Electronic Supplementary Information

A supersandwich multienzyme–DNA label based electrochemical immunosensor

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1. Experimental parts

Chemicals and Materials.

Horseradish peroxidase (HRP, MW 44000), 1-(3-(Dimethylamino)-propyl)-3-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysulfosuccinimide (NHSS), TCEP (tris(2-carboxyethyl) phosphine hydrochloride), and bovine serum albumin (BSA) were from Sigma. Primary anti-IL-6 antibody, IL-6 antigen, detection anti-IL-6 antibody with and without HRP conjugation, IL-8, AFP, PSA, CEA and all DNA sequences were from Shanghai Sagon Biotech Laboratory, Ltd. Carboxyl functionalized carbon nanotubes (CNT) were obtained from Chengdu Organic Chemicals Co. Ltd.

Apparatus

Electrochemical measurements were carried out using a CHI 660C electrochemistry workstation (Shanghai CH Instruments Co., China) at ambient temperature (22$\pm$2 °C). A three-compartment electrochemical cell contained a modified glassy carbon electrode (GCE, d= 3 mm) as working electrode, a platinum wire as auxiliary electrode and a saturated calomel electrode (SCE) as a reference electrode. All potentials were measured and reported versus the SCE.

Measurement procedure

Amperometry was done at -0.25 V *vs.* SCE with the solution stirring for optimum sensitivity. EIS measurements were carried out in the presence of a 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) mixture as redox probe in 0.1 M PBS (pH 7.4) containing 0.1 M KCl at a bias potential of 0.18 V. The alternative voltage was 5 mV and the frequency range was 0.1 KHz-10 KHz.

Preparation of supersandwich multienzyme-DNA-Ab_2 bioconjugate.

First, 50 μL aliquots of the biotinlylated anti-IL-6 antibody at 1.0 μg mL^{-1} was gently

mixed with 50 μL 1.0 μg mL^{-1} S_1 solution for 40 min. The final product was acquired by centrifugation at 15,000 rpm for 20 min. After twice washing with pH 7.4 Tris-HNO_3, the tagged antibodies were obtained and resuspended in 400 μL of pH 7.2 Tris-HNO_3 containing 0.1% BSA. Then 1.0 μM $\mathbf{S_1}$ (capture probe) conjugated anti-IL-6 (S1-anti-IL-6) were hybridized with 100 nM $\mathbf{S_2}$ and 100 nM target DNA $\mathbf{S_3}$ with 5 μM TCEP (tris(2-carboxyethyl) phosphine hydrochloride) for 1 h in Tris buffer (pH 7.4). The product was centrifuged at 15,000 rpm for 20 min at 4 °C and the supernatant was discarded. After centrifugation, the product, named **supersandwich bioconjugate (1)** was washed by pH 7.4 Tris-HNO_3 containing 0.1% BSA and then collected as a homogeneous dispersion storing in the refrigerator at 4 °C. With the same process, **supersandwich bioconjugate (2)** can be prepared with S_4 replacing S_1, S_5 replacing S_2, and S_6 replacing S_3. The rest may be deduced by analogy. **Supersandwich bioconjugates (3), (4)** and **(5)** were prepared with S_7 to S_9, S_{10} to S_{12} and S_{13} to S_{15}, respectively. [Unless specified otherwise, the "supersandwich multienzyme–DNA–Ab_2 bioconjugate" in this paper represented "**supersandwich bioconjugate (1)**".] Traditional sandwich enzyme-DNA label was prepared with the same method, with S_{16}, S_{17} and S_{18}.

Preparation of the immunosensor

For the preparation of the immunosensor, the primary anti-IL-6 (Ab_1) was introduced to the electrode via the schiff-bonds of the interaction between the carboxylic group of carboxylic MWNT and amine groups in Ab_1. Prior to preparation procedure, the GCE was successively polished to a mirror finish using 0.3 and 0.05 μm alumina slurry followed by rinsing thoroughly with water. After successive sonication in 1:1 nitric acid, acetone, and doubly distilled water, the electrode was rinsed with doubly distilled water and allowed to dry at room temperature. Then 5μL 0.50 mg mL^{-1} carboxylated CNT was dipped onto the electrochemical cleaned GCE. The coated surfaces were dried by N_2 gas and rinsed with water to get CNT/GCE. The activation solution (200 mM EDC and 50 mM NHS in MilliQ water) was then cast on the surface to activate the carboxyl group for 10 min at room temperature. And then 20 μL anti–IL–6 antibody solution (50 mM pH 6.8 PBS) was spread onto the activated electrode surfaces of CNT/GCE. The electrode was incubated at 4 °C in a moisture atmosphere. Then the sensors were incubated with 1 M ethanolamine for 10 min to block the residual activated carboxyl groups. After incubation for 6 h, the electrodes were then blocked with 1% BSA solution for 1 h at room temperature, and washed

