

**A novel electrochemical immunosensor for 5-hydroxymethylcytosine  
quantitative detection in genomic DNA of breast cancer tissue**

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### *Reagents and Apparatus*

Anti-5-hmC antibody was obtained from Merck Millipore (Germany). 5-Hydroxymethyl-2'-deoxycytidine-5'-triphosphate (5-hm-dCTP) and 5-methyl-2'-deoxycytidine-5'-triphosphate (5-m-dCTP) were purchased from TriLink BioTechnologies (California, USA). Phos-tag-biotin was provided by Wako Pure Chemical Industries, Ltd. (Japan). Avidin-ALP was obtained from Sangon Biotech (Shanghai) Co., Ltd. DNase I was purchased from Thermo (Massachusetts, USA). Breast cancer tissue and normal breast tissue were supplied by the tumor center of Taian city central hospital (Taian, China). Graphene (GR) was obtained from XFNANO (Nanjing, China). Perylene-3,4,9,10-tetracarboxylic dianhydride (PTCDA), p-nitrophenyl phosphate (PNPP), N-(3-dimethylaminopropyl)-N-ethyl carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Aladdin (Shanghai, China). HiPure Tissue DNA Mini Kit was supplied by Magen (Guangzhou, China).

The stock buffers used in this work were as follows. Phosphate buffer saline (PBS) was prepared by mixing the stock solution of 10mM NaH<sub>2</sub>PO<sub>4</sub> and 10mM Na<sub>2</sub>HPO<sub>4</sub>, and the pH was adjusted by NaOH or HCl. Anti-5-hmC-antibody dilution buffer, 10mM PBS and 30% glycerinum (pH 7.4). 5-Hm-dCTP and 5-m-dCTP stock buffer, 10 mM PBS (pH 7.4). Phos-tag-biotin reaction buffer, 10 mM Tris-HCl (pH 7.0) containing 0.1 M NaCl, 0.1% Tween-20, 0.4 mM Zn(NO<sub>3</sub>)<sub>2</sub>. Blocking buffer, 10 mM PBS (pH 7.4) containing 5 μM ethanol amine. Washing buffer, 10 mM Tris-HCl (pH 7.4). Detection solution, 10 mM Tris-HCl (pH 9.8) containing 1 mM MgCl<sub>2</sub> and 3

mM PNPP.

#### *Preparation of graphene-perylenetetracarboxylic acid (GR-PTCA) nanocomposites*

The 3,4,9,10-erylene tetracarboxylic acid (PTCA) solution was prepared according to previous report<sup>1, 2</sup>. In detail, the PTCA was made by hydrolyzing PTCDA in a minimal volume of 1M NaOH aqueous solution at 80 °C for 1 h. After red deposits appeared in the yellow-green solution, 1 M hydrochloric acid was stepwise added in the mixture and maintained pH at slightly acidity. Subsequently, the red deposits were collected by centrifugation with 12000 rpm and dried under vacuum at room temperature. Afterwards, 10 mg GR was mixed with 2 mg PTCA in 10 mL of double distilled water under the assistance of ultrasonication for 2 h. Then, the solution was stirred continuously for 5 h at ambient temperature and held still overnight. Finally, the mixture was centrifuged and washed three times with doubled distilled water. After dried in a vacuum at 60 °C, the obtained products werere-dispersed in 10 mL distilled water and named as GR-PTCA.

#### *Immunosensor fabrication*

The bare glassy carbon electrode (GCE,  $d=2$  mm) was first polished with 0.03 and 0.5  $\mu\text{m}$  alumina slurry to obtain a mirror-like surface. Then, it was further sonicated in absolute ethanol and double distilled deionized water for 3 min respectively. After dried with nitrogen blowing, 10 $\mu\text{L}$  of GR-PTCA was dropped on the surface of GCE and dry in air (The electrode was named as GR-PTCA/GCE).

