Supporting information

Nanobody-based Electrochemiluminescent Immunosensor for Sensitive Detection of Human Procalcitonin

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1. Experimental Section

1.1. Materials and reagents

Bovine serum albumin (BSA) and Triton X-100 was obtained from SunShine BIO (Nanjing, China). PCT polyclonal antibody (Ab) was provided by Huibiao BIO (Nanjing, China). (3-Aminopropyl)-triethoxysilane (APTS), tetraethoxysilane (TEOS) and chitosan (CS) were purchased from Sigma-Aldrich (Shanghai, China). Cyclohexane, n-hexane, ammonium hydroxide ($NH_3 \cdot H_2O$, 28 % in water), ethanol and glutaraldehyde solution (GA, 25 % in water) were obtained from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). CdTe quantum dots (QDs) were purchased from Shenzhen Biological Tech. Co. (Shenzhen, China). Graphite oxide was obtained from Nanjing XFNANO Materials Tech Co. (Nanjing, China). All other chemicals were of analytical grade and were used as received. The 0.1 M phosphate buffer solutions (PBS) were prepared by mixing 0.1 M NaH_2PO_4 and Na_2HPO_4. Ultrapure water ($\geq$ 18 MΩ cm^{-1}) was used throughout the study.

1.2. Apparatus

Electrochemiluminescence (ECL) measurements were carried out on a MPI-E multifunctional electrochemiluminescent analytical system (Xi'an Remex Analyze Instrument Co. Ltd., China). All ECL measurements were performed in a 5 mL glass cell composed of a modified glassy carbon electrode (GCE, 3 mm in diameter), a platinum wire auxiliary electrode, and an Ag/AgCl (saturated KCl solution) reference electrode. Electrochemical impedance measurements (EIS) were conducted with a CHI Instruments Model 830C electrochemical workstation (Shanghai Chenhua, China). EIS were performed in a 0.1 M KCl solution containing 5 mM $Fe(CN)_6^{3-/4-}$ with a frequency range from 0.01 Hz to 100 kHz at 0.24 V, and the amplitude of the applied sine wave potential in each case was 5 mV. The morphology and size of CdTe@SiO_2 nanoparticles were analyzed with a transmission electron microscope S-2400N (TEM, HITACHI, Japan) and scanning electron microscope JSM-7001F (SEM, JOEL, Japan). Photoluminescence (PL) spectra were obtained on a Model FluoroMax-4

spectrophotometer (Horiba, Japan). UV-vis spectra were performed with a UV-2450 spectrophotometer (Shimadzu, Japan).

1.3. Expression and purification of recombinant PCT

The gene encoding human PCT was synthesized and cloned into pET32a vector and was confirmed by sequencing in Generay Biotechnology Co., Ltd (Shanghai, China). E. coli (DE3) transformed with pET32a-PCT were inoculated in 5 mL LB media containing 100 μg mL^{-1} ampicillin, and cultured at 37 ºC with shaking overnight. On the next day, 1 mL of overnight culture was transferred into 100 mL fresh LB medium containing 100 μg mL^{-1} ampicillin and followed by culturing at 37 ºC for 3 h until OD600 to 0.5. Isopropyl-B-D-thiogalactopyranoside (IPTG) was added into the culture to a final concentration of 1 mM to induce the expression of fusion protein. The purification of recombinant protein was followed as described in the manufacture's protocol of the ProBondTM Purification System (Invitrogen).

1.4. Construction of a nanobody library and selection of PCT-specific Nbs

A two humped camel was immunized by weekly injections of recombinant PCT antigen mixed with Freunds adjuvant. Three days after the last boost (day 42 from start of immunization), 100 mL blood was drawn from the dromedary, lymphocytes were isolated and a phage display library containing the genes coding for the variable domains of the heavy-chain antibodies (nanobodies) was then generated according to established methods.[1] Nanobodies specific for PCT were isolated after phage display and five rounds of biopanning. PCT specific Nbs were then expressed in *E.coli* and purified to homogeneity.

