Design of Electrochemical Detection of Thiols in Cancer Cells Based on the Cleavage of the Disulfide Bond Coupled with Thionine Modified Gold Nanoparticles-assisted Amplification

1 Reagent and Instruments

$HAuCl_4 \cdot 4H_2O$ and trichloroacetic acid (TAC) was obtained from Shanghai Chemical Plant (Shanghai, China). Thionine, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), glutathione (GSH), cysteine (Cys), N-ethylmaleimide (NEM), dopamine hydrochloride, L-adrenaline, 2-mercaptoethanol (β-ME), ascorbic acid (Vc), dithiothreitol (DTT), histamine, uric acid, streptomycin sulfat, and penicillin G sodium salt were purchased from Sigma-Aldrich (USA). Zinc metallothionein (MT) (from rabbit liver) was purchased from Hunan Lugu Biotechnology Co. Ltd. (China). Glutathione reductase (GTR) (from yeast, 1000 U/mL) was purchased from Shanghai Sangon Biological Engineering Technology & Services Co. Ltd. (China). Amido groups modified magnetic nanoparticles (Aminated loaded on MB) (1 μm - 1.5 μm, 10 mg/mL) were purchased from Baseline Chromtech Research Centre (Tianjin, China). All the reagents were analytical grade and used without further purification.

All synthetic oligonucleotides were purchased from SBS Genetech Co. Ltd. (Beijing, China). Their sequences were presented as follows:

DNA1, 3'-NH_2-$(CH_2)_6$-CAG ACT CCA ACT TGC TGG-5',

DNA2, 3'-SH-$(CH_2)_6$-CCA GCA AGT TGG AGT CTG-5'.

2 Preparation of 20 nm Au nanoparticles

AuNPs with a diameter of 20 nm were prepared by reducing $HAuCl_4$ with 1% trisodium citrate according to the method reported previously with a slight modification.[1] Prior to synthesis, all glassware were cleaned with aqua regia ($HCl:HNO_3$ volume ratio = 3:1) [**Caution! aqua regia is corrosive in nature and**

should be handled with care]. $HAuCl_4$ and trisodium citrate solutions were filtered through a 0.22 μm microporous membrane filter prior to use, and then 1.4 mL of 1% trisodium citrate was added to 200.0 mL of boiling 0.01% $HAuCl_4$ solution and stirred for 10 min at the boiling point. The final AuNPs prepared by this method have an average diameter of approximately 20 ± 2 nm as measured by TEM. The prepared 20 nm AuNPs were stored in brown glass bottles at 4 °C.

3 Preparation of AuNP/GR/CILE

Preparation of carbon ionic liquid electrode (CILE). CILE is a new kind of working electrode with many excellent properties such as good resistivity towards electrode fouling, wide electrochemical windows, high electron transfer rate, the inherent catalytic activity and the ability to lower the overpotential of electroactive compounds, which has been proven to be better than the traditional carbon electrode.[1] so CILE is used in this paper as the substrate electrode for the further modification. The process of preparation was following. 3.2 g of graphite powder and 1.0 g of $HPPF_6$ were mixed thoroughly in a mortar to form a homogeneous carbon paste and further heated at 70 °C for 1 h. A portion of the resulting homogeneous paste was packed firmly into a glass tube (4 mm in diameter). Electrical contact was established through a copper wire to the end of the paste in the inner hole of the tube, and the surface of CPE was smoothed on a weighing paper immediately prior to use.

Preparation of electrochemically reduced graphene oxide-carbon ionic liquid electrode (GR/CILE). Graphite oxide (GO) was synthesized from nature graphite by a modified Hummers method.[3] As prepared, graphite oxide was dispersed in phosphate buffer solution (pH 7.0) and sonicated for 2 hours, to form exfoliated graphene oxide (GO). Then the CILE was immersed to the GO solution. The 5 mL GO (0.5 g/ml) in the phosphate buffer solution was electrochemically reduced for 600 s at －1.3 V to obtain electrochemically reduced graphene oxide modified carbon ionic liquid electrode, named GR/CILE.

