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Electrocatalytic reduction of coreactant by highly loaded dendrimer-encapsulated palladium nanoparticles for sensitive electrochemiluminescent immunoassay

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Experimental

Materials and reagents. Single-walled carbon nanohorns (SWNHs) were obtained from Prof. Iijima's group (Japan Science and Technology Agency). Chitosan (≥85%, from crab shell, deacetylation), N-hydroxysulfosuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), potassium tetrachloropalladate (K_2PdCl_4), polyamidoamine dendrimer (ethylenediamine core, generation 4.0 solution, 10 wt.% in methanol) (PMM4), polyamidoamine dendrimer (ethylenediamine core, generation 5.0 solution, 5 wt.% in methanol) (PMM5) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, U.S.A.). Cadmium chloride ($CdCl_2 \cdot 2.5H_2O$), *meso*-2,3-dimercaptosuccinic acid (DMSA), glutaraldehyde (25% aqueous solution) and 2-(N-morpholino)ethanesulfonic acid (MES) were purchased from Alfa Aesar China Ltd. (China). Te rod (4 mm in diameter) was purchased from Leshan Kayada Photoelectricity Co., 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). 0.1 M phosphate buffered salines (PBS) with 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. The clinical serum samples were from Jiangsu Institute of Cancer Prevention and Cure. All other reagents were of analytical grade and used as received. Ultrapure water obtained from a Millipore water purification system (≥18 MΩ, Milli-Q, Millipore) was used in all assays. The O_2-saturated or free solution was prepared by bubbling highly pure O_2 or N_2 into the solution for 30 min.

Apparatus. UV-vis absorption spectra were recorded on a Shimadzu UV-3600 UV-Vis-NIR photospectrometer (Shimadzu Co., Japan). Attenuated total reflection Fourier transform IR (ATR-FTIR) spectra were recorded on a Vector 22 Fourier transform infrared spectrometer (Bruker Optics, Germany). 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. After coated with Au film to improve the conductivity, the morphologies of sample films were examined under an S-4800 scanning electron microscope (Hitachi, Japan). The specimen were also characterized on a carbon-coated 300 mesh copper grid by a JEM-2100 transmission electron microscope (TEM, Hitachi, Japan) with an accelerating voltage of 120 kV for low-resolution and 200 kV for high-resolution TEM images. Tapping mode atomic force microscopic (AFM) images were acquired under ambient conditions by directly casting sample dispersions onto freshly cleaved mica sheets using an Agilent 5500 AFM/SPM system (U.S.A.)

with Picoscan v5.3.3 software. Electrochemical impedance spectroscopic (EIS) measurements were carried out on a PGSTAT30/FRA2 system (Autolab, the Netherlands) in 0.1 M KCl containing 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ with the frequency range of 10^{-1}-10^5 Hz and the amplitude of 5 mV. Cyclic voltammetric 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 Remex Analytical Instrument Ltd. Co., China), with a modified glassy carbon electrode (GCE, 5 mm in diameter) as working, a platinum wire as counter and a Ag/AgCl (saturated KCl) as reference electrodes. All potentials were quoted against this reference electrode. The ECL emission window was placed in front of the photomultiplier tube (detection range from 300 to 650 nm) biased at –800 V. Unless specially stated, the scan rate was 100 mV s^{-1}. The reference levels of CEA in human serum samples were detected with an automation electrochemiluminescent analyzer (Elecsys 2010, Roche).

Preparation of PdNPs@PMM/SWNHs. SWNHs were ultrasonicated in an aqueous solution of 98% H_2SO_4, 68% HNO_3 and double-distilled water (1:3:6 in volume ratio) at 40 ºC for 6 h to generate carboxylated SWNHs. After centrifugation at 8000 *rcf* and decanting the supernatant, the sediment was washed with water until reaching pH 6.0 and dried at 50 ºC overnight. PMM4/5 was used as template to prepare dendrimer-encapsulated PdNPs. To avoid coordination of $PdCl_4^{2-}$ to the terminal primary amines of dendrimers, the solution pH was adjusted below 3 with HCl. Typically, 110 μL of PMM5 (10 wt.% in methanol) solution and 250 μL of 0.1 M K_2PdCl_4 aqueous solution were added to 9.64 ml of pH 3.0 HCl solution, the final molar ratio of $PdCl_4^{2-}$/PMM5 was kept as 120:1. When the ratio of $PdCl_4^{2-}$/PMM4 for preparation of PdNPs@PMM/SWNHs was 40:1. The solution was stirred for 1 h to ensure $PdCl_4^{2-}$ binding to

