Electronic Supplementary Information

Ultrasensitive immunoassay based on dual signal amplification of the electrically heated carbon electrode and QDs functionalized labels for the detection of matrix metalloproteinase-9

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Experimental

Materials. MMP-9 antigen and MMP-9 antibody were purchased from Beijing Boisynthesis Biotechnology CO., LTD. Multiwalled carbon nanotubes (MWCNTs, with an average diameter of 50 nm) were purchased from Shenzhen Nanotech Port Co., Ltd. (Shenzhen, China). The MWCNTs were functionalized with carboxylic acid groups by sonication in a 3:1 sulfuric-acid/nitric-acid mixture for 8 h, in accordance to a previously described protocol.[1] 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), poly(diallyldimethylammonium chloride) (PDDA, 20%, w/w in water, MW 200000-350000), pyrrole(98%), dopamine and lyophilized 99% BSA were from Sigma–Aldrich. Polystyrene (PS) spheres of 200 nm in diameter were purchased from Northwestern Polytechnical University (Xian, China). PBS (10 mM, PH 7.4) was prepared by varying the ratio of NaH_2PO_4 to Na_2HPO_4. The standard MMP-9 antigen solution was prepared in the PBS and stored at 4 °C. All other chemicals were of analytical grade and were used as received. All aqueous solutions were prepared using ultrapure water (Milli-Q, Millipore).

Preparation of water-soluble CdTe QDs. The CdTe QDs were prepared as reported previously.[2] Briefly, freshly prepared NaHTe solution (as Te source, 1.0 mol L^{-1}, 10 μL) was injected through a syringe to N_2-saturated $Cd(NO_3)_2$ solution (as Cd source, 0.005 M, 50 mL) at

room temperature (20 °C) in the presence of 3-mercaptopropionic acid (MPA, 37 μL) as a stabilizing agent and the sulfur source in the later step. The pH was tuned to 12.2 by adding NaOH (1 M). The molar ratio of Cd^{2+}/MPA/NaHTe in the mixture was fixed at 1:1.7:0.04.

Synthesis of PS@PDA. 5 mg of PS spheres (200 nm) were sonicated several minutes in 3 mL tris-HCl and then centrifuged at 16000 rpm for 10 min, repeated the washing process for three times. Finally, PS spheres were dispersed in 10 mL Tris-HCl buffer and sonicated for at least 30 min. PS@PDA was prepared according to reference[3] by adding 20 mg of dopamine into the PS-Tris-HCl buffer and then stirred for 24 h. This dispersion was centrifuged and washed with Tris-HCl and doubly distilled water for two times.

Fabrication of Anti-MMP-9/PS@PDA/CdTe bioconjugates.

Anti-MMP-9 was attached to PS@PDA/CdTe using an EDC/NHS protocol. Briefly, 2 mL PS-PDA solution with 4 mL 0.2% PDDA was sonicated for 20 min, then centrifuged and discarded the upper layer. The solid was washed with ultrapure water for three times. 4 mL CdTe was added into the mixture and sonicated for 20 min, then centrifuged at 15000 rpm for 10 min and washed with ultrapure water for three times to remove excess particles. Half of the mixture in 1 mL water containing 50 mM MES was sonicated for 10 min, then mixed with 0.5 mL PBS containing 400 mM EDC and 100 mM NHS and reacted at room temperature for 1 h, then centrifuged at 15000 rpm and washed several times to remove excessive EDC and NHS. A total of 10 μL 1 mg mL^{-1} Ab_2 was added into the mixture and stirred for 6 h, then centrifuged at 12000 rpm at 4 °C for 10 min. A total of 500 μL PBS (PH 7.4) with 3% BSA was added to the bioconjugate to form a homogeneous dispersion and stored in the refrigerator at 4 °C before use.

Preparation of MWNTs-PPy modified GCE. The GCE with 3 mm in diameter was polished with 1.0, 0.3 and 0.05 μm alumina powder, and sonicated in acetone and water successively. Functionalized MWCNTs (1 mg mL^{-1}) was dispersed in doubly distilled water by sonication to form a concentration of 0.1 mg mL^{-1} solution. Pyrrole (0.1 M) was added to the solution containing water-soluble MWCNTs. The electropolymerization was at 0.7 V and was stopped when the quantity of charge was 0.01 C. Then the modified electrode was washed with doubly distilled water.

