Electrochemical performance of gold nanoparticle–cytochrome c hybrid interface for H2O2 detection

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Here, we describe the formation of a hybrid biointerface consisting of gold nanoparticle (AuNP) and cytochrome c (cyt c) on indium tin oxide (ITO) electrodes using a two-step immobilization procedure. The Au nanoparticles were attached to the ITO electrodes by 3-mercaptopropyl trimethoxysilane (3-MPTMS). The electrode was then incubated with 11-mercapoundecanoic acid (11-MUA) and the nanoparticles were activated to allow for coupling to cyt c. This process resulted in the formation of the AuNP/cyt c hybrid on the ITO electrode. The ITO/AuNP/cyt c substrate surfaces were characterized by scanning electron microscopy (SEM), atomic force microscopy (AFM) and X-ray diffraction analysis (XRD), and cyclic voltammetry (CV) techniques. Further analysis regarding the surface roughness properties of ITO, ITO/AuNP and ITO/AuNP/cyt c were also performed. The ITO/AuNP/cyt c immobilized ITO electrode displayed a pair of well-defined redox peaks (Epa at 0.09 V and Epc at 0.02 V) at pH 7.0 in HEPES buffer solution. Differential pulse voltammetry (DPV) and amperometric i-t measurements on the modified electrode showed a linear response after the addition of hydrogen peroxide (H2O2). The developed electrode sensor had an electron transfer rate constant (k1) of 0.69 s−1 with a detection limit of 0.5 μM. The results of this study suggest that the hybrid layers were well fabricated on the ITO surface and the developed ITO/AuNP/cyt c electrode displayed an excellent electrocatalytic response for the detection of H2O2.

1. Introduction

The detection of hydrogen peroxide (H2O2) is important to many fields including industry, clinical control and environmental protection [1,2]. Many analytical methods have been developed for this purpose but amperometric enzyme-based biosensors have received considerable attention due to its convenience, high sensitivity and selectivity [3,4]. However, a significant challenge in the development of sensitive and stable sensors is the effective immobilization of enzyme and/or any nanostructured materials (such as carbon nanotubes, quantum dots, nanoparticles, etc.) to solid electrode surfaces [5]. Electrochemical biosensors for detection of H2O2 are mostly designed based on immobilization of horseradish peroxidase (HRP) to carbon based electrode materials [6]. Several researchers have used nanostructured materials such as gold nanoparticles (AuNPs), carbon nanotubes (CNTs) coupled with enzymes or proteins to develop a modified electrode [7,4]. In our previous study, we developed novel techniques to detect H2O2 using other biological materials [8–13]. Among the nanostructured materials tested, there has been much interest in using gold nanoparticles (AuNPs) conjugated with redox proteins for direct electron transfer reaction by making a nanostructure film for their use in electronic, optical and sensor applications [14,15]. AuNPs have been widely used to study the direct electrochemistry with redox partners due to its large specific surface area, good biocompatibility and suitability for many surface immobilization mechanisms, so that they could adsorb redox enzymes and proteins without loss of their biological activity [16,17]. Furthermore, these AuNPs can act as tiny conduction centers, which can facilitate the rapid transfer of electrons. Hence, there is an increasing interest in the immobilization of proteins on AuNPs to fabricate biosensors [18,19]. Furthermore, chemical/biochemical sensors require the immobilization of AuNPs from colloidal solutions onto solid substrates.
without aggregation [20,21]. Aggregation would cause the immobilized nanoparticles to lose their unique size-dependent optical and electronic properties. AuNPs in colloidal solutions carry negative charges that prevent the particles from aggregating, and also prevent them from self-assembling into a monolayer. Organic silanes with functional groups, such as amino and thiol, have been widely used to anchor AuNPs to glass slides and metal-oxide films. Layer-by-layer (LbL) assembly, which is based on the alternate adsorption of oppositely charged species, is a powerful technique for preparing AuNPs films combined with biomolecules and has been extensively studied because of its simplicity, flexibility, and effectiveness [22–28].