1.5. Preparation of the anti-PCT nanobody modified GC substrates

1.5.1. Synthesis of chitosan-graphene nanocomposite (GR-CS)

The graphene oxide (GO) was obtained by exfoliation of graphite oxide under ultrasonication in a water bath for 2 h. The obtained brown dispersion was centrifuged at 3000 rpm for 30 min to remove any unexfoliated graphite

oxide. Dialysis was performed to completely remove any residual salts. Then, 10 mL of the prepared GO (1.0 mg mL^{-1}) dispersion was mixed with 1 mL of 0.1 wt% CS in pH 4.0 acetic acid solution. The obtained homogeneous GO-CS mixture was then stirred at 60 ºC for 2 h. After that, 0.5 mL of 1.0 mM ascorbic acid and $NH_3 \cdot H_2O$ were added (to adjust pH to 8.5), followed by continuous stirring at 95 ºC for 15 min. The dispersion was then centrifuged, washed with water and dried in air for 24 h.

1.5.2. Preparation of the nanobody modified GCE

The preparation of the nanobody modified GCE is illustrated in Scheme 1. Prior to each measurement, GCE was carefully polished to a mirror-like surface with 1.0, 0.3 and 0.05 μm alumina slurries, followed by rinsing thoroughly with water. After successive ultrasonication in ethanol and water for 3 min, respectively, the electrode was rinsed with water and dried under nitrogen stream. 5.0 μL of prepared GR-CS dispersion (0.5 mg mL^{-1}) was dropped on the surface of the freshly pretreated GCE and dried in ambient air. Then the modified electrode was incubated with 20.0 μL of 1.0 wt% glutaradehyde solution in pH 7.4 PBS at room temperature for 2 h. After rinsing with water, 10.0 μL anti-PCT nanobody (Nb_1) (500 μg L^{-1}) in 0.01 M pH 7.4 PBS, was dropped onto the surface of the resultant GCE. After 12 h of incubation at 4 ºC, the electrode was washed thoroughly with PBS and then soaked in 1% BSA solution at 4 ºC for 30 min to block the nonspecific binding sites on the electrode surface. The anti-PCT nanobody modified GCE electrode (Nb_1-GR-GS-GCE) was then stored at 4 ºC for subsequent use. For control experiments, PCT polyclonal antibody modified electrode was prepared following the same procedure (Ab-GR-GS-GCE).

1.6. Synthesis of antibody/CdTe@SiO_2 nanosphere labels

1.6.1. Synthesis of CdTe@SiO_2 nanoparticles

Briefly, 1.77 mL Triton X-100, 7.5 mL cyclohexane, 1.8 mL 1-hexanol and 100 μL aqueous solution of CdTe QDs (1 mg mL^{-1}) were mixed under continuous stirring. Then, 100 μL TEOS and 100 μL APTS were added. The

polymerization reaction was initiated by adding 200μL $NH_3 \cdot H_2O$. The reaction was allowed to stir for 24 h at 4 ºC and was terminated by adding ethanol. The CdTe@SiO_2 nanoparticles were collected by centrifugation, washing with ethanol and water, drying in a vacuum at room temperature.

1.6.2. Synthesis of nano-immunological label

To generate nano-immunological labels, the prepared CdTe@SiO_2 nanoparticles were reacted with 5 mL GA solution (1%, in pH 7.4 PBS,) for 1h with stirring. The resultant solution was centrifuged and washed thoroughly with PBS to remove excess GA. The precipitates were dispersed with 1x PBS to a final volume of 5 mL. 1 mL of the above CdTe@SiO_2 suspension (0.5 mg mL^{-1}) was mixed with 20 μL of Nb_{II} or Ab solution (500 μg mL^{-1} in PBS solution) under continuous stirring. The reaction occurred at room temperature during 1 h. Subsequently, unreacted antibodies were removed from the supernatant after centrifugation and the pellet was washed several times with PBS to obtain the Nb_{II}-modified CdTe@SiO_2 nanoparticles (CdTe@SiO_2/Nb_{II}). Finally, the CdTe@SiO_2/Nb_{II} nanoparticles were dispersed by stirring in 5 mL of 1% BSA solution during 2 h, to block excess of aldehyde groups and nonspecific binding sites of the CdTe@SiO_2/Nb_{II} nanoparticles. After being centrifuged and washed with PBS, the resultant CdTe@SiO_2/Nb_{II} nanoparticles were dispersed with PBS to a final volume of 2 mL and stored at 4 ºC for later use.

