

A highly sensitive electrochemical biosensor for evaluation of oxidative stress based on the nano-interface of graphene nanocomposites blended with Au, Fe₃O₄ and Pt nanoparticles

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ABSTRACT: The high level of H₂O₂ is pertaining to high oxidative stress and associated with cancer, autoimmune and neurodegenerative disease, and other related diseases. In this study, a sensitive H₂O₂ biosensor for evaluation of oxidative stress was fabricated on the basis of the reduced graphene oxide (RGO) nanocomposites decorated with Au, Fe₃O₄ and Pt nanoparticles (RGO/AuFe₃O₄/Pt) modified glassy carbon electrode (GCE) and used to detect the released H₂O₂ from cancer cells and assess the oxidative stress elicited from H₂O₂ in living cells. Electrochemical behavior of RGO/AuFe₃O₄/Pt nanocomposites exhibits excellent catalytic activity towards the relevant reduction with high selection and sensitivity, low overpotential of 0 V, low detection limit of ~ 0.1 μM, large linear range from 0.5 μM to 11.5 mM, and outstanding reproducibility. The as-prepared biosensor was applied in the measurement of efflux of H₂O₂ from living cells including healthy normal cells and tumor cells under the external stimulation. The results display that this new nanocomposites-based biosensor is a promising candidate of

nonenzymatic H₂O₂ sensor which has the possibility in the application of clinical diagnostics to assess oxidative stress of different kinds of living cells.

KEYWORDS: hydrogen peroxide, graphene nanosheets, gold nanoparticles, ferroferric oxide nanoparticles, Pt nanoparticles, electrochemical detection, living cell

1. INTRODUCTION

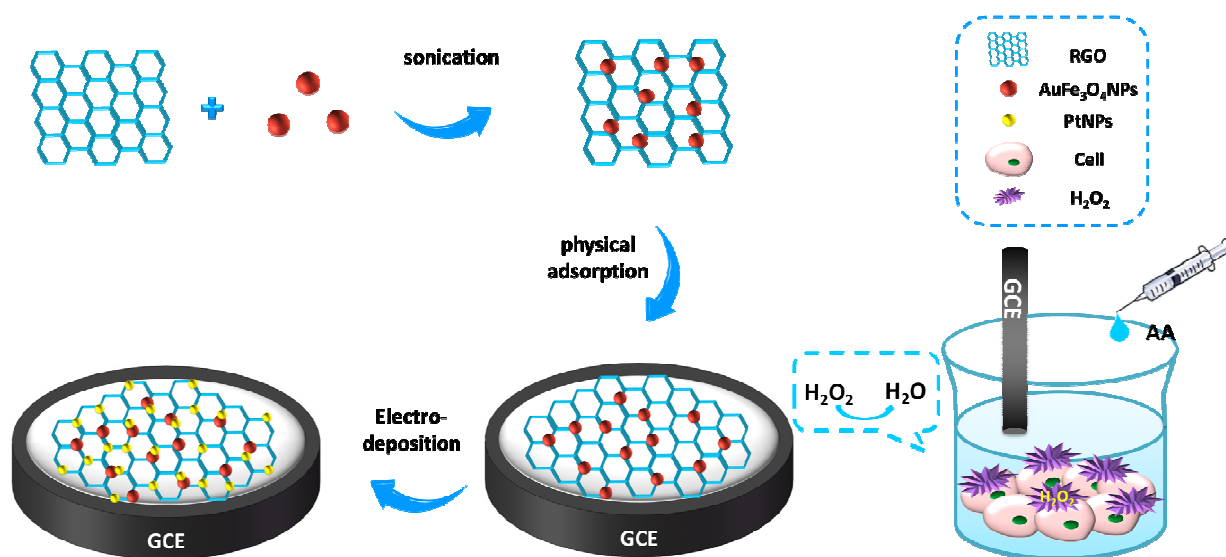
Reactive oxygen species (ROS) are considered as the significant intracellular signaling molecules which can regulate DNA damage, protein synthesis, cell apoptosis, and others living activities, which exists in the aerobic organisms during their whole life.¹⁻³ It is already known that ROS could be endowed *in vivo* with several physiology functions such as immunity activity and signal transition process. However, an excess of ROS accumulation in cells gives rise to internal sabotage activity and leads to oxidative stress which was recognized as the root of caducity and diseases, resulting in destroying the balance of organic redox reaction and biological normal cells and tissues and causing a vary of pathological events such as neurodegeneration, alzheimer disease, autoimmune diseases and cancer.⁴⁻⁶ Hydrogen peroxide (H₂O₂) is one of the major representatives to the oxidative stress damage for its long lifetime to penetrate into other cellular compartment which was distributed in most of the mammalian cells. Therefore, the selective and accurate measure of H₂O₂ in cells and the dynamic observation of living cells are important to illuminate the mechanism of regulating signal transduction pathways and further exploit the potential application in clinical pathological diagnosis.⁷ Therefore, various kinds of H₂O₂ detection methods have been employed such as florescence,⁸ chemiluminescence,⁹ chromatography¹⁰ and electrochemical analysis.^{11,12} Among them, electrochemical technology is considered as one of the most promising strategies on account of its high sensitivity, fast response, production simplicity, low cost and low detection limit.¹³ In the

past several years, enzyme-based electrochemical biosensors have always been the research highlights, which are immobilized with enzymes in the functionalized electrodes, like horseradish peroxidase,¹⁴ glucose oxidase,¹⁵ cytochrome¹⁶ and myoglobin.¹⁷ In spite of the high sensitivity and good selectivity in the moderate condition, the application prospects of enzyme-based sensors are restricted by the inevitable disadvantages such as high costs, a complicated immobilization procedure,¹⁸ the influenced instability and reproduction caused by environment.¹⁹ In compared with enzymatic sensor, nonenzymatic sensors based on nanomaterials reveals many advantages like high electrocatalytic activity, specific surface area and outstanding electron transfer ability. Therefore, we hypothesized to fabricate a highly sensitive nonenzymatic H₂O₂ biosensor to detect the H₂O₂ concentration released from different kinds of cells stimulated by extracellular matters and further to evaluate the oxidative stress of different living cells.

