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Supplementary Information

Double-enhanced Strip Biosensor for Rapid and Ultrasensitive Detection of Protein Biomarkers

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Experimental Details

Materials, Reagents and Apparatus

Hydrogen tetrachloroaurate (III) trihydrate (HAuCl4·3H_2O), trisodium citrate, streptavidin (SA), sucrose, Tween-20, TritonX-100, Tris(2-carboxyethyl) phosphine (TCEP), deoxyadenosine triphosphate (DATP), Na_3PO_4.12H_2O, 30% H_2O_2, horseradish peroxidase (HRP), N,N-2methyl formamide (DMF), 3-amino-9-ethyl carbazole (AEC), phosphate buffer saline (PBS, PH7.4, 0.01M), bovine serum albumin (BSA), carcinoembryonic antigen (CEA) from human fluids (Z95%, SDS-PAGE), human serum, ferritin, thrombin from human plasma were purchased from Sigma-Aldrich (USA). Goat anti Rabbit IgG were purchased from Thermo Scientific. Nitrocellulose membrane (HFB18002) was purchased from Millipore (Billerica, MA). Polyester fiber (VL78), laminated cards (SM31-40), HM3030 dispenser, CTD300P programmable strip cutter, DT2032 portable strip reader were purchased from Shanghai Kinbio Tech. Co., Ltd. Shanghai, China. Hairpin oligonucleotide, target DNA and control DNA probe were synthesized and purified by Sangon (Shanghai, China). All chemicals used in this study were purchased from standard commercial sources and all were analytical reagent grade. All buffer solutions were prepared with ultrapure (18.2 Mcm^{-1}) water from a Millipore Milli-Q water purification system (Billerica, MA).

The oligonucleotide sequences were as follows:

CEA-HO:5'-biotin-C CAC GAT ACC AGC TTA TTC AAT TCG TGG-(CH2)6-SH-3'

CEA-cHO: 5'-biotin-CCA CGA ATT GAA TAA GCT GGT ATC GTG G-3'

MUC1-HO:5'-biotin-ACA CGG CAG TTG ATC CTT TGG ATA CCC TGG CGT GT-(CH2)6-SH-3'

MUC1-cHO:5'-biotin-ACA CGC CAG GGT ATC CAA AGG ATC AAC TGC CGT GT-3'

Thrombin-HO:5'-biotin-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-(CH2)6-SH-3'

Thrombin-cHO:5'-biotin-AGT CAC CCC AAC CTG CCC TAC CAC GGA CT-3'

DNA: 5'-ATC ATA AGC TCA TAC AAT CAC TAA-SH-3'

c-DNA: 5'-TTA GTG ATT GTA TGA GCT TAT GAT-SH-3'

Preparation of gold nanoparticles (AuNPs) and hairpin oligonucleotide (HO).

AuNPs with average diameter 15 nm±3.5 nm were prepared using the citrate reduction method according to the reported.[1] All glasswares used in this study were thoroughly cleaned in aqua regia (three parts HCl and one part HNO_3), rinsed in doubly distilled water, and oven dried prior to use. 4.5 mL of 1% trisodium citrate was put in a 250 mL, round-bottom flask, containing 100 mL of 0.01% boiled $HAuCl_4$ aqueous solution. One minute later, the solution turned deep wine-red. Boiling was pursued for an additional 10 min; then the colloid solution was stirred for another 15 min without heating any more. The resulting AuNPs solution was stored in amber laboratory bottles at 4°C and characterized by an absorption maximum at 520 nm. We condensed the AuNPs to different concentrated solutions (Fig. S1) through the centrifugal and chose the 10-fold AuNPs solution to prepare the HO-AuNPs-DNA, cDNA-AuNPs-HRP and cDNA-AuNPs conjugates.

HO modified with a biotin at its 5' end and a thiol at its 3' end was imposed to synthesize HO-AuNPs compound. The 10 μM solution of the HO were prepared in the buffer (Tris-HCl (20 mM, pH7.4), 100 mM NaCl, and 5 mM $MgCl_2.6H_2O$). The solution was incubated in 95°C water for 5 min and then allowed to slowly cool to room temperature (RT) over 30 min so that the probe HO correctly folded into a hairpin structure. TCEP, a thiol deoxidizer, was used to cut the disulfide bond formed between the thiol groups. DATP, a monomer unit of nucleic acid, was used as sealant to prepare the HO-AuNPs conjugates.[2]

Preparation of HO-AuNPs-DNA Conjugates.