1.7. Sandwich immunoassay with CdTe@SiO_2/Nb_{II} as label

The Nb_I-GR-GS-GCE was incubated with the desired concentration of PCT solution (Ag) in a volume of 100 μL at 37 ºC for 45 min to capture Ag with the first immuno-reactive (Ag-Nb_I-GR-GS-GCE). After being thoroughly washed with PBS, the Ag-Nb_I-GR-GS-GCE was exposed to 0.5 mL of CdTe@SiO_2/Nb_{II} suspension for another 45 min at room temperature. The exposure introduced the CdTe@SiO_2/Nb_{II} label onto the electrode surface through the second immunoreaction. This electrode was thoroughly rinsed again and was gently shaken in

PBS to remove physically adsorbed CdTe@SiO_2/Nb_{II} nanoparticles. Finally, the sandwich immunoassay was completed, and the CdTe@SiO_2/Nb_{II} modified GCE (CdTe@SiO_2/Nb_{II}-Ag-Nb_I-GR-GS-GCE) was thus obtained.

1.8. ECL Detection

The electrolyte was a solution of 0.1 M pH 7.4 PBS containing 0.1 M KCl and 0.1 M $K_2S_2O_8$. The potential range applied to the GC working electrode in the CV measurement was from 0 to –1.7 V at 100 mV s^{-1}. The ECL emission intensity (I_{ECL}) corresponding to CV measurements was recorded by the MPI-E multifunctional electrochemiluminescent analyzer at room temperature. The emission window was placed in front of the photomultiplier tube, which was biased at 600 V.

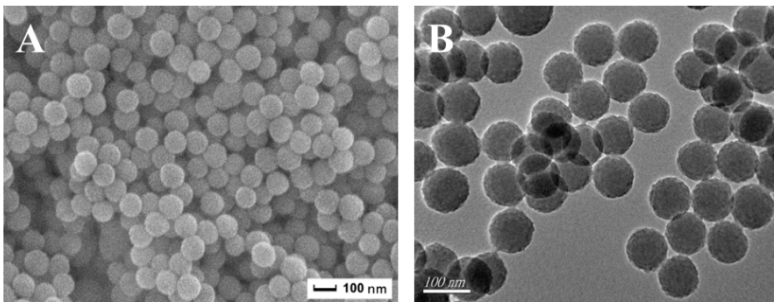

Fig. S1 SEM (A) and (B) TEM image of CdTe@SiO_2 nanoparticles.

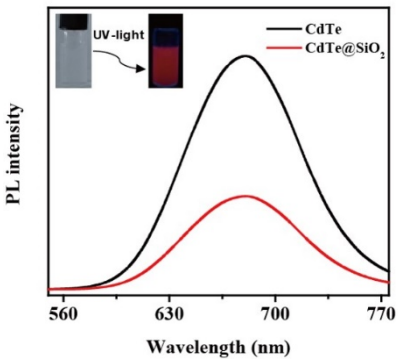

Fig. S2 Photoluminescence spectra of CdTe QDs and CdTe@SiO_2 nanoparticles in aqueous solution, inset is the color change of CdTe@SiO_2 nanoparticles solution with and without UV light illumination.

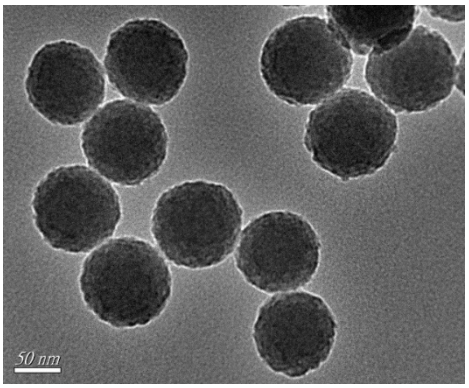

Fig. S3 TEM image of CdTe@SiO_2/Nb_{II}.