Many electrochemical techniques make use of the reduction of \( \text{H}_2\text{O}_2 \) by the catalysis of immobilized horseradish peroxidase (HRP) to construct unmediated HRP-based sensors, which are based on direct electron transfer between an electrode and immobilized HRP [29–33]. It has been reported that proteins containing heme groups, such as hemoglobin, and myoglobin possess peroxidase like catalytic activity, which can reduce \( \text{H}_2\text{O}_2 \) due to the electroactive heme center [34,35] and has also been used for the preparation of \( \text{H}_2\text{O}_2 \) sensors [36,37].

Cytochrome c (cyt c) was used in this study because of its direct electrochemical activity at the electrode. It has been shown that the lysine residues surrounding the heme crevice of the protein plays an important role in binding interactions and electron transfer with its redox partners [38–42]. Cyt c is an excellent model for studying the electron transfer of typical enzymes from a structure point of view. In addition, cyt c is known to have some intrinsic peroxidase activity due to its close similarity to peroxidase. In addition, the performance of a cyt c-based \( \text{H}_2\text{O}_2 \) biosensor will not be altered by the presence of \( \text{O}_2 \) under a reasonable applied potential, since cyt c is not a specific protein for \( \text{O}_2 \) unlike hemoglobin (Hb) and myoglobin (Mb) which are the carriers of \( \text{O}_2 \) in biological systems. In addition to these properties, cyt c has several other advantages for use as a biocatalyst [43].

(a) The heme prosthetic group is covalently bound to the protein. This property may be important for catalysis in the presence of organic solvents; cyt c does not lose its heme catalytic group in these systems, while peroxidases do; (b) Cyt c is active over a wide range of pH [from pH 2 to pH 11]. No other enzyme is active over such a pH range; (c) cyt c is able to perform biocatalytic reactions even at higher temperatures and after chemical modification its thermo stability will be greatly increased; and (d) finally, cyt c is inexpensive. Cost and stability are the two main factors for biocatalysis on a large scale.

Therefore, it is possible to use cyt c containing a heme group that serves as the active center to catalyze the reduction of \( \text{H}_2\text{O}_2 \). However, electron transfer between cyt c and solid electrodes is usually slow [44]. Thus, it is necessary to search for a way to develop a cyt c modified electrode that will enhance electron transfer to the solid surface, while still maintaining well-behaved electrochemistry and good stability. Thus, AuNPs were attached to cyt c and used as an electron shuttle between cyt c and electrode for \( \text{H}_2\text{O}_2 \) detection.

Hence, here we developed an amperometric biosensor of \( \text{H}_2\text{O}_2 \) based on the direct electrochemistry of cyt c immobilized on AuNPs that were conjugated to an ITO surface. The ITO/AuNP/cyt c was characterized by scanning electron microscopy (SEM), atomic force microscopy (AFM), X-ray diffraction analysis (XRD) and cyclic voltammetry (CV). The surface roughness of the ITO, ITO/AuNP, and ITO/AuNP/cyt c was measured with AFM. Direct electron transfer between cyt c and the underlying ITO was observed by cyclic voltammetry. The electrocatalytic reduction of \( \text{H}_2\text{O}_2 \) at ITO/AuNP/cyt c was determined by electrochemical measurements. Furthermore, differential pulse voltammetry (DPV) and chronoamperometric i-t measurements showed a linear response for the detection of hydrogen peroxide (\( \text{H}_2\text{O}_2 \)).

2. Experimental details

2.1. Chemical reagents

Cytochrome c from horse heart was purchased from Sigma–Aldrich (cyt c; purity 99.7% based on \( \text{H}_2\text{O}_2 \) content 7.0%) and used as received without further purification. 3-Aminopropyl triethoxysilane (3–APTES; purity: 99%), 3-Mercaptopropyl trimethoxysilane (3–MPTMS; purity: 85%) and gold colloid solution (−0.01% HAuCl₄) were purchased from Sigma–Aldrich. Glutaraldehyde solution, 11-mercaptooundecanoic acid (MUA, Aldrich) were purchased from Sigma and used as received without any further purification. N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-diethylaminopropyl) carbodiimide hydrochloride (EDC) was obtained from Sigma. 30% hydrogen peroxide was brought from Daijung Materials and Chemicals, Korea and diluted in DI water to prepare molar solutions. 10 mM HEPES buffer solution (pH 7.0) was used in all electrochemical experiments. All solutions were prepared with water (18 MΩ cm) that was purified using a Millipore system (Millipore, Bedford, MA).

