



Contents lists available at ScienceDirect

Analytica Chimica Acta

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

A novel nonenzymatic biosensor for evaluation of oxidative stress based on nanocomposites of graphene blended with CuI

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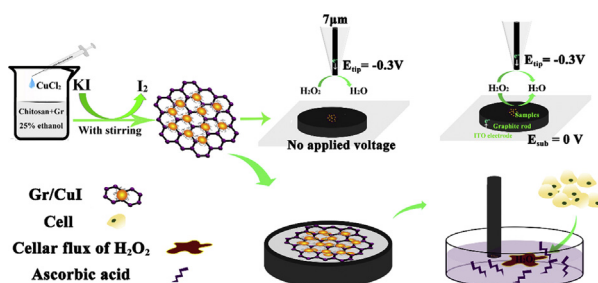
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HIGHLIGHTS

- A novel nonenzymatic H₂O₂ electrochemical biosensor was constructed based on the CuI/Gr composites.
- The biosensor has low detection limit and high sensitivity for H₂O₂ detection.
- SECM imaging study further illustrates the electrochemical catalytic capability for H₂O₂ reduction.
- The H₂O₂ biosensor is used to detect H₂O₂ released from living cells.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:

Received 19 April 2016

Received in revised form

19 May 2016

Accepted 23 May 2016

Available online xxx

Keywords:

Hydrogen peroxide

Cuprous iodide

Graphene

Cells

Oxidative stress

ABSTRACT

A high-sensitive nonenzymatic hydrogen peroxide (H₂O₂) biosensor based on cuprous iodide and graphene (CuI/Gr) composites has been explored for the detection of H₂O₂ released by living cells and monitoring the oxidative stress of cells under exocellular stimulation. The biosensor properties were evaluated by cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS), amperometric i-t curve, and the redox-competition mode of scanning electrochemical microscopy (SECM). Our observations demonstrate that the CuI/Gr nanocomposites modified glassy carbon electrode (GCE) exhibits excellent catalytic activity for H₂O₂ with relatively low detection limit and a wide linear range from 0.5 μM to 3 mM. Moreover, the redox-competition mode of SECM imaging study further illustrates the improved electrochemical catalytic capability for H₂O₂ reduction with CuI/Gr nanocomposites deposited on graphite electrode. Hence, the as-prepared nonenzymatic H₂O₂ biosensor could be used to detect H₂O₂ release from different kinds of living cells under stimulation while eliminating the interference of ascorbic acid.

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1. Introduction

It is well known that reactive oxygen species (ROS) are produced by aerobic cells during metabolic activities, and the related ROS

include O^{•−}, HO[•], HO[•], ·OH, H₂O₂ and singlet oxygen [1], which can somehow enhance the antibacterial activity [2]. The ROS are important intracellular signaling molecules, which are closely related to the fate and signal transduction pathways in different cells that play a significant role in regulating DNA damage, protein synthesis, cell apoptosis, and other living activities [3–5]. The ROS-dependent signaling involves the reversible oxidation and

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reduction of specific amino acids, with crucial reactive Cys residues being the most frequent target [6,7]. Nevertheless, the excessive accumulation of ROS can cause cellular injury that result in altered physiological functions. In general, ROS are maintained at an equilibrium state in normal cells, however, due to any reason once the equilibrium is disturbed, the oxidative stress will occur, which is one of the leading cause of aging and disease [8]. Moreover, the excessive accumulation of ROS can readily destroy the organism's redox reaction equilibrium within the normal cells and tissues, triggering a variety of pathological events such as neurodegenerative diseases, diabetes, cancers, and premature aging [9,10].

Among various kinds of ROS species, H_2O_2 is the most common type of ROS generated from superoxide produced by mitochondria and NADPH oxidases [11,12]. In cells, superoxide results from the one-electron reduction of molecular oxygen (O_2) which is rapidly converted into H_2O_2 by superoxide dismutase [13,14]. The H_2O_2 has a relatively high stability and can readily infiltrate into the cells, where it induce apoptosis or necrosis. The reactive oxygen species role is also well defined in tumor development and in responses to anticancer therapies [15]. Thus, it is crucial to detect intracellular H_2O_2 in order to understand the mechanism of relevant signal transduction and regulation pathways in clinical diagnosis and treatment [16]. Most frequently-used methods to detect H_2O_2 includes chemiluminescence [17], chromatography [18], fluorescence [19,20], electrochemical analysis [16,21,22], etc. In comparison to other detection technologies, the electrochemical method has the characteristics of low cost, low detection limit, high sensitivity, wide detection range and fast response. Over the past few decades, immobilized enzyme biosensor was widely investigated, such as horseradish peroxidase [23], glucose oxidase [24,25] and cytochrome oxidase [26]. However, despite the high sensitivity and selectivity in the moderate condition, the application of immobilized enzyme biosensor is still restricted by its inherent shortcomings including the influenced instability, the rigor of temperature requirements and high cost. Therefore, the nonenzymatic biosensors have the superiority of overcoming the drawbacks of enzyme biosensors and keep their preponderance at the same time, which is gradually manifested. Considering all those above, in this contribution we have explored the possibility of fabricating a high-sensitive nonenzymatic H_2O_2 biosensor based on three-dimensional nanocomposites of graphene (Gr) blended with CuI for rapidly evaluating oxidative stress of different living cells.

