Electronic Supplementary Information

L-cysteine induced hemin/G-quadruplex concatamers electrocatalytic amplification with Pt-Pd supported on fullerene as nanocarrier for sensing the spore wall protein of Nosema bombycis

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1 Experimental

1.1 Reagents and Materials

L-cysteine, hemin, potassium chloropalladate (K_2PdCl_4), gold chloride ($HAuCl_4$), chloroplatinic acid (H_2PtCl_6), bovine serum albumin (BSA, 96-99 %) and polyethylene imine (50%, PEI) in water were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Graphene oxide sheets (GO), fullerene C_{60} (99.5 %) were obtained from Pioneer Nanotechnology Co. (Nanjing, China). Absolute ethyl alcohol and L-ascorbic acid (AA) were obtained from Kelong Chemical Company (Chengdu, China). The spore wall protein of *Nosema bombycis* and its polyclonal antibody (Ab), normal silkworm and infected silkworm blood were obtained from State Key Laboratory of Silkworm Genome Biology of China Southwest University. All synthetic oligonucleotides were synthesized from Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China), the sequence of the oligonucleotides were listed as following:

Table S1 Sequence of Synthesized Oligonucleotides Used in This Work

oligonucleotide	oligonucleotide sequence (from 5′ to 3′)
S_0:	5′-NH_2-$(CH_2)_6$-CCA ACC ACA CCA ACC-3′
S_1:	5′-GGT TGG TGT GGT TGG AGA AGA AGG TGT TTA AGT A-3′
Auxiliary single strand (H_1):	5′-AGG GCG GGT GGG TGT TTA AGT TGG AGA ATT GTA CTT AAA CAC CTT CTT CTT GGG T-3′
Auxiliary single strand (H_2):	5′-TGG GTC AAT TCT CCA ACT TAA ACT AGA AGA AGG TGT TTA AGT TGG GTA GGG CGG G-3′

Phosphate-buffered saline (PBS, 0.1 M) solution were prepared by mixing the stock solutions of NaH_2PO_4, Na_2HPO_4, and KCl as the supporting electrolyte, and 20 mM Tris-HCl buffer (pH 7.4) contaning 140 mM NaCl, 5 mM KCl and 1 mM $MgCl_2$ was used to prepare DNA solutions. Ultrapure water obtained from a Millipore water purification system (≥18 MΩ, Milli-Q, Millipore) was used throughout this experiment. All other chemicals used were reagent grade.

1.2 Instrumentations

Cyclic voltammetry (CV) and differential pulse voltammograms (DPV) were performed on an electrochemical workstation (CHI 660D, Shanghai Chenhua Instrument, China) at room temperature using a conventional three electrode system with a bare or modified glassy carbon electrode (GCE, Φ = 4 mm) as the working electrode, a saturated calomel electrode (SCE) as the reference electrode, and a platinum wire as the counter electrode. The pH measurements were performed with a pH meter (MP 230, Mettler Toledo, Switzerland). The size and surface morphology of prepared nanoparticles were estimated from scanning electron microscope (SEM, S-4800, Hitachi, Tokyo, Japan).

1.3 The pre-treat of the spore wall protein of Nosema bombycis

Before using the method to diagnose Pebrine deisease, we need to acquire the spore wall protein of Nosema bombycis, which selected as the related target biomarker, from the silkworm infected with Nosema bombycis. The necessary pre-

treatment process was listed as follows: Collection of the purified Nosema bombycis. Firstly, the gland of five aged silkworm infected by Nosema bombycis was collected with homogenizer grinding, then the obtained grind liquid was filtered with four layers of gauze under adding physiological saline 0.85%. After that the filter liquor was centrifuged to remove the larger grains in the method of differential velocity centrifugation at 4 °C. Lastly, the highly purified Nosema bombycis was obtained through Percoll density gradient centrifugation for 40 min and stored at -20 °C.

The spore wall protein of Nosema bombycis was extracted *via* the following steps: firstly, adding 50 μL 0.1 mol L^{-1} KOH solution into the pre-purified Nosema bombycis (5×108 spore/tube). Then, the sample tubes were immersed in a constant temperature bath 28 °C for 1 h under oscillating every 10 min. After the obtained homogeneous solution being centrifuged for 5 min at 10000 rpm, the supernatant were collected. And the collected supernatant was the related marker (SWP N.b) of Pebrine disease.