Different molar ratios of the slight modified HO and thiolated DNA (1/1,2/1, 3/1, 4/1, 5/1) and 5 μL 1 mM TCEP were added into 300 μL of the 10-fold concentrated AuNPs solution to prepare HO-AuNPs-DNA conjugates (Fig. S2), respectively. After shaking 1 h at RT, 30 μL of 15 mM DATP were attached to the solution and shook another 30 min at RT. Then, the HO-AuNPs-DNA conjugates were put at 4°C for 4 h to increase the stability of the conjugates. The excess reagents were removed by centrifugation for 10 min at 12,000 rpm. After discarding the supernatant, the red pellets were washed twice with an aqueous solution containing 20 mM $Na_3PO_4.12H_2O$, 5% BSA, 0.25% Tween-20, and 10% sucrose, with the centrifugation time of 10 min at 12,000 rpm; finally, redispersed in 300 μL the same buffer solution and stored at 4 °C before further use.

Preparation of cDNA-AuNPs-HRP and cDNA-AuNPs Conjugates.

Different volumes of the thiolated cDNA and 1% HRP and 5 μL 1 mM TCEP were added into 300 μL of the 10-fold concentrated AuNPs solution to prepare cDNA-AuNPs-HRP conjugates (Fig. S3). Next steps were same as that of preparation of HO-AuNPs-DNA conjugates, except, this conjugates were washed with 300 μL of PBS (pH=7, 0.01M) for three times.

The mixture with 9 μL 10 μM of cDNA and 5 μL 1 mM TCEP were added to 300 μL of the 10-fold concentrated AuNPs solution to prepare cDNA-AuNPs (Fig. S4). Next steps were same as that of preparation of HO-AuNPs-DNA conjugates, except, after shaking 1 h at RT, 50 uL of 15 mM DATP were attached to the solution and this conjugates were washed twice with 10 mM PBSB (pH7.4, 1% BSA). All prepared complexes stored at 4 °C before further use.

Preparation of Streptavidin-biotinylated cHO (cHO-SA) Conjugates.

The streptavidin-biotinylated cHO (cHO-SA) conjugates were obtained using the previously reported method.[3,4] Briefly, five hundred microlitre of 2 mg/ml of streptavidin was mixed with 10 μM biotinylated cHO. The mixture was incubated for 2 h at room temperature. After adding 400 μL PBS (pH=7, 0.01M) into the mixture, the solution was centrifuged in dialysis tube for 10 min at 12000 rpm under RT, then discarded the supernatant. The above step was repeated for three times. The remaining solution in filter was diluted to 600 μL with PBS.

Preparation of the Lateral Flow Strip Biosensor (LFB).

The biosensor consists of three components: sample pad, nitrocellulose (NC) membrane, and absorbent pad. The sample pad (23 mm × 30 cm) is prepared by polyester fiber (PR-VL78) in the sample pad buffer (pH8.0) containing (0.25% TritonX-100, 0.02M Tris-HCl, and 0.15M NaCl) for 1 h, then dried at 37°C for 2 h and at last stored in desiccators at RT before use. SA was utilized to combine with the biotinylated cHO to form the cHO-SA conjugates so that control probes could be immobilized on the NC membrane. So, the test zone and control zone would not be easily washed away by the sample solution if control probes are directly dispensed on the NC membrane. The test zone and control zone on the NC membrane (25 mm × 30 cm) were prepared by dispensing a concentration of 1 mg/mL SA and 1 mg/mL cHO-SA solutions, respectively, with the HM3030 dispenser (Shanghai Kinbio Tech. Co., Ltd. Shanghai, China) (Fig. S5). The distance between two zones was around 0.5 cm. The membrane was dried at RT for 1h and stored at 4°C. Finally, all of the parts were assembled on a plastic adhesive backing layer (60 mm × 30 cm). The sample pad and absorbent pad were fabricated with 2 mm overlapped to ensure the solution migrating through the biosensor during the assay. Instead of choosing a conjugate pad to store HO-AuNPs-DNA conjugates, strips with a 4.0 mm width were cut by using the programmable strip cutter CTD300P and we put them in sample bags at 4°C before use.

Analytical Procedure of Detect CEA Using the Strip Biosensor.