the interior tertiary amines, designated as $PdCl_4^{2-}$@PMM. About 1.0 mg of SWNHs was dispersed in 1 ml of DMF (1.0 mg mL^{-1}) by sonication for 2 h, and then the obtained $PdCl_4^{2-}$@PMM4/5 complex was mixed with the dispersion in the presence of 400 mM EDC and 100 mM NHS for 10 h with stirring to obtain $PdCl_4^{2-}$@PMM anchored SWNHs, which was then washed and centrifuged at 10000 *rcf* for several times to remove excessive $PdCl_4^{2-}$@PMM, EDC, NHS, *etc*. and redispersed in 5 mL water. Finally, PdNPs@PMM/SWNHs were prepared by dropwise addition of 100 μL freshly prepared 1.0 M $NaBH_4$ in the dispersion of $PdCl_4^{2-}$@PMM anchored SWNHs and vigorous stirring for 10 min at 0 ºC.

Preparation of PdNPs@PMM5/SWNHs labeled Ab_2. The PdNPs@PMM5/SWNHs were dissolved in 2 mL 0.1 M pH 6.0 MES and votexed with 20 μL 1 mg mL^{-1} Ab_2 in 40 mM EDC and 10 mM NHS for 20 min at intervals and stirred overnight. The suspension was centrifuged at 6000 *rcf* and washed with 0.01 M pH 7.4 PBST for several times to obtain Ab_2/PdNPs@PMM5/SWNHs. The tracing tag was diluted to 500 μL with 0.01 M pH 7.4 PBS containing 0.5% BSA and stored at 4 ºC. Before use the solution was further diluted by 4 times.

Preparation of quantum dots (QDs). DMSA-stabilized CdTe (DMSA-CdTe) QDs were synthesized according to the electrolysis method and employed as the ECL emitters.[S1] First, 6.0 mg of DMSA was dissolved by stirring in 20 mL ultrapure water containing 200 μL of 1.0 M NaOH to help the dissolution and regulate pH around 10. Then 120 μL of 0.1 M $CdCl_2$ was added dropwisely to obtain a homogeneous solution. After purged through highly pure N_2 for 20 min, potentiostatic hydrodynamic voltammetry, *e.g.* amperometric *i~t* curve was carried out to electrolyze the above solution at about −1.0 V upon a polished Te electrode. The solution remained in the N_2 atmosphere during the whole process, while the applying potential was tuned promptly by monitoring the current around 0.12 mA until a terminal charge quantity of 0.5 C

was reached. The resulting solution was sealed refluxing at 80 °C for 20 h to harvest the DMSA-CdTe QDs and stored at 4 °C prior to use. Before modification, the as-prepared QDs solution was purified and sedimentated in 1:1 (V/V) isopropyl alcohol/water and centrifuged at 5000 *rcf* for 5 min. Decanting the supernatant, the precipitation was then diluted in certain amount of water. According to the Peng's empirical equation,[S2] the concentration of QDs solution was estimated to be 100 μM with a size of 0.97 nm. The QDs could be easily immobilized on the electrode surface by convenient drop-casting due to the relatively low solubility of the capping agent in aqueous solution, which guaranteed the stability of ECL emission.

Preparation of ECL immunosensor and measurement procedure. A 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 DMSA-CdTe QDs was dropped on its surface. After dried in air, 10 μL of 0.025% chitosan solution was coated on the QD film for covalently binding of anti-CEA antibody by activating the chitosan film with 15 μL of 2% glutaraldehyde in 0.01 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 36 °C and 4 °C overnight in a 100% moisture-saturated environment. 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, and then washed with PBST and PBS again to form the ECL immunosensor.