Fabrication of Immunosensors. The preparation of immunosensor is illustrated in Fig. 1B. MWNTs-PPy modified GCE was incubated in 10 μL Ab_1 for 12 h. After washing with PBS buffer, the Ab_1/MWNTs-PPy/GCE was incubated in 3% BSA and PBS solution at 37 °C for 1 h to block excess active groups and nonspecific binding sites on the surface. Then the electrode was incubated in 10 μL Ag for 40 min, after the binding reaction between Ab_1 and Ag, it was incubated with Ab_2/PS@PDA/CdTe bioconjugates for 40 min, the electrode was washed thoroughly with PBS to remove nonspecifically bound Ab_2 conjugates.

Apparatus.

A CHI760 electrochemical workstation and heated carbon pasted electrode (HCPE) were used. The construction of HCPE has been reported.[4] A function generator was used for heating. In all heating experiments the frequency was adjusted to 100 kHz. The temperature of the HCPE was controlled by changing the output of the function generator. A three-electrode electrochemical cell was used. The HCPE was used as the working electrode. Platinum wire and saturated calomel electrode were used as counter and reference electrodes, respectively. The morphology of the modified GCE was verified by field-emission SEM (FESEM, HITACHI S4800).

Transmission electron micrographs (TEM) were measured on a JEOLJEM 200CX transmission electron microscope, using an accelerating voltage of 200 kV. Electrochemical impedance spectroscopy (EIS) was performed with an Autolab electrochemical analyzer (Eco Chemie, The Netherlands) in a 10 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ (1:1) mixture with 1.0 M KCl as the supporting electrolyte, using an alternating current voltage of 5.0 mV, within the frequency range of 0.1 Hz–10 kHz. The static water contact angles were measured at 25 °C by a contact angle meter (Rame-Hart-100) employing drops of pure deionized water. The readings were stabilized and taken within 120 s after the addition

Stripping Voltammetric Analysis.

The CdTe QDs remained at the electrode were dissolved by the addition of 200 μL 0.1 M HNO_3 solution. The solution was transferred into 1800 μL of 0.1 M acetate buffer at pH 4.5, the amount of the dissolved Cd ions were determined by electrochemical stripping technique. The three-electrode system used here contained a HCPE, SCE and a platinum counter electrode. The process involved a 60 s pretreatment at 0.6 V, 200 s electrodeposition at -1.1 V, and stripping from -1.1 to -0.2 V using a DPV, with a 4-mV potential steps, 25-HZ frequency, and 25-mV amplitude

Electrochemical deposition of MWNTs-PPy .

By adjusting the quantity of charge in electropolymerization, the potentiostatic technique can be used to control the formation of MWNTs-PPy. Fig. S1 shows the morphologies of MWNTs-PPy with different charges. Fig.S1 (B) is the SEM image of the film with controlled charge of less than 0.01 C. The MWCNTs is smooth and thin layer of PPy can be seen. It can be observed in Fig. S1 (A) that the diameter of the MWCNTs is wider and the surface is rough,

indicating the formation of polypyrrole. If the controlled charge of electropolymerization was increased further and extra amount of polypyrrole was deposited outside the MWCNTs (Fig. S1 (B)), so the surface area of the film was decreased. Therefore, we chose the controlled charge of 0.01C in the present work.

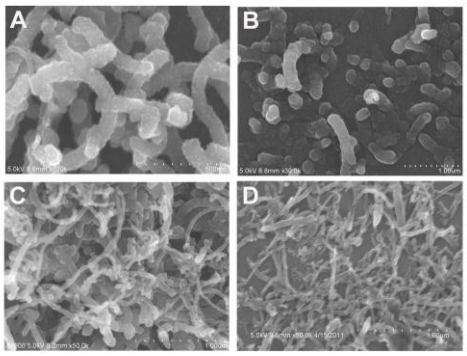

Fig. S1. SEM images of MWNTs-PPy at different charges for electropolymerization (A)-(C). (A) was at 0.01 C, (B) was surmount 0.01 C and (C) was just below 0.01 C. (D) was the pure MWNTs image.

