



Layer-by-layer assembly of graphene, Au and poly(toluidine blue O) films sensor for evaluation of oxidative stress of tumor cells elicited by hydrogen peroxide

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ABSTRACT

High level of oxidative stress is involved in formation of incipient tumor and carcinomatous cells. Here in this contribution we have explored a facile strategy to assess the oxidative stress elicited by hydrogen peroxide (H_2O_2) in cells with amperometric current–time technique *in vitro*. An electrochemical biosensor exhibiting high sensitivity and selectivity to H_2O_2 is fabricated by integration of graphene with gold nanoparticles and poly(toluidine blue O) films. The efflux of H_2O_2 from several representative tumor cells and normal cells on exposure to ascorbic acid could be detected by using the graphene-based nanocomposite films. The results indicate that tumor cells release much more H_2O_2 than do the normal cells. The novel sensor raises the possibility for clinical diagnostic application to evaluate the higher level of intracellular oxidative stress of tumor cells in comparison with normal cells.

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1. Introduction

Reactive oxygen species (ROS), functioning as important intracellular signaling molecules to regulate DNA damage, protein synthesis, cell apoptosis, and others, are generated in the aerobic organisms during their whole life (Halliwell et al., 2000a; Amatore et al., 2008; Ishikawa et al., 2008). But the excessive amount of ROS accumulation in cells leads to oxidative stress, which is involved in the etiology of cognitive dysfunction, myocardial infarction, atherosclerosis, alzheimer disease, autoimmune diseases, and cancer, and is a hazard to living organisms (Halliwell et al., 2000b; Trachootham et al., 2009; Pagliari et al., 2012). Hydrogen peroxide (H_2O_2), formed by disproportionation of unstable ROS superoxide ion (O_2^-), is distributed in most of the mammalian cells (Amatore et al., 2008). It is one of the major contributors to the oxidative stress damage for its long lifetime to diffuse to other cellular compartment (Amatore et al., 2008; Trachootham et al., 2009). Mounting evidence suggests that an increase in ROS generation is associated with the development of incipient tumor and persistent

high oxidative stress existing in carcinomatous cell (Szatrowski and Nathan, 1991; Toyokuni et al., 1995; Lau et al., 2008; Trachootham et al., 2009; Visconti and Grieco, 2009). Quinone-containing anti-neoplastic agents killing tumor cells are partly related to production of large amount of H_2O_2 and hydroxyl radical (Doroshov, 1986). Ascorbic acid (AA) selectively kills cancer cells by formation of H_2O_2 (Chen et al., 2005). Thus real-time monitoring of the level of intracellular H_2O_2 is of great value in elaborating its regulation of signal transduction pathways and searching for new therapeutic strategies for cancer and other diseases (Trachootham et al., 2009).

Several methods have been proposed for quantitative detection of cellular generation of ROS, such as electrochemical analysis (Amatore et al., 2008; Luo et al., 2009; Wu et al., 2011a), electron spin resonance (Brandes and Janiszewski, 2005), chemiluminescence (Wu et al., 2011a) and fluorescence analysis (Doroshov, 1986; Szatrowski and Nathan, 1991). Electrochemical amperometry could offer extracellular noncontact and label-free detection with spatial and temporal resolutions. Initially, the researches in this domain have focused on the fabrication of microelectrodes with appropriate size for investigating the vesicular exocytosis, oxidative stress, neurotransmitter and nitric oxide metabolism at the single cell level (Amatore et al., 2008). Recently, the biosensors based on immobilization of peroxidase onto nanomaterials modified electrode for determination of hydrogen peroxide released by

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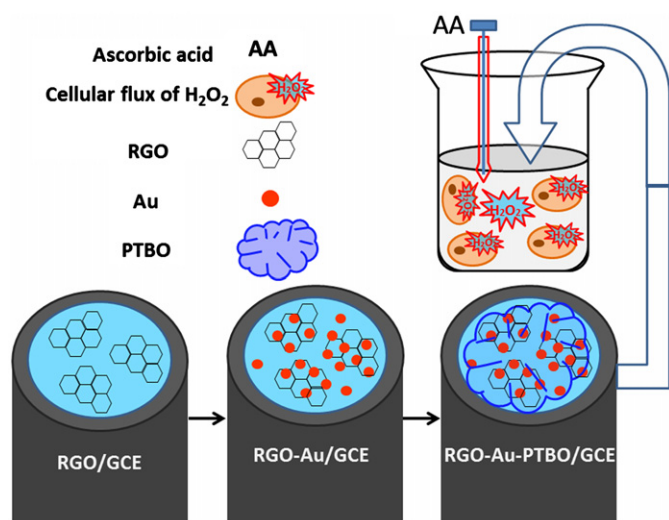


Fig. 1. Schematic of the LBL assembly of RGO–Au–PTBO modified GCE used for detect H_2O_2 efflux from cells stimulated with AA.

tumor and neutrophils *in vitro* have been realized (Luo et al., 2009; Wu et al., 2011b).

