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Investigation of DNA methylation by direct electrocatalytic oxidation

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1. Experimental details

1.1. Chemicals and reagents

Cytosine (C), 5-methylcytosine (5-mC), thymine (T), guanine (G), adenine (A) and calf thymus DNA were purchased from Sigma (USA). Fish sperm DNA and choline chloride (Ch) were obtained from Shanghai Chemical Co. Ltd. (Shanghai, China). They were used as received without further purification. Multiwalled carbon nanotubes (MWNTs; purity >95%) were purchased from Shenzhen Nanotech Port Co., Ltd. (Shenzhen, China). Prior to use, the MWNTs were purified by refluxing the as-received MWNTs in 2.6 M nitric acid for 5 h followed by centrifugation, resuspension, filtration, and air-drying to evaporate the solvent.[1] The purified MWNTs were further heated under vacuum at 400 °C for 2 h. Phosphate buffer solutions (PBS, 0.1 M) of different pH were prepared by mixing stock solutions of 0.1 M KH_2PO_4 and Na_2HPO_4, and adjusted by 0.1 M H_3PO_4 or NaOH (Shanghai Chemical Reagent Company, Shanghai, China).

All other chemicals not mentioned here were of analytical reagent grade. Aqueous solutions were prepared with doubly distilled water at ambient temperature. High purity nitrogen was used for deaeration of the prepared aqueous solutions.

1.2. Apparatus

Electrochemical experiments including differential pulse voltammetry (DPV) and cyclic voltammetry (CV) were carried out on a CHI 760C electrochemical

workstation (Chenhua, Shanghai, China). A conventional three-electrode electrochemical system was used for all electrochemical experiments, which consisted of a working electrode, a platinum wire counter electrode and an Ag/AgCl reference electrode (saturated KCl). A glassy carbon electrode (GCE, 3 mm diameter) was used as the basal working electrode. The electrochemical solutions were thoroughly deoxygenated by N_2 for 15 min before sampling and N_2 atmosphere was maintained throughout the experiments.

Field emission scanning electron microscope (FE-SEM) image was obtained on a JSM-6700F field emission scanning electron microanalyzer (JEOL, Japan). Transmission electron microscope (TEM) image was observed on a JEM-2100 transmission electron microscope (JEOL, Japan).

1.3. Electrode preparation and modification

Prior to use, GCE was carefully polished with 1.0, 0.3, and 0.05 μm alumina powder sequentially and then washed ultrasonically in doubly distilled water and ethanol for 10 min, respectively. The cleaned GCE was dried with nitrogen steam for the next modification. Ch monolayer modified GCE (Ch/GCE) was prepared by immersing the GCE in 0.1 M pH 7.0 PBS containing 2.0 mM Ch and 10 mM KCl and scanning between -1.70 and 1.80 V for 6 cycles at the scan rate of 25 mV s^{-1}. The obtained Ch/GCE was rinsed with distilled water and sonicated for 10 min to remove the physically adsorbed materials. In order to construct MWNTs on the surface of Ch/GCE, 0.50 mg mL^{-1} MWNTs was dispersed into N,N-dimethylformamide (DMF),

and the mixture was sonicated to give a homogeneous dispersion. The MWNTs/Ch/GCE was prepared by casting 10 μL of the MWNTs-DMF suspension onto the Ch/GCE surface and dried under an infrared lamp.

For comparison, Ch/GCE and MWNTs/GCE were also prepared under the same conditions.

1.4. DNA samples preparation

DNA samples were hydrolyzed as follows for the quantification of pyrimidine and purine bases. In brief, accurate amounts of fish sperm DNA (10.0 mg) and calf thymus DNA (15.0 mg) were hydrolyzed in 600 μL of 88% (w/w) formic acid at 170 °C for 30 min in two sealed glass tubes according to the procedure reported in literature.[2] The hydrolysates were then adjusted to neutrality with 2.0 M NaOH. Afterward, the obtained sample solutions were diluted with doubly distilled water. Finally, appropriate amounts of the pretreatment samples were added into 0.1 M pH 7.0 PBS for detection.

