Electronic Supplementary Information:

Enhancing the electrochemical reduction of hydrogen peroxide based on nitrogen-doped graphene for measurement of its releasing process from living cells

Ping Wu, Zhewei Cai,[1] Yang Gao, Hui Zhang and Chenxin Cai*

Jiangsu Key Laboratory of New Power Batteries, Jiangsu Key Laboratory of Biofunctional Materials, College of Chemistry and Materials Science, Nanjing Normal University, Nanjing 210097, P.R. China.

[1] Student of Jinling High School (Nanjing, China) and currently conducting a project of innovation study in College of Chemistry and Materials Science, Nanjing Normal University.

* Corresponding author, E-mail: cxcai@njnu.edu.cn, chenxincai@hotmail.com

Fax: 86 25 85891767; Tel: 86 25 85891780;

1. Experimental details

1.1 Chemicals

All chemicals and solvents were of reagent grade or better. Catalase (E.C. 1.11.1.6, from bovine liver, lyophilized powder, 2000–5000 U/mg, Sigma), phorbol myristate acetate (PMA, ~99%, Sigma), *N*-formylmethionyl-leucylphenylalanine (fMLP,≥ 97%, Sigma), adenosine 5' -diphosphate (ADP,≥ 95%, Sigma), ascorbic acid (AA,≥ 99.0%, Sigma), 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC–5, Sigma), 3-morpholinosydnonimine (SIN–1, Sigma), and graphite powder (99.99995%, 325 mesh, Alfa Aesar) were used as received. Solutions of H_2O_2 were freshly diluted from the 30% solution, and their concentrations were determined using a standard $KMnO_4$ solution. 0.1 M phosphate buffer solution (PBS, pH 7.4) was employed as the supporting electrolyte. All other chemicals were of analytical grade.

1.2 Synthesis of the nitrogen-doped graphene

Nitrogen-doped graphene was synthesized by a facile method involving the steps of graphite oxidation, exfoliation, and chemical reduction. Graphite oxide (GO) was synthesized from commercial graphite powder by a modified Hummers' method.[1,2] Graphite powder (3 g, 325 mesh) was put into a mixture of concentrated H_2SO_4 (12 mL), $K_2S_2O_8$ (2.5 g), and P_2O_5 (2.5 g). The solution was heated to 80 ºC and kept stirring for 4.5 h by using oil-bath. Next, the mixture was cooled to room temperature and diluted with double distilled water (0.5 L) and left overnight. Then, the product was obtained by filtering with use of 0.2 micron Nylon film and washed with double distilled water to remove the residual acid. The product was dried ambient temperature.

The pre-oxidized graphite was then re-oxidized by Hummers' method. Pretreated graphite powder was put into cold (0 °C) concentrated H_2SO_4 (120 mL). Then, $KMnO_4$ (15 g) was added gradually under stirring and the temperature of the mixture was kept to be below 20 °C by ice-bath. Successively, the mixture was stirred at 35 °C for 2 h, and then diluted with double distilled water (0.5 L) by keeping the

temperature at 50 °C. After adding all of 250 mL of double distilled water, the mixture was stirred for 2 h, and then additional water (1 L) was added. Shortly after the dilution, 30% H_2O_2 (20 mL) was added drop by drop, and the color of the mixture changed into brilliant yellow along with bubbling. The mixture was filtered and washed with 1:10 HCl aqueous solution (1 L) to remove metal ions followed by double distilled water (1 L) to remove the acid. The resulting solid was dried in air and diluted to make graphite oxide dispersion (0.5% w/w). Finally, it was purified by dialysis for one week to remove the remaining metal species.

Exfoliation was carried out by sonicating graphite oxide dispersion (0.1 mg/mL) under ambient condition for 20 min. The resulting homogeneous yellow-brown dispersion was used for reduction. The reduction reaction was carried out by adding hydrazine (1.2 mL) into the dispersion of GO (60 mg of GO in 50 mL of water). After sonicated for 1 h and kept stirring for 24 h at 50 ºC, nitrogen-doped graphene (N-graphene) sheets were obtained by filtration of the product and drying in vacuum.

1.3 Synthesis of the nitrogen-free graphene

The N-free graphene was prepared by using $NaBH_4$ as a reducing agent.[3,4] In a typical procedure, 75 mg of GO was dispersed in 75 g of water with sonication. 600 mg of $NaBH_4$ in 15 g of water was added into the GO dispersion after pH being adjusted to 9–10 with 5 wt % sodium carbonate solution. The mixture was then kept at 80 °C for 1 h under constant stirring. During reduction, the dispersion turned from dark brown to black accompanied by outgassing. The N-free graphene was obtained by filtration of the product and drying in vacuum.