Reduced graphene oxide (RGO), with single plane of sp^2 carbon atom networks, exhibits high electrical conductance at room and cryogenic temperatures (Geim, 2009). Numerous studies have shown that gold nanoparticles (Au NPs) possess excellent catalytic ability to H_2O_2 and efficiently improve the electron transfer between analyte and electrode (Peng et al., 2009; Chang et al., 2011). On the other hand, poly(toluidine blue O) (PTBO) films not only provide matrix to enclose the Au NPs, but also prevent some opposite-charged interferences from permeating into the surface of electrode (Sasso et al., 1990). Herein, we have employed the layer-by-layer (LBL) electrochemical deposition technique to modify Au NPs and PTBO films onto the graphene simultaneously to fabricate a RGO–Au–PTBO modified electrode (Fig. 1). Each component layer is characterized by scanning electron microscopy (SEM) and energy diffraction spectrum (EDX). Reaction kinetics of the modified electrode is studied with cyclic voltammetry and electrochemical impedance spectroscopy (EIS). Response of the electrochemical sensor to H_2O_2 in the presence of interfering substance appearing in culture or secretion of cells is examined.

Even if high level of oxidative stress occurs in tumor cells, the quantitative data in this respect have not been reported. In this article, electrochemical sensor based on the RGO–Au–PTBO modified electrode is utilized for detection of H_2O_2 released by cells during their redox homeostasis disrupted by AA (Fig. 1). The intracellular concentration of H_2O_2 could be evaluated by referring to the amount of efflux. Furthermore, analysis on the efflux of H_2O_2 from tumor and normal cell lines proclaims that the level of intracellular oxidative stress elicited by H_2O_2 of tumor cell is much higher than that of normal cell.

2. Experimental

2.1. Reagents and materials

The synthesis of reduced graphene oxide (RGO) was achieved by referring to the procedures in previous works (Li et al., 2008; Marcano et al., 2010). Simply, graphene oxide was reduced by mixture of hydrazine (35 wt%) and ammonia (28 wt%) aqueous solutions at a volume ratio of 10:70, under vigorous shaking and

stirring (95 °C for 1 h). $\text{HAuCl}_4 \cdot 4\text{H}_2\text{O}$, toluidine blue O (3-amino-7-dimethylamino-2-methylphenothiazinium chloride, TBO), catalase (from bovine liver, lyophilized powder, 2000–5000 U/mg), hydrogen peroxide solution (H_2O_2 , 30%), AA, UA, Glu and DA were all purchased from Sigma-Aldrich. All other reagents are of analytical grade. Phosphate buffer saline (PBS) was used as the supporting electrolyte. Ultra-pure water (Millipore-Q, $\geq 18.2 \text{ M}\Omega \text{ cm}^{-1}$, 25 °C) was used in aqueous solution.

Field-emission scanning electron microscopy (FE-SEM; Zeiss, Germany) and energy dispersive X-ray spectrometry (EDX) were utilized to characterize the electrochemical deposition modification of Au nanoparticles (NPs) and PTBO film. Cyclic voltammetry (CV) and amperometric i - t were performed on a CHI660B electrochemical workstation (CHI Incorporation, USA), using a Pt wire as the counter electrode, a saturated calomel electrode (SCE) as the reference electrode, and a 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) using the above three-electrode system. As for the oxygen free electrochemical assay, buffer solution was purged with high purity nitrogen for at least 30 min prior to experiments and the nitrogen airflow was used to prevent oxygen from reaching the solution.

2.2. Electrodeposition modification of Au and PTBO

Bare GCE was carefully polished on a soft lapping pad (Buehler, USA) with 0.3 and 0.05 μm alumina slurries, and followed by ultrasonication cleaning in ethanol and double distilled water for 5 min respectively. As-synthesized RGO was dispersed in 1 mg mL^{-1} chitosan (dissolved ultrasonically in 1% acetic acid of pH 4.8) to form stable graphene dispersions. The RGO modified GCE was prepared by casting 4.5 μL of the RGO dispersions (ca. 4.5 μg RGO) on the surface of the electrode and drying for 2 h at room temperature in a desiccator. CV was employed for the electrodeposition of Au NPs and the electropolymerization of PTBO film onto the bare and RGO modified GCE. Electrodeposition of Au NPs was conducted in PBS (0.05 M, pH 6.8) solution containing 0.2 mM HAuCl_4 and 100 mM KCl from -0.3 to $+1$ V. Electropolymerization of TBO was conducted in PBS (0.05 M, pH 6.8) solution containing 2 mM monomer from -0.5 to $+1$ V. The scan rates for both are 50 mV/s.

