Crumpled Graphene/Poly (azure I) Modified Electrode for Non-Enzymatic Detection of Hydrogen Peroxide Secreted From Tumor Cells

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**Abstract:** Hydrogen peroxide (H$_2$O$_2$), an important representatives of reactive oxygen species, its aberrant expression is related to many diseases including cancers. Therefore, it is very significant to design the reliable method for real-time detection endogenous H$_2$O$_2$. Herein, we prepared a poly (azure I)/crumpled graphene (cGN) modified electrode by electro-polymerization method. Results showed that this electrode presented obvious electrocatalytic effect on the reduction of H$_2$O$_2$. Amperometric method was employed to monitor H$_2$O$_2$, and the amperometric response exhibited a good linear relationship with its concentration in the range of $8.0 \times 10^{-6} \sim 1.25 \times 10^{-3}$ mol/L, the detection limit reached to $6.7 \times 10^{-7}$ mol/L ($S/N = 3$).

Furthermore, this modified also displayed the good selectivity, long-term stability and high anti-interference ability. Excitingly, the established method could be successfully used to detect H$_2$O$_2$ in human serum samples, and measured H$_2$O$_2$ secreted from living MCF-7 cells. It could have potential in cancer diagnosis.

**Key words:** Graphene; Hydrogen peroxide; Poly (azure I); Modified electrode
1. Introduction

Hydrogen peroxide (H$_2$O$_2$), a strong oxidizing agent, is often used to disinfect objects and as a raw material for food processing $^{1,2}$. However, H$_2$O$_2$ accelerates human aging and is a carcinogen, which seriously harms human health $^{3,4}$. Therefore, the sensitive detection of H$_2$O$_2$ is of great significance. Recently, the methods of determination of H$_2$O$_2$ have been developed. Such as chemiluminescence $^5$, high performance liquid chromatography $^6$, fluorescence $^7$, and electrochemical methods $^8$-$^{15}$ and so on. Among these, the electrochemical method has been widely paid attention due to its simple operation, high sensitivity, fast inspection speed, low cost and easy to realize automation.

The conductive polymer can form a stable film on the electrode surface with good electrochemical properties. Recently, conductive polymer-based modified electrodes have been successfully used for the detection of biomolecules $^{16,17}$. Especially, the electroactive conductive polymer film attracts the attention of researchers, because it can be used as an electronic mediator to achieve highly sensitive detection of specific molecules. However, the alone conductive polymer often suffering from some challenge, such as poor conductivity and small specific surface area. To solve these problems, a strategy was developed by introducing conductive materials to form composite to improve their conductivity and adjust the morphology of conductive polymer to increase their specific surface area $^{18,19}$.

Graphene is a two-dimensional carbon nanomaterial with excellent conductivity and stability, and has been used to prepare modified electrode for H$_2$O$_2$ detection $^{20-23}$. 
Herein, we fabricated crumpled graphene (cGN), which was used as “conductivity agent “and “roughening agent” to construct poly (azure I)/cGN modified electrode with a good conductivity and large specific surface area. The prepared poly (azure I)/cGN modified electrode was employed to construct a non-enzymatic H$_2$O$_2$ electrochemical sensor. The results showed that this electrode exhibited obvious electrocatalytic effect on the reduction of H$_2$O$_2$. And could be successfully used to detect H$_2$O$_2$ in human serum samples, and measure H$_2$O$_2$ secreted from MCF-7 cells (Scheme 1). It could have potential in assaying H$_2$O$_2$ in life analysis.

![Scheme 1](image)

**Scheme 1.** Schematic illustration of the constructed electrochemical sensor for monitoring H$_2$O$_2$ released from MCF-7 cells.

### 2. Experimental

#### 2.1. Instruments and reagents

Scanning electron microscope (SEM) images were recorded by Hitachi S-3000N scanning electron microscope (Japan Hitachi ltd). Electrochemical measurements
were operated on a CHI660A electrochemical workstation (Shanghai Chenhua Instruments Co, China). Three electrode system was adopted (Work electrode: bare glassy carbon electrode (GCE) or modified electrode; Reference electrode: saturated calomel electrode (SCE); Counter electrode: platinum wire). High-purity nitrogen was employed to removed oxygen in solution for 15 min and kept over the solution during the measurements.