2. Morphological characterization of modified electrode

The TEM image of MWNTs was shown in Fig. S1A. As can be seen, the average diameter of these nanotubes was about 50 nm. Moreover, in order to characterize the interfacial structure of the constructed MWNTs/Ch/GCE, the morphology of the MWNTs/Ch/GCE surface was observed by FE-SEM. As shown in Fig. S1B, the

MWNTs were assembled to form a porous film on the surface of Ch/GCE. The distribution of MWNTs on the Ch/GCE was uniform and compact, showing that Ch could provide a suitable supporting substrate and favorable local microenvironment for the construction of MWNTs.

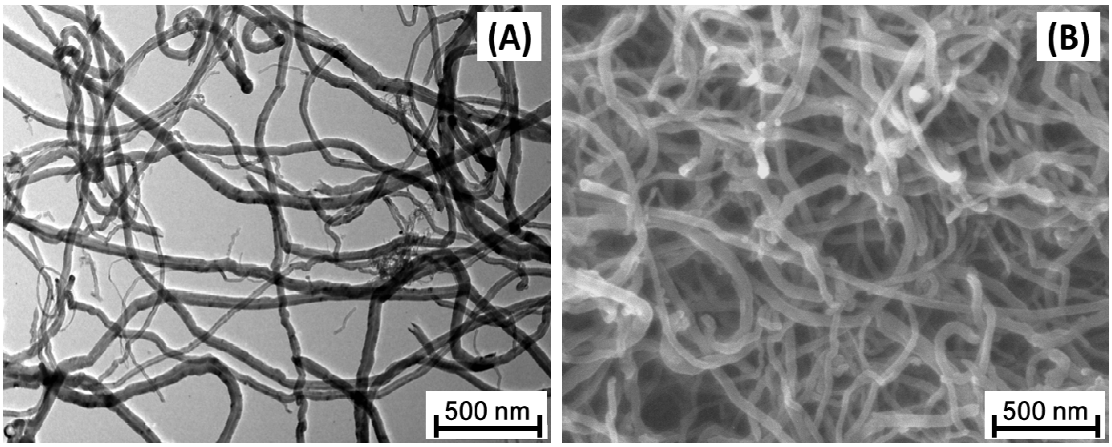

Fig. S1 (A) TEM image of the MWNTs. (B) FE-SEM image of the MWNTs/Ch/GCE.

3. Optimization of solution pH in the electrochemical oxidation of 5-mC and C

The pH value of supporting electrolyte can significantly influence the voltammetric behavior of 5-mC at the MWNTs/Ch/GCE. As shown in Fig. S2A, the oxidation peak potential (E_{pa}) of 5-mC shifted positively with the decrease in solution pH from 10.0 to 3.0, indicating that the electrochemical oxidation of 5-mC was associated with a proton-transfer process. The pH dependence of E_{pa} of 5-mC was described as E_{pa} (V) = 1.578 – 0.0599 pH (n = 10, R = 0.9991). The slope of 59.9 mV pH^{-1} was close to the anticipated Nernstian theoretical value of 59.1 mV pH^{-1} at 25 °C, suggesting that the uptake of electrons was accompanied by an equal number of protons.[3] Moreover,

it can be found that the oxidation peak current of 5-mC was not affected by the solution pH in the range of 10.0-3.0, so pH 7.0 PBS was selected as the supporting electrolyte since it was close to the pH value of physiological environment. Similar phenomena can also be observed for the investigation of C (Fig. S2B), and the linear regression equation between the E_{pa} of C and the solution pH was E_{pa} (V) = 1.764 – 0.0621 pH (n = 10, R = 0.9986).