2.2. Electrode modification

Indium tin oxide [ITO; 20 Ω/cm²] with a thickness of 400 nm were used in these experiments. The transparent electrodes were cleaned by ultrasonic cleaning in successive solutions of Triton X-100/water (1:5, v/v), water, and ethanol for at least 40 min each. The ITO electrode was then heated in a 1:1:5 solution of \( \text{NH}_4\text{OH}:\text{H}_2\text{O}_2 :\text{H}_2\text{O} \) for 40 min at 80 °C, rinsed thoroughly with water, and dried under a stream of nitrogen gas to generate –OH groups on the surface [45].

2.3. Formation of nanoparticles and cytochrome c hybrid on ITO surface

For the formation of AuNP–cyt c hybrid, the –OH terminated ITO substrates were incubated with 2% (v/v) 3-MPTMS in methanol overnight to generate –SH groups on the ITO surface. After rinsing thoroughly, the ITO/MPTMS substrates were immersed in the gold colloidal solution for 2 h, which produced the ITO/AuNPs substrate through metal–thiol interactions. For the construction of ITO/AuNPs/cyt c substrate, the ITO/AuNPs were incubated with 30 mM 11-MUA and activated with EDC and NHS. 20 µL of cyt c was then added to the activated electrode surface. The ITO/AuNP/cyt c were slightly washed with DI water to remove any unbound protein molecules and dried under a N₂ stream.

For the preparation of cyt c/ITO electrodes, ITO electrodes were incubated with a 2% APTES solution in ethanol for 6 h. The samples were then washed with ethanol and DI water. Subsequently the substrates were immersed into 1% glutaric anhydride (GA) overnight at room temperature. The functionalized substrates were washed with DI water and then dried under a N₂ stream. 20 µL cyt c was then incubated on the substrate for 3 h and slightly washed with DI water and dried under a N₂ stream. The electrode modification processes for cyt c immobilization on the ITO/AuNP electrode is represented in the schematic diagram of Fig. 1.

2.4. Characterization of ITO/AuNP/cyt c surface

Surface topography of the bare ITO, ITO/AuNP and ITO/AuNP/cyt c electrode surfaces was investigated by atomic force microscopy (AFM) (Nanoscope IV/Multimode, Digital Instruments). All images were recorded in tapping mode using silicon cantilevers with a resonance frequency (\( f₀ \)) between 250 and 300 kHz. All images
2.5. Electrochemical activity and detection of H₂O₂

Cyclic voltammetry (CV) was performed with a three-electrode system using the ITO/AuNP/cyt c substrate as the working electrode, platinum coil as the counter electrode, and Ag/AgCl/KCl sat as the reference electrode. Experiments were performed using a CHI 660A potentiostat equipped with general purpose electrochemical software and operated in a potential range from 0.2 to −0.4 V at a scan rate 50 mV s⁻¹. The DPV and chronoamperometric experiments were performed using the ITO/AuNP/cyt c modified ITO electrodes with various concentrations of H₂O₂. The potential was set at −0.10 V and the current–time curves were recorded after successive additions of 20 μL aliquots of 200 mM H₂O₂ in 5 mL of 10 mM HEPES, pH 7.0.

3. Results and discussion

3.1. Topographic analysis using SEM and AFM

The topographical features of the ITO, ITO/AuNP and ITO/AuNP/cyt c surfaces were examined by SEM (Fig. 2(a–c)). As shown in Fig. 2(b), the AuNP was well attached on to the ITO substrate and had a particle diameter of ∼40 nm. In addition, the particles in Fig. 2(c), which shows images of the particles after cyt c immobilization, had a larger diameter (∼50 nm), confirming that the cyt c was attached to the AuNP on the ITO surface. Fig. 2(d–f) shows typical AFM images of three different (ITO, ITO/AuNP and ITO/AuNP/cyt c) sample surfaces scanned at a rate of 1 Hz. The sizes of AuNPs immobilized on the MPTMS/ITO were two to three times larger than those of the AuNPs colloids. This was due to the aggregation of AuNPs and the well-known convolution of the AFM tip. However, the images clearly shows that cyt c was conjugated to the AuNPs on the ITO substrates. The roughness parameters obtained from the scanned images were shown in Fig. 2(g–i) and the selected roughness parameters of the three surfaces are given in Table 1.

