Electronic Supplementary Material (ESI) for Molecular BioSystems

Electronic Supplementary Information

Dual-nanogold-linked bio-bar codes with superstructures for in situ amplified electrochemical immunosensing of low-abundance-proteins

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EXPERIMENTAL SECTION

Materials and Reagents. Carcinoembryonic antigen (CEA) was purchased from Biocell Biotechnol. Co., Ltd. (Zhengzhou, China). Monoclonal mouse anti-human CEA antibody (clone II-7, designated as mAb_1, dilution: 1 : 25 – 1 : 50) and polyclonal rabbit anti-human CEA antibody (clone HP-6001, designated as pAb_2) were purchased from Dako Diagnostics (Shanghai, China) Co., Ltd. $HAuCl_4 \cdot 4H_2O$ was purchased from Sinopharm Chem. Reag. Co., Ltd. (Shanghai, China). Gold colloids with 16 nm in diameter were prepared and characterized as described.[S1] All other reagents were of analytical grade and were used without further purification. Ultrapure water obtained from a Millipore water purification system (≥ 18 MΩ, Milli-Q, Millipore) was used in all runs. P_1 and P_2 probes were obtained from Sangon Biotech. Co., Ltd. (Shanghai, China). The sequences of oligonucleotides are designed according the literature[S2] and listed as follows:

P_1: 5'-SH-$(CH_2)_6$-TTTTTCCCTCAGACCCTTTTAGT-3'

P_2: 5'-SH-$(CH_2)_6$-TTTTTACTAAAAGGGTCTGAGGG-3'

Preparation of Electrochemical Immunosensor. A 2-mm glassy carbon electrode (GCE) was polished with 0.3 μm and 0.05 μm alumina, followed by successive sonication in bi-distilled water and ethanol for 5 min and dried in air. Gold nanoparticles were electrochemically deposited on the pretreated GCE by a potential-step electrolysis from + 1.1 to 0 V in 0.5 M H_2SO_4 solution containing 1.0 mM $HAuCl_4$ with different pulse time, i.e., 10, 30 and 60 s.[S3] After washing with distilled water, 30 μL of mAb_1 antibodies (dilution ratio: 1:50) was thrown on the modified electrode, and incubated for 4 h at RT. During this process, mAb_1 antibodies were immobilized on the nanogold-modified GCE due to the strong interaction between gold nanoparticles and proteins. Finally, the as-prepared electrode was immersed into 2.5 wt% BSA for 60 min at room temperature to block the possible remained active sites. The obtained immunosensor was stored at 4 °C for further usage.

Preparation of Ab_2 and P_1-Conjugated Gold Nanoparticles (Ab_2-AuNP-P_1). The Ab_2-AuNP-P_1 nanocomplexes were synthesized and prepared according to the literatures with

a little modification.[S4] Prior to experiment, 5 mL of 16-nm gold colloids (AuNPs, $C_{[Au]}$ = 24 μM) was adjusted to pH 9.0-9.5 by directly using 0.1 M Na_2CO_3 aqueous solution. Then, 200 μL of polyclonal rabbit anti-human CEA antibody (Ab_2, 1 : 100) was added into gold colloids, and incubated for 20 min at room temperature. During this process, the association of Ab_2 antibodies onto the surface of gold nanoparticles is possibly due to the interaction between cysteine or NH_3^+-lysine residues of the proteins and gold nanoparticle.[S5] Afterwards, the alkylthiol-capped barcode DNA P_1 probes (0.5 OD) were injected into the mixture. After gently shaking for 5 min, the mixture was transferred to the refrigerator at 4 °C for further reaction (overnight). Following that, the mixture was centrifuged (14,000 g) for 25 min at RT. The pellet (i.e. P_1/Ab_2-functionalized gold nanoparticles, designated as Ab_2-AuNP-P_1) was re-suspended in 1.0 mL of 2 mM sodium carbonate solution ($C_{[Au]}$ = 120 μM) containing 1.0 wt % BSA and 0.1% sodium azide, pH 7.0, and stored at 4 °C until use.

Preparation of Single-Stranded DNA-Conjugated Gold Nanoparticles. Prior to experiment, 5 mL of 16-nm gold colloids (AuNPs, $C_{[Au]}$ = 24 μM) was adjusted to pH 9.0-9.5 by directly using 0.1 M Na_2CO_3 aqueous solution. Then, 50 μL of the alkylthiol-capped barcode P_1 and P_2 probes (0.5 OD) were injected into the mixture, respectively. After gently shaking for 5 min, the mixture was transferred to the refrigerator at 4 °C for further reaction (overnight). Following that, the mixture was centrifuged (14,000 g) for 25 min at RT. The pellet (designed as P_1-AuNP and P_2-AuNP, respectively) was re-suspended in 1.0 mL of 2 mM sodium carbonate solution ($C_{[Au]}$ = 120 μM) containing 1.0 wt % BSA and 0.1% sodium azide, pH 7.0, respectively, and stored at 4 °C until use.

