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Signal Amplification by Adsorption-Induced Catalytic Reduction of Dissolved Oxygen on Nitrogen-Doped Carbon Nanotubes for Electrochemiluminescent Immunoassay

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Experimental

Materials and Reagents. Nitrogen-doped carbon nanotubes (NCNTs) were synthesized according to the chemical vapor deposition (CVD) method reported previously, and were refluxed in 6 M NaOH at 110 ^{o}C for 4 h to remove the Al_2O_3 support, followed by refluxing in 1 M H_2SO_4 for 8 h to remove residual Fe catalysts.[S1] The purified NCNTs were thoroughly washed with deionized water until the pH value of the filtrate reached 7, and then dried at 70 ^{o}C overnight. Carbon nanotubes (CNTs, CVD method, purity ⩾98%, multi-walled, diameter 40-100 nm and length 1-2 μm) were purchased from Shenzhen Nanotech Port Co. Ltd (Shenzhen, China). Carcinoembryonic antigen (CEA) standard solution (1.0 mg mL^{-1}) was supplied by Shanghai Linc-Bio Science Co., Ltd. (Shanghai, China). Mouse monoclonal capture (Ab_1) and

signal (Ab_2) anti-CEA antibodies (clone No. 27D6 and 28E4) were purchased from Shuangliu Zhenglong Biochem. Lab (Chengdu, China). Bovine serum albumin (BSA), chitosan (≥85%, from crab shell, deacetylation), mercaptopropionic acid (MPA), poly(sodium 4-styrenesulfonate) (PSS, average Mw~70000) and thioacetamide were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Cadmium chloride ($CdCl_2 \cdot 2.5H_2O$) was purchased from Alfa Aesar China Ltd. The clinical serum samples were from Jiangsu Institute of Cancer Research. 0.1 M phosphate buffered salines (PBS) of various pHs were prepared by mixing the stock solutions of 0.1 M NaH_2PO_4 and Na_2HPO_4 containing 0.1 M KNO_3. The washing buffer was 0.05% (w/v) Tween-20 (PBST) in 0.01 M pH 7.4 PBS. The blocking solution was 0.01 M pH 7.4 PBS containing 5% (w/v) BSA and 0.05% Tween-20. All other reagents were of analytical grade and used as received. All aqueous solutions were prepared with ultrapure water from a Millipore water purification system (18 MΩ, Milli-Q, Millipore). The O_2 or N_2-saturated solution was prepared by bubbling highly pure O_2 or N_2 into the solution for 30 min.

Apparatus. X-ray photoelectron spectral (XPS) experiments were operated on an ESCALAB 250 spectrometer (Thermo-VG Scientific Co., U.S.A.) with an ultrahigh vacuum generator. Attenuated total reflection Fourier transformation infrared (ATR-FTIR) spectra were recorded on a Vector 22 Fourier transform infrared spectrometer (Bruker Optics, Germany). The UV-vis absorption spectra were obtained with a UV-3600 UV-vis-NIR spectrophotometer (Shimadzu Co., Kyoto, Japan). Photoluminescence (PL) spectra were obtained on a RF-5301 PC fluorometer (Shimadzu Co., Japan). Tapping mode atomic force microscopic (AFM) images were acquired under ambient conditions by directly casting sample dispersions onto mica sheets using an Agilent 5500 AFM/SPM system (U.S.A.) with Picoscan v5.3.3 software. After coated with Au film to improve the conductivity, the morphologies of sample films were examined

under an S-4800 scanning electron microscope (SEM, Hitachi, Japan) and a JEM-2100 transmission electron microscope (TEM, Hitachi, Japan). Electrochemical impedance spectroscopic (EIS) measurements were carried out on a PGSTAT30/FRA2 system (Autolab, the Netherlands) in 0.1 M KCl containing 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$. The impedance spectra were recorded in the frequency range of 10^{-1}-10^5 Hz with the amplitude of 5 mV. Cyclic voltammetric (CV) experiments were performed on a CHI 812B electrochemical workstation (CH Instruments Inc., USA), and electrochemiluminescent (ECL) measurements were carried out on a MPI-E multifunctional electrochemical and chemiluminescent analytical system (Xi'an, China), with a modified glassy carbon electrode (GCE, 5 mm in diameter, China) as working, a platinum wire as counter and a Ag/AgCl (saturated KCl) as reference electrodes. The ECL emission window was placed in front of the photomultiplier tube (PMT, detection range from 300 to 650 nm) biased at –500 V. Unless specially stated, the scan rate was 100 mV s^{-1}.