2.3. Cell culture

K562 (human leukemia cell), PC12 (rat adrenal medulla pheochromocytoma) and HepG2 (human hepatocarcinoma) cells were obtained from Institute of Hematology, Chinese Academy of Medical Sciences. L02 (human embryo liver cell) and RSC (rat synovial cell) cells were obtained from Third Military Medical University (Chongqing, China). They were maintained in DMEM (high glucose, Gibco) medium supplemented with 10% heat-inactivated fetal calf serum (Sigma, USA), 100 U/mL penicillin (Sigma, USA), and 100 mg mL^{-1} streptomycin (Sigma, USA) at 37 °C with 5% CO_2 in a 95% humidified atmosphere.

2.4. Trypan blue staining of cells

The cells, after treatment with AA, were washed two times with PBS (0.05 M, pH 7.2) solution. 0.2 mL of 0.4% trypan blue stain was added to 1 mL of the cell suspension ($\sim 1 \times 10^5$ cells per mL) with thorough mixing. After allowing it to stand for 8 min at 20 °C, an aliquot of 10 μL mixture of cells suspension filled a hemocytometer for counting.

2.5. Electrochemical detection of H_2O_2 released by cells

Cells were separated from culture medium by 5 min of centrifugation at 1300 rpm, and washed three times with PBS (0.05 M, pH 7.2) solution. Cell number was estimated by a hemocytometer. While the current decreases to a level less than 20 nA, AA was injected to the cells suspension. The amperometric current response of flux of H_2O_2 of about 1×10^6 cells in 1 mL deoxygenated PBS was recorded at RGO–Au–PTBO modified GCE at -0.3 V (vs. SCE).

3. Results and discussion

3.1. Layer-by-layer assembly of the RGO–Au–PTBO modified electrode

Well dispersed RGO in chitosan could strengthen the mechanical tensile strength of the composites film. The robust RGO film covered GCE is convenient for further modification (Chang et al., 2011). In order to integrate Au NPs and PTBO film with RGO, layer-by-layer (LBL) electrodeposition is employed for immobilization of these two components onto RGO. The reduction of chloraurate ion (AuCl_4^-) takes place at ca. 0.45 V (vs. SCE) during electrodeposition (Fig. S1). Au NPs with average diameter about 80 nm uniformly distribute on the surface of RGO sheets, as confirmed by the SEM image (Fig. 2B) and EDX analysis (Fig. S2). Amino group of TBO species is oxidized at potentials higher than +0.9 V, followed by radical dimerization via carbon–nitrogen coupling reactions to form PTBO (Zhou et al., 1998; Yao and Shiu, 2007). Gradual increases in

the peak current at +0.12 V for the positive scan are observed during continuous cycling (Fig. S3), indicating formation of the electropolymerized PTBO film on the RGO–Au modified GCE (Fig. 2C). The amount of Au NPs and PTBO is controlled by the number of CV scanning cycles. We examine the electro-catalytic response of various deposition amounts of Au and PTBO modified RGO electrode to 0.5 mM H_2O_2 . It is suggested that 6 segments of Au together with 20 segments of PTBO deposition under the given condition show optimal response current (Fig. S4).

The RGO–Au–PTBO modified GCE is characterized by two quasi reversible waves (Fig. 2D), corresponding to the functionalities of PTBO. The redox couple at more negative potentials was related to the redox reaction of the heterocyclic nitrogen atom of monomer TBO while the other couple was related to the redox reaction of the nitrogen bridges between monomer units (Zhou et al., 1998; Yao and Shiu, 2007). Fig. 2E depicts the CVs of RGO–Au–PTBO modified GCE in pH 7.4 phosphate buffer saline at different scan rates. The dependence of peak currents of two couples on scan rates is linear (i.e., linear correlation coefficient $R^2 \geq 0.999$), which indicated a surface controlled process at this modified electrode. EIS studies were implemented in order to evaluate the electron-transfer kinetics of the successive layers of modified GCE. Fig. 3 displays the typical Nyquist plots of the impedance of various electrodes recorded in a frequency range of 0.1 Hz – 100.0 kHz in PBS (0.05 M, pH 7.2) containing 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and 0.1 M KCl. Inset of Fig. 3 shows the equivalent circuit for this system. Electron-transfer kinetics such as charge transfer resistance (R_{ct}), solution resistance (R_s), double layer capacitance (C_d) and a mass transfer element W (Warburg impedance) could be extracted by the EIS data fitting. A well-defined quasi-semicircle portion is observed at the Nyquist