Graphene-like two/or three-dimensional materials have attracted much attention due to its extraordinary electronic and optical properties, with ultrahigh carrier mobility, favorable biocompatibility, highest thermal conductivity, high specific surface area, and excellent optical transparency, which is promising in optoelectronic applications [27–30]. Although Gr has giant delocalized π electron system extremely inert in chemistry, several methods have been developed to achieve its efficient chemical modification and keep the original characteristics, such as the formation superlattices materials [31]. The low resistivity and high specific surface area of Gr give it potential application in electrochemical biosensor field [32,33]. Although the poor detection limit of bare Gr restricts its application in living cells, however, the modification of active metals or compounds can obviously improve its catalytic effect [34,35]. Currently, Gr has been conjugated with Pt [36–38], Au [39], Pd [40] and other noble metals to form nanocomposites for detection of H_2O_2 , but its cost is relatively high, and the production process is relatively complicated [4]. In this respect, copper [41–43] and its oxides (such as CuO [44], Cu_2O [45,46] and CuI [47]) have excellent catalytic effect and readily available. Especially, cuprous compounds have been widely applied to electrochemical catalysis such as Cu_2O [45,48,49] and Cu_2S [50,51]. Whereas, another kind of cuprous compounds, i.e., CuI has

extensively being used as an analytical reagent, resin modifier, agent of artificial rainfall and anode tube cover, which also has photoluminescence [52] and catalytic activity. Recently, CuI has been used to catalyze the coupling reaction because of its stability in air and good activity with or without the assistance of supporting ligands [53], such as catalyzed Coupling Reaction of (Hetero) Aryl chlorides and amines [54] and catalyzed cycloaddition reaction of azide [55]. However, bare CuI can be hardly applied to electrochemical biosensors due to its poor electron transfer ability and large electrochemical impedance. Herein, in this study we have explored the possibility to combine the advantages of the Gr with that of CuI for obtaining the synergistic effect of the graphene's super conductivity and high specific surface area with the excellent catalytic activity of the cuprous iodide, and thus fabricating a high-sensitive electrochemical biosensor based on the nanostructured interface of CuI/Gr blending composites.

In the past decades, various kinds of electrochemical biosensors [3,4,16] have been explored to evaluate living cells oxidative stress by stimulation of ascorbic acid. But the interference of ascorbic acid with ROS released from living cells is still unable to eliminate completely. Herein, in this study the highly sensitive CuI/Gr nanocomposites based nonenzymatic H_2O_2 sensor has been exploited and applied to detect the H_2O_2 released from living cells. Due to the special structure of Gr and its strong adsorption for most organics and metal ions, copper ions could be readily adsorbed onto the surface of Gr [56] and reacted with potassium iodide to form CuI/Gr nanocomposites. The as-prepared CuI/Gr nanocomposites could be further utilized to modify electrodes including glassy carbon electrode (GCE) through physical adsorption to form a novel nonenzymatic H_2O_2 electrochemical biosensor for the high-sensitive detection of oxidative stress of living cells under the stimulus of ascorbic acid [57], which can completely eliminate the influence of ascorbic acid itself in the meantime.

2. Experimental

2.1. Reagents and chemicals

Graphene (Gr, 98%) was purchased from Aladdin, Inc. (China). Ascorbic acid (AA), dopamine (DA), β -D(+)-glucose (GLU), uric acid (UA), and chitosan were obtained from Sigma. H_2O_2 , CuCl_2 and KI were obtained from International Laboratory. Chloroplatinic acid ($\text{H}_2\text{PtCl}_6 \cdot 6\text{H}_2\text{O}$) was purchased from Sigma-Aldrich. All the other chemicals were of analytical grade and used as received without any further purification. A physiological PBS solution containing Na_2HPO_4 (10.14 mM), KH_2PO_4 (1.76 mM), KCl (2.28 mM), and NaCl (136.75 mM) was prepared immediately before experiments. All the solutions were prepared by double-deionized distilled water.

The morphological characterizations and surface structures were obtained with the scanning electron microscope (SEM; Zeiss, Germany). The valence state of materials was investigated by X-ray photoelectron spectroscopy (XPS; Ulvac-Phi, Japan). Amperometric i-t curve and cyclic voltammetry (CV) were carried out by CHI660B electrochemical workstation (CHI Inc., USA) and Scanning Electrochemical Microscope (SECM) was performed on a CHI910B electrochemical workstation (CHI Inc., USA). The three-electrode system was constituted by using an Ag/AgCl electrode as the reference electrode, a Pt wire as the counter electrode and bare or modified glass carbon electrode (GCE; 3 mm diameter) as the working electrode. The electrochemical impedance spectrum (EIS) measurements were carried out on an Autolab PGSTAT302N system (Eco Chemie, Netherlands) by using the aforementioned three-electrode system. All amperometric measurements are regularly carried out in PBS buffer solution with stirring and ventilated with high-purity nitrogen for 20 min, and then the electrochemical

measurements were performed inside the deoxygenated atmosphere during experiments.

2.2. Preparation of CuI/Gr composite materials

1 mM CuCl₂, 50 mL of anhydrous ethanol and 150 mL of chitosan solution (2 mg mL⁻¹) was added into the flask with ultrasonic treatment for 20 min, and then 4 mL of 1 M KI was added slowly with stirring and ultrasonic treatment for 30 min. The CuI was isolated via centrifugation at a rate of 12,000 rpm for 15 min and then washed thoroughly and afterwards dispersed in alcohol for 3 times. A 1 mg amount of Gr was suspended in a mixture of anhydrous ethanol (0.25, 0.25, 0.25, 0.5, 2.5, 5, 7.5, and 10 mL) and 2 mg mL⁻¹ chitosan solution (0.75, 0.75, 0.75, 1.5, 7.5, 15, 22.5, and 30 mL) with ultrasonic treatment for 10 min. Then treated with ultrasound for 10 min after a certain volume of 1 M CuCl₂ (0.53, 2.63, 5.26, 26.00, 52.63, 79.00 and 105.00 μ L) was added into the hybrid and ultrasonic treatment for 12 h after KI (2.12, 10.6, 21.2, 106, 212, 318, and 421 μ L) was added. Eventually, the as-prepared CuI/Gr composites were obtained by centrifugation and stored at 4 °C.

