MWCNT-COOH (2 mg/ml) was dropped onto the surface of the electrode, and dried at room temperature. Then, 5 μl AP was dropped on the modified electrode. After drying for 10 h at 4°C , the electrode surface was covered by 3 μl of NF (5% in ethanol).

Results and Discussion

Kinetic parameters of AP

Table 1 gives the kinetic parameters associated with the Michaelis-Menten constant (K_m), the turnover number (catalytic rate constant, k_{cat}) and the catalytic efficiency (k_{cat}/K_m) for

Table 1 Kinetic parameters of typical designed artificial peroxidase

Catalyst system	$K_m/\mu\text{M}$	$k_{\text{cat}}/\text{s}^{-1}$	Catalytic efficiency/ $\mu\text{M}^{-1} \text{s}^{-1}$	Relative catalytic efficiency, ^a %	Ref.
NC ^b	2.5 ± 0.4	0.069 ± 0.001	0.028 ± 0.005	39 ± 5	This work
GSHI ^c	1.52	0.030	0.0198	27	17
Cyt-His-RM ^d	42	0.58	0.0173	24	16
His-hematin-M	3.31	0.0433	0.0131	18	15
Native HRP	5800	420	0.0724	100	15

a. Relative catalytic efficiency = $\frac{\text{Catalytic efficiency of AP}}{\text{Catalytic efficiency of native HRP}} \times 100\%$. b. NC, NF-Cyt c nanocluster at pH 7.0. c. GSHI, Gemini-SDS-hemin-imidazole. d. RM, reverse micelle.

Fig. 1 Typical Lineweaver-Burk plot for AP. Inset: Michaelis-Menten plot. The experiment was carried out in the presence of 1.2 mM H_2O_2 and different concentrations of guaiacol (5 – 200 μM) in PBS (50 mM, pH 7.0).

several artificial peroxidase models.^{15–17} K_m is a function of the guaiacol concentration for various peroxidase-like catalyst models. Here, k_{cat} indicates the maximum moles of substrate converted to product per mole of the catalyst per unit time. Then, the parameters can be obtained from a Lineweaver-Burk linear plot (Eq. (2) and Fig. 1).

$$\frac{1}{V_0} = \frac{K_m}{V_{\text{max}}[S]} + \frac{1}{V_{\text{max}}} \quad (2)$$

where, V_0 is the initial peroxidatic reaction rate. Figure 1 represents the typical Lineweaver-Burk plot for AP, and the inset shows a Michaelis-Menten plot in the presence of 1.2 mM H_2O_2 and different concentrations of guaiacol (5 – 200 μM) at 50 mM PBS at pH 7.0. From Fig. 1, a regression equation, $y = 38.311x + 14.543$, was obtained, and K_m and V_{max} were calculated to be 2.6 μM ($38.311/14.543$) and 0.0688 s^{-1} ($1/14.543$), respectively. The K_m and k_{cat} values of the AP were determined to be $2.5 \pm 0.4 \mu\text{M}$ and $0.069 \pm 0.001 \text{s}^{-1}$, respectively. The catalytic efficiency of the artificial enzyme was calculated to be $0.028 \pm 0.005 \mu\text{M}^{-1} \text{s}^{-1}$, which was $39 \pm 5\%$ as efficient as the native horseradish peroxidase (HRP) ($0.0724 \mu\text{M}^{-1} \text{s}^{-1}$).¹⁵ Moreover, the K_m and k_{cat} values of Cyt c alone at different pH values were very difficult to obtain because the reaction rates were too small to reproducibly detect.

Characteristics of nano-materials

The TEM image (Fig. 2A) shows that the NF-Cyt c particles were in a nano-cluster state, made from the Chain-Ball structure

Fig. 2 Transmission electron microscopies (TEM) of nano-cluster of NF-Cyt c (A) and carboxyl functionalized MWCNTs (B).

with an average ball size of about 40 nm. These results are close to our previous TEM study on MB-NF nano-cluster (60 nm),³² and are consistent with normal predictions of the NF structure, while NF offers a biocompatible microenvironment to confine the biomacromolecules in its ionic cluster region (30 – 50 nm), based on a stylized, semi-empirical view of a polar/nonpolar micro-phase separation in a hydrated ionomer.^{41–43} The nano-cluster complex with an enhanced specific surface area is more effective for catalytic reactions.

The functionalized MWCNTs were also characterized by TEM images; 3 μL of the MWCNTs (2 mg/ml) was dropped onto the surface of fomvar/carbon coated grids (400 meshes), dried, and then viewed under a TEM, as shown in Fig. 2B.

