

Horseradish peroxidase -functionalized Pt hollow nanospheres and multiple redox probes as trace label for sensitive simultaneous multianalyte electrochemical immunoassay

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EXPERIMENTAL SECTION

Reagents and materials

Mouse monoclonal primary anti-CEA (capture anti-CEA, clone no. 27D6) and tracer secondary anti-CEA (signal anti-CEA, clone no. 28E4), and CEA standard solutions were purchased from Biocell Co. (ZhengZhou, China). Mouse monoclonal primary anti-AFP (capture anti-AFP, clone no. 274-1) and tracer secondary anti-AFP (signal anti-AFP, clone no. 179-2), and AFP standard solutions were also obtained from Biocell Co. (ZhengZhou, China). Gold chloride tetrahydrate, sodium citrate, Bovine serum albumin (BSA, 96-99 %) L-cysteine, chitosan (CS, Mw: 100,000-300,000, deacetylating grade: 70-85 %, from crab shells), thionine (Thi), ferrocenemonocarboxylic (Fc-COOH), sodium borohydride (NaBH₄), and horseradish peroxidase (HRP) were obtained from Sigma Chemical Co. (St. Louis., MO., USA). Hydrogen peroxide (30 %, w/v solution) and cobaltous chloride hexahydrate

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($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$) were purchased from Chemical Reagent Co. (Chongqing, China). N-hydroxy succinimide (NHS) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimidehydrochloride (EDC) were purchased from Shanghai Medpep Co. Ltd (Shanghai, China). All chemicals and solvent used were of analytical grade and were used as received without further purification. Double distilled water was used throughout all experiments. Phosphate-buffered solution (PBS, 0.1 M) with pH 7.4 were prepared with stock standard solution of Na_2HPO_4 and NaH_2PO_4 , and 0.1 M KCl was added as the supporting electrolyte. The washing buffer was PBS (pH 7.4) containing 0.05% (w/v) Tween 20 (PBST). Blocking solution was 0.25% (w/v) BSA containing 0.05% Tween 20.

The AFP and CEA was stored at 4 °C, and its standard solutions were prepared with PBS when in use. Gold colloidal nanoparticles with mean size of 16 nm (the graph not shown) were prepared by reducing gold chloride tetrahydrate with sodium citrate at 100 °C for half an hour.¹

Apparatus

Electrochemical measurements, including cyclic voltammetric (CV) and differential pulse voltammetry (DPV), were carried out using a CHI 660C electrochemistry workstation (Shanghai CH Instruments Co., China). A three-compartment electrochemical cell contained a modified glassy carbon electrode (GCE, $\phi = 4$ 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. The pH measurements were made with a pH meter (MP 230, Mettler-Toledo Switzerland) and a digital ion analyzer (Model pHs-3C, Dazhong Instruments, Shanghai, China). The size and morphology of hollow Pt nanoparticles was estimated from transmission electron microscope (TEM, TECNAI 10, Philips Fei Co., Hillsboro, OR). The electrochemical impedance spectroscopy (EIS) measurements were carried out with a Model IM6e (ZAHNER Elektrick Co. Germany).

Preparation of L-cysteine branched chitosan derivatives (CSSH)

The synthesis was performed in the following procedure: First, 50 mg CS was dissolved in 10 mL 1% acetic acid solution under continual stirring to obtain 0.5 wt % CS solution. Second, EDC and NHS were used as coupling agents which catalyze the formation of amide bond between the carboxyl of L-cysteine and the amino of CS. After the L-cysteine solution was activated by the aid of EDC and NHS, 0.5% CS was added and the mixture was allowed to react overnight at room temperature under continuous stirring to produce the L-cysteine branched chitosan derivatives (CSSH). The schematic diagram of the synthesis was shown in Fig. S1.