2.3. Preparation of the modified electrodes

The as-prepared CuI/Gr nanocomposites were dispersed in 0.01% chitosan solution by ultrasonication for 1 h and developing to black suspension of 2 mg mL⁻¹. At first, glassy carbon electrode (GCE, 3 mm in diameter) was carefully polished with 0.05 μ m alumina slurry, and cleaned by ultrasonication in ultrapure water for 5 min to remove microscopic particles on the surface of the GCE. Then, a certain volume of suspension was poured on the surface of GCE as working electrode and allowed to dry in dryer for 2 h at 30 °C.

2.4. Cell culture

Human cervical cancer cell lines (HeLa), human hepatocarcinoma cells lines (HepG2) and human primary glioblastoma cell lines (U-87MG) were bought from the Institute of Hematology, Chinese Academy of Medical Sciences. Human embryo liver cell lines cells (L02) were obtained from the Third Military Medical University (Chongqing, China). The cells cultured in DMEM medium (high glucose, Gibco) containing 10% heat-inactivated fetal calf serum (Sigma, USA), 100 mg mL⁻¹ streptomycin (Sigma, USA) and 100 U mL⁻¹ penicillin (Sigma-Aldrich) at 37 °C with 5% CO₂ in a 95% humid atmosphere.

2.5. SECM studies

SECM was used to further explore and characterize the electrochemical catalytic activity of relevant sensitive interface of the Gr, CuI, and Gr/CuI, respectively, which was modified on graphite electrode (Figure S4d) as substrate electrodes [58]. A carbon fiber microelectrode ($\varnothing$, 7 ± 2 μ m) with glass-to-fiber ratio of 3.0 modified layer of Pt through CV in the range from -0.5 to +1 V at a scan rate of 50 mV s⁻¹ in 2 mM H₂PtCl₆ solution with PBS as the supporting electrolyte and the number of CV cycles was ten, which was used as SECM tip. The SECM cell consisted of four electrodes with the SECM tip as working electrode 1 (W 1), the sample being Gr, CuI, and Gr/CuI modified graphite rod as working electrode 2 (W 2), a Pt wire as counter electrode (C), and an Ag/AgCl as reference electrode (R), respectively.

2.6. Electrochemical detection of H₂O₂ released by cells

The cells suspension were separated from the culture medium by trypsinization (trypsin 0.025%) and centrifugation at 1500 rpm for 3 min and washed three times with the physiological PBS solution and then stored in PBS solution at 37 °C. The number of cells were estimated by using a cell counter. The amperometric i-t curve recorded the current response of adding AA and H₂O₂ in PBS at 0 V. The new configuration of AA 10 μ M was added into 10 mL of deoxygenated PBS until the current become stable. Afterwards 100 μ L cell suspension i.e. 1.2×10^7 cells were added until the current became stable again. The temperature of electrochemical cell was maintained at 37 ± 0.2 °C.

3. Results and discussion

3.1. Surface morphology and microstructure

Surface morphology and relevant microstructure of the modified electrodes were initially characterized by SEM. Chitosan has relatively strong adsorption ability for copper ion due to the existence of the lone pair electrons in nitrogen and oxygen atoms. Moreover, it can get adsorbed on the surface of graphene, which can help CuI to well adsorb on the graphene. Meanwhile, a small quantity of ethanol in feed material can eliminate the influence of solid product I₂ for relatively higher solubility in ethanol than in water, however, the excessive ethanol will cause reaction rate to become too fast. As shown in Fig. 1, SEM studies illustrate the general morphology of electrodes modified with different materials. Fig. 1a displays the surface of CuI which was surrounded by chitosan, forming an approximate spherical particle with the size about 600 nm. Due to the good physical adsorption of Gr, the CuI can readily settle on the surface of Gr. From Fig. 1b, it can be observed that there was a relatively big amount of CuI NPs covered and well distributed on the entire graphene surfaces to form the CuI/Gr nanocomposites. Furthermore, the XPS study confirmed valence states of CuI/Gr. The wide scan XPS spectrum of CuI/Gr in the Figure S1a presents the presence of Cu, I and C on the surface. The emerging C 1s arises from the Gr [59]. Figure S1b and S1c show that Cu 2p_{3/2} and I 3d_{5/2} have the binding energies of 932 and 619 eV, respectively, which are in agreement with those previously reported in the literature for CuI [60,61]. Moreover, no other byproduct, e.g., CuO, was found in Cu 2p spectra.

3.2. CuI/Gr composite modified electrodes

Cyclic voltammetry and electrochemical impedance spectrum (EIS) measurements are initially utilized to characterize the relevant CuI/Gr composites modified electrodes. Fig. 2a displays the typical Nyquist plots of the impedance of various electrodes containing GCE, CuI-GCE, Gr-GCE, and CuI/Gr-GCE recorded in a frequency (ω) ranges from 0.1 Hz to 100.0 KHz in PBS solution which including 0.1 M KCl and 5 mM [Fe(CN)₆]^{3-/4-}. The illustration of Fig. 2a displays equivalent circuit of the electrochemical cell system which involve charge-transfer resistance (R_s), Warburg impedance (Z_w), solution resistance (R_s) and double layer capacitance (C_d). Under high frequency, $(Z_{Re}-R_s-R_{ct}/2)^2 + (Z_{Im})^2 = (R_{ct}/2)^2$ (Z_{Re} was resistance of the circuit impedance and Z_{Im} generated for double layer capacitance impedance), and Warburg impedance contributes less to R_{ct} . Thus the R_{ct} can be directly deprived from the Nyquist plots, the values of bare and CuI modified GCE are 454 Ω and 3358 Ω respectively, and the values of R_{ct} for Gr and CuI/Gr modified GCE could not be accurately determined due to the fast kinetics of these two electrochemical system and small R_{ct} . This also reflect that chemical reaction kinetics of CuI combination on the surface of Gr