Graphene has attracted tremendous attention resulted from the outstanding properties like high surface area, superior electric conductivity and good mechanical strength, and so on.^{20,21} Especially, reduced graphene oxide (RGO) composited through the reduction of graphene oxide owns several functional groups like hydroxyl (-OH) and carboxyl (-COOH) groups resulting in the higher conductivity, which is favored for nucleation and growth of metal nanoparticles in the fabrication of nanocomposites.^{22,23} Graphene is widely used in electrochemically catalysis of H₂O₂, however, the liner range and sensitivity are not enough for H₂O₂ detection in living cells.^{24,25} The combination of graphene and metal nanoparticles usually lead to larger electrochemically active surface areas and higher the electron transfer rate to improve the current response more sensitively.^{26,27} Au nanoparticles possess rapid electron transfer, efficient electrocatalytic properties and good biocompatibility,^{20,21} particularly when dispersed on an oxide support often with much better performance in catalytic activity than the single-component

nanoparticles,²⁸⁻³⁰ due to the synergetic effect at the interface of metal and oxide support which can also provide more active sites.^{31,32} In spite of the observation that the dumbbell-like AuFe_3O_4 nanoparticles display catalytic activity for H_2O_2 reduction,³¹ there is very limited study on the application of AuFe_3O_4 nanoparticles in the area of highly sensitive biosensor and evaluation of oxidative stress. In addition, Pt nanoparticles possess well stability, high electrocatalytic efficiency, good selectivity and have been extensively utilized as the electrochemical electrode material for construction of biosensor.^{33, 34} What's more important is that Pt nanoparticles can readily reduce the H_2O_2 oxidation-reduction overvoltage.³⁴ In view of the above considerations, the integration of graphene, AuFe_3O_4 nanoparticles and Pt nanoparticles are supposed to employ characteristic performance in the application of catalysis, electrons and biosensor, and overcome the existing limitations, resulting from the synergistic effect of each composition.

Scheme 1: Schematic of the RGO/ AuFe_3O_4 /Pt modified GCE used for detecting H_2O_2 efflux from cells stimulated with AA.



Herein, in this study a novel RGO/AuFe₃O₄/Pt nanocomposition based on nonenzymatic H₂O₂ sensor was fabricated and utilized to detect the H₂O₂ released from living cells (Scheme 1). The blending of the nanosheets of reduced graphene oxide (RGO) with Au Fe₃O₄ nanoparticles (AuFe₃O₄ NPs) and Pt nanoparticles (Pt NPs) were modified on glassy carbon electrode (GCE) through physical adsorption and electrodeposition. The as-prepared RGO/AuFe₃O₄/Pt nanocomposites modified GCE (i.e., RGO/AuFe₃O₄/Pt-GCE) reveals much higher current response on the H₂O₂ reduction process compared with the RGO/AuFe₃O₄NPs and Pt NPs modified GCE, exhibiting the high sensitivity, the fast response time, large linear range and low detection limit in the measurement of H₂O₂. This novel nonenzymatic H₂O₂ biosensor can be readily employed to detect the H₂O₂ released from the living cells during the redox homeostasis disrupted by ascorbic acid and further evaluate the intracellular H₂O₂ concentration, indicating that the oxidative stress level in the tumor cells is much higher than that of normal cells, which has significance in the application of acquainting the information of intracellular environment in the tumor developing process.

2. EXPERIMENTAL SECTION

2.1. Reagents and Apparatus. Reduced graphene oxide (RGO) was purchased from XF NANO, Inc. (China). Chloroplatinic acid (H₂PtCl₆·6 H₂O) was purchased from Sigma-Aldrich and was used as received. Ascorbic acid (AA), β-D (+) glucose, uric acid (UA) and chitosan (CHIT) were obtained from Sigma. Hydrogen peroxide (H₂O₂) was obtained from International Laboratory. All other chemicals were of reagent-grade and were used as received without any further purification.

A 0.02 M phosphate buffer solution (PBS, pH 7.4) consisting of NaH₂PO₄ and Na₂HPO₄ was used as the supporting electrolyte. A physiological PBS solution containing KH₂PO₄ (1.76

mM), Na_2HPO_4 (10.14 mM), NaCl (136.75 mM), and KCl (2.28 mM), was mainly used for washing of living cells and observing the H_2O_2 released from the cells. All the solutions were prepared with doubly distilled water.

The morphologies and surface structures were characterized with a scanning electron microscope (SEM) (Zeiss, Germany). The valence states of the relevant nanocomposites were investigated by a PHI Quantera II X-ray photoelectron spectrometer (XPS). All electrochemical measurements were performed on a CHI660B electrochemical workstation (CHI Incorporation, USA). A conventional three-electrode system employing a bare or modified glassy carbon working electrode, an $\text{Ag}|\text{AgCl}$ reference electrode, a platinum wire counter electrode was used. All potentials were measured and reported versus the $\text{Ag}|\text{AgCl}$ reference electrode. All amperometric measurements were regularly accomplished in 0.02 M PBS (pH 7.4) with stirring. The electrolyte was ventilated with highly purity nitrogen for 20 min and then the electrochemical measures were performed inside deoxygenated atmosphere during experiments.

2.2. Preparation of RGO/ AuFe_3O_4 NPs Composite Materials. 0.032 g NaOH was added into 15 mL of DEG and dissolved in ultrasonic for 30 min and then added with 1 mL DEA. 0.0541 g FeCl_3 , 0.0199 g FeCl_2 and 15 mL DEG were added to the three-necked flask and heated to 80 °C in the flow of N_2 . The as-prepared DEG with NaOH was added into the three-necked flask to react for 1 h, and then 1 mL HAuCl_4 (0.01 M) was added into the hybrid. The prepared reaction products was put into the reaction still and heated on 200 °C for 8 h before being cooled to room temperature. The obtained solution was isolated in a centrifugal machine at a rate of 9000 rpm for 10 min and then washed thoroughly and dispersed with twice-distilled water and alcohol for 3 times. Eventually, the AuFe_3O_4 NPs were composed after the processes above.

RGO suspension was dispersed in doubly distilled water under sonication for 2 h. 0.2 mL RGO solution (2 mg/mL) was mixed with 0.2 mL AuFe_3O_4 solution (0.5 mM) and 0.6 mL CHIT solution (1 mg/mL), then was stirred for 20 min to obtain the RGO/ AuFe_3O_4 NPs composite. RGO/ AuFe_3O_4 NPs composite was dispersed by ultrasonication to acquire a suspension before use.

2.3. Preparation of the Modified Electrodes. Glassy carbon electrode (GCE, 3 mm in diameter) was carefully polished with 0.3 and 0.05 μm alumina slurry on a microcloth (Buehler, USA), cleaned by ultra-sonication in ethanol and deionized water for 3min. A certain volume of RGO suspension was cast onto the surface of GCE as working electrode and allowed to dry in air for 3 h at room temperature, the optimal volume is reported below. The Pt NPs were deposited in 2 mM H_2PtCl_6 solution by cyclic voltammetry (CV) in the range between - 1V and + 1 V with a scan rate of 50 mV/s. The modified electrode was cleaned carefully by doubly distilled water in order to remove the remaining H_2PtCl_6 and dried at room temperature. The supporting electrolyte of PBS (0.02 M, pH 7.4) containing NaH_2PO_4 and Na_2HPO_4 was deoxygenated using nitrogen and kept inside nitrogen atmosphere.

