Electronic Supplementary Information

Fe$_3$O$_4$NPs mediated nonenzymatic electrochemical immunosensor for the total protein of *Nosema bombycis* detection without addition of substrate

Hua Xie, Qiqi Zhang, Qin Wang, Yaqin Chai, Yali Yuan*, Ruo Yuan*

**Experimental section**

*Reagents and apparatus*

Gold chloride (HAuCl$_4$), 3-aminopropyltrimethoxysilane (APTES, 97%) and bovine serum albumin (BSA, 96-99%) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Methylene blue (MB) was obtained from Shanghai Aladdin Industrial Corporation (Shanghai, China). Glutaraldehyde (GA), potassium ferricyanide (K$_3$Fe(CN)$_6$) and potassium ferrocyanide (K$_4$Fe(CN)$_6$) were bought from Beijing Chemical Reagent Co. (Beijing, China). Magnetic nanoparticles (Fe$_3$O$_4$NPs, 100 mg·mL$^{-1}$) was provided by Tianjin Base Line Chrom Tech Research Centre (Tianjin, China). Normal silkworm blood, infected silkworm blood, total protein of *Nosema bombycis* (TP N.b) and polyclonal antibody (Ab) were obtained from State Key Laboratory of Silkworm Genome Biology of China Southwest University. All oligonucleotides used in this work were synthesized and purified by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China), and the
corresponding sequences were listed as following:

\[ \text{NH}_2\text{-S}_1: 5'\text{-NH}_2-(\text{CH}_2)_6\text{-CCA ACC ACA CCA ACC-3'} \]

\[ \text{NH}_2\text{-S}_2: 5'\text{-NH}_2-(\text{CH}_2)_6\text{-GGT TGG TGT GGT-3'} \]

Phosphate buffered solution (PBS) (pH 7.0, pH 7.4, 0.1 M) was prepared with 0.1 M \( \text{Na}_2\text{HPO}_4 \), 0.1 M \( \text{KH}_2\text{PO}_4 \), 0.1 M \( \text{KCl} \). 20 mM Tris-HCl buffer (pH 7.4) containing 140 mM \( \text{NaCl} \), 5 mM \( \text{KCl} \), 1 mM \( \text{CaCl}_2 \) and 1 mM \( \text{MgCl}_2 \) was used to prepare DNA oligonucleotide solution. Tetraethoxysilane (TEOS) and other chemical reagents were of analytical grade and used as received. All aqueous solutions were prepared with ultrapure water obtained from a Millipore water purification system (\( \geq 18 \text{ MΩ, Milli-Q, Millipore} \)).

All electrochemical measurements including cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were carried out at room temperature on a CHI 660D electrochemical workstation (Shanghai Chen Hua Instrument, China) using a traditional three electrode electrochemical system with a platinum wire as the auxiliary electrode, a saturated calomel electrode (SCE) as the reference electrode and a bare or modified glassy carbon electrode (GCE, \( \Phi = 4 \text{ mm} \)) as the working electrode. The pH measurements were finished with a pH meter (MP 230, Mettler Toledo, Switzerland). The morphologies of nanomaterials were estimated from transmission electron microscopy (TEM, JEM 1200EX, JEOL, Japan).

**Electrochemical measurements**

CV of stepwise fabrication electrodes was conducted in 5.0 mM \([\text{Fe(CN)}_6]^{3-/4-}\) solution at a scan rate of 100 mV·s\(^{-1}\). DPV was performed in phosphate buffered
solution (pH 7.0) with the potential ranged from -0.6 to 0.1 V, the modulation amplitude of 0.05 V, the pulse width of 0.05 s and the sample width of 0.0167 s.

**Preparation of Fe$_3$O$_4$NPs-Ab-S$_1$, Fe$_3$O$_4$NPs-S$_1$ and Fe$_3$O$_4$NPs-S$_2$ bioconjugations**

The purified Fe$_3$O$_4$NPs (100 mg·mL$^{-1}$) were dispersed in 500 µL PBS (pH 7.4) buffer and then stored at 4 °C for further usage. 100 µL of 50 µg·mL$^{-1}$ Ab and 50 µL of 100 µM GA were added into 300 µL prepared Fe$_3$O$_4$NPs (20 mg·mL$^{-1}$) solution, then 100 µL of 5 µM S$_1$ was added into the above mixture and slightly stirred for 15 min at room temperature. During this process, Ab and S$_1$ could successively be conjugated to Fe$_3$O$_4$NPs with the aid of cross linker GA to obtain Fe$_3$O$_4$NPs-Ab-S$_1$ bioconjugation. According to these steps, we also prepared Fe$_3$O$_4$NPs-S$_1$ and Fe$_3$O$_4$NPs-S$_2$ bioconjugations through cross-linking Fe$_3$O$_4$NPs with S$_1$ and S$_2$. The prepared bioconjugations were stored at 4 °C for subsequent use.