1.4 Instrument and procedures

Transmission electron microscopy (TEM) images were obtained with a JEOL–2010 transmission electron microscope operating at an accelerating voltage of 120 kV. The samples were prepared by dispersing the samples in ethanol and evaporating one drop of the suspension onto a carbon-coated film supported on a copper grid for TEM measurements. Atomic force microscopic (AFM) images were

recorded with a Nanoscope IIIa scanning probe microscope (Digital Instruments, USA) using a tapping mode. The sample used for measurements was prepared by casting the suspension of N-graphene (0.1 mg/mL) on the surface of mica sheet. The solvent was allowed to evaporate before measurements. The surface characteristics of the prepared GO and N-graphene were examined by X–ray photoelectron spectroscopy (XPS), which were recorded on ESCALAB 250 XPS spectrometer (VG Scientifics) using monochromatic Al Kα line at 1486.6 eV. Binding energies were calibrated with respect to the C1s peak at 284.6 eV. Peak fit analysis was performed using the XPS PEAK program (version 4.0).

The electrochemical experiments were performed with a CHI 760B electrochemical workstation (CH Instruments). A two-compartment three-electrode cell with the sample volume of 10 mL was employed. A coiled Pt wire and a saturated calomel electrode (SCE) were used as the counter electrode and the reference electrode, respectively. Buffers were purged with high purity nitrogen for at least 30 min prior to experiments and the nitrogen environment was then kept over the solution to prevent oxygen from reaching the solution. Amperometric measurements were performed under a constant potential of –400 mV (versus SCE). The solution was continuously stirred using a magnetic bar at a rate of 250 rpm.

1.5 Fabrication of the electrode

Several experimental parameters were optimized to obtain the best voltammetric response. For fabrication of N-graphene modified electrode, the N-graphene was dispersed into PBS (pH 7.4) to form a homogeneous suspension (2 mg/mL). Then, the suspension (5 μL) was cast onto the surface of the glassy carbon (GC, 3 mm in diameter, CH Instruments) electrode with a microsyringe, and solvent was allowed to be evaporated before use. The electrode is designated as N-graphene/GC electrode.

By similar procedures, the N-free graphene/GC electrode was also fabricated, and its electrocatalytic characteristic for the reduction of H_2O_2 was compared with that of the N-graphene/GC electrode.

1.6 Purification of neutrophils

The separation of neutrophils from human blood was carried out using the published procedures[5,6] and involved the removal of the erythrocytes by dextran sedimentation to give a supernatant rich in neutrophils and lymphocytes. Neutrophils were subsequently purified by centrifugal separation using a Ficoll (Histopaque 1077, Sigma) density gradient, with the residual plasma and supernatant fractions being discarded. The neutrophil fraction was frequently contaminated with residual erythrocytes, which could be removed by a series of steps beginning with their rapid hypotonic lysis (20 s). The cell lysis step was halted by excess (>10 times) dilution with PBS (0.145 M NaCl, 1.9 mM NaH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4), and the residual neutrophils were concentrated by centrifugal washing. Cell viability (typically in the range 95-98%) was determined using the trypan blue exclusion test. The cell number was estimated by a hemocytometer.

1.7 Measuring the flux of H_2O_2 releasing from neutrophils

The releasing flux of H_2O_2 from neutrophil cells was measured with use of the N-graphene/GC electrode. Neutrophil cells (~1×10^6 cells) were centrifuged to obtain a cell-packed pellet with diameter of ca. 0.5 cm and 1 mL PBS was added for the electrochemical experiments. Before measurements, the buffer was deoxygenated by gently shaking the cells under a humidified nitrogen stream. The N-graphene/GC electrode, which was carefully adjusted to near the cell pellet under a microscope (AxioObserver A1, Carl Zeiss), was biased at –400 mV (vs. SCE). After a steady state background was attained, 100 μL PMA (with the final concentration of 100 ng/mL) was injected into buffer, and response current corresponding to the electrocatalytic reduction of H_2O_2 releasing from the cells was recorded under the physiological pH and temperature (pH 7.4 and 37 °C).

The effects of PMA dose on the release of H_2O_2 from the cells were also evaluated. Various dose of PMA (ranging from 25 to 200 ng/mL) were injected into the buffer to induce the generation of H_2O_2 in the cells, and the flux of H_2O_2 from the cells was measured using the developed electrode.