2.4. Cell Culture. HeLa (human cervical cancer cell lines), U87 (human primary glioblastoma cell lines) and HepG2 (human hepatocarcinoma lines) cells were bought from Institute of Hematology, Chinese Academy of Medical Sciences. L02 (human embryo liver cell lines) cells were obtained from Third Military Medical University (Chongqing, China). They were cultivated at 37 °C with 5 % CO_2 in a 95 % humid atmosphere with DMEM (high glucose, Gibco) containing 10 % heat-inactivated fetal calf serum (Sigma-Aldrich, USA), 100 U/mL penicillin (Sigma-Aldrich, USA), and 100 mg/mL streptomycin (Sigma-Aldrich, USA).

2.5. Electrochemical Detection of H₂O₂ Released by Cells. Cell number was calculated by using a cell counter. Cells were divided from culture medium through centrifugation for 4min at 1500 rpm and washed three times by the physiological PBS (0.02 M, pH 7.4) solution. Ascorbic acid (AA) was injected into cells as the stimulation to motivate cells to generate H₂O₂ after the current reduced to less than 20 nA. The detection of H₂O₂ was not interrupted with the addition of AA. The amperometric current response of H₂O₂ released from approximately 5.0×10^6 cells was measured by RGO/AuFe₃O₄/Pt modified GCE at 0 V in 1 mL of deoxygenated PBS.

3. RESULTS AND DISCUSSION

3.1. Characterization of RGO/AuFe₃O₄ and Pt NPs on Electrode Surface. The SEM images showed in Figure 1 exhibit the general morphology of electrodes modified with different nanomaterials (*i.e.*, Pt, RGO/AuFe₃O₄, RGO/AuFe₃O₄/Pt) employed in this research. Figure 1A displays the external surface of RGO/AuFe₃O₄ modified on the GCE, showing the structure of wrinkled multilayer stacks with small sticking AuFe₃O₄ nanoparticles. Figure 1B reveals the electrodeposited Pt NPs with the size of 50 nm. It can be seen from Figure 1C that a large amount of well distributed Pt NPs electrodeposited densely on the RGO/AuFe₃O₄ surface, composing the RGO/AuFe₃O₄/Pt nanocomposites. From Figure 1D, it can be obtained that EDS result demonstrates the primary composites of RGO/AuFe₃O₄/Pt nanocomposites including C, Pt, Au and Fe elements. Meanwhile, as shown in Figure S1 to Figure S3 of the supplementary information, the XPS studies of relevant nanocomposites of the modified electrode also confirm the corresponding components of the as-prepared nanostructural modification.

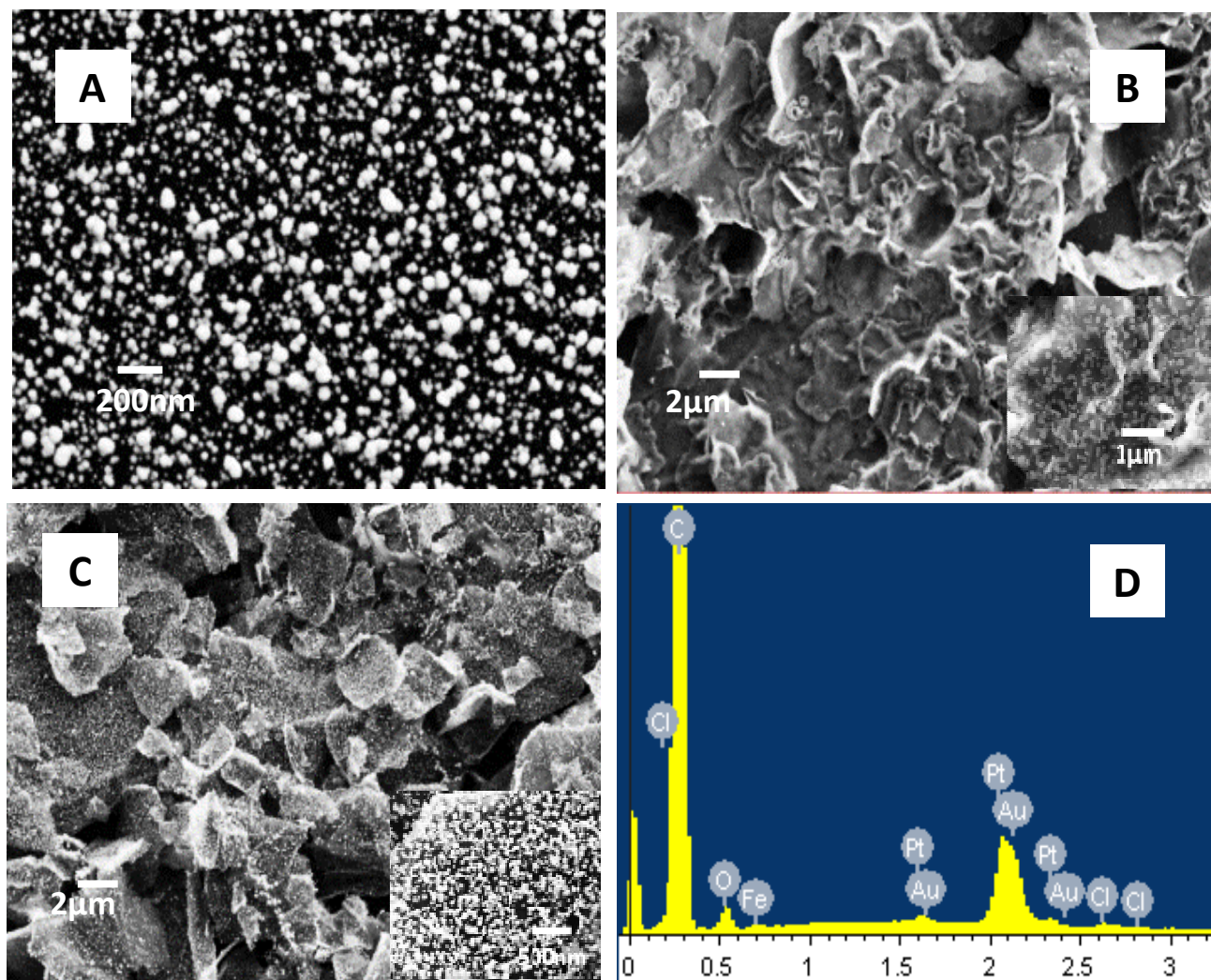


Figure 1. SEM images of Pt NPs (A), RGO/AuFe₃O₄ NPs (B) and RGO/AuFe₃O₄/Pt NPs (C) on GCE. D is the EDS of RGO/AuFe₃O₄/Pt nanocomposites.