1.4 Preparation of rGO-AuNPs and C_{60}@Pt-Pd nanoparticles

The reduced graphene oxide sheets-gold nanoparticles (rGO-AuNPs) were synthesized according to our previous ref[1]. AuNPs could be *in situ* grown onto the rGO without any extra capping agents (such as surfactants[2] and polymers[3]), which may introduce “impurity” into the resultant nanocomposites.

The synthesis of C_{60}@Pt-Pd nanoparticles was performed in the following manners. Firstly, 0.5 mg C_{60} were dispersed in the 1.5 mL positively charged PEI solution (2.5 %) by ultrasonic dispersion. Then, 500 μL K_2PdCl_4 and 500 μL H_2PtCl_6 were dropped into the dispersed solution and stirred for 5 min. Since the $PdCl_4^{2-}$ and $PtCl_6^{2-}$ were absorbed onto the positively charged C_{60} surface, 1 mL 0.3 M AA was thus added to reduce $PdCl_4^{2-}$ and $PtCl_6^{2-}$ with the aim of *in situ* obtaining Pt-Pd

nanoparticles on C_{60} surface. Then, the resulting solution were centrifugally washed several times with ultrapure water and re-dispersed in 2 mL ultrapure water.

1.5 Conjugation of C_{60}@Pt-Pd nanoparticles with Ab_2 and S_0

The preparation of C_{60}@Pt-Pd nanoparticles/Ab_2/S_0 bioconjugates was illustrated in Scheme 1. Firstly, the pre-synthesized 1 mL C_{60}@Pt-Pd nanoparticles was mixed with 500 μL of Ab_2 (50 μg mL^{-1}) and 35 μL 100 μM S_0, and followed by incubation of 16 h at 4 °C under stirring. After that, 35 μL BSA (1 *wt*%) was added and reacted for 40 min to block the possible remaining sites on C_{60}@Pt-Pd nanoparticles. The obtained bioconjugates were re-dispersed in 1.0 mL of PBS (pH 7.4) and stored at 4 °C prior to use.

1.6 Electrode modification

Prior to modification, GCE was polished with 0.3 μm and 0.05 μm alumina, respectively, and then thoroughly cleaned with ultrapure water and ethanol. In order to assembly abundant Ab_1, 10 μL of the as-prepared rGO-AuNPs was coated onto the cleaned GCE surface for drying slowly in air. Subsequently, it was incubated with 20 μL of 10 μg mL^{-1} Ab_1 for 10 h at 4°C. The resulting electrode was rinsed thoroughly to remove the physically absorbed Ab_1 and incubated with 20 μL of 1% BSA for 45 min at room temperature to eliminate nonspecific binding. After that, SWP N.b buffer solution with varying concentrations were added to the electrode surface and incubated for 45 min at room temperature. Based on the specific binding between Ab_1 and SWP N.b, the sandwich-typed format was formed *via* drop-coating 20 μL of prepared C_{60}@Pt-Pd/Ab_2/hemin/G-quadruplex concatamers bioconjugates for 45 min at room temperature. The autonomous assembly of hemin/G-quadruplex concatamers on C_{60}@Pt-Pd/Ab_2/S_0 bioconjugates was previously completed through mixing C_{60}@Pt-Pd/Ab_2/S_0 bioconjugates with 2.5 μM H_1, 2.5 μM H_2, 1 μM hemin and 1 μM

S_1. By monitoring the changed redox peak current of hemin, we could indirectly determine the concentration of target protein with high sensitivity.

1.7 Experimental measurements

The finally obtained immunosensor was placed into in the electrolyte of 1 mL PBS containing L-cysteine (1 mM 60 μL). The differential pulse voltammograms (DPV) measurement was taken: the potential was ranged from -0.6 to 0.1 V, modulation amplitude was 0.05 V, pulse width was 0.06 s, and sample width was 0.02 s. The CV was taken from -0.2~0.6 V (vs. SCE) at 100 mV s^{-1} in 0.10 M PBS (pH 7.0) containing 5 mM $[Fe(CN)_6]^{3-/4-}$. EIS of the electrode fabrication were performed in 5 mM $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 M KCl. EIS measurements were carried out also in the presence of 0.1 M PBS containing 5 mM $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ (pH 7.0). The bias potential was 0.17 V, alternative voltage was 5 mV and frequency range was 0.1 Hz-100 KHz.