Using the offline sectional analysis provided by Nanoscope [Digital Instruments], the height of the AuNP and cyt c was found to be 4 nm and 15 nm, respectively, while the bare ITO contained large clusters of particles. The observed diameter of the AuNP particles was 40 nm, which indicates that these structures were aggregates of ∼2 particles. Further, the size of single cyt c molecule is 3 nm [46]; which means that aggregated AuNPs had attached to the ITO substrate, resulting in the formation of an AuNP–cyt c hybrid with an average diameter of ∼50 nm. From Table 1, it is clear that the RMS roughness decreased for each sample as that for the bare ITO surface. This was expected since the ITO surface is rough and contains many grains with average diameters between 10 and 50 nm separated by deep valleys. In addition, based on the skewness (Rsk) values, it is clear that the peaks dominate the valleys. These combined results confirm that the particles (AuNPs and cyt c) were well immobilized on the surface. In addition, the kurtosis (Rku) increased for all the samples, which further demonstrates that
the high areas dominated over the valleys. Furthermore, the max. peak height ($R_h$) increased in all the three samples. Based on these results, it can be concluded that the cyt $c$ was well attached to the AuNPs through LBL assembly, resulting in the formation of a hybrid (ITO/AuNP/cyt $c$) on the ITO surface.

3.2. X-ray diffraction analysis for confirming the formation of hybrid on ITO electrode

Experimental X-ray diffraction patterns were obtained for AuNPs, cyt $c$ and AuNPs conjugated with cyt $c$ as shown in Fig. 3. The XRD measurement was used mainly to confirm successful conjugation of cyt $c$ to the AuNPs. As shown in Fig. 3, a typical pattern for AuNPs [spectrum (a)] was observed and the structure was confirmed based on the Joint Committee on Powder Diffraction Standards (JCPDS) Card file No.01-073-1234. This pattern confirmed the crystallinity of the particles. In spectrum (b), a broad peak around 2θ = 18 was observed due the Fe heme center of cyt $c$. In the XRD spectrum (c), diffraction peaks can be seen for both the nanoparticles along with a broad peak of cyt $c$. The spectrum of the AuNPs–cyt $c$ conjugate showed a combination of these peaks, which proves that the layer-by-layer assembly process resulted in the formation of a hybrid system on the electrode surface.

3.3. Cyclic voltammetric behavior of ITO/AuNP/cyt $c$ electrode

The cyclic voltammetric studies were carried out to examine the redox behavior of the ITO/AuNP/cyt $c$ electrode in 10 mM pH

<table>
<thead>
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<th>Table 1</th>
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<tr>
<td>Values of selected roughness parameters measured from the surface analysis of AFM topography images for ITO, ITO/AuNP and ITO/AuNP/cyt $c$, respectively.</td>
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<tr>
<th></th>
<th>ITO surface</th>
<th>ITO/AuNP</th>
<th>ITO/AuNP/cyt $c$</th>
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<tbody>
<tr>
<td>Roughness average ($R_a$) (nm)</td>
<td>4.20 ± 0.365</td>
<td>3.35 ± 0.75</td>
<td>3.19 ± 1.09</td>
</tr>
<tr>
<td>RMS roughness ($R_m$) (nm)</td>
<td>5.08 ± 0.386</td>
<td>4.40 ± 0.8</td>
<td>4.01 ± 1.04</td>
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<tr>
<td>Peak to peak height ($R_p$) (nm)</td>
<td>32.3 ± 3.31</td>
<td>45.1 ± 3.2</td>
<td>147 ± 11.73</td>
</tr>
<tr>
<td>Skewness ($R_s$)</td>
<td>-0.36 ± 0.11</td>
<td>0.56 ± 0.3</td>
<td>0.25 ± 0.16</td>
</tr>
<tr>
<td>Kurtosis ($R_k$)</td>
<td>2.60 ± 0.314</td>
<td>3.13 ± 0.41</td>
<td>3.73 ± 0.433</td>
</tr>
<tr>
<td>Max. peak height ($R_h$) (nm)</td>
<td>12.9 ± 0.87</td>
<td>16.8 ± 4.08</td>
<td>18.8 ± 2.01</td>
</tr>
<tr>
<td>Particle height (section analysis) (nm)</td>
<td>3.48 ± 0.74</td>
<td>4.05 ± 0.57</td>
<td>15.0 ± 1.89</td>
</tr>
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</table>
7.0 HEPES buffer at a scan rate of 50 mV s⁻¹. As depicted in Fig. 4(a), no obvious redox peaks were observed for both the electrodes, [ITO (curve i) and ITO/AuNP electrodes (curve ii)] in the potential range of 0.4 to −0.2 V. However, a small increase in the background current was observed for the ITO/AuNP electrode compared to the bare ITO electrode. Furthermore, the ITO/AuNP/cyt c electrode showed a couple of well-defined and quasi-reversible redox peaks (curve iii) because of the Fe³⁺/²⁺ redox center, which was responsible for electron exchange. The anodic peak potential (Epa) and cathodic peak potential (Epc) were located at 0.09 and 0.02 V (vs. Ag/AgCl) respectively, with a peak-to-peak separation (ΔEp) of 70 mV. Moreover, the nanoparticles significantly enhanced the interfacial electrochemical electron transfer rate despite the increase in total distance between the redox center and the electrode surface.