3.2. Optimization Study of RGO/AuFe₃O₄/Pt Nanocomposites-Modified Electrodes. As shown in Figure 2, the deposition parameters of nanomaterials and PH value have a significant impact on the sensitivity of H₂O₂ detection. Figure 2A shows the adding volume of deposited RGO/AuFe₃O₄ leads to the increasing detection sensitivity of H₂O₂, reaching a peak value at the volume of 3 μL. Then the sensitivity declined with the addition of deposition volume and attained a minimum value at 6 μL. Afterwards, the increasing deposition of RGO/AuFe₃O₄

enhanced the H_2O_2 detection sensibility. From Figure 2B, it is noted that the sensitivity response to H_2O_2 detection has a maximum when the deposition CV cycles of Pt NPs was fifteen. Meanwhile, the influence of different pH values of PBS solution on H_2O_2 detection was researched. As shown in Figure 2C, in the range of pH 3.0 - 8.0, the sensitivity of H_2O_2 detection was relatively low in the acidic environment and reached a peak at pH 7.4. The applied potential usually has a large effect on the sensitivity. It can be seen from Figure 2D that, in the range from -0.05 V to 0.1 V, the sensitivity increased with the increase of potential and reached the maximum at 0 V, then decreased with the further rise of the applied potential. To avoid the interference of other substances under high negative potential, 0 V was selected in experiments. Therefore, 3 μL of RGO/ AuFe_3O_4 , 15 CV cycles for Pt NPs deposition, PBS solution of pH7.4 and applied potential of 0 V were employed in this study.

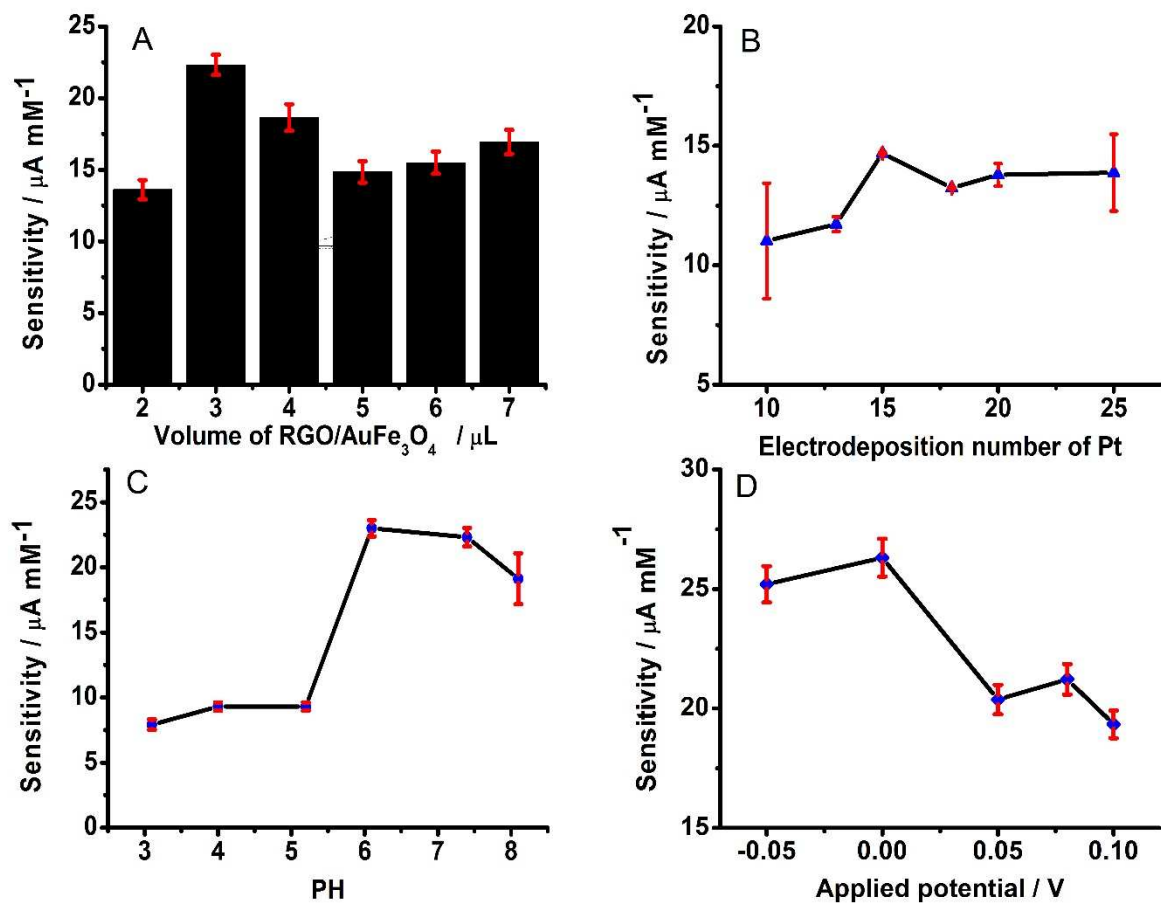


Figure 2. Optimization study of RGO/AuFe₃O₄ NPs volume deposited on the GCE (A), the CV cycles for electrochemical deposition of Pt NPs on the RGO/AuFe₃O₄-GCE (B), the pH value of PBS solution (C) and the applied potential employed (D). Error bars are the standard error of the mean (n = 4 electrodes).

3.3 Electrocatalytic Reduction of H₂O₂ on RGO/AuFe₃O₄/Pt Nanocomposites Modified Electrode. To investigate the electro-catalytic activity of the nano-interface of RGO/AuFe₃O₄/Pt nanocomposites for a nonenzymatic H₂O₂ sensor, cyclic voltammetry (CV) was employed in a range from -0.1 V to + 0.6 V at a scan rate of 0.1 V/s in pH 7.4 PBS on a RGO/AuFe₃O₄/Pt nanocomposites modified glassy carbon electrode (RGO/AuFe₃O₄/Pt-GCE). In this work, CV was employed to quantify the relevant electrochemical parameters and measured in the