2. TEM and AFM images of N-graphene

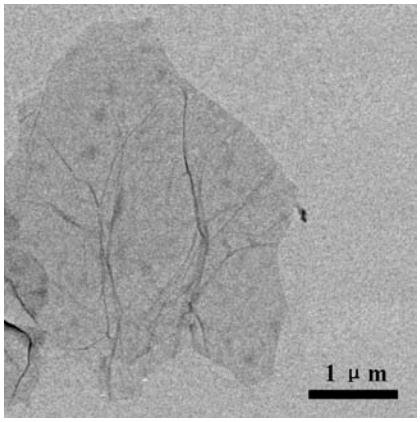

Fig. S1 Typical TEM image of N-graphene

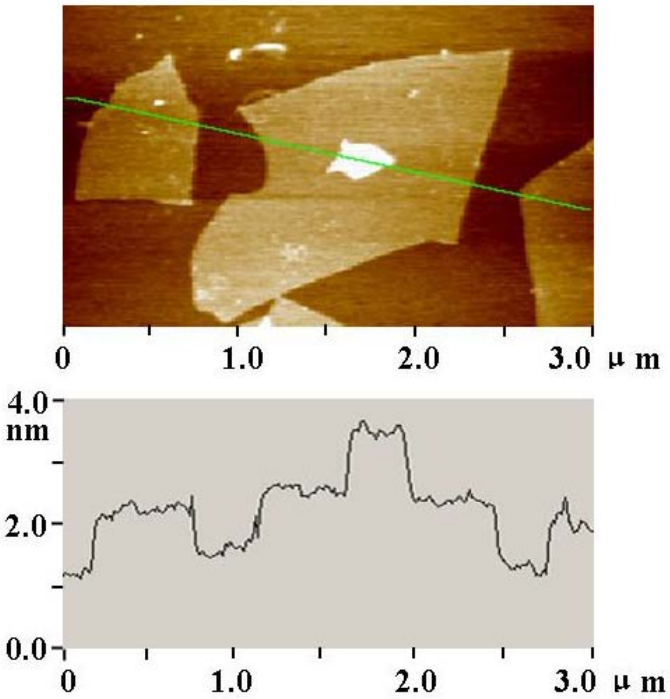

Fig. S2 Typical AFM image of N-graphene

3. The electrocatalytic reduction of H_2O_2 by N-graphene, N-free graphene, and bare GC

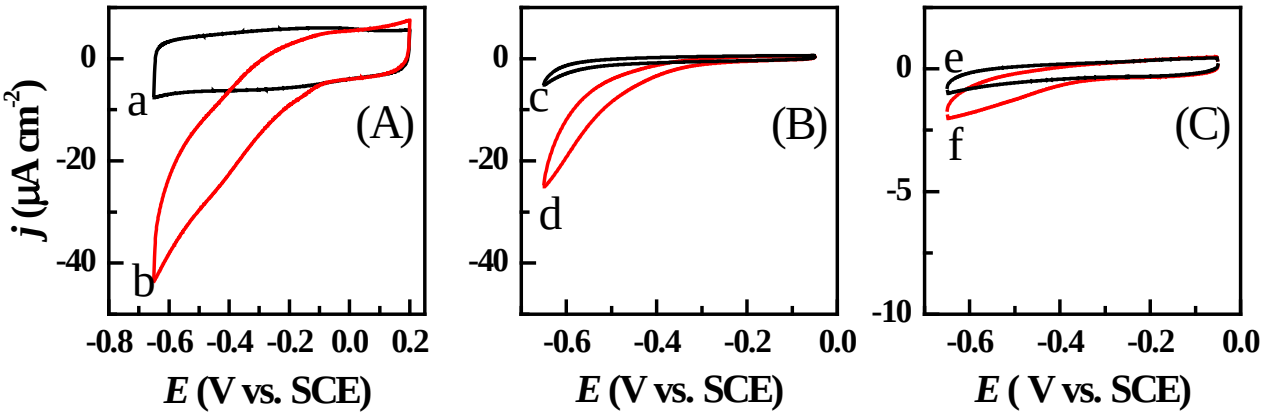

Fig. S3 Voltammetric response of electrocatalytic reduction of 0.2 mM H_2O_2 by N-graphene (A), N-free graphene (B), and bare GC (C). The responses were recorded in N_2-saturated PBS (0.1 M, pH 7.4) with (curves b, d, and f) and without (curves a, c, and e) of 0.2 mM H_2O_2. Scan rate was 100 mV/s.

The electrocatalytic reduction of 0.2 mM H_2O_2 was studied at the N-free graphene and bare GC, respectively. The cathodic current for the reduction of 0.2 mM H_2O_2 catalyzed by N-free graphene and bare GC is less than 1/2 and 1/25, respectively, of that obtained with N-graphene, and the starting potential at N-free graphene (ca.–330 mV, vs SCE, curve d) and bare GC (ca.–380 mV, curve f) is 230, and 280 mV, respectively, more negative than that at N-graphene (ca.–100, curve b).