In our CEA test on LFB, 10 μL of running buffer (10 mM PBSB (0.01M PBS, pH7.4, 1%BSA)) was firstly used to pretreatment the test pad (Fig. S5). Then, 20 μL of HO-AuNPs-DNA conjugates, 20 μL of different concentrations of CEA in PBS, 20 μL of cDNA-AuNPs-HRP conjugates, 20 μL of AEC-H_2O_2 solution were applied to the biosensor at 3 min intervals in sequence. After applying solution to sample pad, the biosensor was washed by adding 20 μL of running buffer (1%PBSB). After reaction for 18 min at room temperature, the solution was applied to the sample application well and the test zone and control zone were evaluated visually within 18 min. Qualitative detection (for high concentration of targets) can be realized by observing the color of test zone with naked eyes. For quantitative measurements, the optical intensity of the red band was recorded using the portable "strip reader" instrument combined with "GoldBio strip reader" software.

For detection of CEA in real human plasma, 1 μL of plasma spiked with different quantities of CEA was applied onto the biosensor, and other steps were same as above said. The results were obtained by recording the optical responses with the strip reader after 18min.

Optimization of Experimental Parameters.

Different running buffers have different wash abilities. Appropriate buffers would minimize the nonspecific adsorption and increase the sensitivity and reproducibility of the LFB. In this study, six types of running buffer

were extensively used to test the assay performance in the presence of 5 ng/mL CEA. The largest peak area and the best S/N ratio were obtained when 1% PBSB was used (Fig. S6A).

To further minimize the nonspecific adsorption, the volume of sealant and the reaction time between the double labelled AuNP conjugates and sealant are significant factors that influence the test performance. The peak areas increased with increasing volumes of DATP and then decreased sharply; it reached a maximum at 30 μL (Fig. S6B), indicating that the spared surface on the AuNPs had completely closed. The reaction time is another issue for electrochemical measurements. The optical responses increased to the peak value with the reaction time up to half an hour (Fig. S6C), indicating a thorough reaction had occurred, resulting in a large response and decreased reaction time.

Additionally, the dispensed volumes of cDNA-AuNP-HRP and AEC-H_2O_2 were optimized, and the results are shown in Fig. S6D-S6E. The investigated results indicated that the peak areas first increased with increasing cDNA-AuNP-HRP conjugate from 5 μL to 20 μL (Fig. S6D), which subsequently decreased. In addition, one could see that the peak areas reached a maximum at 20 μL AEC-H_2O_2 in Fig. S6E. Further taking the saturability of LFSB, the consumption of reagent and the cost per assay into account, we chosen 1/1 CEA-HO to CEA-DNA, 10 μL cDNA, 10 μL HRP, 1% PBSB, 30 μL DATP, 30 min reaction time between DATP and AuNPs labelled conjugates, 20 μL cDNA-AuNP-HRP and 20 μL of AEC-H_2O_2 as the optimized parameters throughout the experiments.

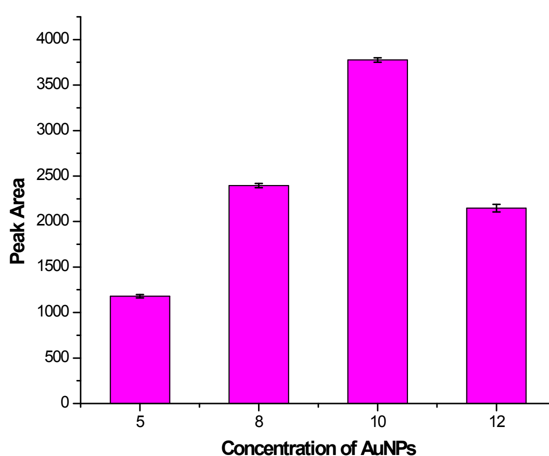

Fig. S1. Plots of the peak areas of red bands on the test zone vs different concentration of AuNPs for 5 ng/ml CEA. The error bars represent the standard deviation of three independent measurements.