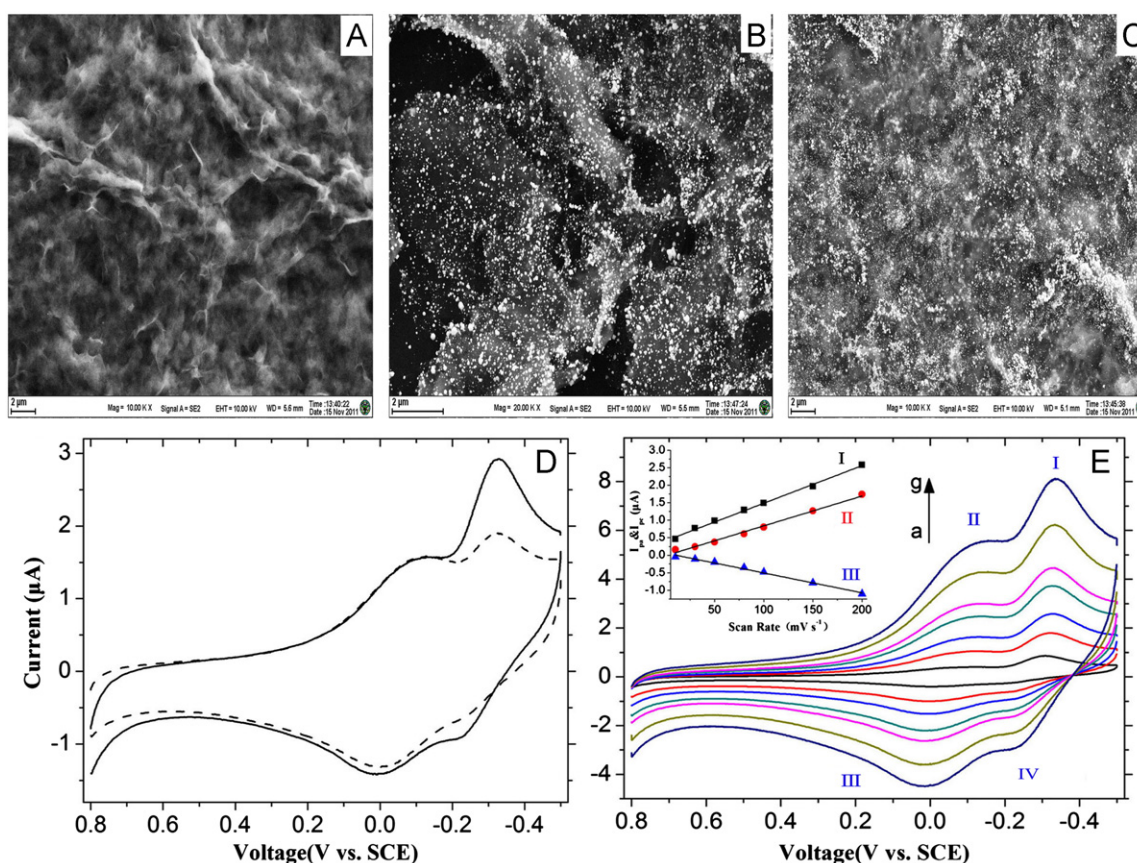


Fig. 2. SEM micrographs of RGO (A), RGO–Au (B), and RGO–Au–PTBO (C) modified GCE. The image (A) shows the graphene-wall structure; (B) illustrates that the Au NPs mainly distribute on the plane of graphene; and (C) manifests the presence of outermost PTBO film (accelerating voltages: 10 kV). CV curves of RGO–Au–PTBO modified GCE (D) in the presence (solid line) and absence (dash line) of 0.5 mM H_2O_2 in N_2 -saturated PBS (0.05 M, pH 7.2), scan rate 50 mV/s; (E) at different scan rates (a–g: 10, 30, 50, 80, 100, 150, 200 mV/s) in N_2 -saturated PBS (0.05 M, pH 7.2). Inset of (E) shows the calibration plots of the peak current of I, II, III vs. scan rate.