Preparation of gold nanoparticles-electrochemically reduced graphene

oxide-carbon ionic liquid electrode (AuNP/GR/CILE). AuNPs modified electrochemically reduced graphene oxide-carbon ionic liquid electrode (AuNP/GR/CILE) was prepared based on a reported procedure.[4] A solution containing 5.0 mmol/L $HAuCl_4$ and 0.5 mol/L KNO_3 (5 mL) was employed for electrodeposition of AuNPs at GR/CILE. AuNPs was electrodeposited onto the newly prepared GR/CILE with the solution by controlled potential at −0.4V for 300 s. The AuNP/GR/CILE was rinsed with doubly distilled water and dried in air for further using.

4 The preparation of captured DNA2 modified working electrode

20 μL of 2.0×10^{-7} M captured DNA2 was dropped onto the surface of *AuNP/GR/CILE* and incubated for 2 h at 37 °C. Then the electrode was washed with 1.0 mL of 0.1 M phosphate buffer solution for three times and stored at 4 °C for using.

5 Conjugation of thionine on Au nanoparticles

Thionine was conjugated onto AuNPs according to the method reported previously with a slight modification.[5] A 4 mL volume of AuNPs solution was mixed with 1 mL of saturated thionine solution and stirred effectively. After 24 h stirring, the resulting solution was centrifuged at 12 000 rpm to obtain the precipitate of thionine-covered AuNPs (EC probe). The precipitate was washed three times with phosphate buffer solution and redispersed in phosphate buffer solution and stored at 4 °C for further use.

6 Preparation of Functioned MB

The cystine was directly immobilized onto the MB with well-established carbodiimide method. The immobilization protocols were the following.[6] Briefly, (1) 1.0 mL of 10^{-3} M cystine was placed in a 15 mL Eppendorf tube (EP tube). Then 20 mg EDAC and 4 mg NHS in 2.0 mL water were added and allow the mixture to react for 30 min. at room temperature with continuous mixing. (2) 1.0 mL suspension of aminated MB (10 mg/mL) was added and incubated at 37 °C for 12 hours. (3) The

resulting MB was separated from the solution on a magnetic rack and washed with 2.0 mL of 0.1 M phosphate buffer solution for three times, and resuspended in 2.0 mL phosphate buffer solution. (4) Then 200 μL 1.0×10^{-7} M of DNA1 was added to the above the MB, and incubated at 37 °C for 12 hours. Finally, the resulting functioned MB was washed with 2.0 mL of 0.1 M phosphate buffer solution for three times, and resuspended in 1.0 mL phosphate buffer solution and stored at 4 °C for further use.

7 Electrochemical GSH biosensor development

The procedure of the fabrication of GSH biosensor and the principle of scission of disulfides-based EC detection of GSH are illustrated in Scheme 1. Briefly, 1.0 mL GSH sample was added to functioned MB solution. After incubated at 37 °C about 60 min, the mixture was separated with a magnetic rack. Then, the captured DNA2 modified electrode was immersed into above solution. After incubation for 0.5 h at 37 °C, the electrode was washed throughout with 100 mM phosphate buffer solution (pH 7.4). Subsequently, the electrode was immersed into the EC probe (thionine-covered AuNPs) solution. After incubated at 37 °C about 60 min, the electrode was taken for EC detection.

8 Electrochemical measurements

The EC detection was carried out with CH instrument Model 660B Electrochemistry Working Station. All EC detections were carried out with a conventional three-electrode system. A Pt wire was used as the counter electrode, and saturated calomel electrode (SCE) was used as reference electrode. The buffer was purged with high-purity nitrogen for 5 min prior to each electrochemical measurement, and the nitrogen environment was then kept over the solution to prevent oxygen from reaching the solution. The differential pulse voltammogram (DPV) parameters were as follows: potential range: −0.70~−0.15 V; pulse amplitude: 0.05 V; pulse width: 0.05 s; pulse period: 0.2 s and quiet time: 2 s.