2 Results and discussion

2.1 The CV and DPV electrochemical characterization of stepwise modified electrode

In Fig. S1A and Fig. S1B showed the CV and DPV curves of stepwise modified electrode in PBS containing L-cysteine (pH 7.0). After the stepwise modified electrode (a-e) being incubated with C_{60}@Pt-Pd/Ab_2/hemin/G-quadruplex DNAzyme concatamers, a stable and obvious reduction peak (f) of hemin/G-quadruplex was obtained. This phenomenon mainly ascribed to the autonomous assembly of the hemin/G-quadruplex DNAzyme concatamers.

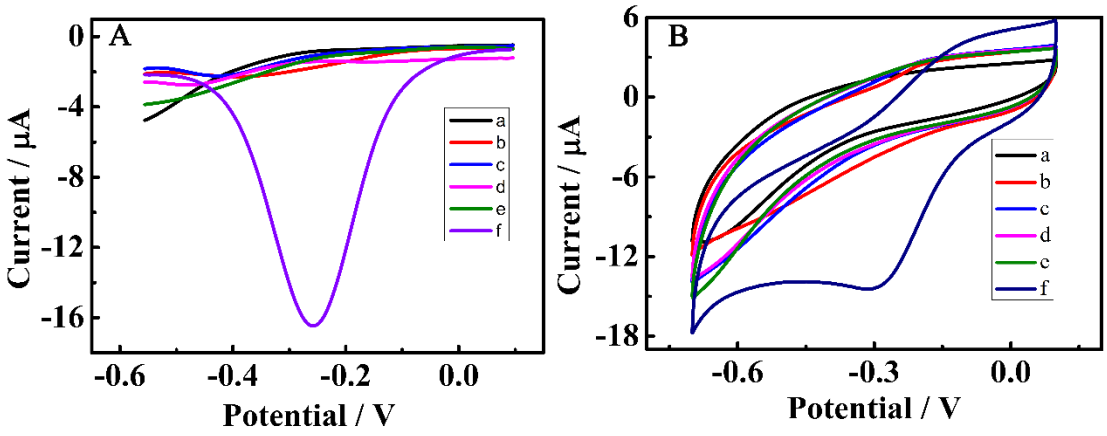

Fig. S1 CV (A) and DPV (B) of bare GCE (a), rGO-AuNPs/GCE (b), Ab_1/rGO-AuNPs/GCE (c), BSA/Ab_1/rGO-AuNPs/GCE (d), and SWP N.b/BSA/Ab_1/rGO-AuNPs/GCE (e) investigated in 5.0 mM $[Fe(CN)_6]^{3-/4-}$, after being incubated with C_{60}@Pt-Pd/Ab_2/hemin/G-quadruplex DNAzyme concatamers in 1 mL PBS containing L-cysteine (60 μL 1 mM) (f).

2.2 The electroactive surface area of the rGO-AuNPs modified electrode

In order to illustrate that the prepared rGO-AuNPs film could improve the surface area and conductivity of the immunosensor, we designed an experiment to quantitatively detect the electro-active surface area of rGO-AuNPs modified electrode by recording CVs at different potential scan rates with $Fe(CN)_6^{4-/3-}$ serving as redox probes (**Fig. S2**). The calculated electro-active surface area (15.82 mm^2) according to the Randles-Sevcik equation[6,7] $I_p=2.69\times10^5 A\times D^{1/2}n^{3/2}v^{1/2}C$, in which n is the number of electrons transferred in the redox reaction (n= 1), A is the electrode area, D is the diffusion coefficient (at 25°C, $D=6.70\times10^{-6}$ cm^2s^{-1}), C is the concentration of the reactant (5.0 mol cm^{-3} $Fe(CN)_6^{3-/4-}$), I_p refers to the redox peak current and v is the scan rate of the CV measurement, was increased compared with some other material decorated electrode[8,9].