**The fabrication of electrochemical immunosensor**

The fabrication of electrochemical immunosensor was performed with the following steps. Prior to fabrication, GCE was polished with 0.3 µm and 0.05 µm alumina powder respectively and sequentially cleaned with ultrapure water, ethanol and ultrapure water under ultra-sonication for 2 min to obtain a mirror-like surface. The well-polished electrode was immersed into 1 wt % HAuCl$_4$ solution for electrochemical deposition under constant potential of -0.2 V for 30 s. Then, 20 µL of 20 µg·mL$^{-1}$ antibody (Ab) was cast onto the AuNPs modified electrode surface, which was preserved in the refrigerator at 4 °C for 10 h. Next, the electrode was rinsed thoroughly with ultrapure water to remove the physically absorbed Ab and incubated
with 20 μL of 0.5 wt % BSA for 40 min at room temperature to block the non-specific binding sites. Subsequently, 20 μL various concentrations of TP N.b was dropped onto the BSA/Ab/AuNPs/GCE electrode surface for 50 min at room temperature. The modified electrode was incubated with Fe₃O₄NPs-Ab-S₁ bioconjugates at room temperature for 50 min. After that, 20 μL the mixture solution containing Fe₃O₄NPs-S₁, Fe₃O₄NPs-S₂ and MB was dripped onto the modified electrode and incubated for 2 h at 37 °C to form Fe₃O₄NPs-DNA dendrimer. Ultimately, the electrodes were washed with double distilled water and prepared for electrochemical measurement.

Results and discussion

Electrochemical characterization of the stepwise modified electrode

The fabrication process of electrochemical immunosensor was characterized by CV and the results were displayed in Fig. S1. A couple of quasi-reversible redox peak of [Fe(CN)₆]³⁻/⁴⁻ was obtained at a bare GCE (curve a). After electrodepositing a layer of AuNPs, the peak current remarkably increased (curve b), which attributed to the fact that AuNPs with superior electronic conductivity could significantly facilitate electron transfer. The peak current decreased obviously (curve c) after incubating with Ab, which illustrated that Ab was successfully assembled onto the AuNPs fabricated electrode. And then, the decorated electrode was blocked with BSA, the peak current further decreased (curve d). After the proposed immunosensor was incubated with the TP N.b, a dramatic decrease in peak current was observed (curve e). It was ascribed to the fact that TP N.b was a kind of inert protein which could hinder the electron
transmission toward the electrode surface.

**Fig. S1** The CVs of electrode at different stages in 5.0 mM \([\text{Fe(CN)}_6]^{3−/4−}\) solution: (a) GCE (b) AuNPs/GCE (c) Ab/AuNPs/GCE (d) BSA/Ab/AuNPs/GCE (e) TP N.b/BSA/Ab/AuNPs/GCE.

*Optimization of experimental conditions for immunosensor*

The concentration of antibody was investigated as it might affect the analytical performance of proposed electrochemical immunosensor. Fig. S2A showed the relationship between increasing antibody concentration and the change of cathodic peak current (\(\Delta I\)) before and after addition of antibody. It was found that the \(\Delta I\) increased rapidly with the increment of antibody concentration from 0.1 to 20 \(\mu\text{g}\cdot\text{mL}^{-1}\), and then tended to level off after over 20 \(\mu\text{g}\cdot\text{mL}^{-1}\). Thus, 20 \(\mu\text{g}\cdot\text{mL}^{-1}\) of antibody was adopted in all experiments.

In our experiments, MB that intercalated into the \(\text{Fe}_3\text{O}_4\text{NPs-DNA dendrimer}\) was utilized as electron mediator and the amount of MB affected the sensitivity of the immunosensor. Therefore, it was significant to investigate the effect of MB concentration. Under the optimal antibody concentration, concentration of MB was
examined from 10 to 600 µM. As seen from Fig. S2B, the ΔI increased rapidly with the increasing concentration of MB until a plateau was reached at 500 µM. Therefore, 500 µM was chosen as the optimal concentration of MB.

![Fig. S2](image)

Fig. S2 The optimization of antibody concentration (A) and MB concentration (B) (inserts: the corresponding trend curves).

**Reproducibility of the proposed immunosensor**

The reproducibility of the immunosensor was evaluated by the relative standard deviation (RSD, n=5) of inter-assay and intra-assay. Five of the same batch proposed immunosensors were used to detect 1.0 ng·mL⁻¹ TP N.b under the same experimental
conditions, all of the five immunosensors showed analogous electrochemical signals achieving an inter-assay RSD of 5.3%. Similarly, the same immunosensor was used to detect 1.0 ng·mL⁻¹ TP N.b for five times and an intra-assay RSD of 5.4% could be obtained. These results demonstrated that the reproducibility of proposed immunosensor was acceptable.

Preliminary analysis of real samples

The feasibility of the proposed immunosensor for TP N.b detection in silkworm blood sample was evaluated with the standard addition method. The sample was directly spiked with different concentrations of TP N.b standard solution. As shown in Table S1, the recovery was ranged from 96.1% to 103.0% and relative standard deviation values varied from 1.0% to 7.8%, which clearly indicated that the proposed electrochemical immunosensor would be suitable for the detection of TP N.b in silkworm blood precisely and accurately.

Table S1 Recovery results of immunosensor in silkworm blood (n = 3).

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Concentration of TP N.b Added/(ng·mL⁻¹)</th>
<th>Concentration of TP N.b Found/(ng·mL⁻¹)</th>
<th>Recovery/%</th>
<th>RSD/%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0010</td>
<td>9.86×10⁻⁴</td>
<td>98.6</td>
<td>6.7</td>
</tr>
<tr>
<td>2</td>
<td>0.010</td>
<td>1.02×10⁻²</td>
<td>102.0</td>
<td>7.8</td>
</tr>
<tr>
<td>3</td>
<td>0.10</td>
<td>9.61×10⁻²</td>
<td>96.1</td>
<td>5.9</td>
</tr>
<tr>
<td>4</td>
<td>1.0</td>
<td>1.03</td>
<td>103.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>