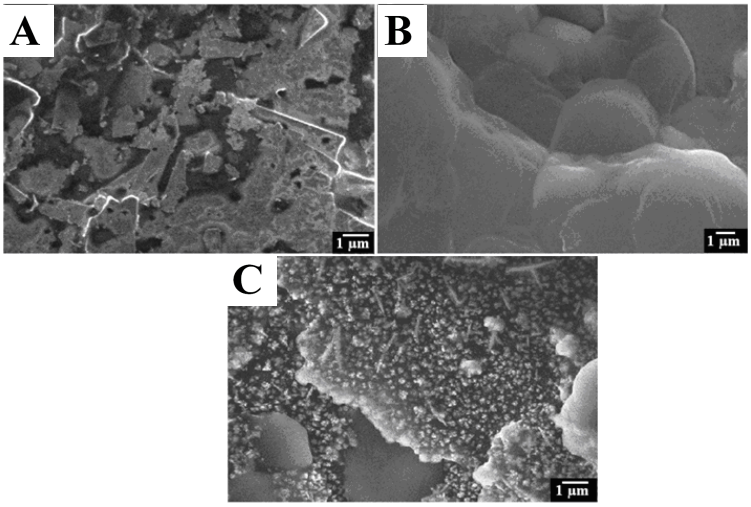

Fig. S1 SEM images of (A) CILE, (B) GR/CILE, (C) Au/GR/CILE

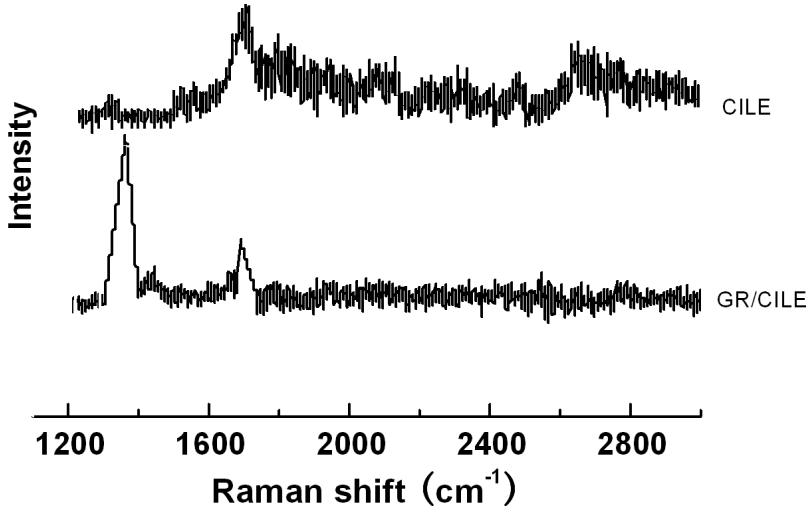

Fig. S2 Raman spectra of GR/CILE and CILE.

9 Electrochemical Behavior of Thionine at the AuNP/GR/CILE

Fig. S2 shows the typical DPVs of thionine obtained at the bare CILE (curve a), GR/CILE (curve b) and AuNP/GR/CILE (curve c), respectively. As can be seen, 4.0×10^{-5} mol/L^{-1} thionine gives a DPV peak current (Ip) of 1.60×10^{-5} A with the peak potential of -0.395 V at the bare CILE (curve a). The Ip of thionine at the GR/CILE is 3.06×10^{-5} A with the peak potential of -0.392 V (curve b), which is 3.2 times as that of at CILE. And the Ip of thionine at the AuNP/GR/CILE is 3.06×10^{-5} A with the peak potential of -0.387 V (curve c), which is 2.8 times as that of at CILE. This may

be attributed to the conductivity and cooperation of AuNP, GR on the electrode surface.