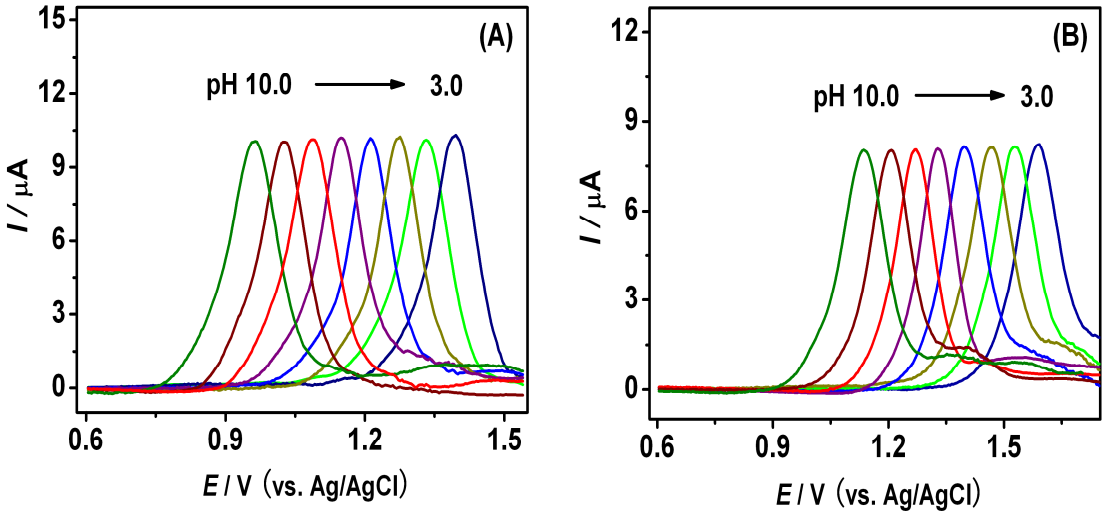

Fig. S2 Background-subtracted DPVs of MWNTs/Ch/GCE in 0.1 M PBS containing 50 μM 5-mC (A) and C (B) at different pH values.

4. Concentration dependence studies of 5-mC and C at different modified electrodes

The concentration dependence studies of 5-mC and C were carried out at Ch/GCE, MWNTs/GCE and MWNTs/Ch/GCE. With regard to the electrochemical behaviors of 5-mC and C at bare GCE, the oxidation peaks of 5-mC and C were completely overlapped at 1.29 V. Therefore, it was difficult to perform the concentration

dependence studies of 5-mC and C at bare GCE due to the slow electron transfer kinetics.[4-7]

Fig. S3A showed the concentration dependences of 5-mC and C at Ch/GCE in static solution. As can be seen, the peak potentials of 5-mC and C were constantly located at 1.20 and 1.36 V, respectively, indicating that the electrocatalytic oxidation processes of 5-mC and C were relatively independent. The oxidation peak currents of 5-mC and C were proportional to their concentrations in ranges of 3.0-420 and 5.0-550 μM. The linear regression equations were $I_{pa,\ 5\text{-}mC}$ (μA) = 0.1038c (μM) + 0.0157 (n=10, R = 0.9985) and $I_{pa,\ C}$ (μA) = 0.0675c (μM) + 0.0184 (n=10, R = 0.9988). The detection limits of 5-mC and C were evaluated to be 0.80 and 1.5 μM (S/N = 3), respectively.

Similarly, the concentration dependence studies of 5-mC and C at MWNTs/GCE were shown in Fig. S3B. The voltammetric currents of 5-mC and C increased linearly with the increase of their concentrations in ranges of 1.5-360 and 3.0-480 μM, respectively. The linear regression equations were $I_{pa,\ 5\text{-}mC}$ (μA) = 0.1454c (μM) + 0.0221 (n=10, R = 0.9991) and $I_{pa,\ C}$ (μA) = 0.1035c (μM) + 0.0283 (n=10, R = 0.9982), with low detection limits of 0.45 and 1.0 μM (S/N = 3), respectively.

The concentration dependences of 5-mC and C at MWNTs/Ch/GCE were depicted in Fig. 3 of the Main Article, and the analytical parameters including calibration curves, linear ranges and detection limits were also discussed. In comparison with the detection performance of Ch/GCE and MWNTs/GCE, the designed MWNTs/Ch/GCE exhibited relatively low detection limit, high sensitivity and wide linear range. The

good performance of MWNTs/Ch/GCE was attributed to the uniform and compact dispersion of negatively charged MWNTs on positively charged Ch assembly, resulting in an extraordinarily high effective surface area and very high electron transfer rate.