Preparation of PSS-functionalized NCNT (PNCNT) labeled Ab_2. PNCNTs were prepared by sonicating 0.01 mg pristine NCNTs in 200 μL of 0.5 M NaCl solution containing 0.5% (w/v) PSS for 30 min to form a homogenous dispersion followed by centrifugation thrice at 12000 rpm at 4 °C for 20 min and washing with water to remove the supernatant. The as-prepared PNCNTs were redispersed in 200 μL of 0.01 M pH 6.0 PBS containing 150 μg mL^{-1} Ab_2. The mixture was allowed to vortex thrice for 5 min every time at an interval of 30min and overnight at 4 °C. After shake for 1 h at room temperature, the mixture was centrifugated twice at 6000 rpm at 4 °C for 10 min and washed with PBST to obtained Ab_2-PNCNTs, which was redispersed in 100 μL of 0.01 M pH 6.0 PBS for immunoassay. PCNT and Ab_2-PCNTs were prepared following the same procedures.

Preparation of quantum dots (QDs). The water-soluble CdS QDs with MPA as stabilizing agent (MPA-CdS QDs) were prepared according to the reported method.[S2] Briefly, 86 μL of MPA was added to 20 mL of 20 mM $CdCl_2$ solution, which was then adjusted to pH 10 with 1 M NaOH. 20 mL of 20 mM thioacetamide solution was added with extensive stirring in air for 30 min. After refluxing at 80 °C for 10 h, the formed CdS QDs solution was dialyzed exhaustively for over one week at 4 °C. Finally, the product was concentrated by ultrafiltration at 10000 rpm at 4 °C for 10 min and diluted with water into a concentration of 14.1 μM. The obtained QDs solution was kept at 4 °C prior to use.

Preparation of ECL immunosensor and measurement procedure. A glassy carbon electrode (GCE) was polished to a mirror using 1.0 and 0.05 μm alumina slurry (Beuhler) followed by sonication in ethanol and water. After the electrode was rinsed with water and allowed to dry, 20 μL of 10 μM MPA-CdS QDs was dropped on its surface. After dried in air, 10 μL of 0.025% chitosan solution was coated on the QD film for covalent binding of CEA by activating the chitosan film with 15 μL of 2% glutaraldehyde in 0.1 M pH 7.4 PBS for 2 h and incubating 20 μL of Ab_1 (50 μg mL^{-1} in 0.01 M pH 7.4 PBS) for 60 min at 37 °C and overnight at 4 °C. The resulting surface was slowly washed with streams of PBST and PBS to remove the physically absorbed Ab_1, and blocked with 20 μL of 5% BSA solution for 1 h at room temperature to block possible remaining active sites against non-specific adsorption, and then washed with PBST and PBS again to form the ECL immunosensor, which was named as GCE/QDs/chitosan-Ab_1.

To carry out the immunoreaction and ECL measurement, the immunosensor was firstly incubated with 20 μL of CEA standard solution or serum sample for 30 min at 37 °C. After washing with PBST and PBS, it was further incubated with 20 μL of Ab_2-PNCNTs for 60 min at 37 °C, followed by washing with PBST and PBS. Finally, the ECL signal was detected in 0.1 M

pH 8.0 PBS containing 0.1 M KNO_3. The reference levels of CEA in the human serum samples were detected with an automation electrochemiluminescent analyzer (Elecsys 2010, Roche).