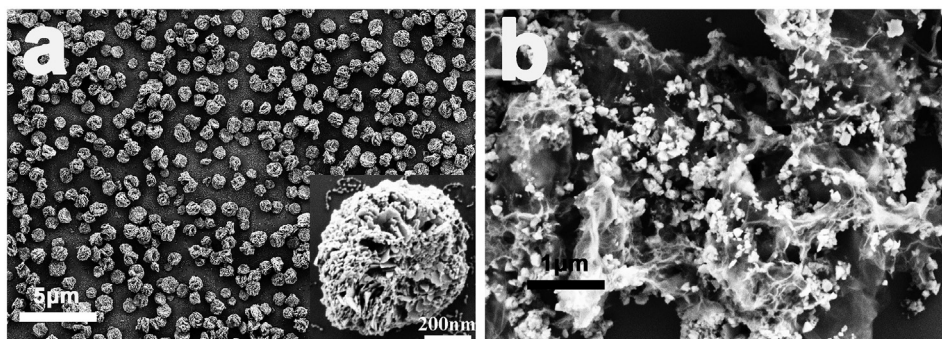


Fig. 1. SEM images of (a) CuI and (b) CuI/Gr nanocomposites.

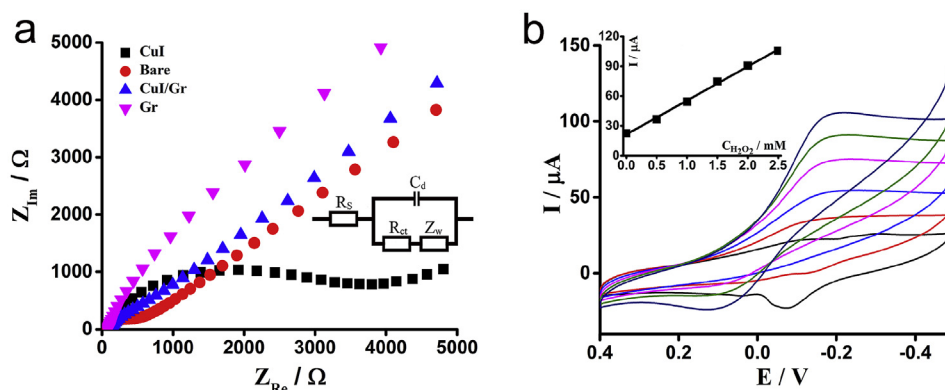


Fig. 2. (a) Nyquist plots at CuI, bare, CuI/Gr and Gr modified GCE in PBS solution with containing 0.1 M KCl and 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$. The frequency range is from 0.1 Hz to 100.0 Hz. (b) CVs of CuI/Gr-GCE in the absence and presence of different concentrations of H_2O_2 in PBS. Voltage range of 0.4 to -0.4 V and Scan rate: 50 mV/s. The illustration is the concentration of H_2O_2 and current linear relationship.

that becomes faster due to the excellent conductivity and large specific surface area of Gr and well catalytic activity of CuI.

Fig. 2b shows the typical CVs curves of CuI/Gr-GCE with various concentrations of H_2O_2 (0, 0.5, 1, 1.5, 2, 5 mM) between potential range of -0.5 – 0.4 V in PBS (pH = 7.2) solution. The illustration shows linear relationship between the reduction currents at -0.2 V and concentration of H_2O_2 . Since the concentration of H_2O_2 increases, the reduction current of negative (electric) potential will also increase, and it also exhibits a linear increase in current with the increase concentration of H_2O_2 , whereas the linear coefficient correlation R^2 was 0.998. It is evident that the electrochemical biosensor modified by CuI/Gr composites has smaller charge transfer resistance and higher reaction rate, and the sensing mechanism was similar to Cu_2O for the reduction of H_2O_2 [48]. In addition, our observations demonstrate that the CuI/Gr-GCE has excellent electrochemical response and linear relationship for different concentration of H_2O_2 , which provides the possibility for CuI/Gr nanocomposites modified glassy carbon electrode as a sensitive biosensor to detect H_2O_2 and relevant biological samples.

3.3. Optimization study of CuI/Gr nanocomposites modified electrodes

The applied potential plays a significant role in three-electrode cell amperometric i -t for CuI/Gr-GCE as biosensor to detect different concentration of H_2O_2 . To make a comparison, five different applied potentials in a range of -0.05 V– 0.05 V were selected as candidates in relevant experiments. As shown in Figure S2, it was observed that the increase in potential will

increase detection sensitivity and the maximum could be attained at 0 V and then going down to minimum at 0.05 V. The potential shows more obvious effect on negative potential than positive potential, 0 V was selected in the following experiments to avoid the interference of other substances under high negative potential.

Meanwhile, it was found that the ratio (mg mg^{-1}) of CuI and Gr, the volume (μL) of electrode modified materials, temperature and pH had significant effect on the sensitivity (Fig. 3). The sensitivity increases at first and afterwards gradually decreases with the increase of the relevant content of CuI (Fig. 3a). The rationale behind this may be attributed to the blending of Gr which possesses excellent electric conductivity and large specific surface area with CuI, which was a good catalyst but with poor conductivity. Thus the relevant electron transfer rate could be slowed down with the increasing amount of CuI in the blending Gr composites. Fig. 3b shows the optimization study of the as-prepared CuI/Gr nanocomposites (2 mg mL^{-1}) volume deposited on the GCE, showing that the modified quantity of 5 μL was an optimal for the relevant study.