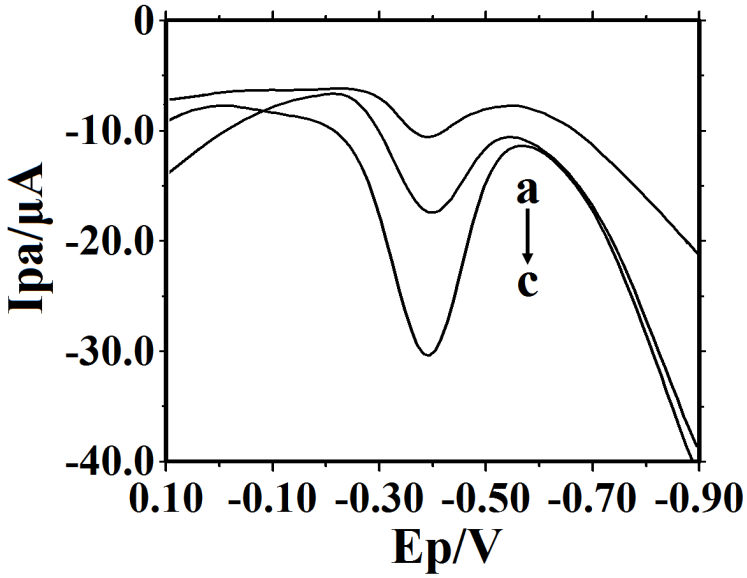

Fig. S3 DPVs of 4.0×10^{-5} mol/L^{-1} Tionine on Au/GR/CILE in pH 6.5 phosphate buffer solution. From a to c were CILE， GR/CILE and Au/GR/CILE, respectively.

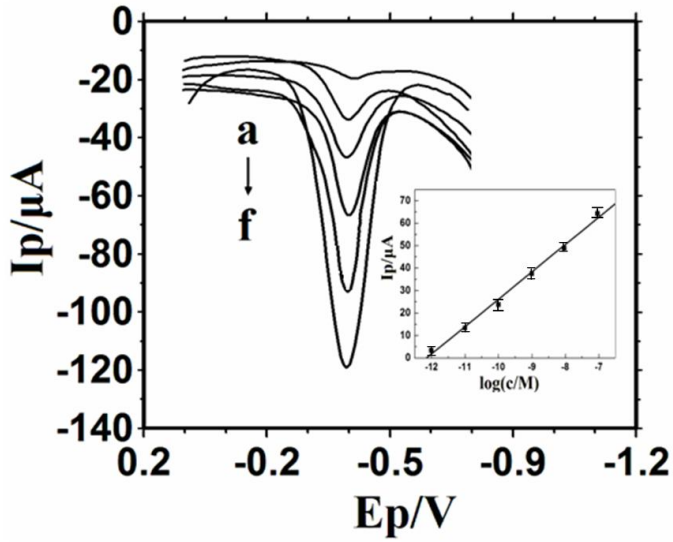

Fig. S4 DPVs of different concentrations GSH (from a to f was 1.0×10^{-12}, 1.0×10^{-11}, 1.0×10^{-10}, 1.0×10^{-9}, 1.0×10^{-8}, 1.0×10^{-7} mol/L) on the Au/GR/CILE in phosphate buffer solution (pH 7.4) at the scan rate of 100mV/s. The calibration curve of peak height versus the concentration of GSH from 1.0×10^{-12} to 1.0×10^{-7} mol/L (insert).