electrolyte solution consisting of 1 mM $K_3[Fe(CN)_6]$ and 0.1 M KCl. It can be seen from Figure 3A that a pair of well-defined redox peaks was observed as the result of the $[Fe(CN)_6]^{3-/4-}$ oxidation-reduction couples. The electroactive surface area of electrodes was calculated on the basis of Randles–Sevcik equation. At the temperature of 25, Randles–Sevcik equation is expressed as $I_p = 2.69 \times 10^5 A D^{1/2} n^{3/2} \gamma^{1/2} C$, where I_p means the peak current(A), A means the effective surface area of electrode (cm^2), D means the diffusion coefficient of reagent which is $6.7 \pm 0.02 \times 10^{-6} cm^2 s^{-1}$ for $K_3[Fe(CN)_6]$, n means the number of transition electron in the oxidation-reduction reaction which is 1 for $[Fe(CN)_6]^{3-/4-}$, γ means the scan rate ($V s^{-1}$) and C is the concentration of the redox reactant ($mol cm^{-3}$). The calculated electroactive surface area of electrodes were in the tendency of bare GCE ($0.06736 cm^2$) < Pt-GCE ($0.07879 cm^2$) < RGO/AuFe₃O₄-GCE ($0.10823 cm^2$) < RGO/AuFe₃O₄/Pt-GCE ($0.14265 cm^2$), revealing that the three-dimensional skeleton, good electrical conductivity of RGO/AuFe₃O₄ and well-distributed and Pt NPs on the GCE surface can bring about the enlarged electroactive surface area.

The admirable three-dimensional structure and uniformly distributed nanomaterials lead to the excellent performance of the molecules transfer and electron conductivity of RGO/AuFe₃O₄/Pt nanocomposites. Therefore, there is significance to research the electrocatalytic activity of H₂O₂ electro-reduction on the RGO/AuFe₃O₄/Pt nanocomposites modified electrode interface. Figure 3B displays the CV curves of RGO/AuFe₃O₄/Pt nanocomposites modified GCE in 0.02 M PBS solution (pH 7.4) in the absence and presence of H₂O₂ of different concentrations. As exhibited in Figure 3B, an obvious reduction peak around 0.15 V emerges in the presence of 0.5 mM H₂O₂. As the concentration of H₂O₂ increases, the reduction current dramatically enhances, accompanied with the reduction peak which becomes sharp and shifts to negative potential slightly, presumably as the result of the torpid electron

transfer kinetics. The obvious reduction current displays that H_2O_2 can be easily reduced on RGO/AuFe₃O₄/Pt nanostructures in a large concentration range. The outstanding electro-catalytic activity of RGO/AuFe₃O₄/Pt-GCE benefit from the characteristic three-dimensional nanostructures, high surface-to-volume ratio, and the collaborative contributions afforded by RGO/AuFe₃O₄ and Pt nanoparticles. It implies that RGO/AuFe₃O₄/Pt nanocomposites have great application potential in H_2O_2 biosensing.

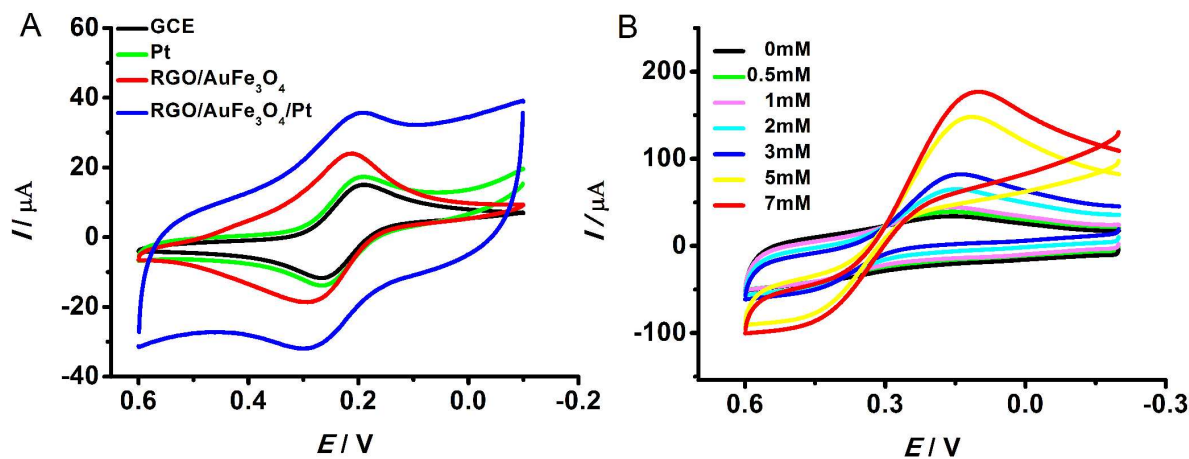


Figure 3. (A) CVs of bare, Pt, RGO/AuFe₃O₄, and RGO/AuFe₃O₄/Pt modified GCEs in potassium ferricyanide solution containing 0.1 M KCl and 1 mM K₃[Fe(CN)₆]. (B) CVs of RGO/AuFe₃O₄/Pt-GCE in the absence and presence of different concentrations of H_2O_2 in 0.02 M PBS. Scan rate: 100 mV/s.

3.4. Electrochemical Properties of RGO/AuFe₃O₄/Pt Nanocomposites Modified GCE.

The CV technology was measured to investigate the charge transport characteristics of the RGO/AuFe₃O₄/Pt nanocomposite film at different scan rates between -0.2 V and 0.6 V vs Ag|AgCl. It is shown in the Figure 4A that the slight peak potential changes from 0.12 V to 0.09 V when scan rate reaches 100 mV/s and then remains at 0.09 V stably with the increase in scan

rate. The negative shift of the related reduction peak potential may be caused by the sluggish electron transfer kinetics.⁴ Meanwhile, the anodic and cathodic peak currents increased with the increase of scan rates from 30 mV/s to 250 mV/s, with a correlation coefficient of 0.996 as exhibited in Figure 4B, which demonstrates that the redox process of RGO/AuFe₃O₄/Pt modified electrode surface is a surface-confined process with the quick electron transfer kinetics. It can be obtained that the AuFe₃O₄/Pt composition was dispersed on the surface of RGO and personality of nice conductivity of RGO/AuFe₃O₄/Pt nanomaterial.