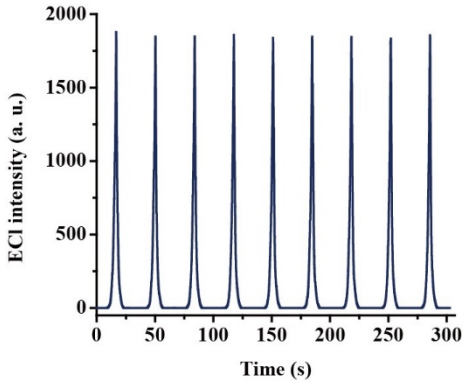

Fig. S4 ECL emission of the immunosensor fabricated using 1 ng mL^{-1} PCT for 9 continuous potential scanning cycles with a scan rate of 100 mV s^{-1} in 0.1 M pH 7.4 PBS-containing 0.1 M KCl and 0.1 M $K_2S_2O_8$.

Electrochemical impedance spectrum (EIS) was employed to provide useful information on the functionalization process. The CdTe@SiO_2 nanoparticles, after attaching NbII onto their surface (without the BSA blocking process), showed a significant increase in diameter, which suggest that the nanobody might form an additional barrier, shielding the $Fe(CN)_6^{3-/4-}$ redox probe from the electrode surface (Fig. S5). The result confirms that CdTe@SiO_2 nanoparticles were successfully functionalized with NbII.

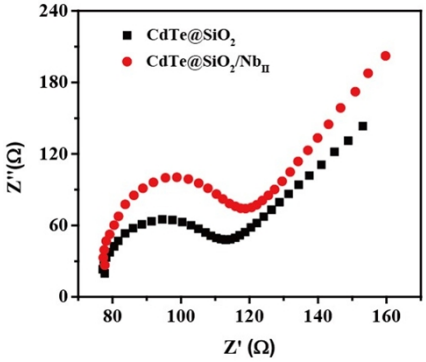

Fig. S5 EIS spectra of CdTe@SiO_2 nanoparticles and CdTe@SiO_2/Nb_{II} label.

A series of experiments have been performed for a better understanding of the ECL signal generation. A clear ECL signal was observed from Nb_{II}/Ag/Nb_I-GR-GS-GCE in a 0.1 M pH 7.4 air-saturated PBS solution containing 0.1 M KCl and 0.1 M $K_2S_2O_8$ (Fig. S6, curve a). Furthermore, the ECL intensity could be greatly enhanced in presence of CdTe@SiO_2/Nb_{II} on the electrode surface (Fig. S6, curve c), while no ECL emission was observed for CdTe@SiO_2/Nb_{II}/Ag/Nb_I-GR-GS-GCE in a 0.1 M pH 7.4, air-saturated, PBS-containing 0.1 M KCl, without $K_2S_2O_8$ (Fig. S6, curve b). This indicated the crucial role played by $S_2O_8^{2-}$ in the cathodic ECL. Two cathodic peaks at –1.36 V and –0.94 V were observed from CdTe@SiO_2/Nb_{II}/Ag/Nb_I-GR-GS-GCE in a 0.1 M pH 7.4 air-saturated PBS solution containing 0.1 M KCl and 0.1 M $K_2S_2O_8$, respectively (Fig. S7). The peak at –1.36 V was attributed to the electrochemical reduction of CdTe QDs to negatively charged radical $CdTe^{-\bullet}$. The other peak at –0.94 V was attributed to the reduction of $S_2O_8^{2-}$ to anion sulfate radical $SO_4^{-\bullet}$. The strong oxidant, $SO4^{-\bullet}$, could react following injection into a hole with the highest occupied molecular orbital of $CdTe^{-\bullet}$ to obtain an excited state ($CdTe^*$), which emitted light in the aqueous solution to obtain an ECL signal. The dissolved oxygen could be a co-reactant in the ECL process.[2] The ECL emission was slightly lower than that of the air-saturated solution, when dissolved oxygen was chased from the solution by bubbling with high-purity nitrogen (Fig. S6, curve d).