UV-Vis spectroscopy was used to determine the AuNPs size. Figure 3 indicates that the spectrum of the prepared AuNPs exhibited maximum and minimum absorptions at 522 and 438 nm, respectively. Curves a and b in the inset of Fig. 3 were plotted based on the maximum and minimum absorptions for AuNPs with different sizes.⁴⁴ Using the regression equations of curves a and b ($y = 0.4275x + 514.76$ and $y = 0.5051x + 431.96$), and by replacing the values for y (for maximum absorption, $y = 522 \text{ nm}$ and minimum absorption, $y = 438 \text{ nm}$), the sizes of the AuNPs were calculated to be 17 and 12 nm, respectively. Thus, the sizes of the prepared AuNPs were characterized to be from 12 to 17 nm.

Electrochemical studies

Figure 4 represents cyclic voltammograms (CVs) of: (a) bare electrode; (b) NF/AuNPs-Cys/GC electrode; (c) NF/MWCNTs/AuNPs-Cys/GC electrode and (d) NF/AP/MWCNTs/AuNPs-Cys/GC electrode at a scan rate of 0.05 V/s. It could be seen that (d) shows a well-defined redox wave with a formal potential ($E^{\circ'}$) of $-45 \pm 2 \text{ mV vs. Ag/AgCl}$.

Fig. 3 UV-vis spectra of AuNPs. Inset: plots of (a) maximum and (b) minimum absorptions in UV spectra versus AuNPs size.⁴⁴

CVs of the NF/AP/MWCNTs/AuNPs-Cys/GC electrode in 50 mM PBS (pH 7.0) at various scan rates are shown in Fig. 5A. The peak currents increased with increasing the scan rate (v), and its linearly was proportional to v (not $v^{1/2}$) in the range of 0.01 – 2.0 V/s (Fig. 5B). The linear-regression equations for cathodic (I_{pc}) and anodic peak (I_{pa}) currents are: $y = 63.3x + 2.81$ (μA) and $y = -54.0x - 4.45$ (μA), respectively. The cyclic voltammograms remained essentially unchanged upon consecutive potential cycling, indicating that the modified electrode is stably confined on the glassy carbon electrode. Figure 5C shows the relationship between the peak potential (E_p) and the natural logarithm of a scan rate ($\ln v$) for a modified electrode. According to Eq. (3),⁴⁵ the cathodic peak potential (E_{pc}) in the range from 1.4 to 2.0 V/s was changed linearly versus $\ln v$ with a linear-regression equation of $y = -0.083x - 0.3607$:

$$E_p = E^{\circ'} + \frac{RT}{\alpha nF} - \frac{RT}{\alpha nF} \ln v \quad (3)$$

Here, α is the cathodic electron-transfer coefficient, n the number of electrons, and T the temperature; R and F are the gas and Faraday constants, respectively. Then, αn could be calculated to be 0.31. According to a reference,⁴⁶ it is usual that $0.3 < \alpha < 0.7$; then, n and α could be concluded to be 1 and 0.31, respectively. From the width of the peak at mid height and a low scan rate, we can also obtain $n = 1$. Thus, the redox reaction between AP and a GC electrode is a single electron transfer process.

The value of the apparent heterogeneous electron-transfer rate constant (k_s) could be calculated using Eq. (4):⁴⁷

$$\ln k_s = \alpha \ln(1 - \alpha) + (1 - \alpha) \ln \alpha - \ln \left(\frac{RT}{nFv} \right) - \alpha(1 - \alpha) \frac{nF\Delta E_p}{RT} \quad (4)$$

As can be seen, in the potential range from 1.4 to 2.0 V/s, the anodic peak potential (E_{pa}) is also linear to $\ln v$ with a linear-regression equation of $y = 0.0669x - 0.1143$. It also indicates that $n\Delta E_p > 0.2$ V; then, the regression equations of

Fig. 4 CVs of different modified electrodes (from inner to outer): (a) bare GC, (b) NF/AuNPs-Cys/GC, (c) NF/MWCNTs/AuNPs-Cys/GC, (d) NF/AP/MWCNTs/AuNPs-Cys/GC. The experiments were carried out in 50 mM PBS (pH 7.0) at a scan rate of 0.05 V/s.

the cathodic and anodic peak potentials will meet at cross point P, where $\ln v = -3.17$; then, k_s was calculated to be 0.65 ± 0.1 s⁻¹.

Considering the reduction peaks and using Faraday's law (Eq. (5)), the average surface concentration of electroactive AP on the surface of GC electrode could be estimated based on the slope of I_p vs. v ,⁴⁸

$$I_p = \frac{n^2 F^2 A \Gamma v}{4RT} \quad (5)$$

where A , T , n , R and F are the electrode surface area, temperature, number of electrons, gas and Faraday constants, respectively. Then, the value of Γ could be calculated to be 7×10^{-10} mol cm⁻², which is about 500-times the theoretical monolayer coverage of 1.4×10^{-12} mol cm⁻².⁴⁹ This can be attributed to the larger surface area of the nano-cluster and the AuNPs-MWCNTs nano complex, which helps to obtain more efficient Cyt *c* immobilization. However, the NF membrane may also contribute to an improvement of the AP electro-catalytic activity. NF may offer a biocompatible micro-environment to confine bio-macromolecules at its ionic cluster region (30 – 50 nm); this view is also consistent with our previous studies.^{20,32,36,37} Moreover, NF may be helpful to restrict and protect the AP/MWCNTs/AuNPs-Cys system on the GC electrode.