After activated by EDC/NHS (25 mM/50 mM) for 1 h, 10  $\mu$ L of anti-5-hmC antibody (20  $\mu$ g/mL) was dripped on the surface of GR-PTCA and incubated for 2 h at room temperature in a humid cell. Then 10  $\mu$ L of blocking buffer was added to passivate the unreacted  $-\text{COOH}$  groups of PTCA for 30 min. After rinsed three times with washing buffer, the electrode was named as Ab/GR-PTCA/GCE. Subsequently, 10  $\mu$ L of different concentration of 5-hm-dCTP was dropped on the surface of Ab/GR-PTCA/GCE and incubated at 37  $^{\circ}\text{C}$  for 2 h. After rinsed three times with washing buffer, the electrode was named as 5-hmC/Ab/GR-PTCA/GCE. Afterwards, 10  $\mu$ L of phos-tag-biotin reaction buffer containing 20  $\mu$ M phos-tag-biotin was dripped on the surface of 5-hmC/Ab/GR-PTCA/GCE and incubated for 1 h in a humid chamber. Then the electrode was rinsed three times with washing buffer and named as biotin/5-hmC/Ab/GR-PTCA/GCE. Finally, the electrode was incubated with 10  $\mu$ L of 10 mM PBS (pH 7.4) containing 50  $\mu$ g/mL avidin-ALP at 37  $^{\circ}\text{C}$  for 2 h and follow rinsed three times with washing buffer. The obtained electrode was named as ALP/biotin/5-hmC/Ab/GR-PTCA/GCE and used for electrochemical detection.

#### *Electrochemical detection*

Electrochemical detection was performed at CHI660C electrochemical workstation (Austin, USA) with three electrode system. Bismuth modified GEC was used as working electrode, which was prepared according to our previous report<sup>3</sup>. The saturated calomel electrode (SCE) was used as reference electrode and the platinum wire electrode was used as the counter electrode.

First, the fabricated ALP/biotin/5-hmC/Ab/GR-PTCA/GCE was immersed into the 10 mL detection solution and incubated for 40 min at 37 °C under magnetic stirring. Then, the working electrode (Bi/GCE), reference electrode and counter electrode were immersed in above solution. Finally the differential pulse voltammetry (DPV) response was recorded. The parameters are as follows: increment potential, 0.004 V; pulse amplitude, 0.05 V; pulse width, 0.05 s; sample width, 0.0167 s; pulse period, 0.2 s; quiet time, 6 s.

#### *Samples treatment and analysis*

In order to analyze the expression level of 5-hmC in breast cancer tissue, the genomic DNA was extracted from breast cancer tissue using HiPure Tissue DNA Mini Kit. Then, the concentration of genomic DNA was measured by Q-5000 UltramicroUV-Vis spectrophotometer (Quawell, USA). After the genomic DNA was diluted to 0.4 ng· $\mu\text{L}^{-1}$  with 10 mM PBS (pH 7.4), 50  $\mu\text{L}$  of the genomic DNA was mixed with 50  $\mu\text{L}$  of DNase I (40 unit· $\text{mL}^{-1}$ ) at 37 °C for 5 h (the mixture solution named as solution 1). Subsequently, 10  $\mu\text{L}$  of solution 1 was used to fabricate electrochemical immunosensor as shown in Section 2.3 and electrochemical detection was performed as shown in Section 2.4. In addition, the genomic DNA extracted from normal breast tissue was used as control group and the detection process was the same as detection of 5-hmC in breast cancer tissue. To investigate whether genomic DNA was degraded successfully by DNase I, all genomic DNA (treated without or with DNase I) were characterized by agarose gel electrophoresis.

*Characterization of modified electrode using electrochemical impedance spectroscopy*