Graphite powder was purchased from Qingdao Hengrui Industrial, China. Formyl methionyl-leucyl-phenylalanine (fMLP) were purchased from Sigma-Aldrich. Trypsin–EDTA were bought from HyClone Corporation. Catalase was bought from Aladin Ltd., H$_2$O$_2$, glucose, ascorbic acid, and KCl were purchased from Alfa Aesar. All reagents were of analytical grade, and used without further purification. All solutions were prepared with doubly distilled water. A mixed solution of NaH$_2$PO$_4$ and Na$_2$HPO$_4$ was used to prepare 0.1 mol/L (pH 7.0) phosphate buffer solution (PBS), and the pH was adjusted with H$_3$PO$_4$ and NaOH.

2.2. Preparation of cGN

Graphene oxide was synthesized according to the previous literature. The cGN was fabricated according to the reported method with some modification. The brief process was as following: 1.0 mg/mL cGN aqueous solution was sonicated for 30 min to form ahomogeneous solution, then 280 μL H$_2$SO$_4$ (98%) was added to the suspension with the total volume of 7 mL, and transferred to a Teflon-lined autoclave and heated at 160 °C for 10 h. The cGN were recovered by filtration, and washed several times with water. Finally, cGN was dried at 50 °C in air for use.
2.2. Preparation of poly (azure I)/cGN/GCE

Before modification, bare GCE surface was polished to be mirror-like by using 1.0, 0.3 and 0.05 μm Al₂O₃ powder suspension. The electrode was then ultrasonically cleaned in ethanol and water for 1.0 min, respectively. Leaving for drying at room temperature for use. 1.0 mg cGN was placed into 1 mL water, and ultrasonically dispersed for 40 min to form a dispersion solution. Finally, 10 μL of cGN dispersion solution were dropped onto the surface of the treated electrode, and dried at room temperature. The loosely adsorbed cGN were removed by immersing into water kept for 10 min and washed with water three times. The cGN/GCE was then placed into 0.1 M PBS (pH 6.0) containing 4.0 mM azure I scanning for 3 cycles in the range of potential of -1.4 - +1.2 V by CV with a scan rate of 100 mV/s. And then, this electrode was continuously scanned 15 cycles in the range of potential of -0.8 - + 0.6 V by CV with a scan rate of 50 mV/s 26. The electrode was then washed with water three times. The prepared electrode was named as the poly (azure I)/cGN/GCE.

2.3. Detection of H₂O₂ released from living cells

MCF-7 cells were harvested with 0.25% trypsin-EDTA, and washed three times with PBS, the obtained cells were resuspended in PBS with a concentration of 1.0 × 10⁶ cells/mL. The measrement of H₂O₂ released from MCF-7 was carried out by using the established method. 0.6 μM fMLP, as inducer, was used to stimulate MCF-7 cells to produce H₂O₂. 700 units/mL catalase was used to consume the produced H₂O₂.

3. Results and discussion

3.1 The characterization of the poly (azure I)/cGN/GCE
Figure 1A displayed the continuous CV curve of azure I on the cGN/GCE surface, a pair of obvious redox peaks were observed, and redox peak currents increased with increasing scan cycle. When the scan reached to 15 cycles, the peak current hardly changed, indicating that the polymerization reaction nearly reached a saturation. To verify that the successful polymerization of azure I on the electrode surface, the CV response of different electrode was tested. As shown in Figure 1B, by comparing with bare GCE and cGN/GCE, a pair of obvious redox peaks were observed, which attributed to the redox of azure I on the cGN/GCE surface, which was similar with previous reports\textsuperscript{26-28}.

![Figure 1. (A) Continuous CVs of azure I on the cGN/GCE surface in 0.1 M PBS (pH 6.0); (B) The CVs of different electrodes in 0.1 M PBS (pH 4.0): (a) poly (azure I)/cGN/GCE, (b) cGN/GCE, (c) bare GCE.](image)

SEM was used to further confirm the formation of poly (azure I)/cGN film. Figure 2B and 2C showed that GN presented a crumpled-like structure. And a polymer film can be obviously observed on the surface of cGN (Figure 2D), indicating that poly (azure I)/cGN film was successfully prepared.
**Figure 2.** SEM images of (A) GO, scale bar: 200 μm; (B) cGN, scale bar: 20 μm; (C) the high magnified cGN, and (D) poly (azure I)/cGN, scale bar: 20 μm.