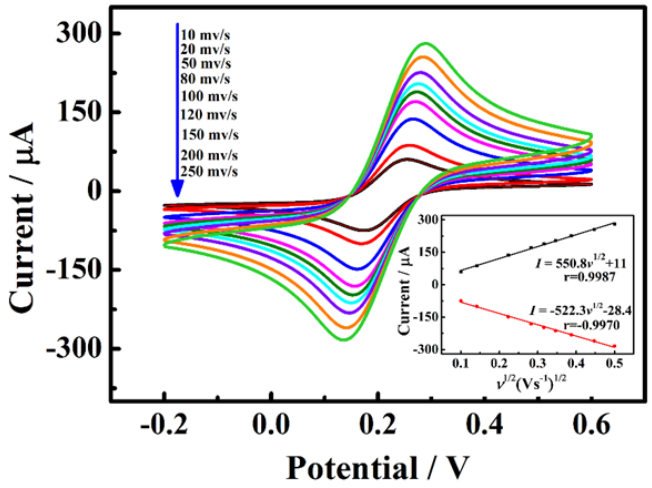

Fig. S2 CVs of AuNPs-rGO modified GCE in 5.0 mM $Fe(CN)_6^{4-/3-}$ at different scan rates from 10 to 250 mV s^{-1}. Insets show the linear relations of the rGO-AuNPs modified GCEs with the anodic and cathodic peak current against the square root of scan rate.

2.3 Amplification performance of L-cysteine substance and C60@Pt-Pd nanocarrier

To evaluate whether the employed L-cysteine substance to generate fixed H_2O_2 and C_{60}@Pt-Pd as nanocarrier to load amount antibody and hemin/G-quadruplex DNA concatamers could efficiently amplify the electrochemical signal and improve the sensitivity of proposed immunosensor, the control experiment were processed at the same but without L-cysteine substance (Fig. S3A) or without C_{60}@Pt-Pd nanocarrier (Fig. S3B) were studied. It can be seen that the role of L-cysteine and C_{60}@Pt-Pd are vital in signal amplification and sensitivity improvement. Furthermore, the amplified DPV current with NADH as substance was compared with DPV current with L-cysteine substance (Fig. S3C). It can be seen that the amplification efficiency of L-cysteine was better than NADH, which are firstly adopted in electrochemical signal amplification system for constructing the just hemin/G-quadruplex induced pseudobienzyme electrochemical biosensor. And hence the developed immunosensor for SWP N.b detection owns high sensitivity.