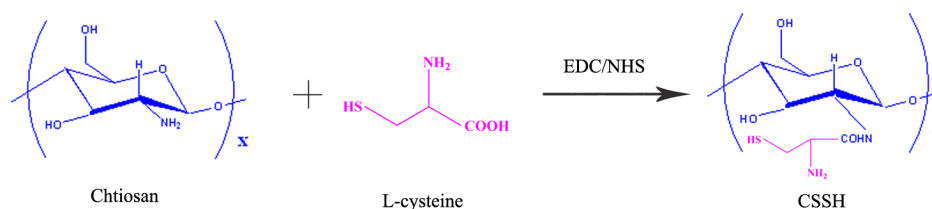


Fig. S1 Preparation of L-cysteine branched chitosan derivatives (CSSH)

Preparation of Pt hollow nanospheres (HPtNPs)

The HPtNPs with mean 24 nm were synthesized according to the literature with

slightly modification.² Briefly, 150 mL of 0.4 mM $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ was mixed with 0.4 mM citric acid solution, which was used as a stabilizer. The solution was treated with N_2 bubbling for 10 min to remove the dissolved O_2 , and then 10 mL of 40 mM NaBH_4 was added dropwise into the above solution under vigorous stirring to produce a brown Co hydrosol. Afterwards, 50 mL of 0.8 mM H_2PtCl_6 was added dropwise into the Co hydrosol. The colour of the solution became blackish brown gradually, which showed that a trans-metallation reaction occurred. The Co nanoparticles were oxidized to Co^{2+} by PtCl_6^{2-} according to the equation as follow: $2 \text{Co} + \text{PtCl}_6^{2-} = \text{Pt} + 2 \text{Co}^{2+} + 6 \text{Cl}^-$. The structure and morphology of HPtNPs were investigated by TEM (Scheme 1B). It was found that the centers of nanoparticles were brighter than the edges and the mean size was about 24 nm. As a comparison, the Pt stuffed nanospheres, whose mean size was about 24 nm, were also synthesized.³

Measurement procedure

The stepwise modified processes of the wok electrode were characterized by cyclic voltammetric (CV) and electrochemical impedance spectroscopy (EIS). CV measurements were carried out in an unstirred 5 mL 0.1 M PBS (pH 7.4) containing 5 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ / $\text{K}_4[\text{Fe}(\text{CN})_6]$ (1:1) with the potential range from -0.2 to -0.6 V at 50 mV/s. EIS measurements were carried out in the presence of a 5 mM $\text{K}_3[\text{Fe}(\text{CN})_6]$ / $\text{K}_4[\text{Fe}(\text{CN})_6]$ (1:1) mixture as redox probe in 0.1 M PBS (pH 7.4) containing 0.1 M KCl at a bias potential of 0.17 V. The alternative voltage was 5 mV and the frequency range was 50 MHz-10 KHz.