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with they were rinsed with PBS and 0.05% Tween (PBST) to remove physically absorbed Ab_1. After aspiration, Ab_1 modified electrodes were incubated with 10 μL of detecting Ag samples for 1 h at 37 °C. After the binding reaction between Ab_1 and Ag, the electrodes were immersed into 10 μL supersandwich multienzyme-DNA-Ab_2, traditional sandwich multienzyme-DNA-Ab_2 or HRP-Ab_2 bioconjugates solution for an incubation of 50 min. Finally, the electrodes were washed thoroughly with PBST to remove nonspecifically bound conjugations, which could cause a background response before measurement.

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Table S1. DNA sequences

S_1	***5'-avidin-ACGAA AGATA GCCAC TCGTA TTCAT CACTG GACCG ATACG CGACA TATCG TGCCA ATTAG-3'***
S_2	***5'-HRP-TGACA TTTGC TCGAT TCCTA TACGA GTGGC TATCT TTCGT CTAAT TGGCA CGATA TGTCG-3'***
S_3	***5'-ACGAA AGATA GCCAC TCGTA TTCAT CACTG GACCG ATACG CGACA TATCG TGCCA ATTAG-3'***
S_4	**5'-avidin-GCTAT ACAGC ATGCT CACCG-3'**
S_5	**5'-HRP- TGACA TTTGC TCGAT TCCTA GCTGT ATAGC CGGTG AGCAT-3'**
S_6	**5'-GCTAT ACAGC ATGCT CACCG-3'**
S_7	**5'-avidin-ACCGT GCTAT ACAGC ATGCT CACCG ATAGT-3'**
S_8	**5'-HRP- TGACA TTTGC TCGAT TCCTA GCTGT ATAGC ACGGT ACTAT CGGTG AGCAT-3'**
S_9	**5'-ACCGT GCTAT ACAGC ATGCT CACCG ATAGT-3'**
S_{10}	**5'-avidin-GCTAT ATATC GATTA ACCGT GCTAT ACAGC ATGCT CACCG ATAGT AAGCA TGCTT ACTAT-3'**
S_{11}	**5'-HRP- TGACA TTTGC TCGAT TCCTA GCTGT ATAGC ACGGT TAATC GATAT ATAGC ATAGT AAGCA TGCTT ACTAT CGGTG AGCAT-3'**
S_{12}	**5'- GCTAT ATATC GATTA ACCGT GCTAT ACAGC ATGCT CACCG ATAGT AAGCA TGCTT ACTAT-3'**
S_{13}	**5'-avidin-GCTAT ATATC ACAGT TAATA GCTGT ATAGC ACGGT TAATC TGCTT ACTAT CGGTG AGCAT TACTT AATAT CGATG ATCAT-3'**
S_{14}	**5'-HRP-TGACA TTTGC TCGAT TCCTA GATTA ACCGT GCTAT ACAGC TATTA ACTGT GATAT ATAGC ATGAT CATCG ATATT AAGTA ATGCT CACCG ATAGT AAGCA-3'**
S_{15}	**5'-GCTAT ATATC ACAGT TAATA GCTGT ATAGC ACGGT TAATC TGCTT ACTAT CGGTG AGCAT TACTT AATAT CGATG ATCAT-3'**
S_{16}	**5'-avidin- ATGCA CGATA CGCAT CGGCT ATTAC TAGCG GCAAT-3'**
S_{17}	**5'- ACGGA ACCGG CCCTT GGCTT CCGCA GGAGA TTATA ATTGC CGCTA GTAAT AGCCG ATGCG-3'**
S_{18}	**5'-AAGCC AAGGG CCGGT TCCGT ACGAG ATTGC GACGA-HRP-3'**