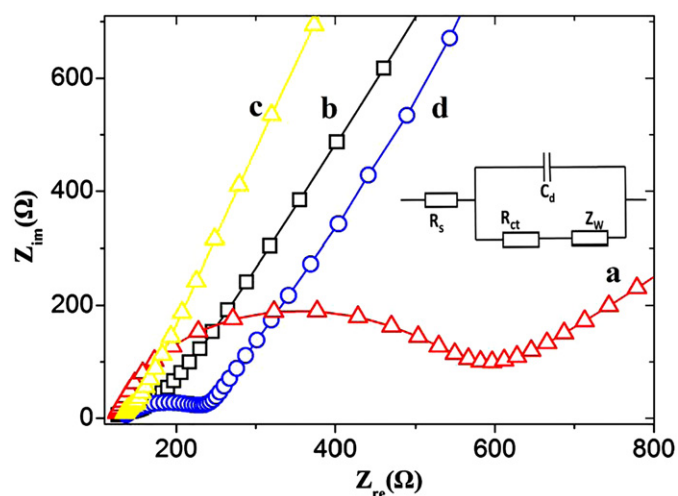


Fig. 3. Nyquist plots at bare (a), RGO (b), RGO-Au (c), and RGO-Au-PTBO (d) modified GCE in PBS solution containing 5 mM $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and 0.1 M KCl. The frequency range is from 0.1 Hz to 100.0 kHz. The equivalent circuit is shown in the right inset.

plots of bare and RGO-Au-TBO modified GCEs, with R_{ct} values of 520 and 150 Ω respectively. As for the plots of RGO and RGO-Au modified GCE, an ill-defined semicircle and a well-defined linear portion within most of the Z_{re} range were observed. This suggested that their R_{ct} is negligible, i.e., the heterogeneous reactions at the RGO or RGO-Au interface are so kinetically facile that mass transfer in solution plays a significant role.

3.2. Electrocatalysis of RGO-Au-PTBO film to hydrogen peroxide

RGO has been previously employed for the electro-catalytic reduction of hydrogen peroxide (Zhou et al., 2009). However, the amperometric sensor of graphene for H_2O_2 held a narrow linear range and sensitivity to the other electroactive compounds. It has been reported that the linearity of the current response could be extended by coverage of a specific membrane possessing high diffusion limitation with respect to the substrate (Cocheguerente et al., 1994). Besides, the polymer film could block some interfering species from access to electrode surface. So we explored the LBL assembly of Au NPs and PTBO film onto RGO with electro-deposition method to form sandwich-like structure for improved performance of RGO based sensor. Fig. 2D shows the CV response of H_2O_2 at RGO-Au-TBO films modified GCE. The cathodic current at -0.3 V increased by 241% in the presence of 0.5 mM H_2O_2 compared to that without H_2O_2 , demonstrating the efficient catalytic effect of RGO-Au-TBO film toward the electrochemical reduction of H_2O_2 .

Amperometric response to the change of H_2O_2 concentration was surveyed to illustrate the superior biosensor performance of RGO-Au-PTBO composites film. Fig. 4A displays amperometric traces recorded at RGO, Au-PTBO and RGO-Au-PTBO modified GCE that was polarized at -0.3 V in a stirred deoxygenated PBS (0.05 M, pH 7.2), spiked with gradual increase of aliquots of H_2O_2 stock solution. In the concentration range of 0.01–1 mM, the current response at RGO approximated to that of RGO-Au-PTBO modified GCE, while the Au-PTBO modified GCE generated little response. This denoted that RGO in the composite film was highly sensitive to H_2O_2 for its unique electron transfer features and edge plane graphite structures. As depicted in the Raman scattering spectrum (Fig. S5), the sharp intensity enhancement at the D peak sites (1350 cm^{-1}) of RGO compared with the raw graphite flakes demonstrated the reduction of graphene oxide and the presence of abundant defects on the RGO (Chang et al., 2011).