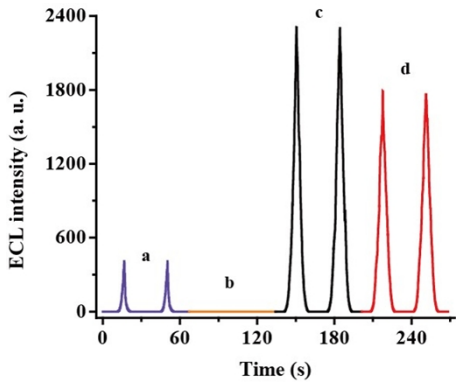

Fig. S6 (A) ECL response of Nb_{II}-Ag-Nb_I-GR-GS-GCE (a) and CdTe@SiO_2/Nb_{II}-Ag-Nb_I-GR-GS-GCE in air-saturated 0.1 M pH 7.4 PBS-containing 0.1 M KCl and 0.1 M $K_2S_2O_8$ (c) or without the presence of 0.1 M $K_2S_2O_8$ (b); (d) CdTe@SiO_2/Nb_{II}-Ag-Nb_I-GR-GS-GCE in 0.1 M pH 7.4 PBS-containing 0.1 M KCl and 0.1 M $K_2S_2O_8$ after displacing oxygen with pure nitrogen for 30 min.

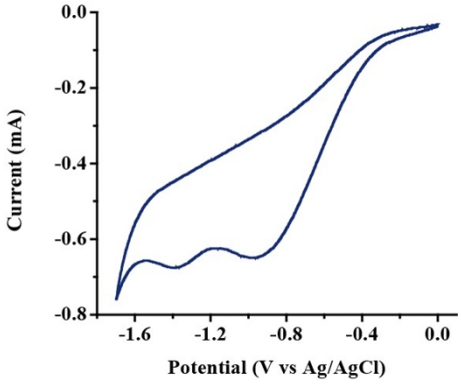

Fig. S7 Current Voltage diagram of CdTe@SiO_2/Nb_{II}-Ag-Nb_I-GR-GS-GCE at a PCT concentration of 1 ng mL^{-1} at 100 mV s^{-1}.

An intra-assay relative standard deviation (RSD) was used to evaluate the repeatability of the different electrodes. Fig. S8 showed RSD of 1.75%, 0.73%, 0.85%, and 0.37%, as examined by five determinations, respectively. The results indicated the acceptable repeatability of the modified electrodes.

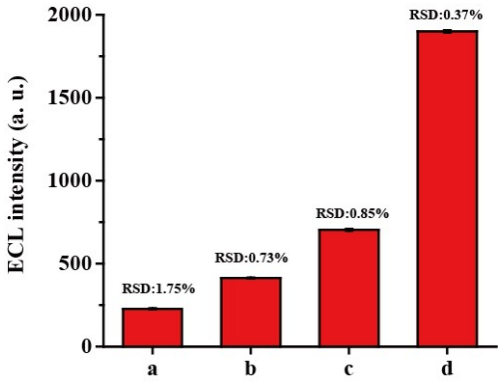

Fig. S8 The repeatability of ECL intensity obtained for (a) bare GCE, Nb_I-GR-GS-GCE (b-d) incubated in 1ng mL^{-1} PCT solution (b), followed by incubation with QDs/Nb_{II} (c) or with CdTe@SiO_2/Nb_{II} suspension (d).

Table 1. Comparison of Serum PCT Levels in Clinical Samples of the Proposed Method and the Commercial Roche Cobas Electrochemiluminescence Immunoassay (ECLIA)

Sample	ECL method (ng mL^{-1})[a]	Roche Cobas ECLIA (ng mL^{-1})	Relative deviation (%)
1	0.72	0.68	5.88
2	1.04	0.97	7.22
3	1.14	1.10	3.64
4	2.3	2.15	6.98
5	2.84	3.07	-7.49
6	4.46	4.14	7.73
7	7.01	6.47	8.35

[a] Each data point presents an average of five independent measurements.

Reference:

1 C. Vincke, C. Gutiérrez, U. Wernery, N. Devoogdt, G. Hassanzadeh-Ghassabeh and S. Muyldermans, In *Antibody Engineering: Methods and protocols Antibody Engineering*; 2nd ed.; Springer, 2012; pp 145-176.

2. H. Cui, G.-Z. Zou and X.-Q. Lin, *Anal. Chem.* 2003, **75**, 324-331.