Ternary Hybrid of Carbon Nanotubes/Graphitic Carbon Nitride Nanosheets/Gold Nanoparticles as Robust Substrate Electrodes in Enzyme Biofuel Cell

Panpan Gai, Rongbin Song, Cheng Zhu, Yusheng Ji, Yun Chen, Jian-Rong Zhang, and Jun-Jie Zhu

a State Key Laboratory of Analytical Chemistry for Life Science and Collaborative Innovation Center of Chemistry for Life Sciences, School of Chemistry and Chemical Engineering, Nanjing University, Nanjing 210093, P. R China.

b School of Chemistry and Life Science, Nanjing University Jinling college, Nanjing 210089, P. R China.

* Corresponding authors. E-mail address: jrzhang@nju.edu.cn (J.-R. Zhang); jjzhu@nju.edu.cn (J.-J. Zhu).
Experimental Section

Materials and chemicals.

Pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQ-GDH; E.C. 1.1.5.2, from microorganism–not specified by the company) was purchased from Toyobo Co., Japan, and used as supplied. Bilirubin oxidase (BOD, E.C. 1.3.3.5, from *Myrothecium verrucaria*), poly(diallyldimethylammonium chloride) (PDDA, 20%, w/w in water, MW=200 000-350 000), 1-ethyl-3-(3-dimethyl-aminopropyl) carbodimiide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS) and 3-(N-morpholino) propanesulfonic acid (MOPS-buffer) were purchased from Sigma-Aldrich. Both of the enzymes were used as received without further purification. Chloroauric acid (HAuCl$_4$·4H$_2$O) was obtained from Shanghai Chemical Reagent Co. Ltd. (Shanghai, China). 0.1 M pH=7.4 phosphate buffer solutions (PBS) consisting of Na$_2$HPO$_4$ and NaH$_2$PO$_4$ were employed as the supporting electrolyte. Au NPs were prepared according to the literature by adding a sodium citrate solution to a boiling HAuCl$_4$ solution.$^1$ All the other reagents were of analytical grade and used without further purification. Ultrapure fresh water was obtained from a Millipore water purification system ($\geq$18 MΩ, Milli-Q, Millipore) was used throughout the whole experiment.

Apparatus.

X-ray powder diffraction (XRD) measurements were performed on a Japan Shimadzu XRD-6000 diffractometer with Cu Kα radiation ($\lambda$=0.15418 nm). Field emission scanning electron microscopy (FESEM) images and transmission electron microscopy (TEM) images were measured by a HITACHI S4800 SEM and a JEOL 2010 TEM, respectively. X-ray photoelectron spectroscopy (XPS) analysis was carried out on a Thermo Fisher X-ray photoelectron spectrometer system. Electrochemical impedance spectroscopy (EIS) was performed with an
Autolab electrochemical analyzer (Eco Chemie, The Netherlands) in 2.5 mM K$_3$Fe(CN)$_6$/K$_4$Fe(CN)$_6$ aqueous solution with 0.5 M KNO$_3$ as the supporting electrolyte, within the frequency range of 0.01 Hz to 100 kHz. Electrochemical measurements were performed on a CHI 660D workstation (Shanghai Chenhua Apparatus Corporation, China) with a conventional three-electrode system, which was composed of a platinum wire as the auxiliary electrode, a saturated calomel electrode (SCE) as the reference electrode, and a bioanode or biocathode as the working electrode, respectively. The open circuit potential of the electrodes were tested with a two-electrode configuration. After a stable $E_{ocp}$ was observed, the variable external load ranged from 50 $\Omega$ to 100 k$\Omega$ was connected in series between bioanode and biocathode. When the external load was connected in external circuit, the output voltage was decreased because of electrode polarization. The output voltage was tested after the EBFC operating 5 s. Then the power output was obtained by the equation $P=U^2/R$.

**Synthesis of the protonated g-C$_3$N$_4$ NSs.**

g-C$_3$N$_4$ NSs were prepared according to a previously published procedure with some modifications: bulk g-C$_3$N$_4$ was prepared by heating unipolar ratio of melamine and urea from room temperature to 550 °C with a ramp rate of 3°C min$^{-1}$ under in a nitrogenous atmosphere, and then the temperature was kept at 550°C for 2 h. To protonate the obtained bulk g-C$_3$N$_4$, 1 g of bulk g-C$_3$N$_4$ powder was dispersed in 100 mL of 5 M HNO$_3$ and refluxed for 12 h. The white product was centrifuged, washed with water to near-neutral pH, and re-dispersed in water. The resultant suspension solution was centrifuged at 3000 rpm for 15 min to remove the residual un-exfoliated g-C$_3$N$_4$ to obtain the g-C$_3$N$_4$ NSs, and then the solution was freeze-dried and dissolved with water to a concentration of 1 mg mL$^{-1}$.