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 36 °C. After washing with PBST and PBS, it was incubated with 20 μL of Ab_2/PdNPs@PMM5/SWNHs for 60 min at 36 °C, followed by washing with PBST and PBS again. Finally, the ECL signal was detected in air-saturated 0.1 M pH 9.0 PBS containing 0.1 M KNO_3.

Morphology characterization of Ab_2/PdNPs@PMM5/SWNHs

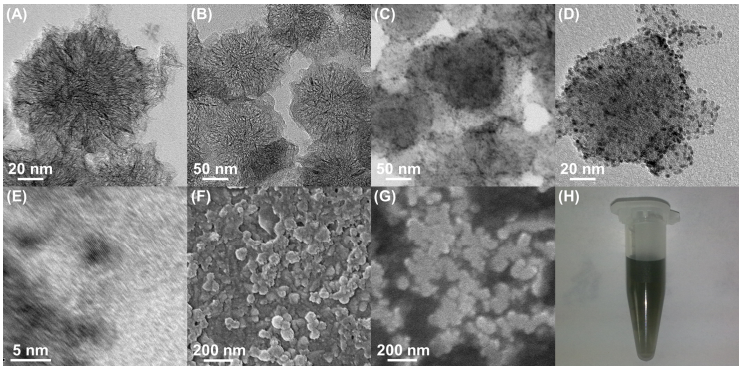

Fig. S1. TEM images of (A) SWNH, (B) PMM5/SWNHs, (C) PdNPs@PMM5/SWNHs, and (D) individual nanohybrid of PdNPs@PMM5/SWNH. (E) HRTEM image of PdNPs@PMM5. SEM images of (F) PdNPs@PMM5/SWNHs and (G) Ab_2/PdNPs@PMM5/SWNHs. (H) Photograph of PdNPs@PMM5/SWNHs suspension.

Spectroscopic characterization of Ab_2/PdNPs@PMM5/SWNHs

The preparation process of tracing tag could be confirmed by ATR-FTIR spectra. Both PMM4 and PMM5 showed two transmittance peaks with a slight difference in peak positions (Fig. S2A, curves a and b). The peaks for C=O stretching (amide I) and N−H bending/C−N stretching (amide II) vibrations of PMM4 were at 1632 and 1553 cm^{-1}, while those for PMM5 were at 1629 and 1552 cm^{-1}. The broad transmittance band of these dendrimers at 3271 cm^{-1} was assigned to the N−H stretching mode of terminal amines. The spectrum of the mildly oxidized SWNHs with acid treatment illustrated two broad peaks at 1181 and 1462 cm^{-1} (Fig. S2A, curve c), which were respectively associated with C−O stretching of saturated aliphatic ethers and the stretching of aromatic rings or C=C conjugated with nearby C=O groups. After PdNPs@PMM5/SWNHs nanohybrids were formed, the peak for amide I red shifted compared with free PMM5 (Fig. S2A, curve d), indicating that the interior functional groups were sensitive to the association with PdNPs in the dendrimer. The spectrum of Ab_2/PdNPs@PMM5/SWNHs showed the vibration of

amide I and amide II of proteins around 1633 and 1549 cm^{-1} and the broad coupling of O–H at 3254 cm^{-1}, which confirmed the successful labeling of Ab_2 to nanohybrids (Fig. S2A, curve e).

XPS was used to characterize the formation of PdNPs@PMM/SWNHs nanohybrids. Compared with pristine SWNHs (Fig. S2B, curve a), the acid treatment obviously increased the relative atomic ratio of O/C (Fig. S2B, curve b), indicating the generation of oxygenated species. In comparison with the spectrum of SWNHs and PMM/SWNHs (Fig. S2B, curves a and c), the spectrum of PdNPs@PMM/SWNHs showed two new strong peaks at 336 and 342 eV (Fig. S2B, curves d and e), which correspond to $Pd3d_{5/2}$ and $Pd3d_{3/2}$ of nanometric palladium in metallic state, respectively.[S3] This result confirmed that PdNPs were successfully anchored onto PMM/SWNHs. The XPS analysis further revealed PdNPs@PMM5/SWNHs (Fig. S2B, curve e) contained higher contents of Pd than PdNPs@PMM4/SWNHs (Fig. S2B, curve d). Hence, the high loading PdNPs in PMM5/SWNHs could be a good candidate for electrocatalytic reduction toward O_2 in sensitive immunoassay.

