Electronic Supporting Information (ESI)

Ultrasensitive electrochemical immunoassay of proteins based on in-situ duple amplification of gold nanoparticles biolabel signal

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Contents

1. Experimental Section

1.1. Materials and Reagents

Goat anti-human IgG (anti-hIgG) and hIgG (MW=150,000 D) were purchased from Beijing Dingguo Changsheng Biotechnology Co., Ltd. Monoclonal mouse anti-human PSA (anti-hPSA) and hPSA (MW=34,000 D) were purchased from Beijing Key Biotech. Co., Ltd. Note that anti-hIgG and anti-hPSA were used here as both Ab_1 and Ab_2 in their immunoelectrodes for convenience, respectively, as reported before.[1-4] Bovine serum albumin (BSA) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Trisodium citrate and hydroxylamine hydrochloride were purchased from Shanghai Chemicals Station (Shanghai, China). Chloroauric acid ($HAuCl_4$), *N*-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethyllaminopropyl)carbodiimide hydrochloride (EDC) were purchased from Aldrich. MWCNTs (purity ≥ 95%, diameter 20-40 nm, and length 5-15 μm) were purchased from Nanoport Co. Ltd. (Shenzhen, China). The washing and blocking buffer solution for immunoassay was 0.01 M PBS (pH 7.4, 10 mM NaH_2PO_4–Na_2HPO_4 + 0.15 M NaCl). Hydrochloric acid (38%) and hydrobromic acid (≥40%) were supplied by Sinopharm Chemical Reagent as analytical grade reagents. The gold staining solution composed of 18 mM NH_2OH and 3 mM $HAuCl_4$ was prepared daily. All other chemicals were of analytical grade or better quality. Milli-Q ultrapure water (Millipore, ≥18 MΩ cm) was used throughout. The clinical serum samples were donated by the Liuzhou Chinese Medicine Hospital of Guangxi University of Chinese Medicine, China, and the hPSA level in each sample was analyzed by electrochemical luminescence in the hospital.

1.2. Apparatus

All electrochemical experiments were conducted on a CHI660C electrochemical workstation and a three-electrode electrolytic cell was used. A disk glassy carbon electrode (GCE) with 3.0 mm diameter and a platinum wire of 0.1 mm diameter (CH Instruments, Inc.) served as the working electrode (WE) and the counter electrode (CE), respectively. The reference electrode (RE) was a KCl-saturated calomel electrode (SCE), which was separated from the electrolytic solution by a Luggin capillary filled with the test solution. All potentials are cited versus SCE. The semi-derivative treatment is a pure mathematical one and can be completed in seconds by simply clicking the semi-derivative option on the CHI660C software after running linear sweep voltammetry (LSV). A computer-interfaced HP4395A impedance analyzer was employed in the quartz crystal microbalance (QCM) experiments.[5] AT-cut 9 MHz piezoelectric quartz crystals (PQCs) with 12.5-mm wafer diameter (Model JA5, Beijing Chenjing Electronics Co., LTD, China) were used. The Au electrode of 6.0-mm diameter (key-hole configuration, area = 0.29 cm^2) on one side of the PQC was exposed to the solution and served as the working electrode, while that on the other side faced air. The UV-Vis spectra were recorded on a UV-2450 spectrophotometer (Shimazu Co., Japan). Scanning electron microscopy (SEM) images and EDX spectra were collected on a JSM-6360 field emission scanning electron microscope equipped with an energy-dispersive X-ray spectrometer.

1.3. Preparation of Ab_2-AuNPs

All glassware were soaked in aqua regia (V_{HNO3} : V_{HCl} =1:3), rinsed with ultrapure water, and then oven-dried prior to use. Considering the loading of antibody and the steric hindrance effect in the immunoreactions, we prepared Au nanoparticles (AuNPs) of 13 ± 4 nm diameter

for biolabeling as reported.[6] Briefly, 100 mL of 0.01% (w/v) aqueous $HAuCl_4$ was brought to boiling under vigorous stirring, and 2.5 mL of 1% (w/v) aqueous trisodium citrate was quickly added to the boiling solution. When the solution turned deep red in color, indicating the formation of AuNPs, boiling was pursued for an additional 10 min. The heating source was removed, and the solution was left stirring and cooled down to room temperature. Then, 10 μg of Ab_2 was added to 1.0 mL of this AuNPs dispersion and gently mixed at 4 ^oC overnight. After centrifugation at 4,800 rpm for 30 min, the supernatant was discarded and the sediment was washed with 0.01 M pH 7.4 PBS. After another centrifugation and discarding of the supernatant, the resulting Ab_2-AuNPs were redispersed into 0.5 mL of 0.01 M pH 7.4 PBS containing 1.0% (w/v) BSA to block the nonspecific sites, which was stored at 4 ^oC prior to use.