4. The H_2O_2 concentration-dependent electrocatalytic current

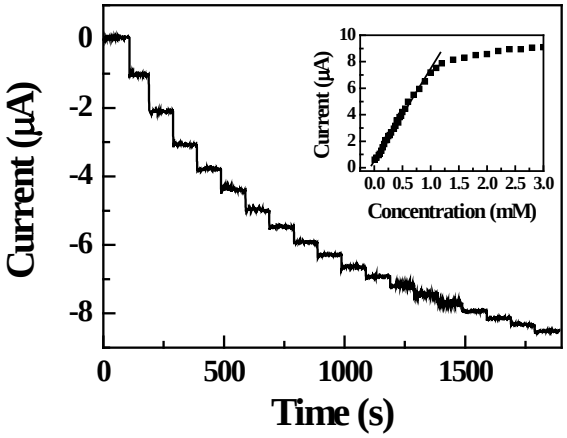

Fig. S4 Typical steady state current response of the electrocatalytic reduction of H_2O_2 by N-graphene on successive addition of 0.1 mM H_2O_2 in solution (PBS, pH 7.4) under a constant potential of –400 mV. Inset shows the calibration curve of the electrocatalytic current on the concentration of H_2O_2.

5. The selectivity and anti-interference characteristics of the N-graphene toward the electrocatalytic reduction of H_2O_2

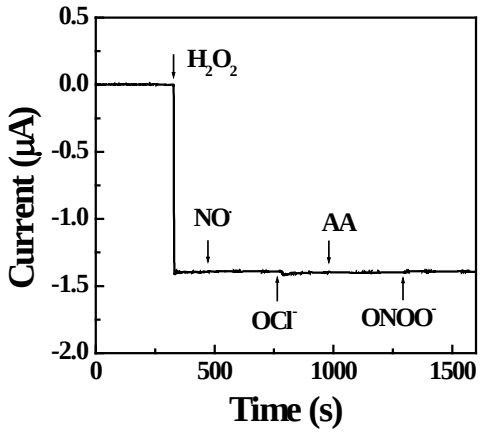

Fig. S5 Amperometric response of H_2O_2 (0.1 mM), NO· (0.1 mM), AA (0.1 mM), OCl^- (0.1 mM), and $ONOO^-$ (0.1 mM) at the N-graphene/GC electrode under an applied potential of –400 mV (versus SCE).

Interference experiments were performed to examine the selectivity of the N-graphene toward the reduction of H_2O_2. The response of the common coexisting ROS and compounds in biological system such as OCl^-, NO·, $ONOO^-$, and AA was recorded at the electrode. NO· was produced by dissolving 3-(aminopropyl)-1-hydroxy-3-isopropyl-2-oxo-1-triazene (NOC–5) into solution.[7,8] $ONOO^-$ was generated by dissolving 3-morpholinosydnonimine (SIN–1) into PBS (pH 7.4). OCl^- was formed by directly dissolving NaClO in solution. As shown in Fig. S5, a well-defined H_2O_2 response is observed at the N-graphene/GC electrode, whereas the same levels of OCl^-, NO·, $ONOO^-$, and AA result in negligible signals, suggesting the biosensor performs with high selectivity to H_2O_2 over these biological relevant ROS and biological compounds, indicative the suitability of the proposed biosensor to practical applications.

References

1 N. I. Kovtyukhova, P. J. Ollivier, B. R. Martin, T. E. Mallouk, S. A. Chizhik, E. V. Buzaneva and A. D. Gorchinskiy, *Chem. Mater.*, 1999, **11**, 771.

2 W. S. Hummers, Jr. and R. E. Offeman, *J. Am. Chem. Soc.*, 1958, **80**, 1339.

3 Y. Si, and E. T. Samulski, *Nano Lett.*, 2008, **8**, 1679.

4 R. Muszynski, B. Seger and P. V. Kamat, *J. Phys. Chem. C*, 2008, **112**, 5263.

5 S. Chettibi, A. J. Lawrence, R. D. Stevenson and J. D. Young, *FEMS Immunol. Med. Microbiol.*, 1994, **8**, 271.

6 A. W. Griffith and J. M. Cooper, *Anal. Chem.*, 1998, *70*, 2607.

7 X. C. Gong, Q. L. Li, K. H. Xu, X. Liu, H. M. Li, Z. Z. Chen, L. L. Tong, B. Tang and H. B. Zhong, *Electrophoresis*, 2009, **30**, 1983.

8 H. Madea, Y. Fukuyasu, M. Y. Fukuda, K. Saeki, H. Matsuno, Y. Yamauchi, K. Yoshida, K. Hirata and K. Miyamoto, *Angew. Chem. Int. Ed.*, 2004, **43**, 2389.