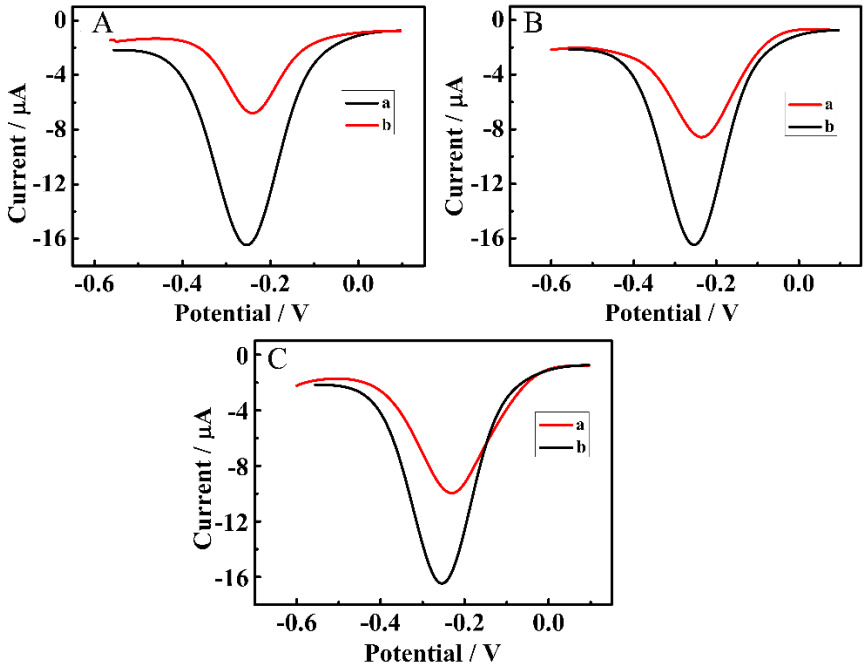

Fig. S3 DPV current of different strategies: (A) The signal of proposed immunosensor without (a) and with (b) L-cysteine in PBS (pH 7.0), (B) the signal of proposed immunosensor without (a) and with (b) C_{60}@Pt-Pd in PBS containing L-cysteine (pH 7.0), (C) the signal of proposed immunosensor in PBS containing NADH (a) and containing L-cysteine (b) (60 μL 1 mM).

2.4 Optimization of experimental condition for immunosensor

The added concentration of L-cysteine was an important factor in the amplifying system. Therefore, before testing the target biomarker (SWP N.b), the relationship between electrochemical signal of immunosensor and L-cysteine concentration was studied. As shown in Fig. S2, the peak current increased with the increasing addition of L-cysteine, and then tended to level off after the L-cysteine amount more than 60 μL (1 mM). Thus, to obtain high sensitivity, 60 μL (1 mM) of L-cysteine was used in the subsequent work.

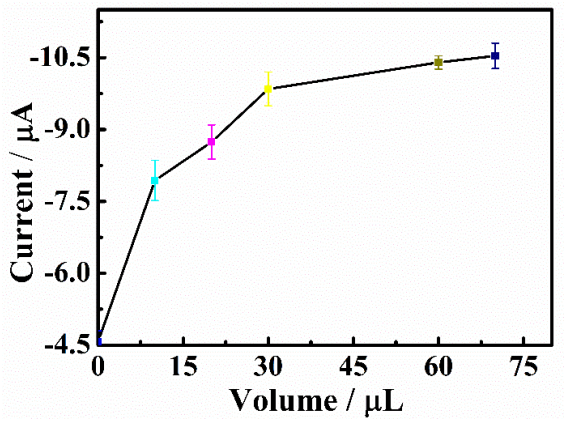

Fig. S4 The effect of L-cysteine concentration on DPV current of the immunosensor in 1 mL PBS (pH 7.0).

2.5 Real sample analysis

The analytical reliability and potential application of the proposed immunosensor was investigated *via* a standard addition method. The proposed SWP N.b immunosensor was applied to tested silkworm blood spiked with five different concentrations of SWP N.b: 0.001, 0.010, 0.050, 0.100 and 0.500 ng mL^{-1}. As shown in Table S2, the acceptable recovery (101.7-112.7 %) demonstrated that the detection of SWP N.b in diluted silkworm blood samples is quite feasible and may be of great value in real sample applications.

Table S2 Determination of SWP N.b in normal silkworm blood samples (n = 3).

Sample number	Added / ng mL^{-1}	Found / ng mL^{-1}	Recovery / %	RSD / %
1	0.001	0.00127	112.7	4.2
2	0.010	0.01034	103.4	5.7
3	0.050	0.0542	108.4	3.8
4	0.100	0.1017	101.7	6.8
5	0.500	0.5202	104.0	7.1

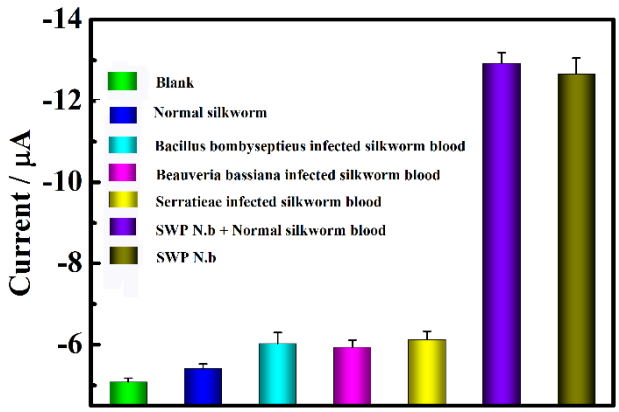

Fig. S5 The specificity of immunosensor for SWP N.b detection and the SWP N.b concentration was 1 ng mL^{-1}.

References

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