1.4. Preparation of immunoelectrodes

The GCE was carefully polished with aqueous alumina slurries (Al_2O_3) by stepwise decreasing the particle size (0.5 and 0.05 μm). After being thoroughly rinsed with water, the polished electrode was ultrasonically treated sequentially in water, ethanol, and water, each for 5 min, to remove residual alumina powder. Then, the GCE was treated with concentrated sulfuric acid for 15 s. Afterwards, the GCE was subjected to electrochemical rinsing in 0.5 M aqueous H_2SO_4 to remove possible contamination, i.e., the GCE was scanned between -1.0 to 1.0 V vs. SCE at 100 mV s^{-1} for a sufficient number of cycles to obtain reproducible cyclic voltammograms. The GCE was dried with nitrogen gas before use.

Firstly, the carboxylated MWCNTs were prepared by 4 h sonication treatment with 3:1 H_2SO_4/HNO_3 and repeated washing with water until pH 7.0 was reached.[7] Next, as shown in

Scheme 1, a dispersion of carboxylated MWCNTs in water was prepared by addition of 1 mg of carboxylated MWCNTs powder to 2 mL of ultrapure water followed by ultrasonication for 15 min. 4 μL of 0.5 mg mL^{-1} carboxylated MWCNTs solution was dropped onto the pretreated GCE and dried overnight at room temperature to yield the carboxylated MWCNTs-modified GCE (MWCNTs/GCE). Then, the electrode was immersed in 50 μL of an aqueous solution containing 400 mM EDC and 100 mM NHS for 15 min, and then incubating with 6 μL of 1 mg mL^{-1} primary antibody in pH 7.4 PBS buffer for 3 h at room temperature. At last, excess Ab_1 was washed away with the washing buffer, and the Ab_1/MWCNTs/GCE was exposed to PBS (6.0 μL) containing 3% BSA for 1 h to block the nonspecific binding sites (BSA/Ab_1/MWCNTs/GCE). After another washing with the buffer, the BSA/Ab_1/MWCNTs/GCE was stored in PBS at 4 ^oC, when not in use.

The assay of antigen is illustrated in Scheme 1. First, the immunoelectrode was incubated in 6 μL PBS containing antigen (a tube with a PBS drop was covered on the WE to avoid solvent evaporation) at 37 ^oC for 1 h to form antigen/BSA/Ab_1/MWCNTs/GCE. After being rinsed with the washing buffer, this electrode was incubated in 6 μL PBS containing Ab_2-AuNPs at 37 ^oC for 40 min to form Ab_2-AuNPs/antigen/BSA/Ab_1/MWCNTs/GCE and then rinsed thoroughly with PBS to remove the nonspecific binding species. After removing the rinsing solution, 6 μL of gold-staining solution was dropped on the Ab_2-AuNPs/antigen/BSA/Ab_1/MWCNTs/GCE, which was incubated at room temperature for 20 min, yielding a gold/Ab_2-AuNPs/antigen/BSA/Ab_1/MWCNTs/GCE by the AuNPs-catalyzed gold-staining reaction. The gold staining solution was composed of 18 mM NH_2OH and 3 mM $HAuCl_4$ (fresh prepared). While NH_2OH is thermodynamically capable of reducing

$AuCl_4^-$ ions to atomic Au, the reaction is dramatically accelerated by the AuNPs surfaces. As a result, no new particle nucleation occurs in solution and all the reactive $AuCl_4^-$ ions go into the production of larger particles.[8] Hence, when the gold-staining solution was incubated with AuNPs on the Ab2-AuNPs/antigen/BSA/Ab1/MWCNTs/GCE surface, the catalyzed deposition of gold occurred only on the surfaces of AuNPs captured on the sensing surface,[8-12] yielding the gold/Ab2-AuNPs/antigen/BSA/Ab1/MWCNTs/GCE. The electrode was then washed with ultrapure water three times. The final immunoelectrode was obtained and stored in a dry environment prior to use.

Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) in PBS containing 1.0 mM $K_3Fe(CN)_6$ + 1.0 mM $K_4Fe(CN)_6$ as well as QCM experiments were performed for better understanding of various electrode-modification steps. For the EIS measurements, the working electrode potential was fixed at the formal potential of the $Fe(CN)_6^{3-/4-}$ redox couple after being conditioned at this potential for at least 100 s.