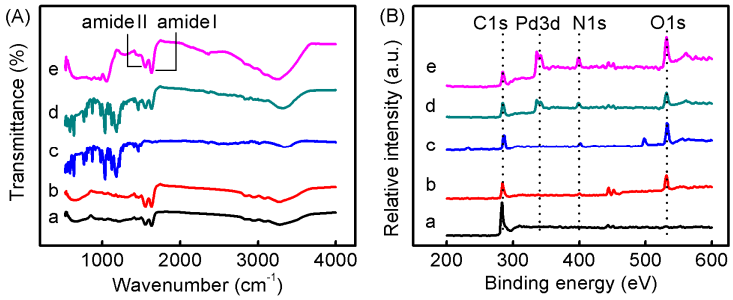

Fig. S2. (A) ATR-FTIR spectra of PMM4 (a), PMM5 (b), SWNHs (c), PdNPs@PMM5/SWNHs (d) and Ab_2/PdNPs@PMM5/SWNHs (e). (B) XPS survey scans of SWNHs (a), SWNHs (b), PMM/SWNHs (c), PdNPs@PMM4/SWNHs (d) and PdNPs@PMM5/SWNHs (e).

AFM characterization of the immunosensor

AFM images were utilized to monitor the sequential fabrication of QD-based ECL immunosensor and the immunological recognition. QDs film on a glass substrate displayed

smooth morphology with a height less than 100 nm (Fig. S3A). After chitosan was covered on the QDs film and Ab_1 was then covalently bound to the chitosan surface, the height become around 150 nm and some aggregation of Ab_1 could be observed (Fig. S3B). The blocking with BSA led to much more aggregation of proteins and an undulate height of 250 nm (Fig. S3C). After immunoreaction of the immobilized capture antibody with CEA, the height increased to over 300 nm (Fig. S3D). The different surface morphologies with increasing height confirmed the specific conjugation of antigen to the immobilized Ab_1.

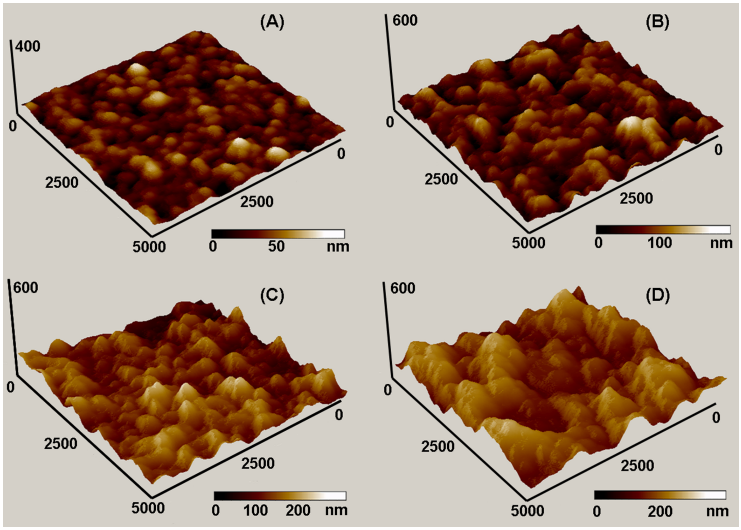

Fig. S3 AFM images of (A) QDs, (B) QDs/Ab_1, (C) QDs/Ab_1/BSA and (D) QDs/Ab_1/BSA/CEA.