3.2 The effect of pH and scan rate

pH value of the solution affects the electrochemical performances of poly (azure I)/cGN/GCE. 0.1 M PBS with different pH (3.0-9.0) were chose to investigate the effect of pH. Results showed that the peak potential gradually shifted negative with the increase of pH value, which indicated that protons participated in the process of electrode reaction. Meanwhile, the peak current value was largest in pH 4.0. Thus, 0.1 M PBS (pH 4.0) was selected to carry out following experiments.

We also investigated the effect of the scan rate on the electrochemical behaviors of poly (azure I)/cGN/GCE by CV in the range of 0.06 - 0.14 V/s. The results were shown in **Figure 3.** It could be seen that the oxidation peak and reduction peak currents gradually increased with the increase of scan rate, and presented a good linear relationship. The linear equations were $i_p (\mu A) = 3.85 + 116.70 \nu$ and $i_p (A) =$
-3.38 -138.15 v, the correlation coefficients were R² = 0.9966 and R² = -0.9983, indicating that the reaction process was controlled by adsorption.

Figure 3. (A) CVs of the poly (azure I)/cGN/GCE in 0.1 M PBS (pH=4.0) with different scan rates: (a) 0.06 V/s, (b) 0.08 V/s, (c) 0.1 V/s, (d) 0.12 V/s, (e) 0.14 V/s; (B) The relationship between peak current and scan rate.

3.3. Electrocatalytic mediated reduction of H₂O₂

The electrocatalytic ability of the poly (azure I)/cGN/GCE for the reduction of H₂O₂ was studied. It can be seen from Figure 4A, an obvious change in the CV response of the poly (azure I)/cGN/GCE was observed with adding H₂O₂. And the reduction peak current continuously increased while its oxidation peak current continuously decreased, which revealed a typical electrocatalytic reduction process for H₂O₂. The possible mechanism of H₂O₂ reduced on this modified electrode was as following 28:

\[
\text{Poly(azurine I) (R)+H}_2\text{O}_2 \rightarrow \text{Poly(azurine I) (O)+H}_2\text{O} \quad (1)
\]

\[
\text{Poly(azurine I) (O)+ne}^- + n\text{H}^+ \rightleftharpoons \text{Poly(azurine I) (R)} \quad (2)
\]

To verify that the poly (azure I)/cGN film had more better performances. The amperometric response of poly (azure I)/cGN film was about 4.3 times higher than
that of the poly (azure I) film under the same concentration H₂O₂ (Figure 4B), revealing that poly (azure I)/cGN had more higher activity than that of poly (azure I) alone.

**Figure 4.** (A) CVs of the poly (azure I)/cGN/GCE in the presence of the different concentrations of H₂O₂ in 0.1 M PBS. The concentrations of H₂O₂: 0 mol/L (a), 2.0×10⁻⁵ mol/L (b), 4.0×10⁻⁵ mol/L (c), 6.0×10⁻⁵ mol/L (d), 8.0×10⁻⁵ mol/L (e). (B) Amperometric i-t curves for H₂O₂ reduction at the poly (azure I)/cGN/GCE and poly (azure I)/GCE, respectively.

3.4 Working curve and detection limit

We investigated the work curve and detection limit of this sensor by testing its amperometric response to different concentrations H₂O₂. The results were shown in Figure 5, a well-defined steady-state current response to H₂O₂ was presented. Amperometric response of H₂O₂ exhibited a good linear relationship with its concentrations in the range of 8.0×10⁻⁶~1.25×10⁻³ mol/L. The linear equation was \( i_p = 17.90 + 2.91 \lg c \) \( (R^2 = 0.9870) \) with the detection limit of 6.7×10⁻⁷ mol/L \( (S/N=3) \). By
comparing with previous reported methods \textsuperscript{29-32}, the established method displayed a good performances (Table 1).

Figure 5. (A) Amperometric i-t curve of H\textsubscript{2}O\textsubscript{2} reduction at the poly(azure I)/cGN/GCE in stirring 0.1 M PBS (pH 4.0) (Applied potential: -0.2 V); (B) The relationship between the current values and concentrations.