1.5. ASV analysis procedures

Linear sweep anodic stripping voltammetry (ASV) for metal immunoassay was conducted as follows. First, we applied 0 V cathodic potential in air, and this potential is sufficiently negative to realize diffusion-controlled gold electrodeposition in the three-electrode system under our experimental conditions. Then, the gold label was dissolved by addition of 8 μL of 1.0 M aqueous HBr-Br_2, and the cathodic preconcentration of atomic gold was simultaneously started and lasted for 400 s. The use of an acidic bromine-bromide solution has been proved to be more efficient than the traditional cyanide reagent for the extraction of gold from ores.[13] Even commercialized as a guarantee grade hydrobromic acid, it must contain a significant

amount of bromine, and Dequaire have confirmed that Br_2 is necessary for gold dissolution in the 1.0 M HBr medium.[14] We also tried to use aqua regia to dissolve gold but the reduction products of concentrated HNO_3 interfered with the electrochemical detection. At the last 30 s of the preconcentration time, 2 μL of a fresh solution of 3-phenoxypropionic acid solution (4 mM in 1.0 M HBr) was added. The presence of bromine should decrease the efficiency of the electrodeposition of gold through its reoxidation after 0.7 V. Therefore, the excess of bromine was suppressed after oxidative treatment and before ASV recording by addition of a solution bromine-trapping reagent, i.e., 3-phenoxypropionic acid, which, as many phenol derivatives, has the property to react spontaneously with bromine.[14] LSV from 0 to 0.9 V with 100 mV s^{-1} scan rate was performed to record the ASV currents of atomic gold.

1.6. Simulation experiments

To compare the signaling efficiency (δ, Eq. (1)) of our $MLAI_{sa}$ protocol with other protocols, we conducted simulation experiments simply by cast-coating an appropriate amount of AuNPs ($n_{AuNPs\text{-}cast}$ in mol) on a GCE and then detecting the recovered amount of the cast-coated AuNPs ($n_{AuNPs\text{-}LSV}$ in mol) by LSV and the Faraday law. The detailed procedures are as follows. First, 10 μL of 0.24 mM AuNPs dispersion was cast-coated on GCE and dried in air in each case, then (1) for the protocol similar to $MLAI_{sa}$ but the cathodic potential was applied soon after AuNPs dissolution (curve a in Fig. 1), the GCE-supported AuNPs were chemically dissolved by adding 8 μL of 1.0 M HBr-Br_2, a 0-V cathodic potential was applied soon after the AuNPs dissolution (ca. 10 s after the addition of HBr-Br_2) for cathodic preconcentration of atomic Au for a given time length, and LSV at 100 mV s^{-1} from 0 V to 1 V was then conducted to record the anodic stripping LSV curve; (2) the conventional

solution-replacement method. A solution of the chemically dissolved $AuCl_4^-$ ions (8 μL) was transferred from the electrode surface into a 5 mL beaker containing 2 μL 3-phenoxypropionic acid solution and 990 μL of 1.0 M HCl as the electrolyte solution, followed by cathodic preconcentration on another GCE well-treated as above under solution-stirred condition and then linear sweep ASV analysis at 100 mV s^{-1} from 0 V to 1 V; (3) A direct anodic stripping of the cast-coated AuNPs from 0 V to 1 V in 8 μL of 1.0 M aqueous HCl injected on the electrode surface; (4) Another reported electroanalysis protocol involving an electrooxidation step at 1.25 V for 150 s in 8 μL of 1.0 M aqueous HCl injected on the AuNPs modified GCE surface, followed by LSV at 100 mV s^{-1} from 1.25 to 0 V in the same solution.

Table S1. Comparison of analytical performance of some hIgG and hPSA immunosensors

Analyte	Label	Analytical technique	LDR / ng mL^{-1}	LOD / pg mL^{-1}	Ref
hIgG	Glucose oxidase	Chronoamperometry	0.005-1	2	1
	CdS QDs	Photoelectrochemical	5×10^{-4}–5	0.5	15
	HRP	Chronoamperometry for H_2O_2	0.1-200	50	16
	CdTe QDs	Fluorometry/SWV	0.1−500 /5×10^{-3}−100	30/5	17
	AuNPs	ASV for catalytically deposited Ag	1.66−2.725×10^{4}	1000	18
	AuNPs	ASV for Au(III)	0.5−100	500	14
	AuNPs and ALP	ASV for catalytically deposited Ag	0.01-250	4.8/6.1	19
	AuNPs	Immunodot assay	0.01-100	10	8
	AuNPs	Our $MLAI_{sa}$ method with gold staining	4×10^{-7}–400	0.3×10^{-3}	This work
hPSA	Glucose oxidase	Colorimetric immunoassay	1×10^{-5}-0.1	3.1×10^{-3}	20
	Gold nanorods-glucose oxidase	Electrogenerated Chemiluminescence	0.01-8	8	21
	AuNPs	Scanometric immunoassay	1×10^{-3}-1	1	12
	HRP	Square wave voltammograms	1×10^{-3}-10	0.4	22
	Silica nanoparticles−$[Ru(bpy)3]^{2+}$	Electrochemiluminescence	1×10^{-3}-10	1	23
	Dopamine-Fe_3O_4-ferrocene monocarboxylic acid	Square wave voltammograms	0.01-40	2	24
	Superparamagnetic Particle	Surface plasmon resonance (SPR)	1×10^{-5}-5×10^{-3}	1×10^{-2}	25
	Magnetic beads	Personal glucose meter	0-100	400	26
	AuNPs	Our $MLAI_{sa}$ method with	1.8×10^{-7}–450	0.1×10^{-3}	This work