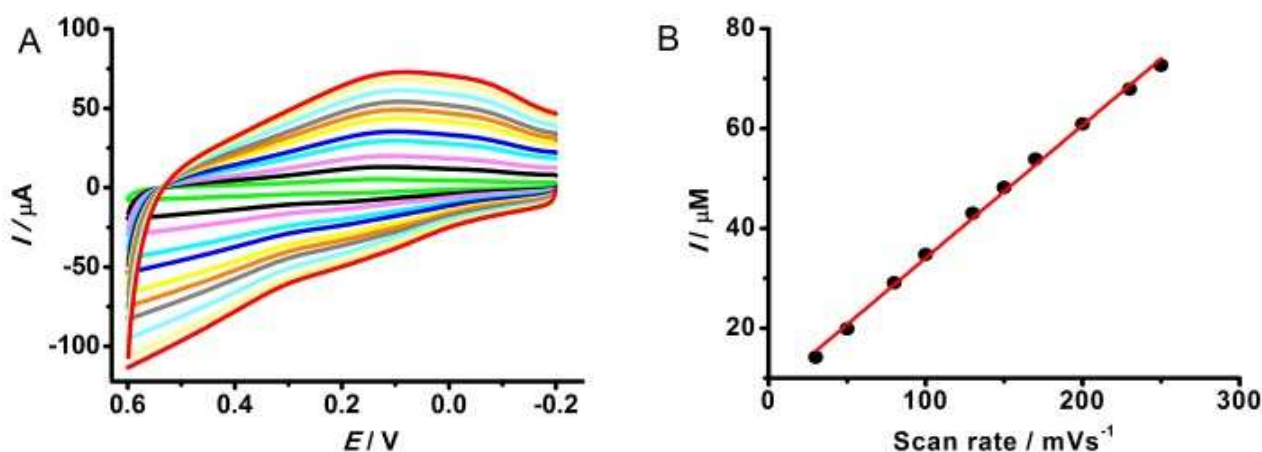


Figure 4. (A) CVs of RGO/AuFe₃O₄/Pt-GCE in 0.02 M PBS at a scan rate of (from inner to outer): 30, 50, 80, 100, 130, 150, 170, 200, 230 and 250 mV/s, respectively. (B) The linear relationship between the peak currents and the scan rates

3.5. Amperometric Response to H₂O₂ Detection. Figure 5A displays the amperometric response of the RGO/AuFe₃O₄/Pt modified electrode to the successive addition of varying concentrations of in 0.2 M pH 7.4 PBS solution and the typical i-t curves at 0 V. It can be seen that RGO/AuFe₃O₄/Pt-GCE proved enhanced amperometric response for H₂O₂ detection compared with RGO/AuFe₃O₄-GCE and Pt-GCE. No obvious signal or only weak amperometric

response to the addition of H_2O_2 was received on the RGO/ AuFe_3O_4 -GCE (the blue curve) and Pt-GCE (the black curve) respectively. Owing to the excellent electrocatalytic activity of RGO/ AuFe_3O_4 nanocomposites to H_2O_2 , a nonenzymatic sensor was constructed for the detection of H_2O_2 . It was obtained that the sensor responded quickly to the change of H_2O_2 concentration and achieved 95 % of the steady-state current within 4 s after the injection of H_2O_2 (the red curve).Figure 5B exhibits the corresponding calibration curve for the nonenzymatic H_2O_2 sensor, indicating that RGO/ AuFe_3O_4 /Pt-GCE reveals a great increased sensitivity. The RGO/ AuFe_3O_4 /Pt modified sensor has had a linear relationship with the concentration of H_2O_2 in the range of 0.025 – 2 mM with a correlation coefficient of $R^2 = 0.998$. The linear regression equation for RGO/ AuFe_3O_4 /Pt-GCE was $I (\mu\text{A}) = 26.31C + 1.31$ (where C is the concentration of H_2O_2) with a sensitivity of $184.43 \pm 5 \text{ mA M}^{-1}\text{cm}^{-2}$ based on $n = 5$ electrodes. The linear regression equation for Pt-GCE was $I (\mu\text{A}) = 4.6917C + 7.23$ with a sensitivity of $59.55 \pm 3 \text{ mA M}^{-1}\text{cm}^{-2}$ based on $n = 5$ electrodes.

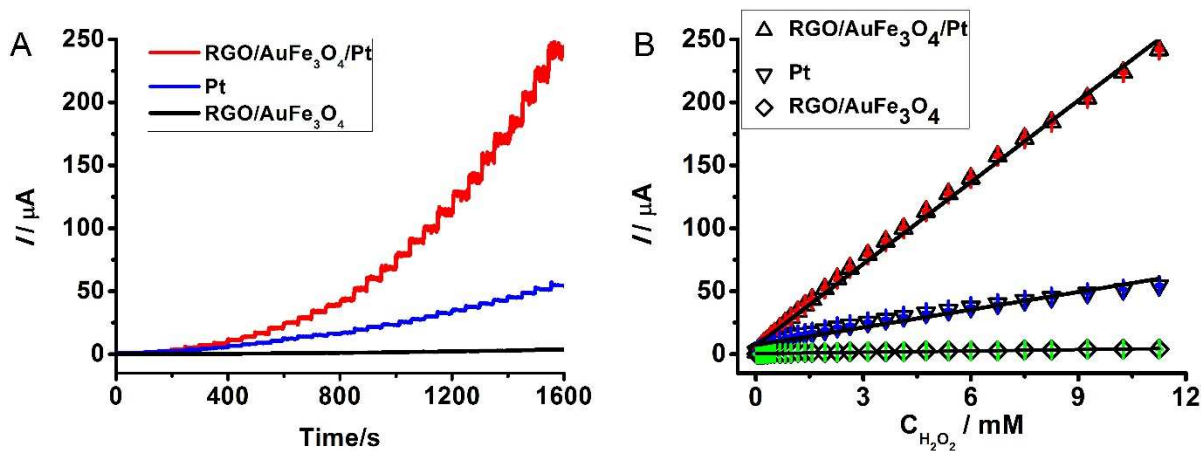


Figure 5. (A) Amperometric response of RGO/ AuFe_3O_4 /Pt-GCE, Pt-GCE, and RGO/ AuFe_3O_4 -GCE to successive additions of H_2O_2 at 0 V in 0.02 M PBS. (B) Linear relation between the

amperometric response and H_2O_2 concentration. Error bars are the standard error of the mean ($n = 4$ electrodes).