Static Contact Angle Measurement

The hydrophilicity of an electrode surface is commonly used to characterize its biocompatibility, which can be measured with the contact angle of the substrate. As shown in Fig. S2, the contact angles of the bare GCE, CNTs, and MWNTS-PPy were 71.2 ± 1.9 °, 42.2 ± 1.5 °, and 23.4 ± 0.8 °, respectively. The MWNTs-PPy film showed the lowest contact angle, indicating better hydrophilicity, which was attributed to the presence of PPy. Thus, the improved biocompatibility of MWNTs-PPy film was in favor of enhancing protein loading and retaining the bioactivity, which thus improves the sensitivity of the immunosensor.

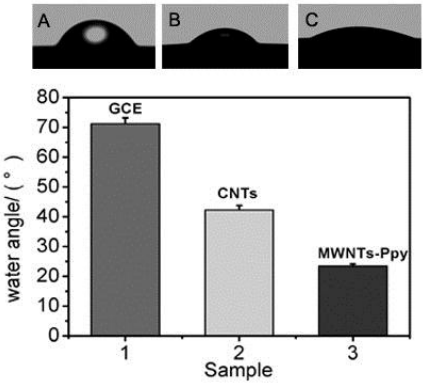

Fig. S2. Contact angle of bare GCE (A), CNTs (B), and MWNTs-PPy (C).

Electrochemical characteristics of the stepwise modified electrodes

The conductive property of electron film can be seen from the change of electrode-transfer resistance of $[Fe(CN)_6]^{3-/4-}$as redox probes with EIS. As shown in Fig. S3, compared with the bare GCE (a) and MWNTs/GCE (c), the EIS of MWNTs-PPy/GCE showed a lowest resistance (b), implying that MWNTs-PPy was an excellent electric conducting material and accelerated electron transfer.

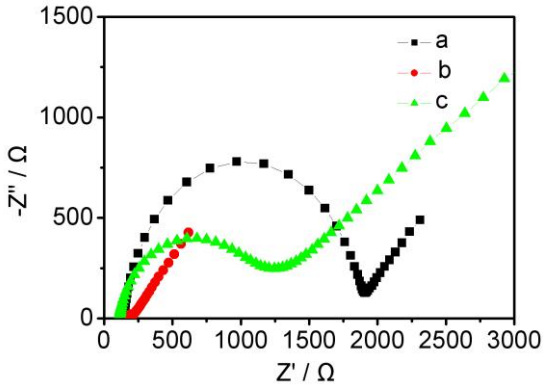

Fig. S3. EIS of (a) GCE, (b) MWNTs-PPy/GCE, and (c) MWNTs/GCE in 10.0 mM $[Fe(CN)_6]^{3-/4-}$ containing 1.0 M KCl.

Zeta Potential Measurement

In order to monitor the assembly process of the Anti-MMP-9/PS@PDA/CdTe, the zeta potential of the material at different stage was shown in Fig.S4, it showed that when PDA was formed

outside the PS spheres, the zeta potential was changed, after PS@PDA was treated with PDDA, the zata potential was positive indicating the existence of PDDA. After the negative CdTe was sonicated with PS@PDA/PDDA, the zeta potential turned negative again, showing the successful assembling of CdTe. According to Ab_2 probe attaching to the bioconjugate, the potential was changed again.

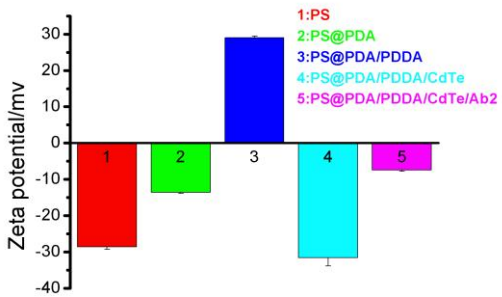

Fig. S4. Zeta potential of PS@PDA/PDDA/CdTe/Ab_2 at different stage.

Optimization of Conditions for Electrochemical Detection

The concentration of the Ab_2 and the time of the antigen-antibody reaction conjugates could affect the analytical performance of the proposed immunosensor. As shown in Fig. S 5(A), with the increasing concentration of Ab_2 from 10 to 40 mg mL^{-1}, the peak current showed an increasing response until the Ab_2 concentration of 32.16 mg mL^{-1}, indicating that all available recognition sites of immobilized MMP-9 were match with the nanoprobe. Thus, 32.16 mg mL^{-1} was chosen as the optimal nanoprobe concentration. Moreover, at the optimized nanoprobe concentration, peak current changed with incubation time in the range from 10 to 60 min, and reached a maximum value at 40 min (Fig. S 5(B)),. For sufficient recognition of target protein, 40 min was chosen as the optimal incubation time.