gold staining

Table S2. Immunoassay of hPSA in clinical serum samples using our method and the reference method

Serum samples	Reference method [a] / ng mL^{-1}	Our $MLAI_{sa}$ method [b] / ng mL^{-1}	RSD / %
1	1.10	1.17	6.36
2	0.97	1.01	4.12
3	2.04	2.15	5.39
4	10.69	9.97	-6.73
5	39.81	40.52	1.78
6	27.58	25.89	-6.13
7	> 100	114	-

[a] The reference method was electrochemical luminescence conducted on an Roche Cobas E601 Immunology Analyzer in the hospital.

[b] Given as the average value of three repeated assays.

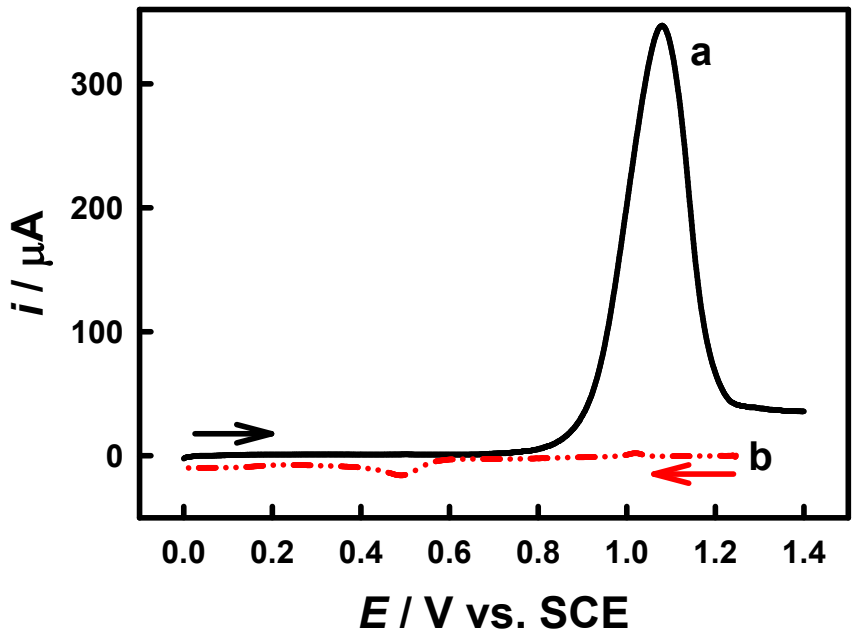

Fig. S1. LSV curve for direct anodic stripping of AuNPs cast-coated on the GCE (a) and electrooxidation of the AuNPs at 1.25 V for 150 s and subsequent reduction to Au^0 at 0.49 V (b). Conditions: 10 μL of 0.24 mM AuNPs dispersion for cast coating. LSV at 100 mV s^{-1} was performed in an aqueous solution of 1 mL 1.0 M HCl. According to Eq. 1, we have obtained $\delta = Q_p/(zFn_{AuNPs\text{-}cast}) = 6.68\times10^{-4}/(3\times96485.3\times10\times10^{-6}\times0.24\times10^{-3}) = 96.2\%$ for curve a, here Q_p (=6.68×10^{-4} C) is the electrical charge under the anodic stripping LSV peak (curve a).