2. EIS

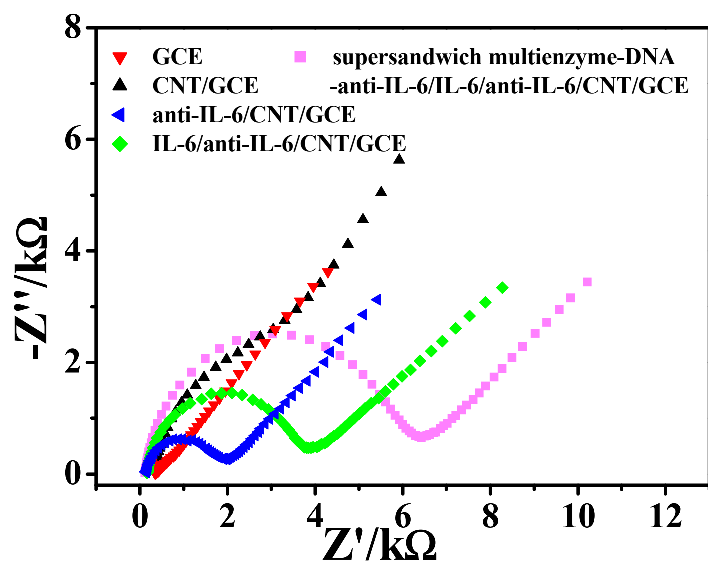

Fig. S1 EIS of different electrodes

3. The response of different supersandwich multienzyme-DNA label

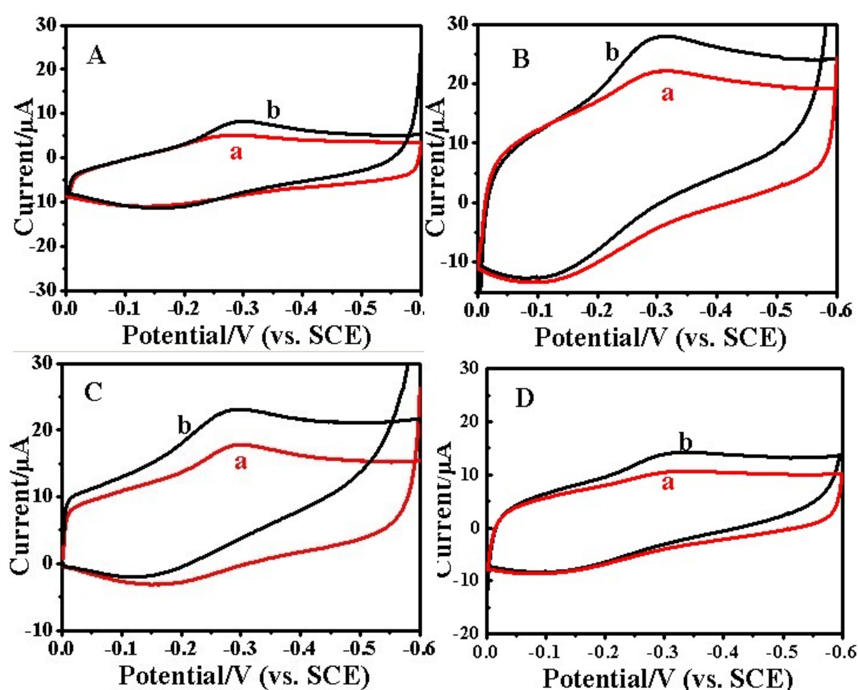

Fig. S2 The CV response of different supersandwich multienzyme-DNA label of the immunosensor with supersandwich bioconjugate (2) (A); (3) (B); (4) (C) and (5) (D) in the supersandwich unit in 5 mL 0.1 M PBS (pH 7.4) with 0 (a) and 5 (b) μM H_2O_2.

In the experiment of optimization, we have studied the size effect of the supersandwich on the response of the sensor as shown in Fig. S2. We tried sequences of different sizes in table S1 and studied their response. The result showed that the size of the supersandwich DNA structure has some effect on the response. We found that the use of supersandwich bioconjugates (2) or (3) with 10 or 15 base pairs in one supersandwich DNA unit [Fig. S2 (A) and (B)], decreased current by 65% or 25%, respectively than which used supersandwich bioconjugates (1) with 20 base pairs in the unit (see Figure 1). This result is consistent with the fact that the stability of 10 or 15 base pairs in one supersandwich DNA unit is lower, leading to a lower content of HRP on the electrode surface. And the use of supersandwich bioconjugates (4) or (5) with 30 or 40 base pairs in the unit [Fig. S2 (C) and (D)], also decreased current by 32% or 59%. Thus we chose S_1 to S_3 for the fabrication of supersandwich DNA in the whole paper.