Characterization of CdS QDs

The UV-vis spectrum of the as-prepared CdS QDs showed an absorption inflection point at 371 nm (Fig. S1, curve a), from which the size and concentration of CdS QDs could be estimated to be 3.5 nm and 4.2 μM with Peng's empirical equations, respectively.[S3] The PL spectrum (excited at 365 nm) of CdS QDs solution showed a strong emission peak with a maximum intensity at 560 nm (Fig. S1, curve b). The similar PL excited wavelength and absorption wavelength indicated the emitter was contributed to the excited state of QD core (QD*).

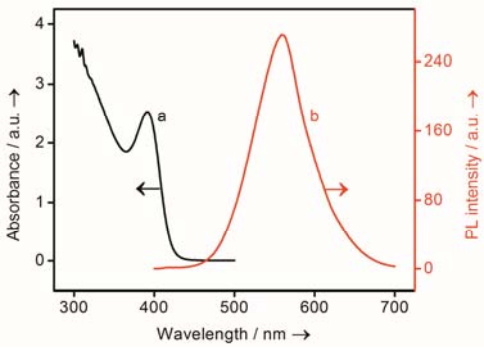

Fig. S1. UV-vis (a) and PL (b) spectra of MPA-CdS QDs.

Characterization of PSS-functionalized NCNT labeled Ab_2

The nitrogen covalently dopes in two forms of pyridinic N (401.8 eV) and graphitic N (400.0 eV) with an N/C atomic ratio of 6.30% calculated from the XPS (Fig. S2A). After functionalizing NCNTs with PSS by the wrapping method, the NCNTs could well disperse in water. PNCNTs could be validated by the presence of S at the content of 1.63% from the XPS curve (Fig. S2C) and the peaks at 1282 cm^{-1}, 1233 cm^{-1}, and 1159 cm^{-1} (two ν_{S-O} and one $\nu_{S-phenyl}$) corresponding to sulfonic acid group in the ATR-FTIR spectra (Fig. S2D), in which the

peaks at 1033 cm^{-1} (ν_{C-H} in-plane bending) and 973 cm^{-1} (out-of-plane hydrogen wagging) were the characteristic vibrations of a p-substituted phenyl group.[S4] The electron-withdrawing of N-dopants, resulting in net positive charge on the adjacent C atoms in the graphene plane, facilitated the functionalization of negatively-charged PSS by wrapping around the pristine NCNTs.[S5] The XPS survey scan of Ab_2-PNCNTs displayed an increasing N content up to 13.0%, while the XPS spectrum of distinct N1s peaks exhibited a N ratio of pyridinic (402.1 eV)/graphitic (400.5 eV) below one (Fig. S2B), showing a growing relative abundance of N in amino groups of protein. The ATR-FTIR spectra of PNCNTs and Ab_2-PNCNTs also confirmed the successful labeling of PNCNTs to Ab_2 (Fig. S2D). PNCNTs did not display obvious peak, while Ab_2-PNCNTs showed the vibration of amide I and amide II of protein around 1650 and 1539 cm^{-1} and the broad coupling of O–H at 3279 cm^{-1}.

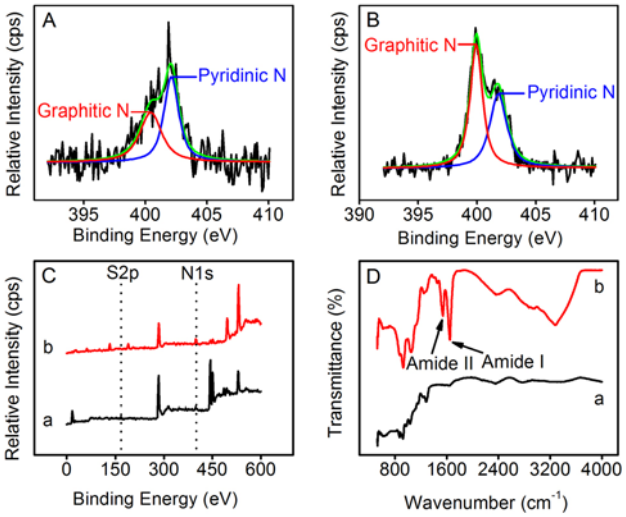

Fig. S2 XPS N1s spectra of (A) PNCNTs and (B) Ab_2-PNCNTs. (C) XPS survey scan of PNCNTs (a) and Ab_2-PNCNTs (b). (D) ATR-FTIR spectra of PNCNTs (a) and Ab_2-PNCNTs (b).