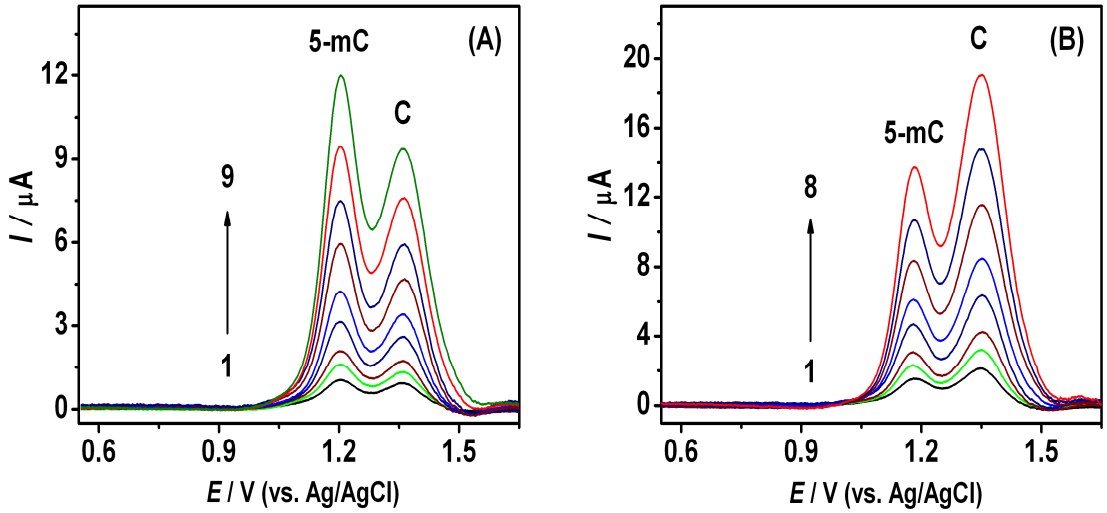

Fig. S3 (A) Background-subtracted DPVs of Ch/GCE in 0.1 M pH 7.0 PBS containing 10, 15, 20, 30, 40, 55, 70, 90, 110 μM 5-mC; and 12, 18, 24, 36, 48, 66, 84, 108, 132 μM C (from 1 to 9, respectively). (B) Background-subtracted DPVs of MWNTs/GCE in 0.1 M pH 7.0 PBS containing 10, 15, 20, 30, 40, 55, 70, 90 μM 5-mC; and 20, 30, 40, 60, 80, 110, 140, 180 μM C (from 1 to 8, respectively).

5. Comparison of different detection methods for simultaneous detection of 5-mC and C

The detection performance of the proposed method was compared with literature results in Table S1. As can be seen, the analytical parameters of the present system were comparable with those of chromatography methods reported in literatures.[8-11]

Moreover, the proposed electrochemical technique was rapid, convenient, low-cost and easy of miniaturization for small volume samples.

Table S1 Comparison of analytical parameters of different detection methods for simultaneous detection of 5-mC and C

Detection method	Analyte	Linear range (μM)	Detection limit (μM)	Reference
HPLC	5-mC	0.1-10	0.082	[8]
	C	0.1-10	0.162	
Capillary chromatography	5-mC	5.0-5000	2.17	[9]
	C	7.5-5000	5.02	
Ion chromatography	5-mC	2.4-1571	0.64	[10]
	C	1.8-856	0.45	
Liquid chromatography	5-mC	2.0-64	0.52	[11]
	C	30-960	0.52	
Electrochemical technique	5-mC	0.60-450	0.15	This work
	C	1.5-600	0.40	