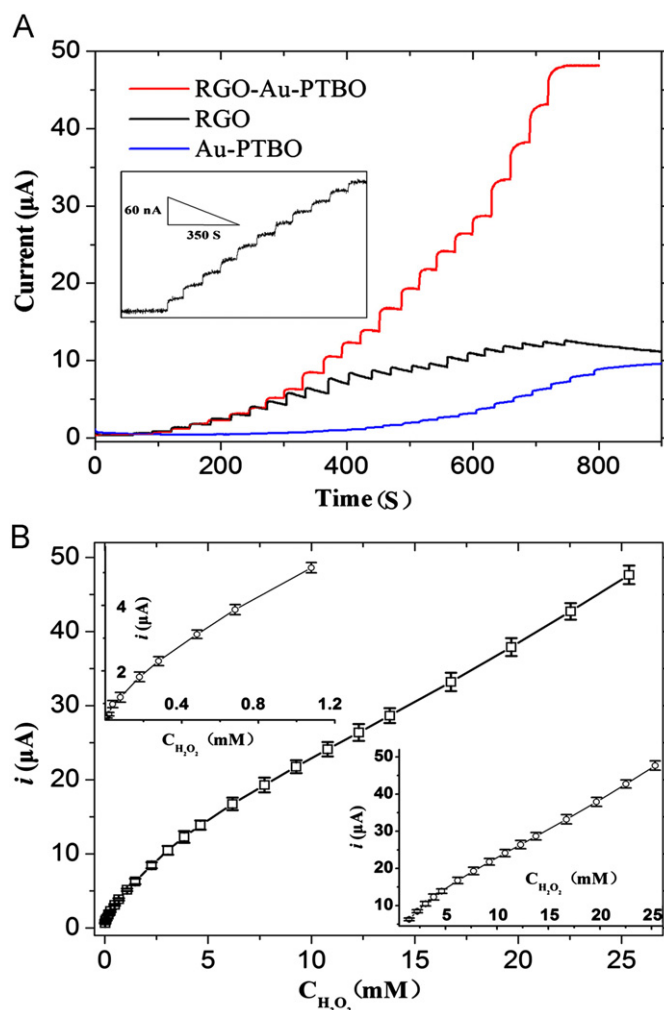


Fig. 4. (A) Amperometric $i-t$ curves of Au-PTBO (blue), RGO (black), and RGO-Au-PTBO (red) modified GCE in N_2 -saturated PBS (0.05 M, pH 7.2) at -0.3 V with successive addition of H_2O_2 . Inset: $i-t$ curve shows the response of RGO-Au-PTBO modified GCE to addition of aliquots of 0.2 μM H_2O_2 . (B) Corresponding calibration curves for concentrations of H_2O_2 from 5 μM to 25.362 mM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

When the concentration of H_2O_2 exceeded 1 mM, the current response got unstable on RGO modified GCE and decreased rapidly to 60% of the maximum in 30 s. In contrast, the enduring catalytic current at the RGO-Au-PTBO modified GCE was retained. Moreover, the amperometric current density at RGO-Au-PTBO was 5 times greater than that of Au-PTBO modified GCE. It is inferred that the electro-catalysis of Au-PTBO toward the reduction of H_2O_2 was augmented by the RGO substrate (Fig. 4A).

In view of the above observations, it is evident that H_2O_2 sensor based on the LBL assembly of RGO-Au-PTBO film can greatly extend the range of linear response. The linear range for detection of H_2O_2 at the applied potential of -0.3 V contained two segments: 5.0×10^{-6} – 1.0771×10^{-3} M (correlation coefficient $R=0.992$ ($n=8$), with sensitivity of $63.39 \pm 1.56\text{ }\mu\text{A mM}^{-1}\text{ cm}^{-2}$) and 1.4745×10^{-3} – 2.5362×10^{-2} M ($R=0.992$ ($n=15$), with sensitivity of $24.52 \pm 2.30\text{ }\mu\text{A mM}^{-1}\text{ cm}^{-2}$) (Fig. 4B). The sensors responded very rapidly and attained 96% of the steady-state currents in less than 2 s over the calibration range (Fig. 4A). Some reports claimed that the multi-layer modified electrode would diminish the sensitivity to the analytes (Cocheguerente et al., 1994). Owing to the optimal amount of different components in the composite films and

electro-catalytic properties of the outer layer PTBO to H_2O_2 , this loss of sensitivity was avoided at the RGO–Au–PTBO films modified electrode. A low value of limit of detection (LOD) for the sensor is $0.2 \mu\text{M}$ (ratio of signal to noise $S/N=5$; inset of Fig. 4A), which matched that of nitrogen-doped graphene ($0.05 \mu\text{M}$, $S/N=3$; Wu et al., 2011b). Consequently, the RGO–Au–PTBO films based sensor was competent in real-time monitoring of the flux of H_2O_2 from cells.

The potential interference of electroactive compounds in physiological fluid at the RGO–Au–PTBO films electrode was extensively examined. Fig. 5 illustrated the amperometric response of the sensor upon addition of 0.3 mM H_2O_2 , 1 mM dopamine (DA), AA, glucose (Glu), uric acid (UA) and a second 0.3 mM H_2O_2 , successively. DA, AA, Glu, and UA yielded little current response at the RGO–Au–PTBO films modified electrode under the applied negative potential. The current response of 0.3 mM H_2O_2 was also not affected in the presence of the other compounds. The low potential (-0.3 V) applied for detection and ingenious design of the modified electrode (the outer PTBO films) endowed the sensor with high selectivity.