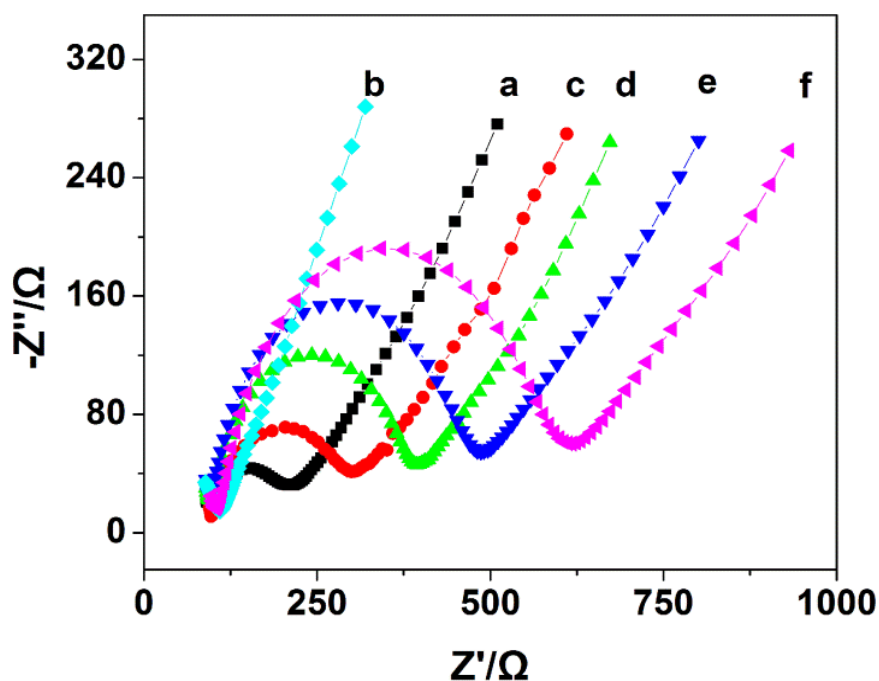


Fig.S1. The electrochemical impedance spectroscopy of different electrodes in 5 mM  $\text{Fe}(\text{CN})_6^{3-/4-}$  solution containing 0.1 M KCl. (a) GCE, (b) GR-PTCA/GCE, (c) Ab/GR-PTCA/GCE, (d) 5-hmC/Ab/GR-PTCA/GCE, (e) biotin/5-hmC/Ab/GR-PTCA/GCE, (f) ALP/ biotin/5-hmC/Ab/GR-PTCA/GCE.

The fabrication process of the electrochemical immunosensor was characterized by electrochemical impedance spectroscopy (EIS) in 5 mM  $\text{Fe}(\text{CN})_6^{3-/4-}$  (1:1) solution containing 0.1 M KCl with the frequency range of  $10^{-1}$  -  $10^5$  Hz. As shown in Fig. S 1, the bare GCE shows the electron transfer resistance ( $R_{et}$ ) value of 124  $\Omega$  (curve a). However, the  $R_{et}$  value decreased (curve b) significantly and only a straight line was observed when GR-PTCA was immobilized on the surface of GCE. The phenomenon

can be explained that GR-PTCA increased the effective surface area of GCE electrode. After 5-hmC antibody was further captured on the electrode surface, the  $R_{et}$  value (curve c) increased obviously due to the steric hindrance effect of the antibody. Subsequently, the  $R_{et}$  further increased (curve d) when 5-hm-dCTP was assembled on the electrode surface. It can be attributed to the electrostatic repulsion between phosphate group of 5-hm-dCTP and  $Fe(CN)_6^{3-/4-}$ . Afterwards, when the electrode was incubated with phos-tag-biotin and avidin-ALP in turn, the  $R_{et}$  value (curve e and curve f) increased successively due to the steric-hindrance effect of phos-tag-biotin and ALP. This proved that phos-tag-biotin and avidin-ALP were captured on the electrode surface successfully. According to the changes of  $R_{et}$ , we can demonstrate that the immunosensor was fabricated successfully.

#### *Optimum of detection conditions*

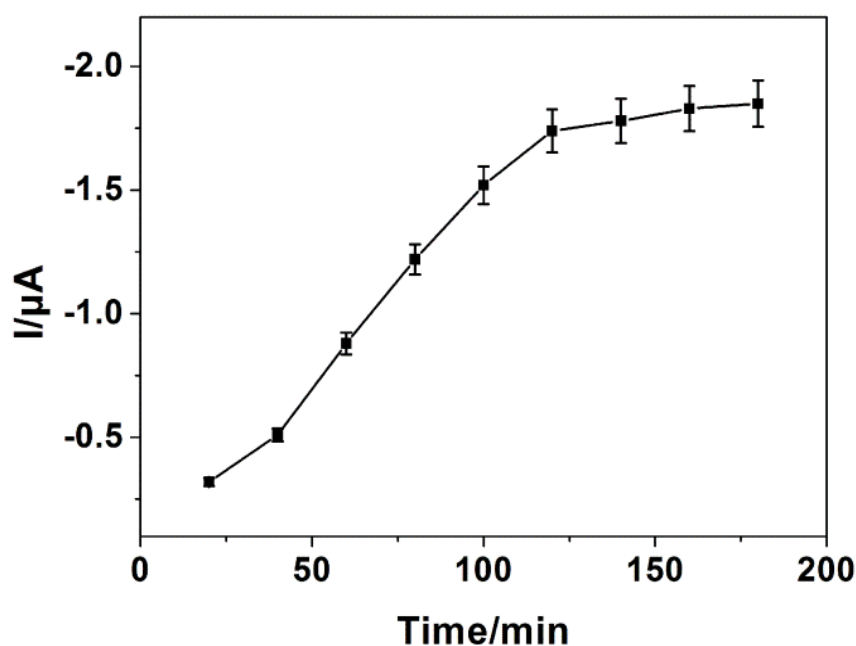


Fig.S2. The effect of 5-hm-dCTP reaction time.