RESULTS AND DISCUSSION

Electrochemical characterization of the immunosensor

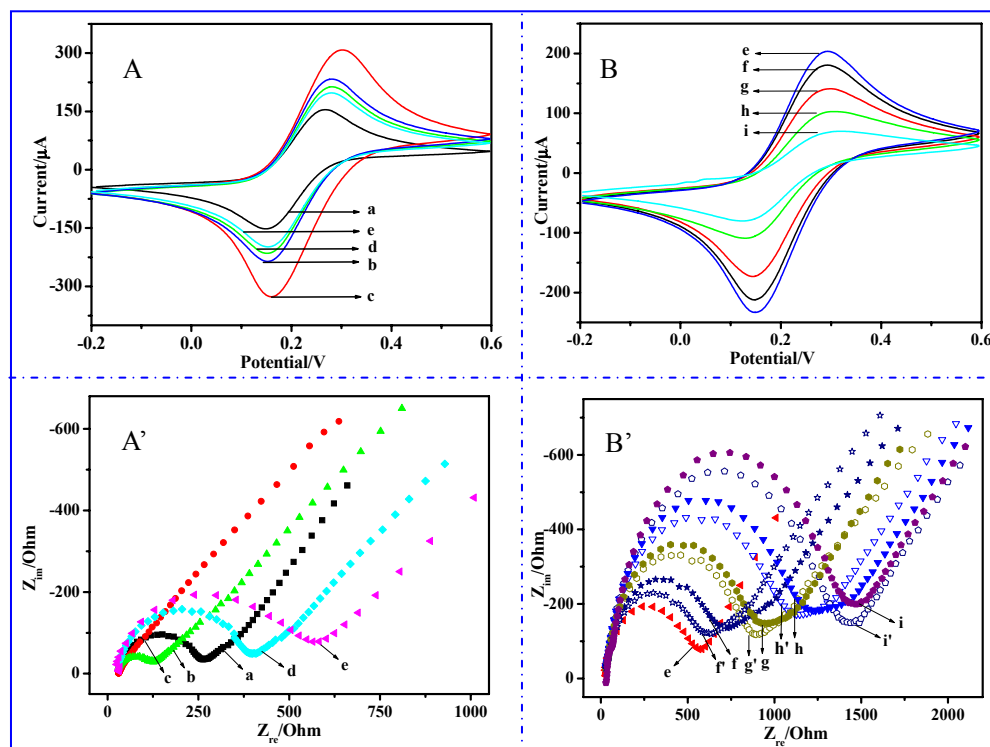


Fig.S2 CVs (A, B) and EIS (A', B') of different modified electrodes in pH 7.4 PBS containing 5 mM $K_3[Fe(CN)_6]$ / $K_4[Fe(CN)_6]$ (1:1) as redox probe: (a) bare GCE, (b) CSSH/GCE, (c) GNP/CSSH/GCE, (d) Ab/GNP/CSSH/GCE, (e) BSA/Ab/GNP/CSSH/GCE. Curves f-i were the CVs and EIS after the BSA/Ab/GNP/CSSH modified electrodes were incubated in 1, 5, 10 and 20 ng/mL mixture solution of AFP and CEA, respectively. Curves f'-i' were the EIS after the sandwich format immunoreaction of the corresponding immunosensors.

The electrochemical characteristics were investigated by CV and EIS. Fig. S2A show the CVs of the modification procedure in 5 mM $K_3[Fe(CN)_6]$ / $K_4[Fe(CN)_6]$ (1:1) solution containing 0.1 M KCl. The redox probe $K_3[Fe(CN)_6]$ / $K_4[Fe(CN)_6]$ reveals a reversible CV at the bare GCE (a). After the pretreated GCE was modified with CSSH film, the peak current increased greatly (b) due to the fact that the positively charged CSSH film facilitated the diffusion of the negatively charged $K_3[Fe(CN)_6]$ / $K_4[Fe(CN)_6]$ redox probe towards the electrode surface. A further increase of peak current could be found (c), which was in accordance with the enhancer of electron transfer of GNPs. When antibodies (d) and BSA (e) were adsorbed on the GNPs

surface, the peak current decreased, which was ascribed to the inhibition effect of the antibody and BSA biomolecules for electron transfer. The curve e in Fig. S2B showed the CV of BSA/Ab/GNPs/CSSH film modified GCE and curve f-i in Fig. S2B represented the CV of the resulted immunosensor which was incubated with the mixture solution containing 1, 5, 10, and 20 ng/mL AFP and CEA, respectively. With the increase of the concentration of antigen, more antigen-antibody complex forming on the electrode surface inhibited the electron transfer. So the current of curves f-i decrease consecutively.

EIS is an effective method for probing the interfacial properties of modified electrodes. It is well known that the high frequency region of the impedance plot shows a semicircle related to the redox probe $\text{Fe}(\text{CN})_6^{3-/4-}$, followed by a Warburg line in the low frequency region which corresponds to the diffusion step of the overall process. The semicircle diameter in the impedance spectrum equals to the electron-transfer resistance (R_{et}). As shown in Fig. S2A', the resistance of CSSH/GCE (a) was smaller than that at a bare GCE (b). When GNPs was assembled on the electrode surface, the R_{et} further decreased (c), which proves that GNPs is beneficial to the electron transfer. After the electrode was modified with antibodies, an increase in R_{et} was observed (d) and a further increase was noticed (e) when the Ab/GNPs/CSSH/GCE was blocked with BSA. The curves f-i in Fig. S2B' were the Nyquist diagrams of the resulted immunosensors which were incubated with the mixture solution containing 1, 5, 10, and 20 ng/mL AFP and CEA, respectively. The proportionate increase of the semicircle diameter with the concentration of antigen

suggested that the electrode interface has captured antigen as the immunocomplex hydrophobic protein layer would obstruct the electron transfer of electrochemical probe. When the electrodes captured CEA and AFP were incubated with secondary antibodies via sandwich format immunoreaction, the curves $f''-i'$ demonstrated the EIS of the corresponding immunosensors, respectively. As shown in Fig. S2B', the resistances for $f''-i'$ decreased comparing with $f-i$. The reason might be the fact that the multilabeled secondary antibodies (Pt hollow nanospheres and redox probe) have high charge transfer efficiency and facilitate the electrolyte to penetrate the immunocomplex layer.