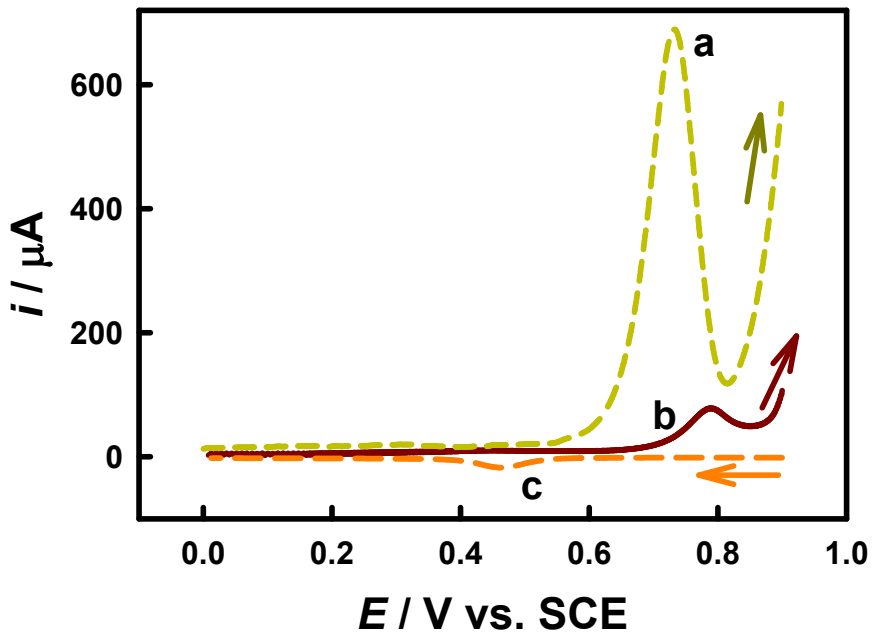

Fig. S2. LSV curves for immunoassay of 400 ng mL^{-1} hIgG by our $MLAI_{sa}$ method (a), direct anodic stripping of AuNPs label in 1.0 M aqueous HCl (b) and electrochemical oxidation of the AuNPs at 1.25 V for 150 s and subsequent reduction to Au^0 at 0.49 V (c).

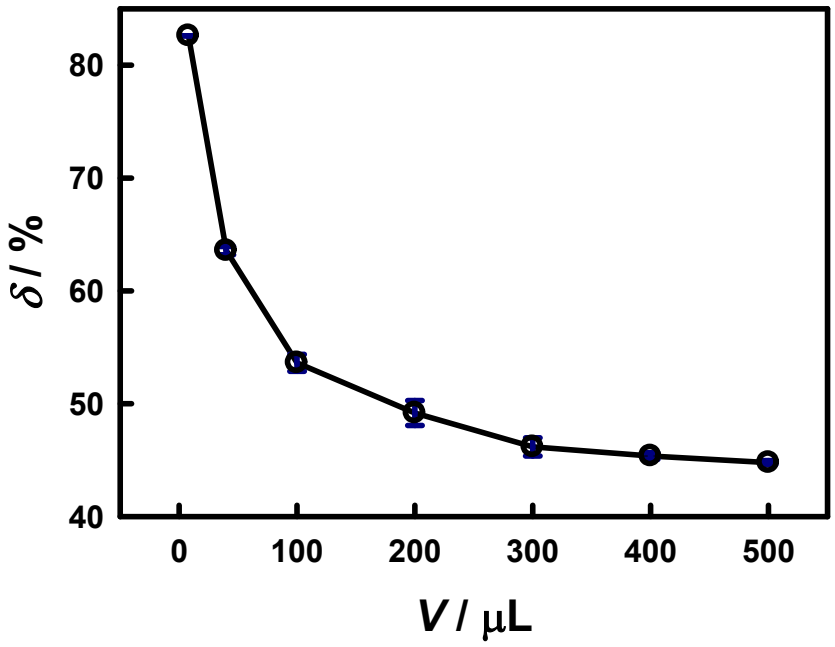

Fig. S3. δ versus volume of 1.0 M HBr-Br_2 used to dissolve AuNPs in the simulation experiments for our $MLAI_{sa}$ protocol (3 repeated runs). Conditions: 400-s cathodic preconcentration; others are the same as in Fig. 1 except for varying volume of HBr-Br_2.