SEM and TEM images were also used to display the morphology change during the functionalization of PNCNTs. NCNTs showed defective bamboo-like structure with distinctive compartment layers and a diameter distribution of 40-60 nm (Fig. S3A). The doping of carbon

nanotubes with nitrogen can improve the biophilicity of carbon nanomaterials[S6] and is beneficial to the coverage of proteins in correct conformation.[S7] After PNCNTs were further labeled to Ab_2, the formed Ab_2-PNCNTs showed a rough surface due to the adsorption of protein along the sidewall (Fig. S3C), which led to a larger tube diameter than PNCNTs (Fig. S3B).

Characterization of the immunosensor

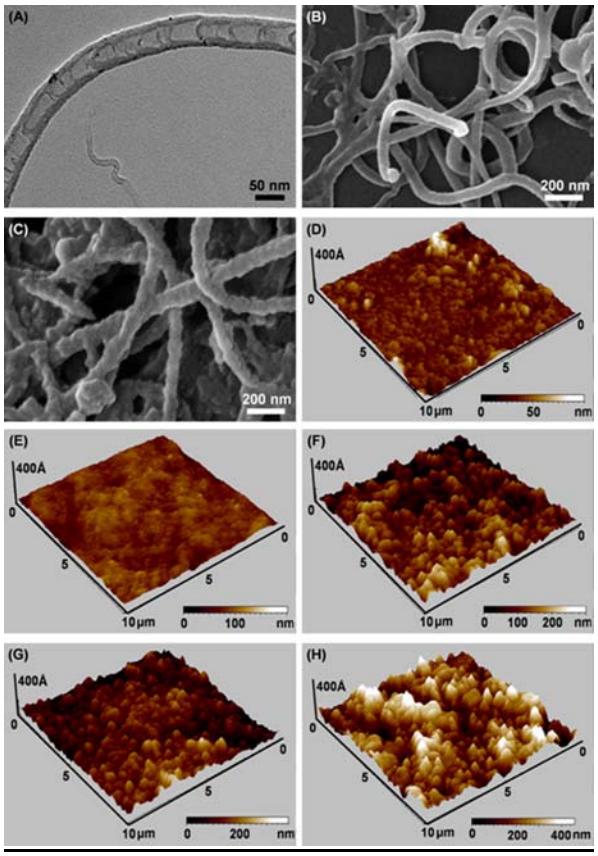

Fig. S3 TEM image of (A) NCNTs, SEM images of (B) PNCNTs and (C) Ab_2-PNCNTs, and AFM images of (D) QDs, (E) QDs/chitosan, (F) QDs/Ab_1, (G) QDs/Ab_1/BSA and (H) QDs/Ab_1/BSA/CEA.

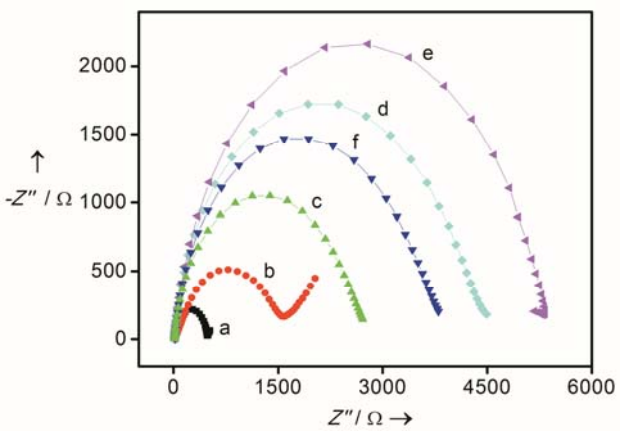

Fig. S4 EIS plots of bare GCE (a), GCE/QDs (b), GCE/QDs/Ab_1 (c), GCE/QDs/Ab_1/BSA (d) GCE/QDs/Ab_1/BSA/CEA (e) and GCE/QDs/Ab_1/BSA/CEA/Ab_2-PNCNTs (f).