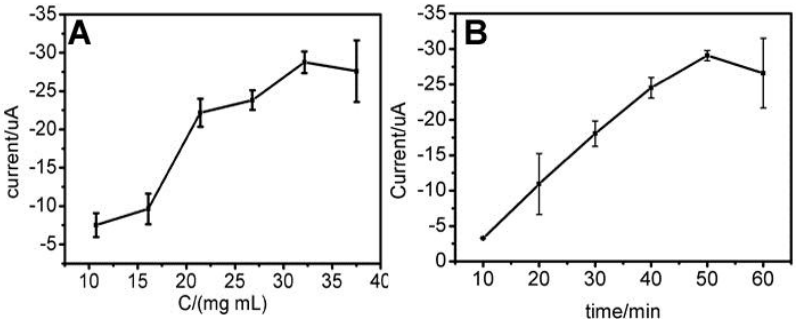

Fig. S5. Effects of PS@PDA/PDDA/CdTe/Ab_2 concentration (A) and incubation time (B) on peak currents of DPV on HCPE for 10 pg mL^{-1} MMP-9.

Selectivity, reproducibility and stability

To investigate the specificity of the immunosensor, we mixed 10 pg mL^{-1} MMP-9 with 100 pg mL^{-1} AFP, 100pg mL^{-1} human IgG or 100 pg mL^{-1} BSA respectively, and then detected the current response of the mixture. Compared with the response of the immunosensor in 10 pg mL^{-1} pure MMP-9, no significant difference (R.S.D ranged from 4.1 % to -2.3 %) was observed, indicating that the human AFP, IgG and BSA could not cause the observable interference (Fig. S 6(A)). Furthermore, when the immunosensor was stored at 4 °C for 14 days, it remained more than 90 % of initial responses for MMP-9. This result indicated the good stability of the immunosensor which might be attributed to the good biocompatibility of MWNTs-PPy since they could retain the bioactivity of proteins. The slow decrease of responses might be due to the gradual deactivation of the immobilized biomolecules (Fig. S 6(B)).

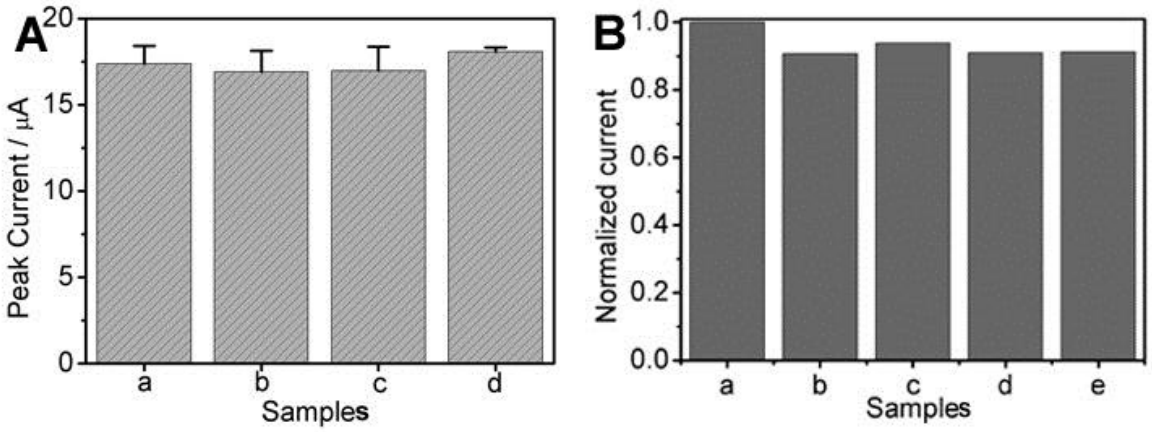

Fig.S6. (A) The specificity of the immunosensor towards other proteins. The peak currents to (a) 10 pg mL^{-1} MMP-9, (b) 100 pg mL^{-1}AFP, (c) 100 pg mL^{-1} BSA, and(d) 100 pg mL^{-1} IgG. (B) Normalized currents of the immunosensor for 100 pg mL^{-1}MMP-9 after 14 d storage: (a) control, (b)-(e) 14 d.

References

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4. **J. J. Zhang, Y. Liu, L. H. Hu, L. P. Jiang and J. J. Zhu, *Chem. Commun.*, 2011, 47, 6551-6553.**