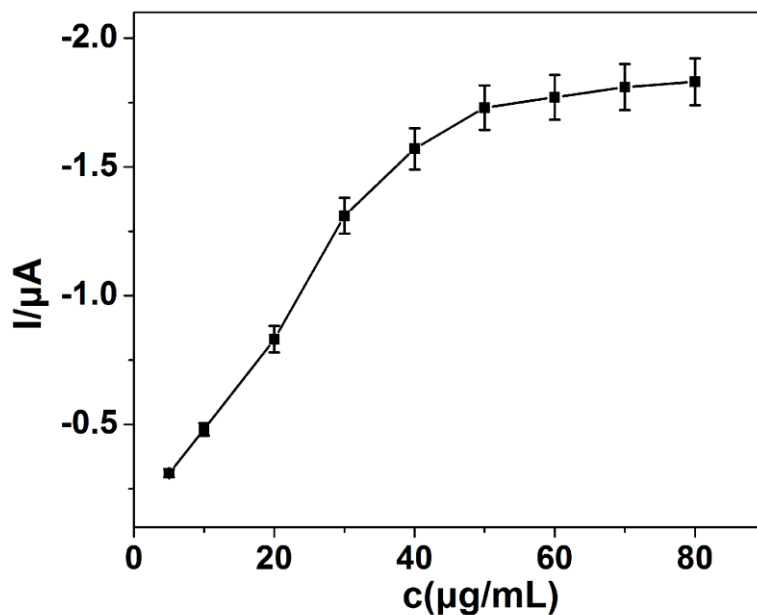


Fig. S3. The effect of avidin-ALP concentration on the DPV response.

#### *Selectivity and reproducibility*

In order to confirm the good selectivity of the electrochemical immunosensor, 5-m-dCTP was selected as the interference factor. The detection procedure was as same as Section 2.3 except that 5-hm-dCTP was replaced by 5-m-dCTP. As shown in Fig S4 A, after detection buffer was incubated by different electrodes, DPV response for 5-m-dCTP (5 nM) was much lower than that obtained from 5-hm-dCTP (5 nM). The results indicate that anti-5-hmC antibody can't recognize 5-mC. Therefore, the developed electrochemical immunosensor has good detection selectivity. The reproducibility assay was performed by six different and freshly fabricated immunosensors using 30 nM 5-hm-dCTP. The result was shown in Fig. S4 B. The Relative Standard Deviation (RSD) for six detections is 5.26 %, which indicate that the fabricated immunosensor has an acceptable reproducibility.



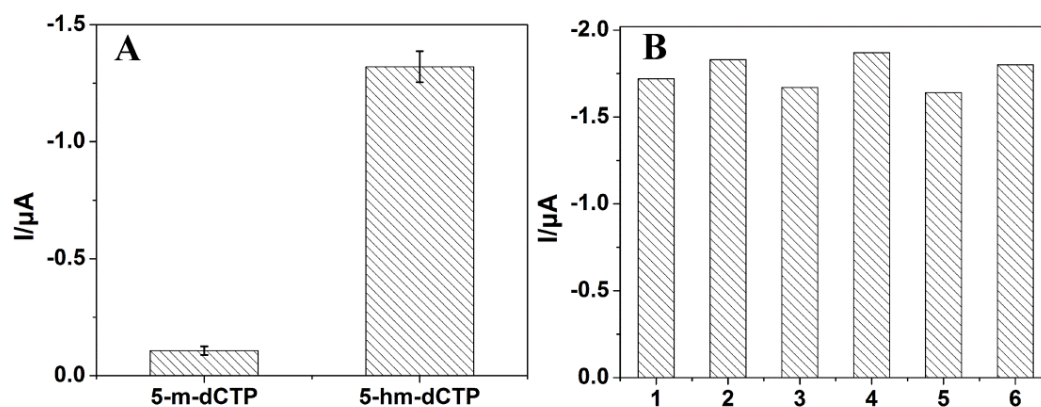


Fig. S4. (A) DPV response for 5-m-dCTP and 5-hm-dCTP, (B) The histogram for the DPV response of six immunosensors using 30 nM 5-hm-dCTP.

### References

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