Optimization of conditions

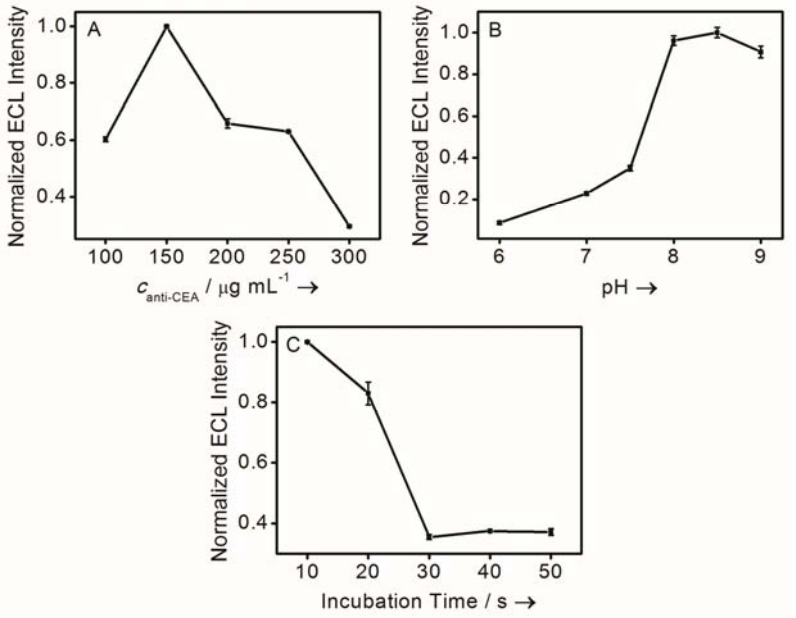

Fig. S5 Effects of (A) Ab_2 concentration used for preparation of Ab_2-PNCNTs, (B) pH of detection and (C) incubation time for CEA on ECL intensity in air-saturated 0.1 M PBS. 150 μg mL^{-1} Ab_2, pH 8.0 PBS and 30 min for CEA capture are the optimal conditions.

Stability and reproducibility

Both the intra-assay and inter-assay precisions of the ECL immunosensor were examined at 5 ng mL^{-1} CEA for five times. The relative standard deviations (RSD) were 4.5% and 6.6%, respectively, showing the good precision and acceptable fabrication reproducibility. Nine measurements of ECL emission upon continuous cyclic scans of the ECL immunosensor at 5 ng mL^{-1} CEA showed coincident signal with RSD of 0.81% (Fig. S6), indicating acceptable reliability and stability of the detection signal.

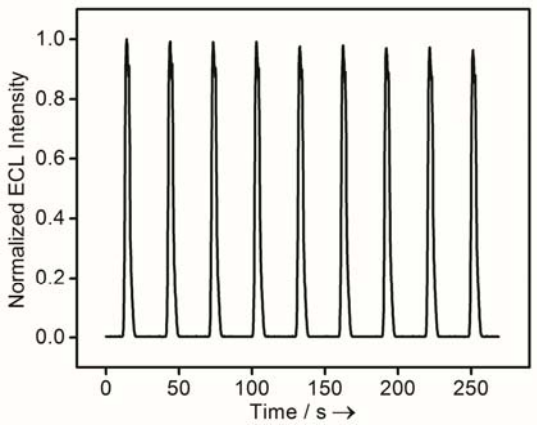

Fig. S6 Continuous cyclic scans of the immunosensor in the air-saturated detection solution after incubation with 5 ng mL^{-1} of CEA and then Ab_2-PNCNTs.

Table S1. Comparison of CEA determinations in human serum samples with the proposed immunosensor and turbidimetric immunoassay (in ng mL^{-1}).

Sample	proposed method	reference method	relative error (%)
1[a]	12.77	12.45	2.57
1[b]	1.286	1.245	3.29
1[c]	0.112	0.125	–9.96
2	5.74	5.32	7.89
3	3.17	2.98	6.38

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