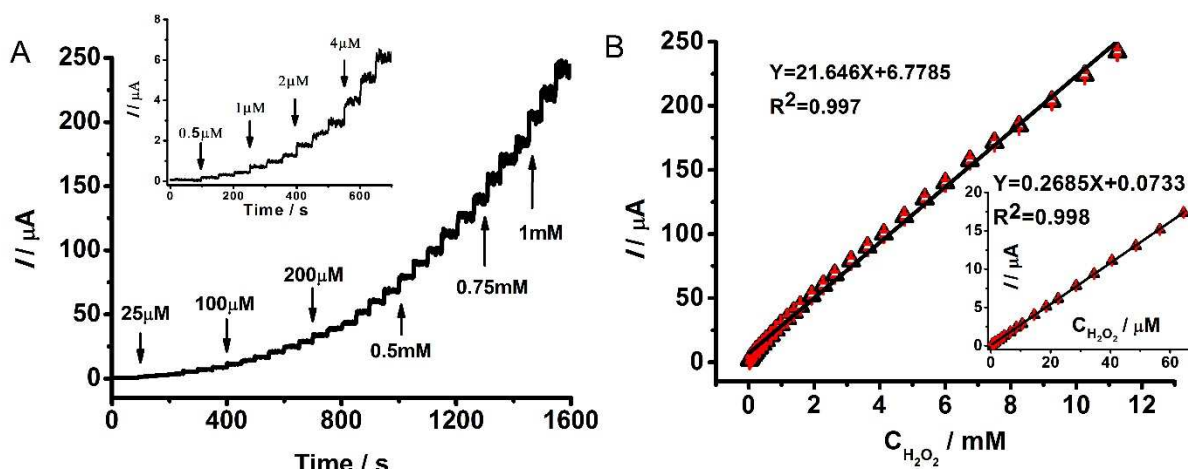


Figure 6. (A) Amperometric response of RGO/AuFe₃O₄/Pt-GCE to successive additions of H_2O_2 at 0 V in 0.02 M PBS (H_2O_2 was added at the point indicated by arrows to the concentrations mentioned in A). (B) Linear relation between the amperometric response and H_2O_2 concentration ranging from 0.025 mM to 11.5 mM and from 0.5 μM to 22.5 μM (Inset B).

From Figure 6, there were two segments in the relevant linear range for the detection of H_2O_2 at the applied potential of 0 V, which were $0.5 \times 10^{-6} - 6.45 \times 10^{-4}$ M (correlation coefficient $R^2 = 0.998$) and $8.25 \times 10^{-4} - 11.5 \times 10^{-3}$ M (correlation coefficient $R^2 = 0.997$). The limit of detection for the H_2O_2 sensor was calculated to be $\sim 0.1 \mu\text{M}$ (ratio of signal-to-noise $S/N = 3$). These results indicate that the excellent performance of a wide linear range, low detection potential, and high sensitivity endow RGO/AuFe₃O₄/Pt nanocomposites the promising material for nonenzymatic H_2O_2 amperometric sensor.

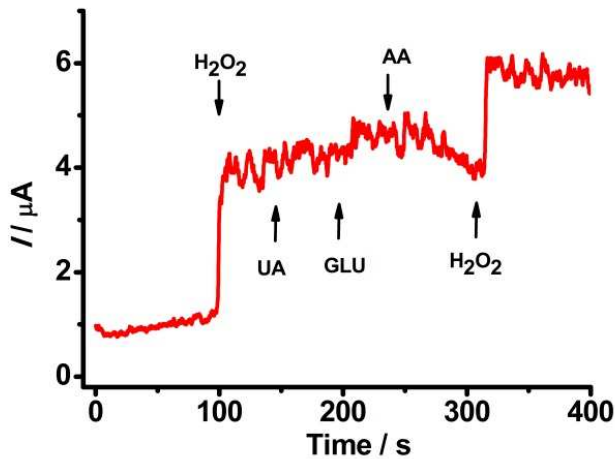


Figure 7. Amperometric response to successive addition of 0.1 mM H₂O₂, 1.0 mM UA, 1.0 mM GLU, 0.5 mM AA, and a second 0.1 mM H₂O₂ of RGO/AuFe₃O₄/Pt-GCE at 0 V in 0.02 M PBS.

The anti-interference ability is one of the most important analytical factors for a sensor. The influence of some electroactive species on the response of a sensor to 0.1 mM H₂O₂ was evaluated at the potential of 0 V. 0.1 mM H₂O₂, 1.0 mM uric acid (UA), 1.0 mM glucose (GLU), 0.5 mM AA and a second injection of 0.1 mM H₂O₂ were added into N₂-saturated PBS to investigate the anti-interference ability of the RGO/AuFe₃O₄/Pt modified nonenzymatic sensor. Figure 7 displays that there was no obvious current response after the addition of UA, AA, and GLU in the same sample. The current response to the addition of H₂O₂ was not affected in the presence of other electroactive species, indicating that UA, AA, and GLU have no interference in the determination of H₂O₂. The high selectivity of this sensor is primarily due to the appropriate applied potential and nanocomposites used.

The tests of reproducibility and storage stability are very important for H₂O₂ sensor. The excellent reproducibility with a relative standard deviation less than 3.94 % (RSD) for the H₂O₂ sensitivity was obtained on the basis of six independent RGO/AuFe₃O₄/Pt-GCEs. The as-prepared RGO/AuFe₃O₄/Pt-GCE was stored at room temperature while the sensor was out of use.

The storage stability of the constructed sensor was received by measuring the current response to 0.25 mM H_2O_2 once a day. As reported in the variation of the response sensitivity at the RGO/AuFe₃O₄/Pt-GCE declined to about 86 % of its initial response sensitivity and kept steady in 2 weeks, indicating long-term stability. All the research data implies that the outstanding performance of wide linear range, low detection, low detect limit and high sensitivity can bring about the reliability of RGO/AuFe₃O₄/Pt-GCE in the area of H_2O_2 sensitive detection.

3.6. Measurements of H_2O_2 Release from Living Cells. This novel nonenzymatic biosensor based on the RGO/AuFe₃O₄/Pt nanocomposites was explored to detect H_2O_2 in living cells. Scheme 1 shows the scheme of electrochemical detection of cellular flux of H_2O_2 under the stimulation of AA on the RGO/AuFe₃O₄/Pt modified GCE. Three kinds of tumor cell lines and one kind of normal cell lines were selected as model in our study, *i.e.* human hepatocyte line L02, human cervical cancer cell HeLa, human hepatoma cell HepG2 and human glioma cell U87.