Fig. 3c shows the relevant influence of temperature. The results manifested that the detection sensitivity of electrochemical system rises with the increase in temperature. At ambient, the reaction rate of H_2O_2 disproportion accelerated as temperatures increased. Since the detection of biological sample under conditions of 310.15 K, and the changes of sensitivity form 298.15 K–298.15 K was $4.35 \mu\text{A mM}^{-1}$, the influence of temperature on the experiment reaches to extremely significant level, therefore the control of temperature in the experiment was very important. Fig. 3d displays the influence of different pH (ranged of 3.0–8.0) for CuI/Gr-GCE to

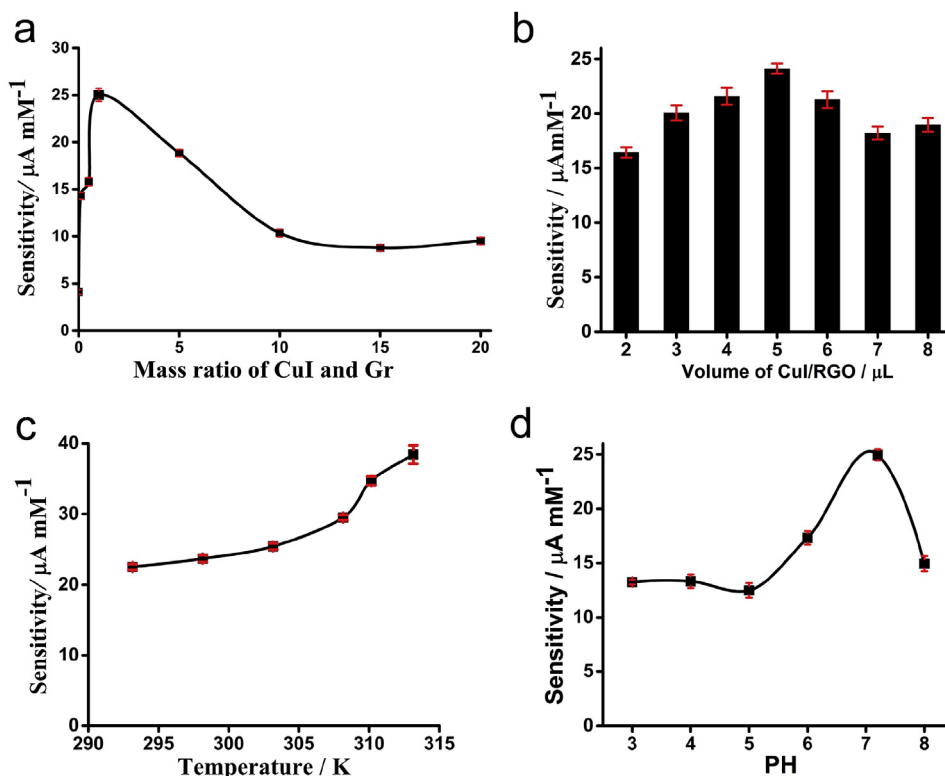


Fig. 3. Optimization study on the experimental conditions for the electrocatalytic reduction of H_2O_2 . (a) Optimization study of the mass ratio for CuI and Gr, (b) the CuI/Gr composite volume deposited on the GCE, and (c) the influence of different temperature, (d) effect of different pH values for CuI/Gr-GCE to detect H_2O_2 . Error bars are the standard error of the mean ($n = 3$ electrodes).

detect H_2O_2 . It was noticed that the sensitivity of H_2O_2 detection is relatively lower in the acidic environment and reached a maximum at $\text{pH} = 7.4$ which provides the possibility for biological applications. Based on the above results, it is apparent that the experimental conditions for the applied potential is 0 V, and the modified volume is 5 μL , whereas pH is 7.4, while the mass ratio of relevant CuI and Gr is 1 and the temperature is 310.15 K.

3.4. Amperometric response to H_2O_2 detection

Fig. 4 displays the amperometric response of CuI, Gr and CuI/Gr modified GCE under the optimization conditions to the successive addition of varying concentrations of H_2O_2 in PBS solution. It can be seen that CuI/Gr-GCE has enhanced amperometric response for

H_2O_2 detection compared with Gr-GCE and CuI-GCE (Fig. 4a). Only weak amperometric response to the addition of different concentrations of H_2O_2 was detected on the Gr-GCE and no obvious signal on the bare CuI modified in GCE, which correspond to the typical Nyquist plots of the impedance due to slower electron transfer rate. Owing to the excellent electrochemical catalytic activity of CuI/Gr composites to H_2O_2 , a new sensitive nonenzymatic sensor could be constructed for the detection of H_2O_2 . It is observed that this sensor responded quickly to the change in H_2O_2 concentration and achieved the steady-state current within 4 s after the injection of H_2O_2 . As illustrated in Fig. 4b, the corresponding calibration curve for the nonenzymatic H_2O_2 sensor demonstrates that the CuI/Gr-GCE has an excellent detection sensitivity, which also confirms the CuI/Gr-GCE can be used as high-sensitive electrochemical biosensor to

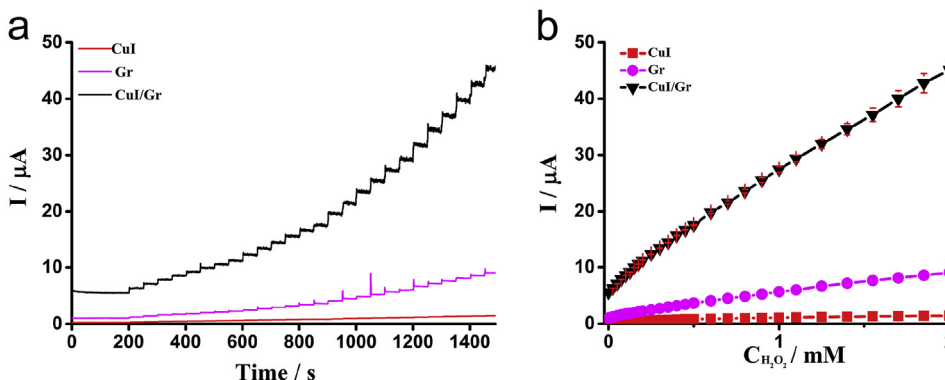


Fig. 4. (a) Amperometric $i-t$ curves of Gr-GCE, CuI-GCE and CuI/Gr-GCE in N_2 -saturated PBS at 0 V with successive addition of H_2O_2 . (b) Corresponding calibration curves of current versus H_2O_2 concentrations for Gr-GCE, CuI-GCE and CuI/Gr-GCE. Error bars are the standard error of the mean ($n = 5$ electrodes).