Electrochemical Measurements. Electrochemical measurements were performed with an AutoLab (Eco Chemie, The Netherlands) system. A three-electrode system comprising a prepared working electrode, a platinum wire as auxiliary electrode and a saturated calomel electrode (SCE) as reference was employed for all electrochemical experiments. The assay was performed as follows:

(i) *Immunoreaction*: 10 μL of mixture solution comprising various concentrations of target CEA sample/or standards and 1.0 mg mL^{-1} Ab_2-AuNP-P_1 colloids was

dropped onto the surface of the mAb_1-modified GCE, and incubated for 25 min at RT. The aim of this step was to form a sandwiched immunocomplex on the GCE surface.

(ii) *Hybridization reaction for the formation of superstructures*: After washing with pH 7.0 PBS, 20 μL of P_1-AuNP suspension ($C_{[Au]}$ = 120 μM) and 20 μL of P_2-AuNP suspension ($C_{[Au]}$ = 120 μM) were dropped onto the modified GCE in turn, and re-incubated for 30 min at RT. During this process, the hybridization reaction between P_1-AuNP-Ab_2 and P_1-AuNP/P_2-AuNP was automatically progressed to form the superstructures on the GCE surface.

(iii) *Intercalation of methylene blue*: After washing with pH 7.0 PBS, the modified electrode was suspended into the 0.5 mM methylene blue aqueous solution, and incubated for 30 min at RT. During this process, the methylene blue molecules were intercalated into the grooves of the double-helix.

(iv) *Electrochemical measurement*: After rinsing thoroughly with pH 7.0 PBS to remove the un-intercalated methylene blue, the electrochemical characteristics of the resulting immunosensors were investigated in pH 7.0 PBS by square wave voltammetry (SWV) from -500 mV to 0 mV (*vs*. SCE) (Amplitude: 25 mV; Frequency: 15 Hz; Increase *E*: 4 mV). Analyses are always made in triplicate.

For comparison, HCR-based immunoassay was also carried out, as described in detail in our most recent paper.[S3]

Characteristics of immunoreaction process. Fig. S1 shows the electrochemical impedance spectroscopy (EIS) of variously modified electrode after each step. These EIS data were fitted to a Randles equivalent circuit (inset of Fig. S1), which contains electrolyte resistance (R_s), the lipid bilayer capacitance (C_{dl}), charge transfer resistance (R_{et}) and Warburg element (Z_w). The complex impedance can be presented as the sum of the real, Z_{re} and imaginary, Z_{im}, components that originate mainly from the resistance and capacitance of the cell. The two components of the scheme, R_s and Z_w, represent bulk properties of the electrolyte solution and diffusion of the applied redox probe in solution, respectively. Thus, they are not affected by chemical transformations occurring at the electrode interface. The

other two components of the circuit, C_{dl} and R_{et}, depend on the dielectric and insulating features at the electrode/electrolyte interface. In EIS, the semicircle diameter of EIS equals the electron transfer resistance, R_{et}. This resistance controls the electron transfer kinetics of the redox-probe at electrode interface. Its value varies when different substances are adsorbed onto the electrode surface. As seen from curve '*a*' in Fig. S1, a very small R_{et} was obtained at nanogold-deposited GCE. However, when the mAb_1 antibodies were immobilized onto the nanogold-modified GCE, a large resistance (R_{et} = 537 Ω) was observed (curve '*b*' in Fig. S1). Moreover, the resistance further increased when the as-prepared immunosensor was reacted with target CEA (curve '*c*' in Fig. S1). The reason might be attributed to the formation of antigen-antibody immunocomplex. Furthermore, when the sandwiched immunocomplex with the Ab_2-AuNP-P_2 was formed on the electrode, the resistance re-increased (curve '*d*' in Fig. S1). This is most likely as a consequence of the fact that the negatively charged single-stranded DNA hindered the negatively changed $Fe(CN)_6^{4-/3-}$ ions. These results revealed that the sandwiched immunocomplex could be formed on the electrode.

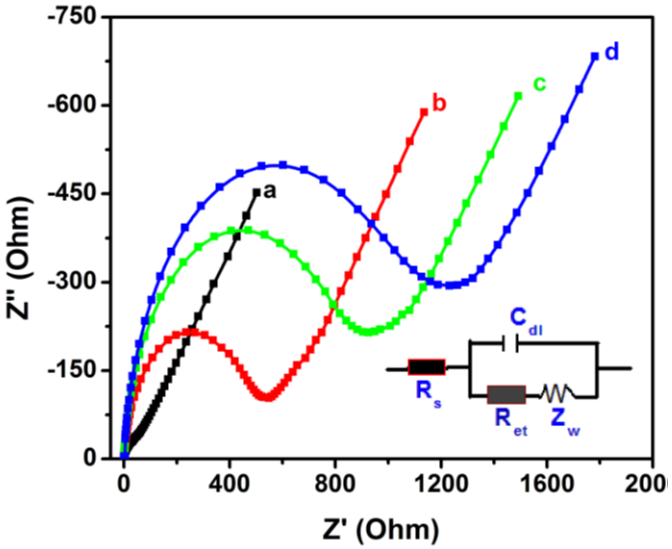

Fig. S1 Nyquist diagrams for (a) nanogold-modified GCE, (b) mAb_1-modified nanogold/GCE, (c) electrode '*b*' after incubation with 0.1 pg mL^{-1} CEA, and (d) electrode '*c*' after incubation with Ab_2-AuNP-P_1 in 5 nM $Fe(CN)_6^{4-/3-}$ + 0.1 M KCl with the range from 10^{-2} Hz to 10^5 Hz at an alternate voltage of 5 mV (*Inset*: equivalent circuit).