The synergistic action of HRP and Pt hollow nanospheres towards the reduction of H_2O_2

It is well known that Pt possessed the catalytic activity of toward the reduction of H_2O_2 ⁴⁻⁶. The porous and rough Pt hollow nanospheres provided a larger surface area than smooth Pt stuffed nanospheres, and the incomplete shell of hollow nanospheres may also provide the interior surface for the catalytic reaction². Under the same experimental conditions, we used “redox probes@Ab₂ and BSA conjugated Pt hollow nanospheres” and “redox probes@Ab₂ and BSA conjugated Pt stuffed nanospheres” as the secondary antibody for sandwich-type immunoassay (the same concentration of analyte), respectively. The comparison of electrocatalytic activity was shown in Fig. 1(a) in the manuscript. The Pt hollow nanospheres labeled antibody (curve c) exhibited a higher catalytic activity than Pt stuffed nanospheres labeled antibody (curve d), which suggested the incomplete and porous shells of the Pt hollow nanospheres have a higher surface area (interior and external surface both participated the catalytic reaction) and therefore exhibit enhanced electrocatalytic performance.

Further, we used “redox probes@Ab₂ and HRP conjugated Pt hollow nanospheres” as labeled probes for assay. As shown in Fig. 1(a), the catalytic activity of HRP and Pt hollow nanospheres labeled antibody (curve a) was higher than that of BSA and Pt hollow nanospheres labeled antibody (curve c). From the experimental results, we concluded the higher catalytic activity was because of the synergistic action of HRP and Pt hollow nanospheres.

Reproducibility and stability

Table S1 The reproducibility of the proposed immunosensor.

C _{AFP} (ng/mL)		1	5	10	20	C _{CEA} (ng/mL)		1	5	10	20
variation coefficients (%) ^a	intraassay	1.9	2.3	3.8	4.7	variation coefficients (%) ^a	intraassay	2.3	3.2	3.3	5.1
	interassay	4.2	4.9	5.6	6.6		interassay	3.8	5.2	6.1	5.9

^a Each sample was measured five times using five parallel prepared immunosensors and each value was mean value of three measurements.

In the meanwhile, the repeatability and reproducibility of the proposed immunoassay were assessed by the variation coefficients of intra- and interassays. The intra-assay precision of the developed immunoassay was evaluated by detecting four samples containing 1, 5, 10 and 20 ng/mL AFP and CEA, respectively. Each sample was measured five times using five parallel prepared immunosensors and the variation coefficients were 1.9 % and 2.3 % for 1 ng/mL AFP and CEA, 2.3 % and 3.2 % for 5 ng/mL AFP and CEA, 3.8 % and 3.3 % for 10 ng/mL AFP and CEA and 4.7 % and 5.1 % for 20 ng/mL AFP and CEA. Similarly, the variation coefficients of interassay were 4.2 % and 3.8 % for 1 ng/mL AFP and CEA, 4.9 % and 5.2 % for 5 ng/mL AFP and CEA, 5.6 % and 6.1 % for 10 ng/mL AFP and CEA and 6.6 % and 5.9 % for 20 ng/mL AFP and CEA. And the results were listed in Table S1. These results indicated the proposed simultaneous multianalyte immunoassay had acceptable precision and reproducibility. In addition, the immunosensor could be stored at 4 °C.

In this way, over 90 % of the initial responses remained after one week and 81 % of the initial responses remained after one month for both AFP and CEA, which indicated the stability of the proposed immunosensor was acceptable.

Application

Table S2 The recovery of the proposed immunosensor in human serum.

Sample number	Standard values (ng/mL)		Found values (ng/mL) ^a		Recovery (%)	
	AFP	CEA	AFP	CEA	AFP	CEA
1	1	1	0.97 ± 0.02	1.02 ± 0.03	97	102
2	5	5	5.21 ± 0.11	5.12 ± 0.15	104.2	102.4
3	10	10	10.2 ± 0.28	10.1 ± 0.19	102	101
4	20	20	19.5 ± 0.31	20.1 ± 0.25	97.5	100.5
5	30	30	31.0 ± 0.56	31.2 ± 0.47	103.3	104

^a Mean value ± S.D. of three measurements

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