Effect of the pH value

Figure 6 represents the CVs of the modified electrode in 50 mM PBS at various pH values. An increase in the pH of the solution from 5.0 to 10.0 led to a negative shift in both reduction and oxidation peak potentials. Figure 6 (inset) shows that the formal potential of the electrode is pH dependent. These results indicate that the slope was 57.9 mV/pH over a pH range from 5.0 to 10.0. This value was close to the ideal Nernst's value of 59.2 mV/pH for the one-electron, one-proton process.⁴⁸

Detection limit

To determine the detection limit (minimum concentration of H₂O₂ that could be detected by this method), the steady cathodic peak current (I_s) of each addition was measured, while the final concentration of H₂O₂ ($C_{H_2O_2}$) (from 0.05 nM to 10 nM) was increased gradually (Fig. 7, inset). The detection limit was determined to be 0.05 nM from the cross point of lines fitted to the linear segments of the I_s vs. $C_{H_2O_2}$ (Fig. 7).^{50,51}

Fig. 5 (A) CVs of NF/AP/GC electrode at different scan rates (from inner to outer 0.01, 0.02, 0.03, 0.04...1.9, 2.0 V/s). (B) Relationships between the anodic peak current, I_{pa} (cathodic peak current I_{pc}) and the scan rate (v). (C) Relationship between the peak potential (E_p) and the natural logarithm of the scan rate ($\ln v$) for the NF/AP/GC electrode (linear fitting at scan rates from 1.4 – 2.0 V/s).

Fig. 6 CVs of NF/AP/MWCNTs/AuNPs-Cys/GC electrode in 0.05 M PBS at different pH values (from left to right): 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0, respectively. Inset: plot of formal potential (E°) vs. pH value.

Electrochemical kinetic parameter

The apparent Michaelis-Menten constant (K_m^{app}) is a reflection of both the enzyme affinity and the ratio of microscopic kinetic constants. This value can be obtained from the electrochemical version of the Lineweaver-Burk,³⁷

$$\frac{1}{I_s} = \frac{1}{I_{max}} + \frac{K_m^{app}}{I_{max}C}, \quad (6)$$

where, C is the substrate concentration in the bulk solution, I_s the steady-state current after the addition of a substrate and I_{max} the maximum current measured under saturated substrate conditions. Figure 8 represents a Lineweaver-Burk plot for the modified electrode in the presence of different concentrations of H_2O_2 . The K_m^{app} value of the modified electrode was calculated to be 0.23 nM, which is lower than most reported values for Cyt *c* immobilized on different modified electrodes: e.g., 0.23 and 0.08 mM for native and denatured Cyt *c* on nanotextured diamond surface,⁵² 0.45 mM for Cyt *c* on Chi-RTIL-MWNTs film.⁵³ A low K_m^{app} value indicates strong substrate binding, and exhibits a higher affinity of H_2O_2 for a NF/AP/MWCNTs/AuNPs-Cys modified GC electrode. The MWCNTs-AuNPs nano-composite and the nano-cluster structure may help to reduce the bridge length between the

Fig. 7 Determination of the H_2O_2 detection limit for a NF/AP/MWCNTs/AuNPs-Cys modified GC electrode. The detection limit was determined from the cross point of the lines fitted to the linear segments of the steady current, I_s vs. the concentration of H_2O_2 ($C_{H_2O_2}$). Inset: Steady cathodic peak current I_s vs. $C_{H_2O_2}$. The inset has the same units as the figure.

Fig. 8 Lineweaver-Burk plot for K_m^{app} determination.

electroactive center of the AP and GC electrode,³⁷ and result in the high sensitivity of the modified electrode to H_2O_2 .

Conclusions

Negative-charged NF may interact with positive-charged Cyt *c*

through an electrostatic interaction and form a nano-cluster structure with peroxidase activity in 50 mM PBS. The mean value of the ball sizes in Chain-Ball structure of nano-cluster was about 40 nm. Thus prepared nano-cluster may be used as an artificial peroxidase (AP) instead of HRP with good stability and activity. Direct electron transfer was achieved using an immobilized NF/AP/MWCNTs/AuNPs-Cys on GC electrode. MWCNTs/AuNPs-Cys acts as a promoter and bridge between the electroactive center of the AP and the electrode. The AP modified electrode could be used as a third-generation biosensor of H_2O_2 with good sensitivity and stability.

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