Long-term stability and reproducibility is quite essential for nanomaterial modified electrode to construct the sensor. The LBL assembly fabrication of four independently modified electrodes

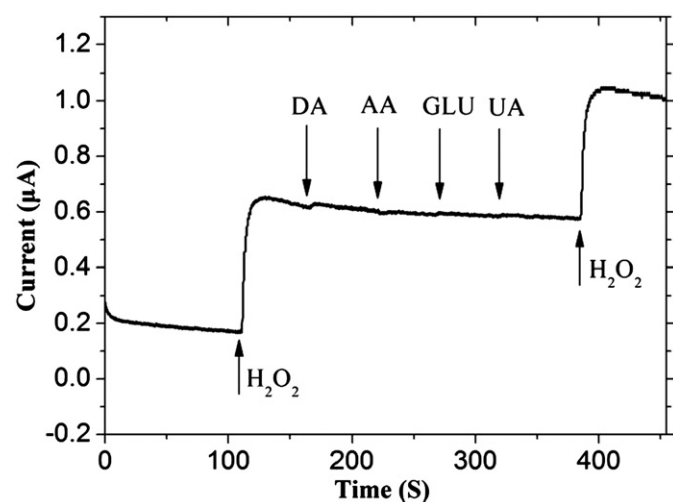


Fig. 5. Amperometric i - t response of RGO–Au–PTBO modified GCE at -0.3 V in PBS to successive addition of 0.3 mM H_2O_2 , 1.0 mM DA, 1.0 mM AA, 1.0 mM GLU, 1.0 mM UA, and a second 0.3 mM H_2O_2 .

showed an acceptable reproducibility with a deviation less than 5.0% (RSD) for the CV response to 0.5 mM H_2O_2 . When the as-prepared RGO–Au–PTBO modified GCE was stored in airtight and dark spaces, the CV response to 0.5 mM H_2O_2 declined by ca. 5% within 2 months (data not shown).

3.3. Electrochemical determination of H_2O_2 flux from tumor and normal cells

Intracellular H_2O_2 could be generated from the metabolism of oxygen radical, oxidase enzymes catalytic reaction, etc. (Halliwell et al., 2000b; Trachootham et al., 2009; Auchincloss et al., 2012). The concentration of H_2O_2 keeps a value favored for cellular proliferation. The way to maintain redox homeostasis inside cells is implemented not only by catabolism but also by excretion of H_2O_2 (Liu and Zweier, 2001; Bienert et al., 2006). Artificial stimulation is exerted on cells to disrupt intracellular redox homeostasis, which leads to efflux of H_2O_2 from cells. As discussed above, AA motivates cell generation of large amount of H_2O_2 , and can be chosen as the stimulus. Fig. 1 shows the scheme of electrochemical determination of cellular flux of H_2O_2 on exposure to AA by using the RGO–Au–PTBO modified GCE. We select 3 kinds of tumor cell lines and 2 kinds of normal cell lines as model, i.e., human leukemia cells K562, human hepatoma cell HepG2, rat adrenal medulla pheochromocytoma PC12, human hepatocyte line L02, and rat synovial cell RSC.

First of all, a control experiment was designed to certify that the efflux from cells stimulated with AA is hydrogen peroxide. In the previous discussion, AA produced no response at the RGO–Au–PTBO films modified electrode under the applied potential -0.3 V (Fig. 5 and curve c in Fig. 6A). When AA was added to PBS solution containing 1×10^6 cells, the current increased sharply in 7 s to a peak value, and then decreased to ca. 10% of the maximum in 20 s (curve a, Fig. 6A). However, if 200 U/mL catalase was added to the PBS in advance, the current remained nearly constant (curve b, Fig. 6A). This observation is in agreement with that reported in the literature (Liu and Zweier, 2001). Since catalase is known to specifically disintegrate H_2O_2 (Chen et al., 2005; Wu et al., 2011b), the instant current response should be attributed to the fast cellular secretion of H_2O_2 upon the stimulation of AA. Moreover, the current response or amount of flux of H_2O_2 increased upon increasing concentration gradient of AA. An initial concentration of $0.5 \mu\text{M}$ AA could induce detectable current signal. The current increased nonlinearly with the concentration of AA (Fig. S6). When 1×10^6 K562 cells are treated with $4 \mu\text{M}$ AA, the flux of H_2O_2 is estimated to be about $3 \mu\text{M}$.