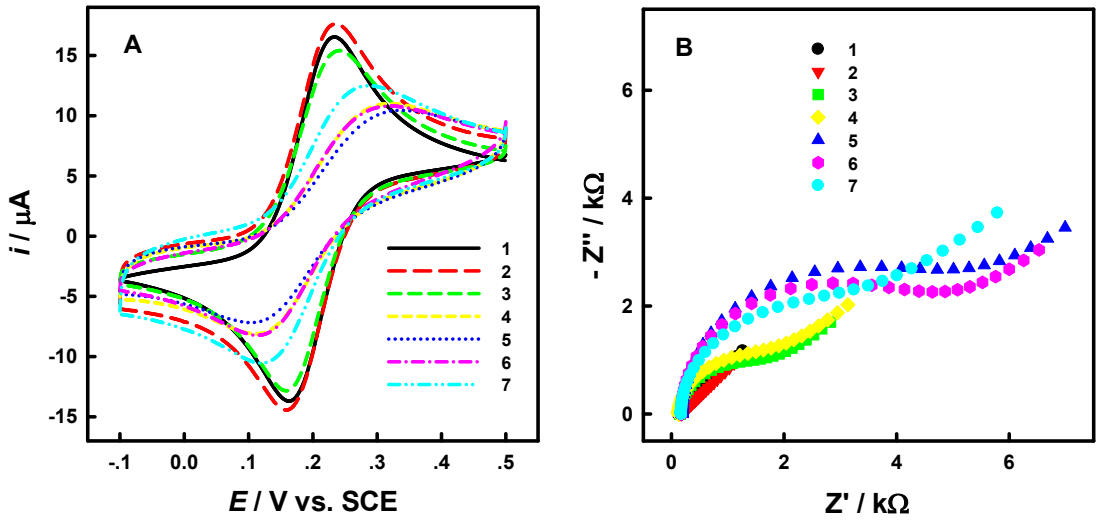

Fig. S4. CV (A) and EIS (B) at bare GCE (1), MWCNTs/GCE (2), Ab_1/MWCNTs/GCE (3), BSA/Ab_1/MWCNTs/GCE (4), antigen/BSA/Ab_1/MWCNTs/GCE (5), Ab_2-AuNPs/antigen/BSA/Ab_1/MWCNTs/GCE (6), and gold/Ab_2-AuNPs/antigen/BSA/Ab_1/MWCNTs/GCE (7) in 0.1 M PBS containing 1.0 mM $K_3Fe(CN)_6$ + 1.0 mM $K_4Fe(CN)_6$. Scan rate: 50 mV s^{-1}. EIS: 100 kHz~1 Hz, 5 mV rms, 0.21 V bias. Here, Ab_1, antigen and Ab_2 refer to anti-hIgG, hIgG and anti-hIgG, respectively. Since either a larger peak-to-peak separation of anodic and cathodic peak potentials or a larger diameter of the EIS semicircle should indicate a decreased electrochemical reversibility of the $Fe(CN)_6^{3-/4-}$ probe,[27-29] the electrochemical reversibility of the $Fe(CN)_6^{3-/4-}$ probe should, from the CV and EIS results, satisfy the order bare GCE ≈ MWCNTs/GCE > Ab_1/MWCNTs/GCE > BSA/Ab_1/MWCNTs/GCE > gold/Ab_2-AuNPs/antigen/BSA/Ab_1/MWCNTs/GCE > Ab_2-AuNPs/antigen/BSA/Ab_1/MWCNTs/GCE > antigen/BSA/Ab_1/MWCNTs/GCE, demonstrating that modification of insulating proteins can decrease the electrode activity, and the electron-conducting AuNPs can somewhat recover the electrode activity. The gold staining can enlarge the size of the Au nanoparticles on the electrode surface, as implied by the increased reversibility of the probe. These experiments have proven that the AuNP-

catalyzed gold deposition reaction on the immunoelectrode was successful. Carboxylated MWCNTs were used here. The carboxylation of MWCNTs should decrease the electron-conductivity of pristine MWCNTs.[30] However, from the present CV and EIS data before and after modification of carboxylated MWCNTs (labels 1 and 2), our carboxylated MWCNTs were still sufficiently electron-conducting for subsequent amperometric immunoassay.