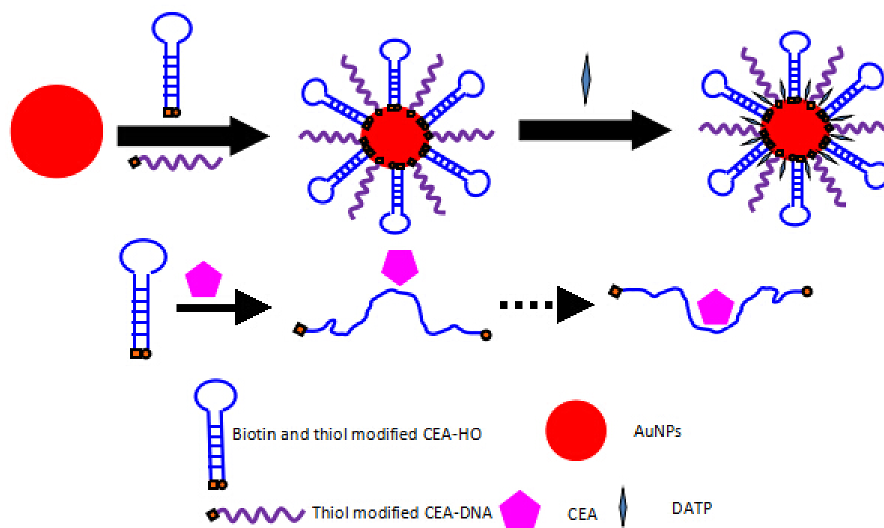

Fig. S2. Schematic illustration for preparation of HO-AuNPs-DNA conjugates and conformational switch of HO.

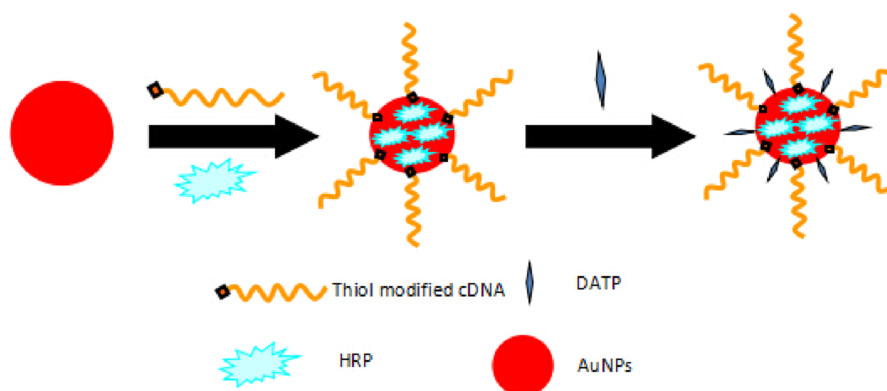

Fig. S3. Schematic illustration for preparation of cDNA-AuNPs-HRP conjugates.

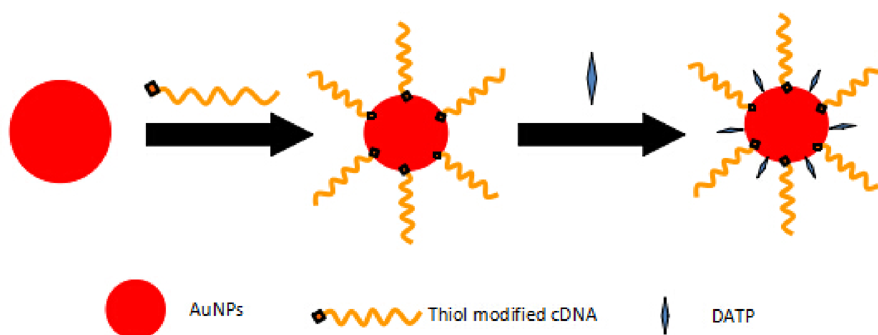

Fig. S4. Schematic illustration for preparation of cDNA-AuNPs conjugates.

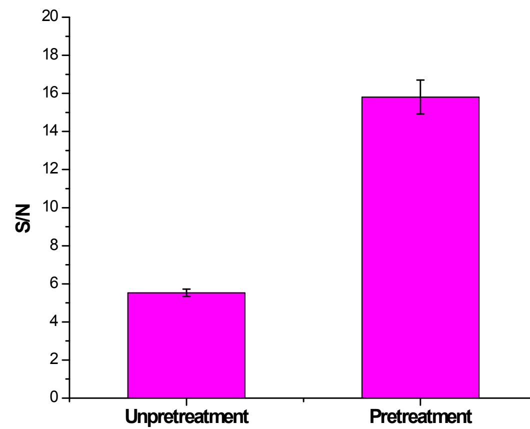

Fig. S5. Plots of the signal to noise of peak areas of red bands on the test zone vs different treatment methods of the strip biosensor for 5 ng/ml CEA.The error bars represent the standard deviation of three independent measurements.