Cyclic voltammograms of the ITO/AuNP/cyt c electrode in 10 mM pH 7.0 HEPES at different scan rates from 0.01 to 0.1 V s⁻¹ were collected and are shown in Fig. 4(b). All scan rates resulted in well-defined enhanced reduction and oxidation peaks with a slight shift in both cathodic and anodic peak potentials at different scan rates.

Furthermore, investigations on the ITO/AuNP/cyt c electrode revealed a linear plot for cathodic and anodic peak currents against the scan rate and logarithm of the scan rate as shown in Fig. 5(a and b), respectively. Based on this analysis, an increase in scan rate resulted in a shift of the oxidation wave to a more positive potential, while the reduction wave shifted to a more negative potential. The anodic and cathodic peak potentials were linearly dependent on log v. A graph of Epc = f(log v) yielded two straightlines with a slope equal to −2.3 RT/(αnF) for the cathodic peak, and a slope of 2.3 RT/(1 − α)nF for the anodic peak, as shown in Fig. 5c. The charge-transfer coefficient (α) value was determined to be 0.456 from the slope of the straight lines based on the following equation:

$$\log \frac{k_a}{k_c} = \log \left( \frac{\alpha}{1-\alpha} \right)$$  \hspace{1cm} (1)

where kₐ (0.037) is the slope of the line derived from Epc = f(log v); kₐ (0.044) is the slope of the line derived from Epc = f(log v); α is the charge-transfer coefficient. The apparent electron-transfer rate constant (k₀) for electron transfer between the electrode and the surface deposited layers was also to be 0.69 s⁻¹ according to the following equation [41]:

$$\log k_0 = \alpha \log(1 - \alpha) + (1 - \alpha) \log \left( \frac{RT}{nF} \right)$$  \hspace{1cm} (2)

Furthermore, the surface concentration (Γ) of the cyt c molecules on the ITO/AuNP/cyt c electrode could be estimated based on the slope of Iₚ vs. v using the following equation:

$$I_p = \frac{n^2F^2AΓv}{4RT}$$  \hspace{1cm} (3)

where Iₚ is the reduction peak current, v is the scan rate and A is the electrode surface area, respectively. F is the Faraday constant, R is the gas constant and T is the absolute temperature. The value of Γ was calculated to be 4.2 × 10⁻¹⁰ mol/cm².