**Synthesis of CNTs/g-C$_3$N$_4$ nanocomposite.**
CNTs (10 mg) were added to the as-made C$_3$N$_4$ NSs solution (1 mg/mL, 10 mL), and then the mixture was sonicated for 2 h in an ice bath to obtained a uniform and opaque black solution, followed by microwave heating at 180$^\circ$C for 20 min. The sonication and hydrothermal aging process resulted in the assembly of g-C$_3$N$_4$ NSs and CNTs. The obtained products were collected by centrifugation and dried at 110$^\circ$C overnight, denoted as CNTs/g-C$_3$N$_4$ NSs nanocomposite. Similarly, a series of CNTs/g-C$_3$N$_4$ NSs nanocomposite could be prepared by changing the mass ratio of CNTs and g-C$_3$N$_4$ NSs (i.e. 2:1, 1.5:1, 1:1, 1:2, 1:5, 1:10), that is, the mass percentage of g-C$_3$N$_4$ NSs in the CNTs/g-C$_3$N$_4$ NSs nanocomposite was 33.3 wt.%, 40 wt.%, 50 wt.%, 76.7 wt.%, 83.3 wt.% and 90.9 wt.% respectively.

**Preparation of CNTs/g-C$_3$N$_4$ NSs/Au NPs ternary hybrid and CNTs/Au NPs.**

Briefly, 5.0 mg of the CNTs/g-C$_3$N$_4$ NSs nanocomposite was dispersed in 1% PDDA salt solution (0.02 M NaCl, 5.0 mL) and then was sonicated for 30 min to form a homogeneous suspension of positively charged CNTs/g-C$_3$N$_4$ NSs/PDDA. Residual PDDA polymer was removed by centrifugation (15000 rpm, 10 min), and the obtained precipitate was washed with water at least three times. Subsequently, the purified CNTs/g-C$_3$N$_4$ NSs/PDDA was dispersed in 50 mL of the prepared negatively charged Au NPs solution, and stirred at room temperature for 2 h. After that excessive Au NPs were removed by centrifugation (8000 rpm, 10 min) and the CNTs/g-C$_3$N$_4$ NSs/Au NPs ternary hybrid precipitate was recovered and dissolved with water to a concentration of 1 mg mL$^{-1}$. The preparation of CNTs/Au NPs was similar to the ternary hybrid, in which CNTs were instead of CNTs/g-C$_3$N$_4$ NSs nanocomposite. The quantity of Au in the ternary hybrid and CNTs/Au NPs was 0.28±0.02 g/g, which were determined by ICP-MS technology.

**Preparation of PQQ-GDH or BOD modified ternary hybrid electrode.**
The PQQ-GDH modified CP/CNTs/g-C$_3$N$_4$ NSs/Au NPs ternary hybrid bioanode was prepared by first casting 50 μL of as-prepared ternary hybrid suspension on the surface of the carbon paper. Then, the ternary hybrid electrode was dried at 37°C for 2 h and immersed in a solution containing 1 mg mL$^{-1}$ EDC and NHS for 30 min to activate the carboxyl group of Au NPs. After rinsing with ultrapure water to eliminate excess EDC and NHS, the activated electrode was coated by 50 μL of PQQ-GDH solution (2 mg mL$^{-1}$, dissolved in MOPS-buffer (50 mM, pH 7.0) containing Na$_2$SO$_4$ (100 mM) and CaCl$_2$ (1 mM)) for 12 h at 4°C to obtain the PQQ-GDH modified ternary hybrid bioanode. The BOD modified ternary hybrid biocathode was fabricated similar to the bioanode, in which 50 μL of BOD solution (2 mg mL$^{-1}$, dissolved in 0.05 M pH 7.0 PBS solution) was instead of PQQ-GDH. Before the fabrication of the EBFC, both the prepared bioanode and biocathode were purged with ultrapure water to wipe off unbound enzymes. The electrodes were stored at 4°C when not in use. As a control, these enzymes modified CNTs/Au NPs electrodes were prepared in a similar process except that CNTs/Au NPs was used as the substrate instead of CNTs/g-C$_3$N$_4$ NSs/Au NPs ternary hybrid.