Table. S1

General chart of the volumes of HO and DNA

Test Number	Ratio	HO/ul	DNA/ul	T-Peak Area	S/N
1	1:1	9.0	9.0	3926.11	16.45
2	2:1	12.0	6.0	3836.28	16.07
3	3:1	13.5	4.5	3769.71	15.79
4	4:1	14.4	3.6	2238.62	9.38
5	5:1	15.0	3.0	917.44	3.89

Table. S2

General chart of the volumes of cDNA and HRP

Test Number	cDNA /ul	HRP/ul	Peak Area	S/N
1	5	10	2155.09	9.03
2	20	10	1979.89	8.30
3	10	10	3500.06	14.67
4	10	20	2121.36	8.89
5	10	5	1584.88	6.64

Fig. S6. Effect of (A) types of running buffer (B) volumes of DATP (C) different reaction time of HO-AuNPs-DNA and DATP (D) volumes of cDNA-AuNPs-HRP (E) volumes of AEC+H_2O_2 on the responses of 5 ng/ml CEA. The error bars represent the standard deviation of three independent measurements.

Fig. S7. Plots of (A) their corresponding optical responses of red bands on the strip biosensors with different concentrations of CEA using a portable strip reader **(B)** the peak areas on the test zone vs different concentrations of CEA. Inset displays the linear relationship between the peak areas on the test zone vs the logarithm of CEA concentrations.

Fig. S8. Plots of the peak areas of red band on the test zone (A) detected different proteins. (CEA, 5 ng/ml; other proteins, 100 ng/ml) (B) detected with 5 ng/ml CEA at one week, four weeks, and seven weeks, respectively. The error bars represent the standard deviation of ten independent measurements.

Table. S3. Analytical Performances Different Methods

Method	Probe	Protein	LOD	Linear Range	Reference
LFB	HO-AuNP	CEA	0.5 ng/mL	1-200 ng/ml	This Work
LFB	HO-AuNP-DNA	CEA	9.1 pg/mL	5×10^{-2}-200 ng/ml	This Work
LFB	HO-AuNP-DNA-HRP	CEA	2.9 fg/mL	5×10^{-6}-20 ng/mL	This Work
LFB	HO-AuNP-DNA-HRP	MUC1	1.2 ng/ml	4.8-1×10^{3} ng/ml	This Work
LFB	HO-AuNP-DNA-HRP	Thrombin	18 pM	19-8×10^{5} pM	This Work
LFB	Apatmer-AuNP	Thrombin	2.5 nM	5-100 nM	Xu et al (2009)[5]
LFB	Antibody-AuNP	Thrombin	5 nM	7.5-500 nM	Xu et al (2009)[5]
EI	Antibody-AuNP-HRP	CEA	1.09 pg	5×10^{-2}-20 ng/ml	Wang et al (2013)[6]
ECL	Au@Pt-GQD-H2O2	CEA	0.6 pg/ml	1×10^{-3}-10 ng/ml	Li et al (2014)[7]
QCM	AuNP	CEA	1.5 ng/ml	3-50 ng/ml	Tang et al (2006)[8]
SPR	SAM-Au Chip	CEA	3 ng/ml	3-480 ng/ml	Altintas et al (2011)[9]

Table. S4. Determination of CEA (ng/ml) in Human Plasma Samples Using the Strip Biosensor and CL.

Sample Number	CL-Value (ng/ml)[a]	SB-Value (ng/ml)[b]	Relative Error (%)[c]
1	14.380	14.079	-2.095
2	13.080	13.023	-0.433
3	7.420	6.865	-7.483
4	3.394	3.228	-4.898

5	2.680	2.594	-3.206
6	$1.710*10^{-1}$	$1.812*10^{-1}$	5.944
7	$1.160*10^{-1}$	$1.078*10^{-1}$	-7.079
8	$3.070*10^{-2}$	$2.921*10^{-2}$	-4.849
9	$2.220*10^{-2}$	$2.287*10^{-2}$	3.000
10	$1.790*10^{-3}$	$1.895*10^{-3}$	5.857
11	$1.600*10^{-3}$	$1.581*10^{-3}$	-1.175
12	$1.590*10^{-4}$	$1.615*10^{-4}$	1.547
13	$1.370*10^{-4}$	$1.421*10^{-4}$	3.746
14	$9.800*10^{-5}$	$1.057*10^{-5}$	7.900
15	$8.600*10^{-5}$	$8.700*10^{-5}$	1.167

[a]The concentration of CEA in real human plasma samples was certified using chemiluminescence (CL). [b]Each sample was analyzed using our proposed strip biosensor, and all values were obtained as an average of ten repetitive determinations±standard deviation (mean±SD). [c]DSB vs CL method.

Note and References

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