EIS and ECL characterization of the immunosensor

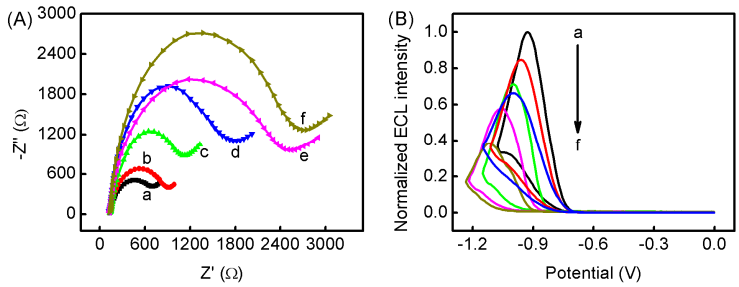

Fig. S4 (A) EIS plots of bare GCE (a), QDs (b), QDs/Ab_1 (c), QDs/Ab_1/BSA (d), QDs/Ab_1/BSA/CEA (e) and QDs/Ab_1/BSA/CEA/Ab_2-tag (f) modified GCE. (B) ECL-potential curves of QDs (a), QDs/Ab_1 (b), QDs/Ab_1/BSA (c), QDs/Ab_1/BSA/CEA (d), QDs/Ab_1/BSA/CEA/Ab_2 (e) and QDs/Ab_1/BSA/CEA/Ab_2-tag (f) modified GCE in air-saturated pH 9.0 PBS.

Formula of the QD-based ECL route

$$O_2 + 2e^- + 2H_2O \rightarrow H_2O_2 + 2OH^- \quad (1)$$

$$n\mathrm{QD} + ne^- \rightarrow n\mathrm{QD}^{\bullet -} \quad (2)$$

$$O_2 + 2\mathrm{QD}^{\bullet -} + 2H_2O \rightarrow 2\mathrm{QD}^* + 2H_2O_2 \quad (3)$$

$$H_2O_2 + 2\mathrm{QD}^{\bullet -} \rightarrow 2\mathrm{QD}^* + 2OH^- \quad (4)$$

$$\mathrm{QD}^* \rightarrow \mathrm{QD} + h\nu \quad (5)$$

Optimization of detection and synthetic conditions

The cathodic ECL peak intensity of QDs depended on the pH of detection solution. As shown in Fig. S5A, in the pH range from 6.0 to 10.0, the ECL intensity increased with the increasing pH value and then reached a plateau at pH 9.0. Although the ECL response slightly increased at pH 10.0, considering the bioactivity of immunoreagents, pH 9.0 PBS was selected as the detection solution throughout the following experiments.

The quantity of the excited state R* essentially depended on the amount of QDs. The strongest ECL intensity was obtained when 20 μL of 100 μM QDs solution was used for preparation of immunosensor (Fig. S5B), and more QDs led to a decrease of ECL response due to the inhibited electron exchange by the thicker QDs film.

Incubation time was an important parameter in immunoassay. With the increasing incubation time up to 30 min, the ECL emission quickly decreased due to the increasing amount of Ab_2/PdNPs@PMM5/SWNH assembled onto immunosensor surface, which blocked the electron

transfer and, more importantly, catalyzed the electro-reduction of dissolved oxygen, and thus quenched the ECL emission (Fig. S5C). At the incubation time of 30 min, the ECL emission trended to a minimum value, indicating a saturated binding between Ab_1 and CEA. Therefore, 30 min of incubation time was chosen for sandwich-type immunoassay.