10 Preparation of free thiols extracts from cancer cells

Cancer cells were prepared as described in the published paper.[7] Briefly, Murine P388 Lymphocytic Leukemia cell was cultured in DMEM supplemented with 10% fetal bovine serum and a mixture of 5 μg/mL insulin, 5 μg/mL transferring, 40 ng/mL dexamethasone, 3.7 g/L $NaHCO_3$, 100 units/mL penicillin and 100 μg/mL streptomycin, and maintained at 37ºC in a humidified atmosphere containing (95% air and 5% CO_2). Cells were collected in the exponential phase of growth, and 1.0×10^5 cells were dispensed in a 1.5 mL EP tube, washed twice with ice-cold PBS, and resuspended in 3 mL sodium phosphate-EDTA buffer (PBSE, pH 7.4, 0.10 M). After homogenization, the 3% perchloric acid was added into the cell homogenate to precipitate protein. With the mixture centrifuged 20 min at 12 000 rpm, 4 °C, to pellet insoluble material. Without disturbing the pellet, carefully transfer the cleared lysate to a fresh 1.5 mL EP tube. The lysate was used immediately for free thiols assay or frozen at -70 °C.

For the intracellular protein thiols detection, the protein pellet was collected by centrifugation and washed with TCA-EDTA solution firstly. Then the protein was redissolved in 3 mL Tris–HCl buffer (pH 7.4, 0.10 M) containing 5 mM EDTA and 0.5% sodium laurylsulfonate (SDS). Aliquots (each 20 μL) were used immediately for free thiols assay or frozen at -70 °C.

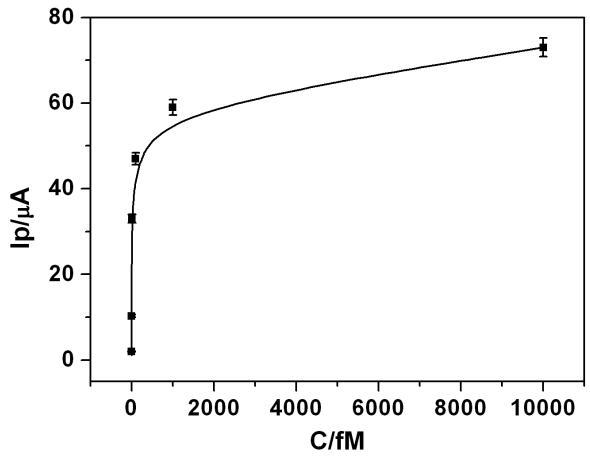

Fig. S5 EC responses of MT. All data were obtained at 25°C.

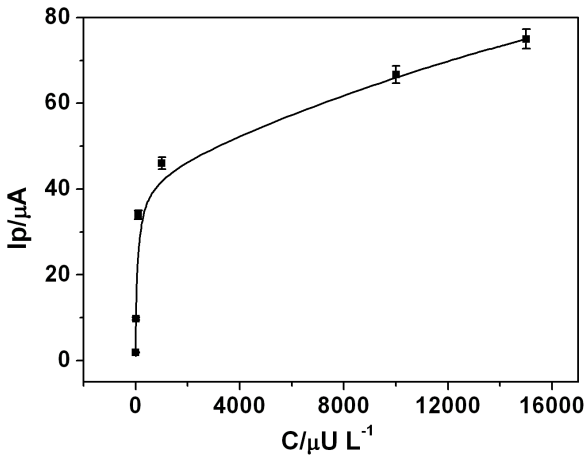

Fig. S6 EC responses of GTR. All data were obtained at 25°C.

Table S1. Results of Determination of Thiols in Cells

Sample	Detected [a] ($\times 10^{-9}$ M)	Added ($\times 10^{-9}$ M)	Found [a] ($\times 10^{-9}$ M)	Recovery (%)
Non-protein thiols	6.9 ± 0.12	2.00	8.95 ± 0.11	102.5
		5.00	11.78 ± 0.14	97.6
		10.00	16.88 ± 0.12	99.8
Protein thiols	12.1 ± 0.18	2.00	14.11 ± 0.20	100.5
		5.00	17.02 ± 0.25	98.4
		10.00	22.17 ± 0.23	100.7

[a] The average of five determinations (±SD).

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