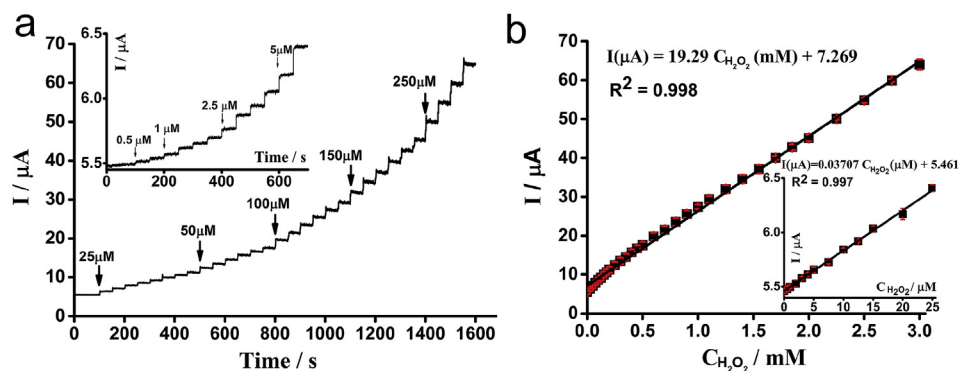


Fig. 5. (a) Amperometric *i*–*t* curves of CuI/Gr-GCE in N_2 -saturated PBS at 0 V with successive addition of H_2O_2 and which was added at the point indicated by arrows to the concentrations mentioned. (b) Corresponding calibration curves with H_2O_2 concentrations ranging from 0.5 to 25 μM and 0.025–3 mM.

Table 1
Comparison of the performance of various hydrogen peroxide sensors.

Electrode materials	Detection potential (V)	Linear range (μM)	Detection limit (μM)	Ref
Graphene-CdS nanocomposites	0.32 (vs Ag/AgCl)	5–1000	1.7	[63]
Porous graphene Pt nanocomposite	–0.1 (vs Ag/AgCl)	1–1477	0.5	[64]
Au nanoparticle (GNP)–dotted TiO_2 nanotubes	–0.4 (vs Ag/AgCl)	15–750	0.22	[65]
Cuprous sulfide nanoplates	–0.35 (vs. Ag/AgCl)	15–3750	1.3	[50]
Bimetallic Pt–M (M = Cu, Ni, Pd, and Rh) nanoporous	0.3 (vs. Ag/AgCl)	0–4000	12.2	[41]
Bimetallic Ag@Cu nanowires	–0.6 (vs. Ag/AgCl)	1000–10,000	10	[42]
Cu_2S nanoparticles/ordered mesoporous carbons	–0.1 (vs. Ag/AgCl)	1–3030	0.2	[51]
Cu_2O –reduced graphene oxide	–0.3 (vs. SEC)	30–12,800	21.7	[48]
Graphene/Au nanoparticles/toluidine blue O films	–0.3 (vs SCE)	5–25,362	0.2	[3]
CuI/Gr nanocomposite	0 (vs Ag/AgCl)	0.5–3000	0.2	this work

detect the reactive oxygen species.

Fig. 5 displays amperometric *i*–*t* curves of CuI/Gr-GCE in N_2 -saturated PBS at 0 V with successive addition of H_2O_2 0.025, 0.05, 0.1, 0.15 and 0.25 mM and corresponding calibration curves with H_2O_2 concentration. This illustrates another segment of CuI/Gr-GCE to detect lower concentration of H_2O_2 . The two segments in the relevant linear range for the detection of H_2O_2 are 5×10^{-7} – 2.5×10^{-5} M (correlation coefficient $R^2 = 0.998$ and sensitivity is $424.41 \mu A mM^{-1} cm^{-2}$) and 2.5×10^{-5} – 3×10^{-3} M (correlation coefficient $R^2 = 0.998$ and sensitivity is $273.04 \mu A mM^{-1} cm^{-2}$), whereas the detection limit of H_2O_2 is 0.2 μM (noise than the $S/N = 3$). A comparison of linear range, detection limit, and detection potential for CuI/Gr with other H_2O_2 sensors reported in the literature are shown in Table 1, which indicates that the analytical parameters for CuI/Gr are comparable and even better than those obtained at several electrodes reported

recently. Therefore, these results suggest that the CuI/Gr composites modified electrodes can be fabricated as a kind of excellent non-enzyme H_2O_2 electrochemical biosensor, characterizing with low detection limit, wide linear range and high sensitivity, moreover, the CuI as a common catalyst is very cheap and easy reach.