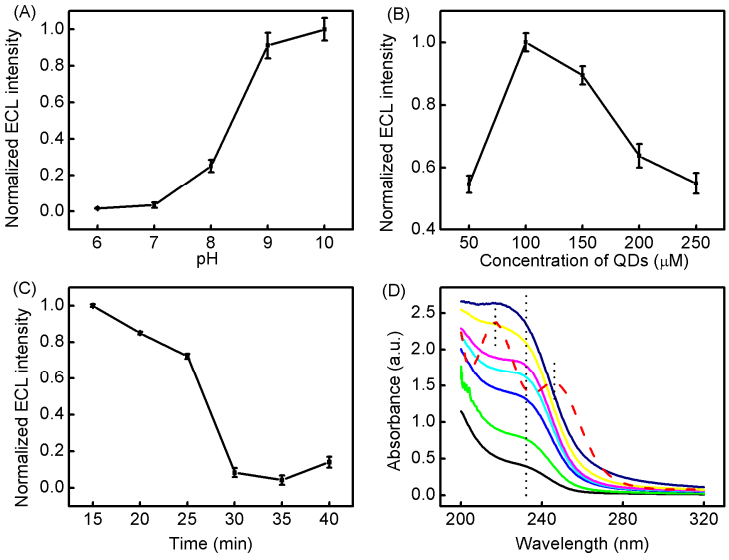

Fig. S5 Effects of (A) the buffer pH, (B) the concentration of QDs with a fixed volume of 20 μL and (C) the incubation time of CEA on ECL intensity. (D) UV-vis absorbance spectra of pH 3.0 HCl containing 10 μM PMM5 and $PdCl_4^{2-}$ with molar ratios of 1:20, 1:40, 1:60, 1:80, 1:100, 1:120 and 1:140 (solid lines from bottom to top) and 0.2 mM $PdCl_4^{2-}$ solution (dashed line). pH 9.0 PBS, 20 μL of 100 μM QDs and 30 min for CEA capture are the optimal detection conditions, while 120:1 for complexation of $PdCl_4^{2-}$ to PMM5.

K_2PdCl_4 solution (pH 3.0) showed the two strong ligand-to-metal charge-transfer bands at 217 and 246 nm (Fig. S5D, dashed), corresponding to the hydrolysis product of free $PdCl_3(H_2O)^-$ due to the relative low first-order cumulative formation constant ($\log K_1$=6.1) of $PdCl_4^{2-}$. After PMM5 was added in $PdCl_4^{2-}$ solution at a molar ratio of 1:40 and reaction for 1 h, the ligand-to-metal charge-transfer bands disappeared and a new single band occurred at about 235 nm,

suggesting negligible $PdCl_3(H_2O)^-$ in the solution and a new ligand-to-metal charge transfer band associated with the complex of Pd anion to interior tertiary amine of the dendrimer.[S4] When raising the ratio of $PdCl_4^{2-}$/PMM5 from 40:1 to 140:1, the absorbance band at about 235 nm increased significantly, indicating more complex of Pd anion to the amine was formed. The peak of the absorbance band showed an obviously blue shifting to 225 nm when the ratio changed from 120:1 to 140:1, which could be ascribed to the superposition of peaks corresponding to dendrimer-encapsulated $PdCl^{3-}$ (235 nm) and free $PdCl_3^-$ (217 nm), indicating that K_2PdCl_4 concentration exceeded its saturated binding to the interior amine of dendrimer, and the molar ratio of 120:1 became a critical point. At the ratio of 140:1, the homogeneous $PdCl_4^{2-}$/PMM5 solution became suspension after standing for 0.5 h at room temperature due to the interaction of exceeded $PdCl_3^-$ and exterior amine of dendrimer, which decreased the solubility of the complex. Thus this work chose 120:1 for complexation of $PdCl_4^{2-}$ to PMM5.

Reproducibility, stability and applications of the immunosensor

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 7.0% and 8.1%, respectively, demonstrating 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 1.2% (Fig. S5), indicating acceptable reliability and stability of the detection signal.

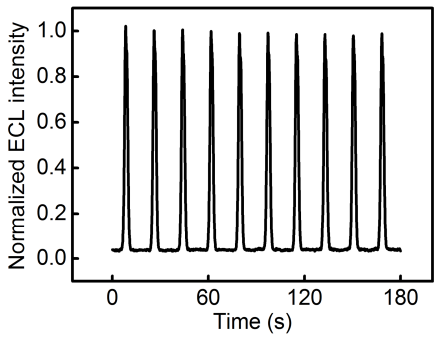

Fig. S6 Continuous cyclic scans of the immunosensor in air-saturated detection solution after incubation with 5 ng mL^{-1} of CEA and then Ab_2-labeled tracing tag.

Table S1. Assay results of CEA (ng mL^{-1}) in clinical serum samples using the proposed and reference methods.

Sample no.	1	2	3
Proposed method	1.19	2.84	5.74
Reference method	1.29	3.17	5.32
Relative error (%)	−7.8	−10.4	7.31

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