Table 1. Comparison of the performances with some published works

| Electrode                              | Linear range (M)       | Detection limit (M) | Ref. |
|----------------------------------------|------------------------|---------------------|------|
| prussian blue/hollow polypyrrole       | 5.0×10\textsuperscript{-6} - 2.775×10\textsuperscript{-3} | 1.6 × 10\textsuperscript{-6} | 29   |
| montmorillonite-supported copper sulfide | 3.0×10\textsuperscript{-5} - 2.0×10\textsuperscript{-4} | 2.47 × 10\textsuperscript{-5} | 30   |
| Vertical alpha-FeOOH nanowires         | 5.0×10\textsuperscript{-5} - 5.0×10\textsuperscript{-4} | 1.8 × 10\textsuperscript{-7} | 31   |
| Au nanoparticles                       | 1.0×10\textsuperscript{-4} - 0.16 | 2.3 × 10\textsuperscript{-7} | 32   |
| poly(azure I)/cGN                      | 8.0×10\textsuperscript{-6} - 1.25×10\textsuperscript{-3} | 6.7 × 10\textsuperscript{-7} | This work |

3.5 The stability, reproducibility and anti-interference ability

To explore the reproducibility and stability of the poly (azure I)/cGN/GCE, three poly(azure I)/cGN/GCE were prepared and used to detect H\textsubscript{2}O\textsubscript{2} with the same concentration, the relative standard deviation was calculated to be about 4.9%. The
modified electrode was placed at room temperature for 4d. The same concentration of H$_2$O$_2$ was measured, no significant change was found in the response current. Furthermore, the modified was continuously scanning for 20 cycles, no significant change happened in the response current for the same concentration H$_2$O$_2$. The above results indicated that the proposed electrode had good stability and reproducibility.

The anti-interference ability of this modified electrode was studied. The potential interfering substances including glucose, ascorbic acid and KCl were selected to verify its anti-interference performances. A stable current increase could be observed with the continuous addition of H$_2$O$_2$. While glucose, ascorbic acid, and KCl were continuously injected, no obvious changes in current signal, indicating that this electrode had good anti-interference ability.

3.6 Sample assay

This electrode was used to detect different concentrations H$_2$O$_2$ ($5.0 \times 10^{-5}$ mol/L, $1.0 \times 10^{-5}$ mol/L) in 5% human serum samples which were obtained from healthy person. The recoveries were 96.00% and 100.60%, respectively (Table 2), indicating that the sensor could be used to detect H$_2$O$_2$ in complex samples.

**Table 2. Determination of H$_2$O$_2$ in human serum samples**

| No. | Added (10 μmol/L) | Found (10 μmol/L) | Recovery (%) |
|-----|-------------------|-------------------|--------------|
| 1   | 1.00              | 0.96              | 96.00        |
| 2   | 5.00              | 5.03              | 100.60       |

*The values were obtained from the results of three measurements.

3.7 Determination of H$_2$O$_2$ released from living cells
We also studied the practical application of this electrode by real-time detecting H$_2$O$_2$ released from living MCF-7 cells used fMLP as inducer (Figure 6). Compared with cells or fMLP alone, an obvious amperometric response was observed in the presence of cells and fMLP. However, no amperometric response was found in the presence of catalase, cells and fMLP, confirming that the detective H$_2$O$_2$ was secreted only from the MCF-7 cells, which were consistent with the previous reports. Therefore, the proposed sensor had potential in assaying H$_2$O$_2$.

**Figure 6.** (A) Amperometric i-t curve of the poly(azure I)/fRGO/GCE at an applied potential of −0.2 V for detecting endogenous H$_2$O$_2$ released from living cells via adding fMLP, cells without fMLP, fMLP without cells, and catalase injected into cells with fMLP; (B) Confocal images of MCF-7.

4. Conclusion

In this work, the poly (azure I)/cGN film was fabricated and employed to construct a non-enzymatic electrochemical H$_2$O$_2$ sensor. The proposed electrode exhibited an excellent electrocatalytic ability for H$_2$O$_2$ reduction, and displayed good selectivity, stability and anti-interference ability. In addition, this modified could
detect H$_2$O$_2$ in human serum samples, and successfully measure H$_2$O$_2$ secreted from living cells. It could have potential in assaying H$_2$O$_2$ in life analysis.

**Conflicts of interest**

There are no conflicts to declare.

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