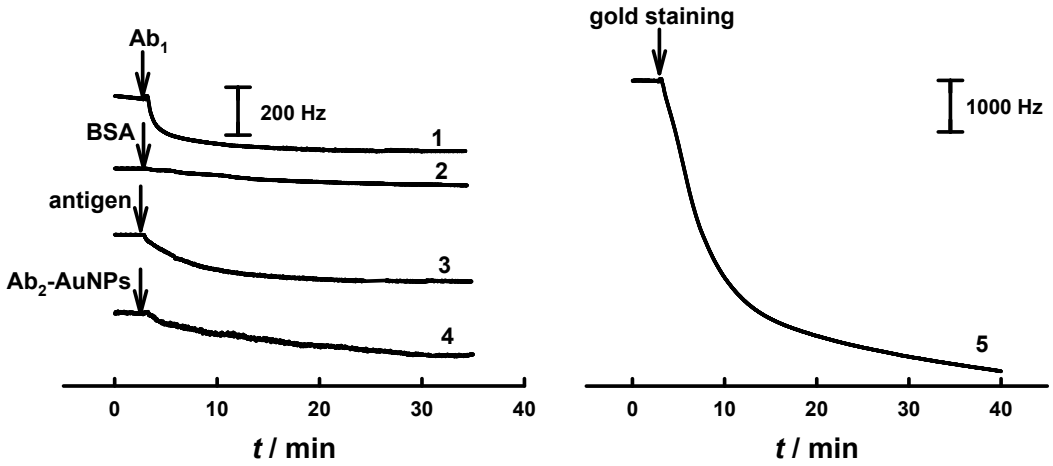

Fig. S5. Time-dependent QCM-frequency responses of a bare QCM Au electrode before and after Ab_1 addition into stirred 0.1 M PBS (1), of the Ab_1/QCM Au electrode before and after BSA addition into stirred 0.1 M PBS (2), of the BSA/Ab_1/QCM Au before and after antigen addition into stirred 0.1 M PBS (3), of the antigen/BSA/Ab_1/QCM Au before and after Ab_2-AuNPs addition into stirred 0.1 M PBS (4), and of the Ab_2-AuNPs/antigen/BSA/Ab_1/QCM Au before and after addition of gold staining solution into stirred ultrapure water (5). Here, Ab_1, antigen, and Ab_2 refer to anti-hIgG, hIgG and anti-hIgG, respectively. The experimental procedures are as follows. A bare QCM Au electrode was placed in stirred 0.1 M PBS. After the injection of 0.05 mg mL^{-1} (final concentration) Ab_1 in a stirred 2 mL PBS solution (1), followed by 1-h incubation, the QCM frequency decreased by 220 Hz, indicating the successful adsorption of Ab_1 on QCM Au electrode. Excess antibody molecules were washed away with the washing buffer. Subsequent blocking of the nonspecific binding sites by incubation with 3% BSA in a stirred 2 mL PBS for 20 min decreased the QCM frequency by 107 Hz (2). After washing with buffer, 25 μg mL^{-1} (final concentration) antigen was added into a new stirred 2 mL PBS (3), the QCM frequency decreased by 204 Hz, indicating the successful immunoreaction of Ab_1 with antigen and precipitation of the product on QCM Au electrode. After the QCM electrode was washed with buffer, 1 mg mL^{-1} (final concentration) Ab_2-AuNPs dispersion was added into a new stirred 2 mL PBS (4), the QCM frequency decreased by 141 Hz, indicating the successful formation of the sandwiched immunological

structure on the QCM Au electrode. Finally, after another washing with buffer, 18 mM NH_2OH and 3 mM $HAuCl_4$ (final concentrations) were added into a new stirred ultrapure water (5), the QCM frequency decreased by 5.2 kHz, indicating the successful formation of gold shells on the labeled AuNPs. Note that the deposition of gold on the Ab_2-AuNPs/antigen/BSA/Ab_1/QCM electrode was almost finished after ca. 20 min, demonstrating saturation of catalytic surface sites of AuNPs for gold staining.

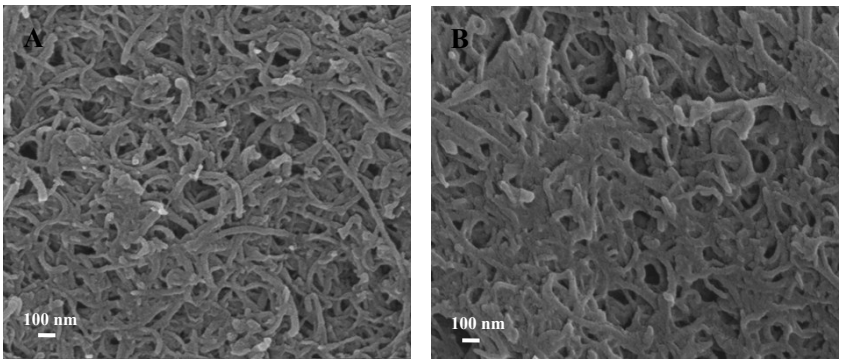

Fig. S6. SEM images of antigen/BSA/Ab_1/MWCNTs/GCE (left) and after gold staining treatment (right). Concentration of hIgG: 400 ng mL^{-1}.