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3. Amperometric results for the immunosensors without CNT

We also played the control experiment without CNT in the immobilization platform. The result in Fig. S3 showed the sensitivity and the linear relationship without CNT immobilization platform of Ab_1 are poorer than those with CNT.

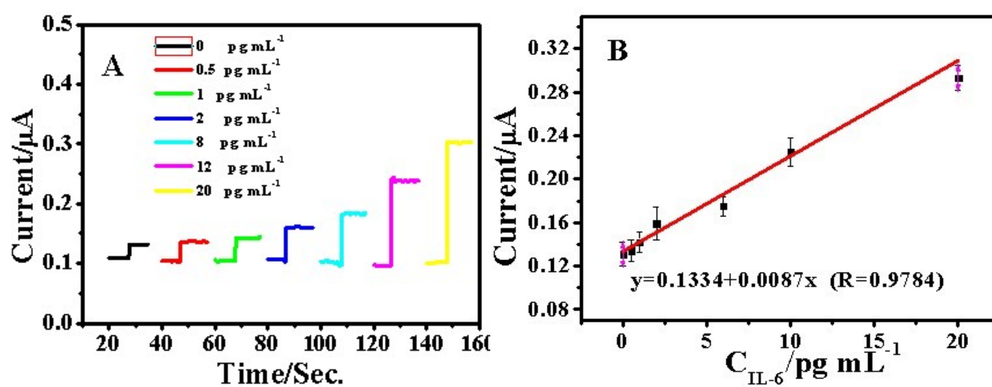

Fig. S3 Amperometric results for the immunosensors without CNT in the immobilization platform incubated with different concentrations of IL-6: (A) steady state amperometric current at -0.25 V in pH 7.4 PBS with 1.0 μM H_2O_2; (B) calibration plot (n = 3).

4. The gel-electrophoresis of the supersandwich structure and the traditional structure.

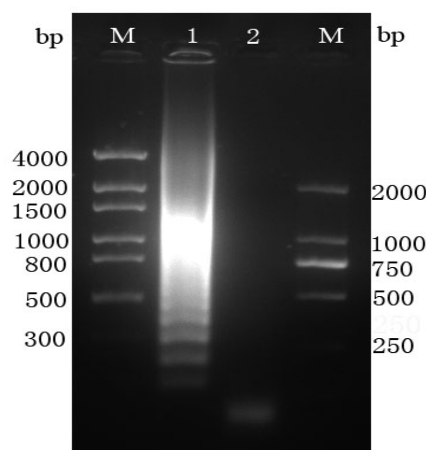

Fig. S4 The gel-electrophoresis results of supersandwich multienzyme-DNA (1) and traditional sandwich DNA-HRP (2)

The gel-electrophoresis results confirm the formation of the supersandwich multienzyme-DNA structure. As we can see, this supersandwich multienzyme–DNA label (Figure S4, 1) produces a ladder of different lengths of the supersandwich structure, with the maximum in the range 1000 to 1500 base pairs. According to the number of base pairs in one unit (cycle), the redox centers incorporated into single supersandwich was calculated as 25 to 32. While Figure S4, 2 exhibits the traditional sandwich DNA-HRP structure's bands with much less base pairs than 1.

The amount of HRP molecules in the supersandwhich DNA structure was determined by Bradford protein assay. The Bradford protein assay is a simple procedure for determination of protein concentrations in solutions that depends upon the change in absorbance at 595 nm in Coomassie Blue G-250 upon binding of protein [1]. By the Bradford protein assay the amount of HRP was estimated to be 0.36 μg in normal sandwich DNA structure, and the amount of HRP in the supersandwich DNA structure was estimated to be 11.12 μg, which is 30.89 times of that in normal sandwich DNA structure.

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6. Determination of IL-6 added in normal human serum

Table S2 Determination of IL-6 added in normal human serum with the proposed immunosensor. All the results were measured in 0.1 M PBS (pH 7.4) solution containing 1.0 μM H_2O_2.

Number	Add (pg mL^{-1})	Found (pg mL^{-1})	Recovery(%)
1	1	1.25	1.039
2	4	4.35	1.157
3	8	9.62	1.014
4	16	15.38	0.9815
5	20	19.46	0.8823

Reference

1. M. M. Bradford, *Anal. Biochem.* 1976. **72**, 248.