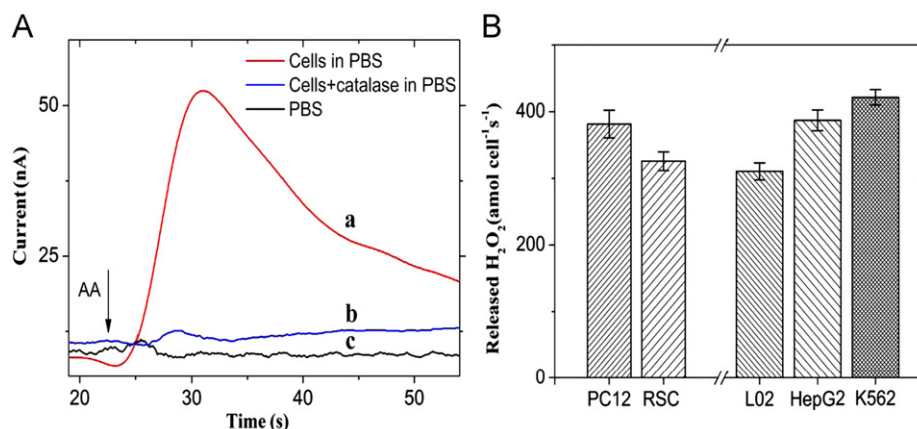


Fig. 6. (A) Groups of control experiments are designed to verify the efflux of H_2O_2 from cells stimulated by AA. Current response of RGO–Au–PTBO modified GCE upon addition of AA at -0.3 V in (a) PBS containing 1×10^6 K562 cells, (b) PBS containing 1×10^6 K562 cells and 200 U/mL catalases or (c) PBS. (B) Amount of H_2O_2 released by PC12, RSC, L02, HepG2, and K562 cell lines stimulated by $4 \mu\text{M}$ AA. The values are expressed as means $\pm$ SD of at least three independent measurements.

This level of H_2O_2 causes no cytotoxicity to almost all cells (Halliwell et al., 2000b; Clement et al., 2001; Chen et al., 2005). Trypan blue staining reveals that above 99.9% cells survive, with intact cell membrane after interaction with $4\text{ }\mu\text{M}$ AA for 1 h. Therefore, in subsequent treatment of tumor and normal cells, $4\text{ }\mu\text{M}$ AA is applied.

The efflux amount of H_2O_2 from K562, HepG2, L02, PC12, and RSC cell lines are analyzed with the RGO–Au–PTBO film modified GCE. According to the amperometric current response to the standard sample, the calibration equation between the current (I) and H_2O_2 concentration (C) is expressed as follows:

$$I(\text{nA}) = 9.5889 + 30.0328C (\mu\text{M})$$

The H_2O_2 of cellular efflux may re-enter into cells or be scavenged by secretion of enzyme (Lopez-Lazaro 2007; Trachootham et al., 2009); the peak value of current response indicates the maximum of H_2O_2 efflux. As for normal cell lines (L02 and RSC), the H_2O_2 amounts were calculated to be $\sim 310.43 \pm 22.76$ and $325.69 \pm 23.95\text{ amol cell}^{-1}\text{ s}^{-1}$, respectively, with no significant difference between these two groups. It is clear that all 3 kinds of tumor cell lines released more H_2O_2 than did normal cell lines (Fig. 6B). For example, the amount of H_2O_2 by hepatic tumor cells (HepG2) was $\sim 25\%$ more than that of the normal hepatic cells (L02). The maximum efflux of H_2O_2 was acquired by K562 cell line, which reached $421.46 \pm 29.70\text{ amol cell}^{-1}\text{ s}^{-1}$.

Two potential hypotheses have been deduced for the production and distribution of H_2O_2 in cells stimulated by AA. One claims that the tumor and normal cells may generate equal quality of H_2O_2 in response to AA. Accordingly more amount of H_2O_2 flux from tumor cells reflected a decline in enzymatic ROS-scavenging capacity. An alternative viewpoint argues that the tumor cells generated more H_2O_2 than normal cell lines, as a result of an elevation of ROS production capacity in abnormal growth of tumor (Halliwell et al., 2000b; Trachootham et al., 2009). On the other hand, the transport routine of H_2O_2 through the cell membranes is fundamental for understanding of its distribution. It is acknowledged that H_2O_2 diffuses freely through the lipid bilayer of membrane on the basis of early studies. However, current researches reported that H_2O_2 permeates rapidly across biomembranes but the permeation is limited by some channel proteins, like aquaporins (Bienert et al., 2006, 2007). In either case, its diffusion from high to low concentration regions is well-recognized. When intracellular redox homeostasis was interrupted by artificial stimulation, the extracellular and intracellular concentrations of H_2O_2 were regulated to remain at the same level. Thus the amount of flux of H_2O_2 supplied a reference for evaluation of oxidative stress in cells. For instance, as verified in this work, the level of oxidative stress elicited by H_2O_2 in hepatic tumor cell is 25% higher than that of its normal cell.

4. Conclusion

In summary, an LBL assembly of Au NPs and PTBO films onto graphene modified electrode was fabricated by using the electro-deposition technique. Each layer in the composites film exhibited enhanced catalytic effect toward electro-reduction of H_2O_2 . The low potential (-0.3 V) for detection and PTBO films rendered the sensor adequate selectivity for quantitative detection of flux of H_2O_2 from cells. The real-time monitoring of H_2O_2 released by cells during their disturbed redox homeostasis was achieved with the sensor. The observations of the efflux of H_2O_2 indicated the high level of H_2O_2 existing in tumor cells and demonstrated the quantitative evaluation of intracellular oxidative stress elicited by H_2O_2 of tumor cells.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2012.10.001>.

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