The tests of reproducibility and long-term stability are quite essential for CuI/Gr modified electrode to construct the biosensor. Five independent electrodes illustrate an excellent reproducibility with a standard deviation less than 2.3% for the sensitivity to H_2O_2 . When the as-prepared CuI/Gr-GCE is stored at room temperature, the sensitivity response H_2O_2 is found to decline 14.6% within one week (Figure S3a). Figure S3b illustrates the amperometric response of the relevant biosensor upon addition of 0.05 mM H_2O_2 , 0.2 mM GLU, 0.2 mM UA, 0.2 mM DA, 10 μM AA, and repeated injection of 50 μM H_2O_2 . The studies show that addition of GLU, UA and DA during the experiments have almost no obvious effect for

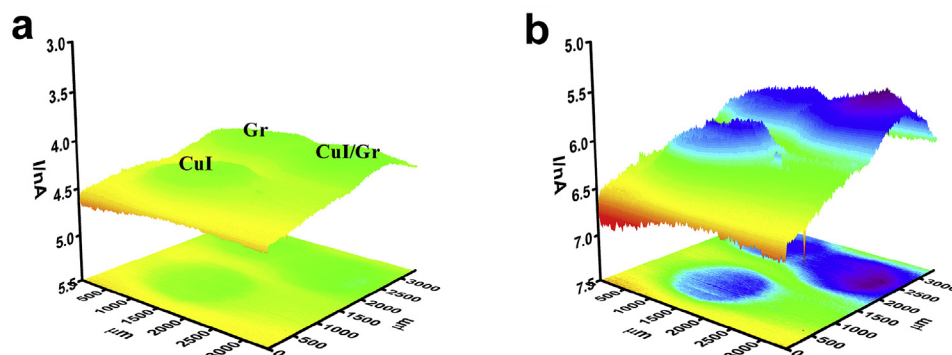


Fig. 6. Redox-competition mode of SECM imaging displaying the tip currents of H_2O_2 reduction over CuI, Gr and CuI/Gr in N_2 -saturated PBS at tip potential of -0.3 V and substrate potentials of (a) no applied voltage, (b) 0 V (vs. Ag/AgCl).

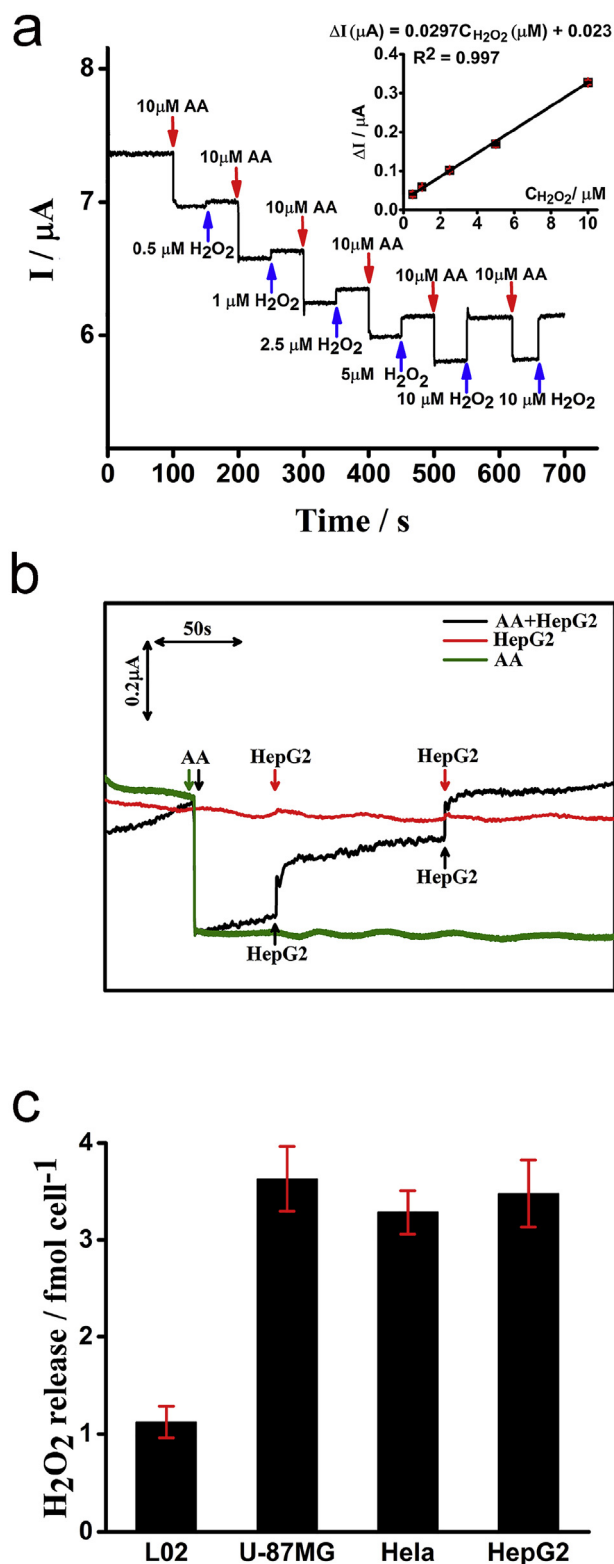


Fig. 7. (a) Amperometric *i-t* curves of CuI/Gr-GCE with successive addition of H_2O_2 and AA, and which was added at the point indicated by arrows to the concentrations mentioned. And corresponding calibration curves with H_2O_2 concentrations ranging from 0.5 to 10 μM . (b) Amperometric response upon addition of AA and HepG2 (black), HepG2 (red) and AA (green). (c) Amount of H_2O_2 released by L02, U-87MG, HeLa, and HepG2. The values are expressed as means $\pm$ SD of at least four independent measurements. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the relevant current response, and the current apparently decreased while adding AA again, which could be attributed to the good electrocatalytic activity of the Gr for AA at 0 V (vs Ag/AgCl) [62], and the CuI adsorption on the surface of Gr can further enhance the catalytic effect. Therefore, the results indicate that the electrochemical biosensor of CuI/Gr-GCE displays a very good stability and reproducibility, dual response to H_2O_2 and AA, with little effect on other substances.