Figure 8 depicts the amperometric responses obtained at the RGO/AuFe₃O₄/Pt-GCE in the presence of HeLa cells stimulated by 1 μM AA in physiological PBS buffer at 0 V versus Ag|AgCl. To be the control groups, the cells with catalase (blue line, Figure 8A) and the buffer solution without the cells (green line, Figure 8A) were also employed under the same conditions and there was no current change obtained. After the addition of AA, the current caused by H_2O_2 elimination in HeLa cells was increased prominently, and the current reached a peak and then decreased to ca. 50 % of the maximum in 30 s. However, the current in the control group pre-added in the PBS with 500 U/mL catalase almost showed no changes (blue line, Figure 8A). The invariable current response can be resulted from the catalase introduced unique decomposition of released H_2O_2 from HeLa cells under the stimulation of AA.² The current change of HeLa was obtained by integration of the curve peak after AA irritation, which was about 10.86 μA ,

corresponding to 40.55 μM H_2O_2 in HeLa cell solution, as calculated from the standard curve displayed in the inset of Figure 8B. The volume of cell solution was 1 mL and continuing time was 100 s, which is equivalent to 0.578 nmol of released H_2O_2 . The amounts of measured HeLa cells were approximately 5.0×10^6 , and the H_2O_2 liberated from each cell was almost 335 ± 28.5 amol. The efflux amount of H_2O_2 from HepG2, L02 and U87 cell lines were analyzed by the same method discussed above. As for normal cell lines L02, the H_2O_2 amount were calculated to be $\sim 151 \pm 20.6$ amol. In the case of two other tumor cells HepG2 and U87, the H_2O_2 released from cells were respectively $\sim 440 \pm 24.3$ amol and $\sim 375 \pm 33.5$ amol. It is obvious that there are more released H_2O_2 from three kinds of tumor than that of normal cell lines, as exhibited in Figure 8B. For instance, the amount of H_2O_2 by human cervical cancer cell HeLa was almost twice as much as that of the normal hepatic cells L02.

The production and distribution of H_2O_2 in cells irritated from AA can be attributed to the following views. The intracellular concentration of H_2O_2 is held at a stable level in favor of cellular activity and proliferation, the process of maintain of intracellular redox homeostasis is not only regulated by the catabolism but also affected by the H_2O_2 elimination.² When living cells were stimulated by external elements, the intracellular redox homeostasis was destroyed and excreted H_2O_2 induced by stress reaction. The diffusion of relevant H_2O_2 from high to low concentration regions was promoted by the method of free molecule diffusion readily cross the lipid bilayer of membrane³⁵ or through the means similar to water which was facilitated by some channel proteins, such as aquaporins.^{36, 37} Then the extracellular and intracellular concentrations of H_2O_2 were regulated to remained at the same level, achieving the state of redox homeostasis again. What's more, it is considered 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.⁵

Therefore, the detection of the flux of H_2O_2 is meaningful for evaluation of living cell oxidative stress and has possibility in the application of investigation of oxidative stress capability distinctions in different living cells. It is also of certain significance for exploration of environment in tumor cells and therapy of cancer.

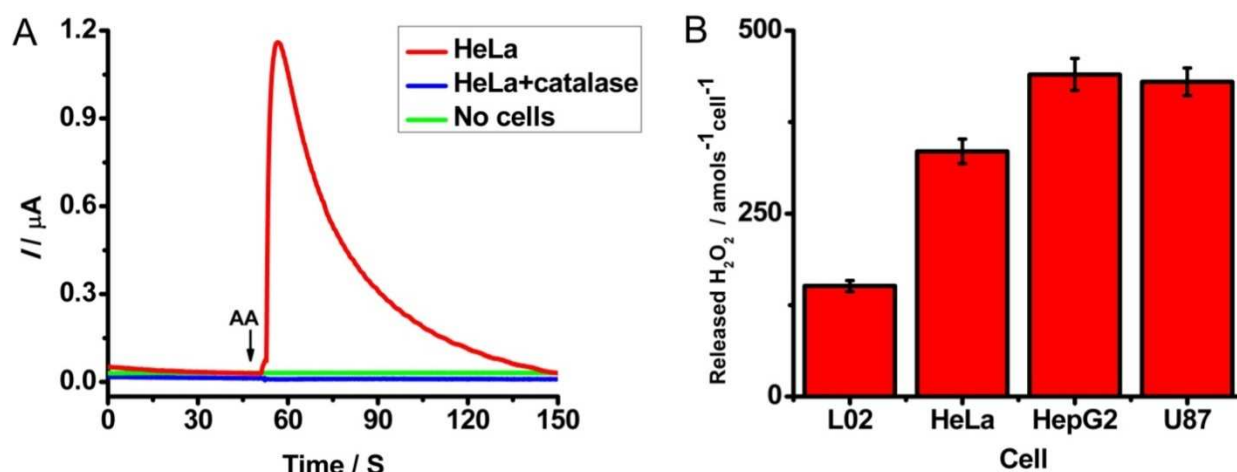


Figure 8. (A) Amperometric response upon addition of AA at 0 V in PBS containing HeLa cells (red), HeLa cells and catalases (blue), without cells (green). (B) Amount of H_2O_2 released by L02, HeLa, HepG2, and U87 cell lines stimulated by 1 μM AA. The values are expressed as means $\pm$ SD of at least three independent measurements.

4. CONCLUSIONS

In summary, we have reported a nonenzymatic H_2O_2 sensor constructed on the basis of hybrid nano-electrochemical catalysts of RGO/Au/ Fe_3O_4 /Pt nanocomposites, which were obtained through electrochemically deposited by high amount of Pt nanoparticles. The robust nonenzymatic hybrid nanocatalysts RGO/Au/ Fe_3O_4 /Pt modified GCE possess much better electrochemical catalysis properties and higher sensitivity for the measurement of H_2O_2 than that of one elemental modification of GCE (*i.e.*, relevant GCE electrode decorated by single nanomaterial alone). The results demonstrate that the nonenzymatic hybrid nanocatalysts

RGO/Au/Fe₃O₄/Pt based biosensor display high selectivity and sensitivity for oxidative stress like H₂O₂ release, with relatively low overpotential of 0 V, low detection limit of ~ 0.1 μM, largelinear range from 0.5 μM to 11.5 mM and outstanding reproducibility. This robust nonenzymatic biosensor has shown great potential applications in the determination of H₂O₂ released from the living cells including cancer cells, which is significance in the development of ultrasensitive nonenzyme biosensors for clinical diagnostics to rapidly assess oxidative stress of different kinds of diseased cells.

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Notes

The authors declare no competing financial interest.

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SUPPORTING INFORMATION

The XPS studies of relevant nanocomposites to characterize the valence states of main elements of relevant nanocomposites and the figures for EDS results of RGO/AuFe₃O₄ and Pt NPs

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3 displayed in supporting information. This information is available free of charge via the Internet
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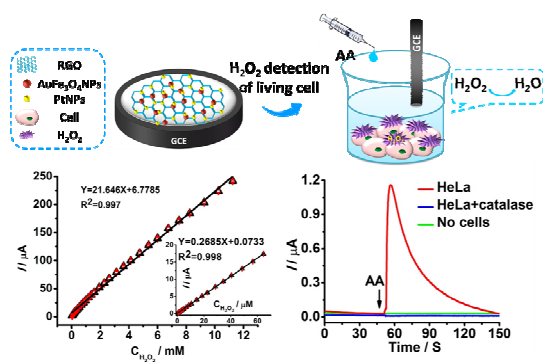
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