**Biofuel cell assembly.**

A membrane-less glucose/O$_2$ EBFC could be constructed by selecting the above bioanode and biocathode at the room temperature (25°C). The supporting electrolyte was oxygen-saturated 0.1 M PBS (pH=7.4) containing 5 mM of glucose.
Fig. S1 TEM of g-C₃N₄ NSs
**Fig. S2** SEM of CNTs/g-\(\text{C}_3\text{N}_4\) NSs nanocomposite with different mass percentage of g-\(\text{C}_3\text{N}_4\) NSs in CNTs/g-\(\text{C}_3\text{N}_4\) NSs nanocomposite. The mass percentage of g-\(\text{C}_3\text{N}_4\) NSs was labeled in SEM image.
Fig. S3 TEM of CNTs (A) and CNTs/Au NPs (B)
**Fig. S4** (A) XRD patterns of g-C$_3$N$_4$ NSs (a), CNTs (b), CNTs/Au NPs (c), CNTs/g-C$_3$N$_4$ NSs nanocomposite (d) and CNTs/g-C$_3$N$_4$ NSs/Au NPs ternary hybrid (e). (B) The changes of the zeta-potentials during the assembly process. The bars represent g-C$_3$N$_4$ NSs (a), CNTs (b), CNTs/g-C$_3$N$_4$ NSs nanocomposite (c), CNTs/PDDA (d), CNTs/g-C$_3$N$_4$ NSs/PDDA (e), CNTs/Au NPs (f) and CNTs/g-C$_3$N$_4$ NSs/Au NPs ternary hybrid (g). (C) XPS of the g-C$_3$N$_4$ NSs (a), CNTs/g-C$_3$N$_4$ NSs nanocomposite (b) and CNTs/g-C$_3$N$_4$ NSs/Au NPs ternary hybrid (c). (D) FT-IR spectra of the g-C$_3$N$_4$ NSs (a), CNTs/g-C$_3$N$_4$ NSs nanocomposite (b) and CNTs/g-C$_3$N$_4$ NSs/Au NPs ternary hybrid (c).
Fig. S5 The high resolution XPS of C and N in g-C$_3$N$_4$ NSs (A, C) and CNTs/g-C$_3$N$_4$ NSs nanocomposite (B, D)
Fig. S6 (A) EIS of bare CP electrode (a), CP/CNTs/g-C₃N₄ NSs/Au NPs ternary hybrid electrode (b), PQQ-GDH modified ternary hybrid bioanode (c) and BOD modified ternary hybrid biocathode (d). Insets are the enlarged view of curve b (left) and the Randles equivalent circuit used to fit the EIS data (right). (B) EIS of bare CP electrode (a), CP/CNTs/Au NPs electrode (b), PQQ-GDH modified CNTs/Au NPs bioanode (c) and BOD modified CNTs/Au NPs biocathode (d). Inset is the enlarged view of curve b.
Fig. S7 (A) The enlarged view of the defined segment of Fig. 2(A). (B) The enlarged view of the defined segment of Fig. 2(B).
**Fig. S8** (A) CVs of the ternary hybrid electrode (a), PQQ-GDH modified ternary hybrid electrode (b), CP/CNTs/Au NPs electrode (c) and PQQ-GDH modified CP/CNTs/Au NPs electrode (d) in PBS (pH=7.4) containing 0 mM glucose. (B) CVs of the ternary hybrid electrode (a), BOD modified ternary hybrid electrode (b), CP/CNTs/Au NPs electrode (c) and BOD modified CP/CNTs/Au NPs electrode (d) in PBS (pH=7.4) saturated with N\(_2\). Scan rate =50 mV s\(^{-1}\).
Fig. S9 (A) Plot of the anodic current density from the CV measurements at the potential of 0.4 V at PQQ-GDH modified ternary hybrid electrode with different mass percentage of g-C$_3$N$_4$ NSs in CNTs/g-C$_3$N$_4$ NSs nanocomposite. (B) Plot of the cathodic current density from the CV measurements at the potential of 0.1 V at BOD modified ternary hybrid electrode with different mass percentage of g-C$_3$N$_4$ NSs in CNTs/g-C$_3$N$_4$ NSs nanocomposite. The mass percentage of g-C$_3$N$_4$ NSs in CNTs/g-C$_3$N$_4$ NSs nanocomposite was 0 wt.%, 33.3 wt.%, 40 wt.%, 50 wt.%, 76.7 wt.%, 83.3 wt.% and 90.9 wt.%, respectively.
Fig. S10 (A) CVs of PQQ-GDH modified the ternary hybrid bioanode in the PBS (pH=7.4) solution containing 5 mM glucose saturated with N$_2$ (black curve) or O$_2$ (red curve). (B) CVs of BOD modified the ternary hybrid biocathode in the PBS (pH=7.4, saturated with O$_2$) solution in the absence (black curve) or presence (red curve) of 5 mM glucose. (C) CVs of PQQ-GDH modified CNTs/Au NPs bioanode in the PBS (pH=7.4) solution containing 5 mM glucose saturated with N$_2$ (black curve) or O$_2$ (red curve). (D) CVs of BOD modified CP/CNTs/Au NPs biocathode in the PBS (pH=7.4, saturated with O$_2$) solution in the absence (black curve) or presence (red curve) of 5 mM glucose.
Fig. S11 The polarization curves of ternary hybrid based EBFCs (a) and CNTs/Au NPs based EBFCs (b) in the oxygen-saturated 0.1 M PBS (pH=7.4) containing 5 mM of glucose. (c) Ternary hybrid based EBFCs in the nitrogen-saturated 0.1 M PBS (pH=7.4) containing 0 mM of glucose. Every point corresponds to the average value of three independent measurements.
Fig. S12 The stability of ternary hybrid based EBFCs (a) and CNTs/Au NPs based EBFCs (b) in the oxygen-saturated 0.1 M PBS (pH=7.4) containing 5 mM of glucose.
References