3.5. SECM evaluation for H_2O_2 electrochemical catalytic reduction

The SECM has been used to further evaluate local electrocatalytic activity of relevant inter-surfaces of the modified electrodes. The electrochemical characteristic of microelectrode was initially evaluated by cyclic voltammetry in potassium ferricyanide solution, which was illustrated as s-shape in Figure S4a. As shown in Figure S4b, the relevant microelectrode had good response to different concentration of H_2O_2 , with deoxidization peaks at -0.3 V. Before scanning, the probe approach curve was recorded in N_2 -saturated PBS solution containing 1 mM H_2O_2 . It was noted that the H_2O_2 could be readily reduced on the surface of microelectrode at -0.3 V. Moreover, when the tip's proximity was approaching closely to the sample surface, the reduction current increased rapidly, displaying positive feedback effect (Figure S4c). The approach automatically stopped at a predefined increase in current response and then the tip risen 50 μm in order to reduce the risk of tip crash. Meanwhile, the tip currents of H_2O_2 reduction over CuI, Gr and CuI/Gr interface were similar to the base current in N_2 -saturated PBS at tip potential of -0.3 V without applying voltage to substrates (Fig. 6a), indicating that there are almost no catalytic activity for H_2O_2 without applying voltage. It was also observed that the reduction in current of H_2O_2 apparently decreased over the samples at substrate potentials of 0 V (Fig. 6b), suggesting that the amount of H_2O_2 in the gap between sample and tip was relatively small due to competitive effect of two electrodes. In addition, it was found that the concentration of H_2O_2 over CuI/Gr interface was significantly lower than other two groups at substrate potentials of 0 V, implying that much more H_2O_2 reduced on the surface of CuI/Gr nanocomposites modified electrode due to the relatively better electrochemical catalytic activity, when compared with that of CuI or Gr alone.

3.6. Measurements of H_2O_2 release from living cells

On the basis of the anti-interference study, it is evident that CuI/Gr-GCE has good response to H_2O_2 and ascorbic acid (AA), thus it is possible to detect ROS released from living cell by stimulation with AA [16,57]. However, AA itself can react with ROS, and the stability of ROS is very poor, therefore, the detection result of ROS released from living cell by dropping AA into the cells suspension will be lower due to dual influences from reducibility of ascorbic acid and poor stability of ROS. In this study we have fabricated a novel nonenzymatic H_2O_2 electrochemical biosensor that also has a response to AA. A certain amount of AA has been added in to the PBS solution and then added a certain amount of cell suspension, and the whole process is recorded by amperometric *i-t* curves. Fig. 7a shows the amperometric *i-t* curves of CuI/Gr-GCE with successive addition of AA and H_2O_2 . The response current decreased quickly ($0.3703 \pm 0.0438 \mu\text{A}$) when the PBS solution containing 10 μM AA (i.e., the concentration of AA was nonlethal to cells [57]), meanwhile, it apparently increased upon addition of H_2O_2 . The successive addition of AA again led to the decrease in relevant current, and the increment of current (ΔI) presents a liner relationship (correlation coefficient $R^2 = 0.998$) with the increase in H_2O_2 concentration. The results also indicate that the two

compounds do not affect each other in this electrochemical system. And there exists a good liner relationship between a series of concentrations of H_2O_2 and corresponding response currents even under interference of different concentration of AA. Therefore, through this strategy the evaluation of the oxidative stress of living cells could be performed with high sensitivity, which can also readily eliminate the interference of AA simultaneously.

Considering above results, three kinds of cancer cells (i.e., U-87MG, HepG2 and HeLa cancer cells) and normal hepatic cells (i.e., L02) were selected as relevant cellular models to investigate their oxidative stress under the stimulation of AA. Fig. 7b demonstrates the amperometric response for the related detection, and the arrows represent the sites of adding AA and relevant cells. It was found that approximately 3.479 ± 0.347 fmol H_2O_2 was liberated from one HepG2 cancer cell, and the efflux amounts of H_2O_2 from one L02 normal cell, U-87MG and HeLa cancer cell were 1.122 ± 0.163 fmol, 3.631 ± 0.335 fmol and 3.285 ± 0.022 fmol, respectively (Fig. 7c). It is evident that the cancer cells generated relatively higher ROS than that of normal cells, which may result in abnormal growth of the cancer cells or tumor tissues. Therefore, this raises possibility of the *in situ* quantitative detection of the flux of H_2O_2 from living cells and thus realizing the accurate evaluation of oxidative stress in target cells.

4. Conclusion

In summary, in this study we have fabricated a nonenzymatic H_2O_2 electrochemical biosensor based on CuI/Gr nanocomposites modified electrode. The results indicate that the as-prepared CuI/Gr modified GCE electrodes exhibit excellent electrochemical catalytic property and high sensitivity for rapid detection of H_2O_2 , with outstanding reproducibility, low overpotential of 0 V (vs. Ag/AgCl) as well as low detection limit, and a wide linear range from 0.5 μM to 3 mM. Meanwhile, the relevant nonenzymatic H_2O_2 biosensor could be readily utilized for the high-sensitive determination of H_2O_2 released from the living cells under stimulation of ascorbic acid to assessment of oxidative stress in different cells and eliminate the interference of AA simultaneously. Moreover, the redox-competition mode of SECM imaging also demonstrates improved electrochemical catalytic capability for H_2O_2 reduction of the as-prepared CuI/Gr deposits on graphite, which could be further utilized to exploit the potential single cell recognition in future clinical diagnostics.

Acknowledgments

This work is supported by National High Technology Research & Development Program of China (2015AA020502) and the National Natural Science Foundation of China (81325011, 21327902 and 21175020).

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.aca.2016.05.043>.

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