3.4 Electrocatalytic reduction of H₂O₂ at the modified electrode

Differential pulse voltammetry technique has the ability to determine the highest sensitivity and the lowest detection limit of any electrochemical sensor. Hence, the electrochemical response of the ITO/AuNP/cyt c electrode was observed as a function of H₂O₂ concentration. Fig. 6 shows the differential pulse voltammogram in a buffer solution of 10 mM HEPES (pH 7.0), which clearly shows a well-defined higher differential pulse peak for the H₂O₂...
catalytic reduction current at $-0.1 \text{V}$. The potential of $-0.1 \text{V}$ was selected because the complete reduction of cyt c occurs at $-0.1 \text{V}$ even though the reduction starts at $0.02 \text{V}$. The current response increased when the $\text{H}_2\text{O}_2$ concentration was increased from 0.1 mM to 0.4 mM. The magnitude of the current also increased linearly upon the addition of several concentrations of $\text{H}_2\text{O}_2$. Thus, these results clearly demonstrate that by using the differential pulse technique, the modified electrode can be used as for the accurate and sensitive detection of $\text{H}_2\text{O}_2$.

3.5. Amperometric determination of $\text{H}_2\text{O}_2$ at the modified cyt c/AuNP/ITO electrode

Cyt c possesses intrinsic peroxidase activity due to its similarity with peroxidase for catalysis of $\text{H}_2\text{O}_2$ [42]. The electrocatalytic response of the ITO/AuNP/cyt c to $\text{H}_2\text{O}_2$ was investigated and the principle of catalyzing $\text{H}_2\text{O}_2$ can be described using the following equation.

\[
\text{cytc–Fe(III)} + e^- \rightarrow \text{cytc–Fe(II)}
\]  

\[
2\text{cytc–Fe(II)} + 2\text{H}^+ + \text{H}_2\text{O}_2 \rightarrow 2\text{cytc–Fe(III)} + 2\text{H}_2\text{O}
\]

The chronoamperometric experiment on the ITO/AuNP/cyt c electrode was carried out to measure the catalytic response of the hybrid molecules for $\text{H}_2\text{O}_2$ detection. Fig. 7(a) illustrates a typical current–time plot for the ITO/AuNP/cyt c on successive additions of 20 $\mu$L aliquots of 200 mM $\text{H}_2\text{O}_2$ in 5 mL of 10 mM HEPES at pH 7. When an aliquot of $\text{H}_2\text{O}_2$ was added to the buffer solution, where the working electrode potential was set at $-0.1 \text{V}$, the reduction current increased steeply and then reached a stable value and this response was observed for every addition over a long period. As shown in Fig. 7(b), a chronoamperometric curve was obtained for the cyt c/ITO electrode after the addition of 20 $\mu$L aliquots of 200 mM $\text{H}_2\text{O}_2$ in 5 mL of 10 mM HEPES at pH 7. However, the addition of $\text{H}_2\text{O}_2$ resulted in only a slight increase in the current and the response was not as stable as compared with the AuNP/cyt c electrode. In addition, saturation was quickly reached when this system was used.

Fig. 7(c) shows the amperometric curve for the ITO/AuNP in the absence of cyt c. When this system was used, the addition of $\text{H}_2\text{O}_2$ only resulted in a slight increase in the current but the response quickly saturated. Overall, the developed electrode sensor showed a linear response to $\text{H}_2\text{O}_2$ concentration up to 6 mM. The current response vs. $\text{H}_2\text{O}_2$ concentration shows a linear increase in...
the reduction current. The detection limit of the present electrode sensor was 0.5 μM, which was comparable to other electrodes in terms of detection limit. Moreover, due to the incorporation of AuNP, the electrode achieved a faster electron transfer rate. Table 2 summarizes the detection limit of the present electrode compared with previous studies. Furthermore, this system reached 95% of the steady-state current in less than 10 s after the addition of H2O2. Thus, the electrode developed in this study showed a fast response for the detection of H2O2.

4. Conclusions

In conclusion, ITO/AuNP/cyt c was fabricated and used as an electrode for direct electrochemistry of cyt c by forming a hybrid to detect H2O2. The surface morphologies and the roughness parameters were investigated by AFM. The electrochemical properties of the ITO/AuNP/cyt c were characterized by using CV and other electrochemical methods. ITO/AuNP/cyt c displayed good redox behavior with enhanced peak currents due to the incorporation of AuNPs. Moreover, ITO/AuNP/cyt c displayed a good response in the electrocatalytic reduction of H2O2. Thus, the hybrid system with nanometer-sized AuNPs achieved direct electron transfer of cyt c, which allowed for the construction of an efficient biosensor for the detection of H2O2.

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