Optimization of Experimental Conditions. To ensure an optimal analytical performance of the developed immunoassay, some experimental parameters including incubation time and

incubation temperature for the antigen–antibody reaction, and pH of the assay solution should be investigated. Usually, the antigen-antibody reaction is adequately carried out at human normal body temperature (37 °C). Considering the possible application of the proposed immunoassay in the future, we selected room temperature (25 ± 1.0 °C) for the antigen-antibody interaction throughout the experiment. At this condition, we monitored the effect of incubation time on the currents of the immunosensors from 10 min to 40 min (*Note*: To avoid confusion, the incubation times of the immunosensor with CEA were paralleled with those of the immunosensor-CEA with Ab_2-AuNP-P_1). As shown in Fig. S2, the peak currents increased with the increment of incubation time, and tended to level off after 25 min. Hence, an incubation time of 25 min was selected for sensitive determination of CEA at acceptable throughput.

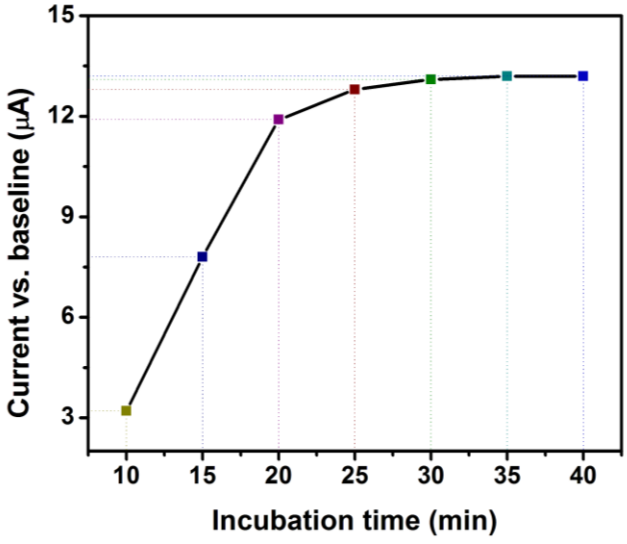

Fig. S2. Dependence of the electrochemical signal of the immunosensors on incubation time for the antigen-antibody reaction by using 0.1 pg mL^{-1} CEA.

In this work, the SWV peak current mainly derives from the redox characteristic of the intercalated methylene blue. Usually, the signal is relative to pH value of supporting electrolyte. Fig. S3 displays the dependence of the currents on pH of PBS by using 0.1 pg mL^{-1} CEA as an example. As indicated from Fig. S3, an optimal current was obtained at pH 7.0 PBS. Higher or lower pHs resulted in the decrease of the peak currents. Thus, a pH 7.0 with PBS was chosen as the supporting electrolyte.

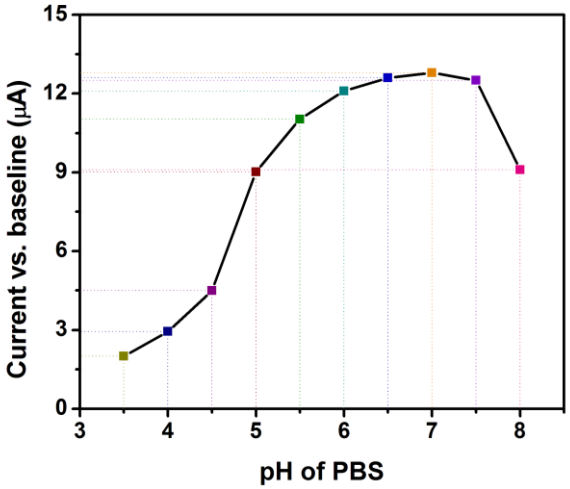

Fig. S3. Dependence of the electrochemical signal of the immunosensors on pH of PBS by using 0.1 pg mL^{-1} CEA.

Table S1 Comparison of the assayed results for clinical serum samples using the electrochemical immunoassay and the referenced ELISA method

Sample no.	Method; concentration[a]		RSD (%)
	Immunosensor	ELISA[b]	
1	23.5 fg mL^{-1}	26.7 fg mL^{-1}	6.4
2	84.3 pg mL^{-1}	76.8 pg mL^{-1}	4.7
3	3.15 ng mL^{-1}	2.82 ng mL^{-1}	5.5
4	24.7 ng mL^{-1}	28.6 ng mL^{-1}	7.3
5	78.9 ng mL^{-1}	76.2 ng mL^{-1}	1.8

[a] The average value of three assayed results.

[b] The data of samples 1-2 were calculated according to dilution ratio.

Reference

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