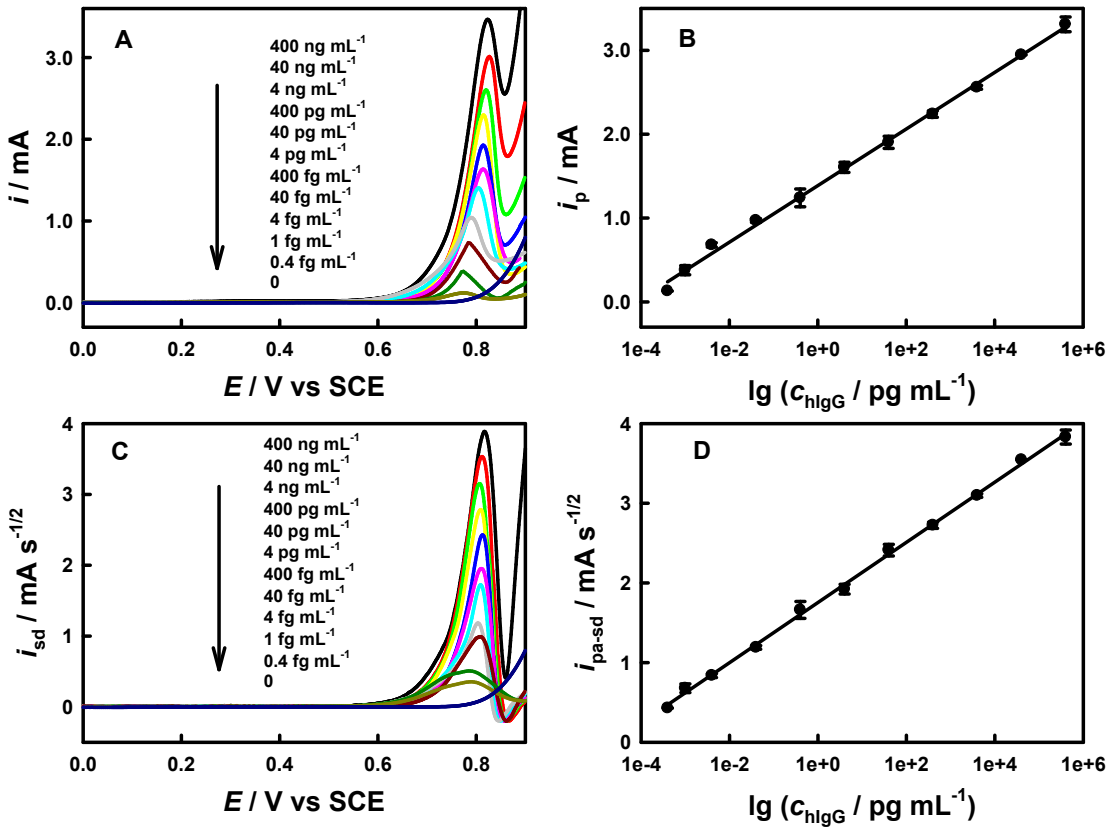

Fig. S7. Linear sweep ASV curves for hIgG immunoassay using our MLAI$_{sa}$ protocol (A) and the corresponding calibration curve (B), the voltammetric curves after semi-derivative treatment (C) and the corresponding calibration curve (D, typical linear regression equation: $i_{pa\text{-}SD}$ (mA s$^{-1/2}$) = 0.378lgc_{IgG} (pg mL^{-1}) + 1.75, R^2=0.9985) (n=3). Scan rate: 100 mV s^{-1}.

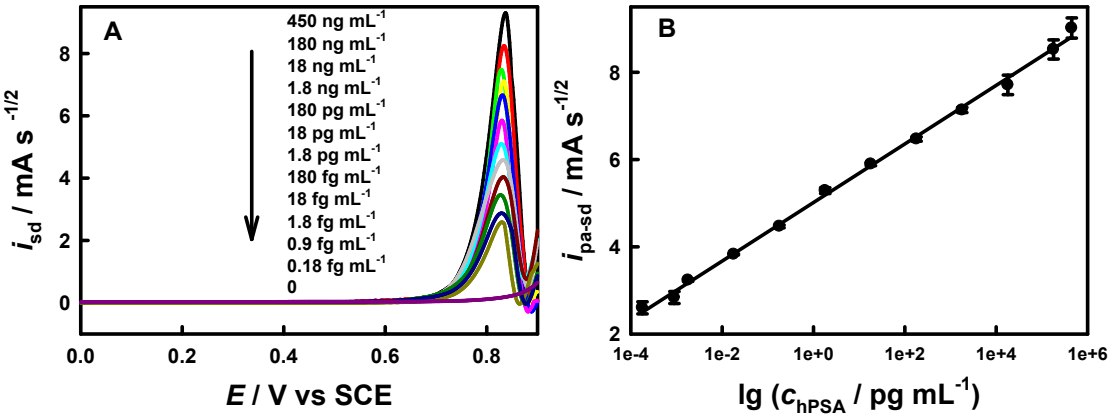

Fig. S8. The voltammetric curves after the semi-derivative treatment of linear sweep ASV curves (Fig. 4A) for hPSA immunoassay using our $MLAI_{sa}$ protocol (A) and the corresponding calibration curve (B, typical linear regression equation: $i_{pa\text{-}SD}$ (mA $s^{-1/2}$) = 0.671lgc_{IgG} (pg mL^{-1}) + 5.02, R^2=0.9978) (n=3). Scan rate: 100 mV s^{-1}.

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