6. The data of the evaluation of C methylation statuses in calf thymus and fish sperm DNA samples

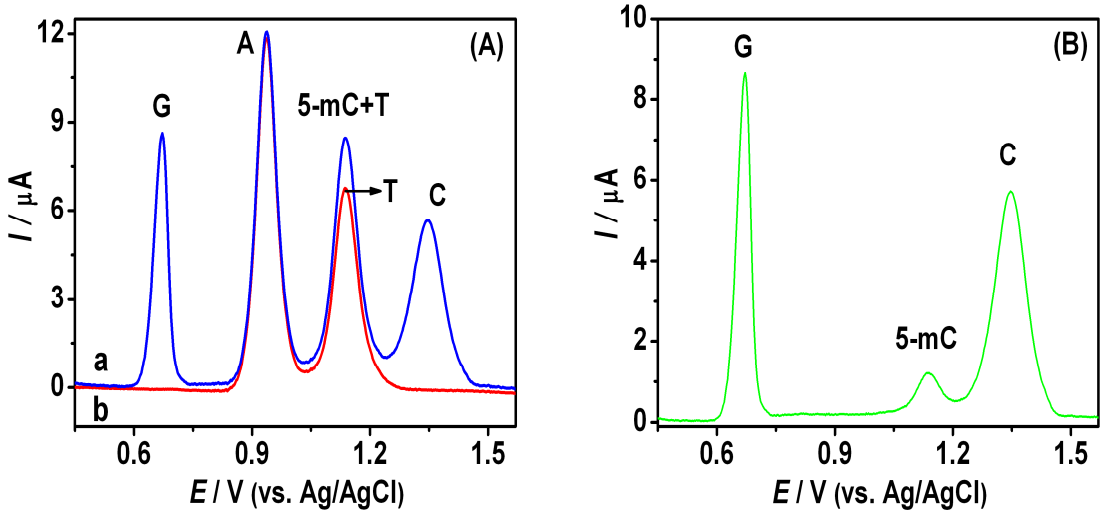

Fig. S4 (A) Background-subtracted DPVs of 0.10 mg mL^{-1} calf thymus DNA (a, blue) and equivalent A and T (b, red) at MWNTs/Ch/GCE in 0.1 M pH 7.0 PBS. (B) Subtraction of the DPV curves of a and b.

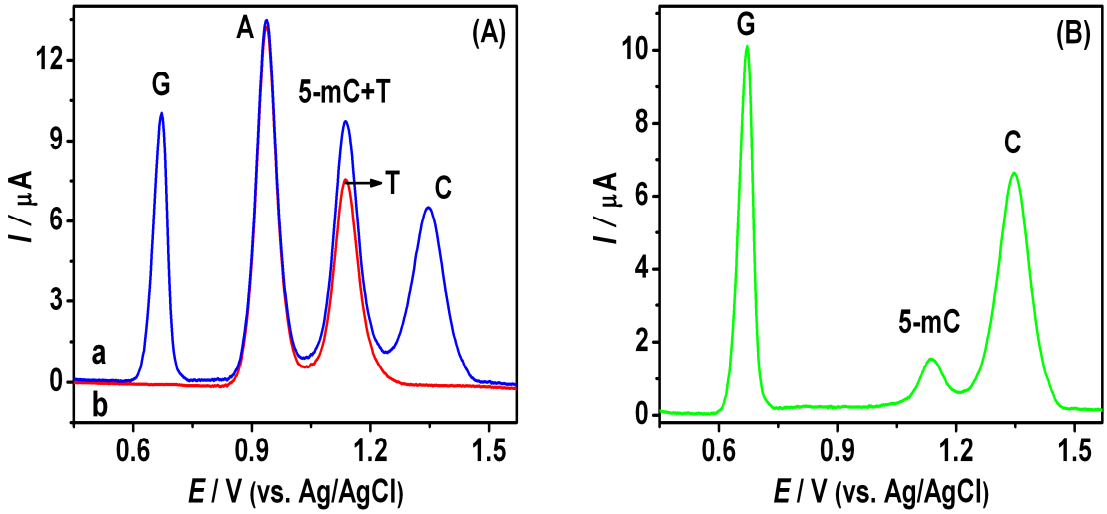

Fig. S5 (A) Background-subtracted DPVs of 0.10 mg mL^{-1} fish sperm DNA (a, blue) and equivalent A and T (b, red) at MWNTs/Ch/GCE in 0.1 M pH 7.0 PBS. (B) Subtraction of the DPV curves of a and b.

7. Detection of C methylation status in ssDNA

With regard to the detection of C methylation status in ssDNA, a fragment of p53 tumor suppressor gene was investigated. As shown in Fig. S6A, for the oxidation of wild-type p53 tumor suppressor gene (curve a), four well-defined oxidation peaks were observed at 0.67, 0.94, 1.18 and 1.33 V, corresponding to the current signals of G, A, T and C, respectively. When the p53 gene was methylated (Fig. S6A, curves b-d), the peak located at 1.18 V increased evidently, while the peak located at 1.33 V decreased obviously, indicating the conversion of C to 5-mC. Therefore, the methylated mutation of C can be clearly distinguished from the difference between the obtained DPVs. Moreover, subtraction of the voltammetric responses of wild type p53 gene from that of methylated p53 genes would result in more clear resolution of 5-mC and C (Fig. S6B). The resulting curves showed that both the increased peak currents of 5-mC and decreased peak currents of C were proportional to the molecular number of methylated C in p53 gene, revealing the promising application of the proposed method in the detection of the methylation status of ssDNA.

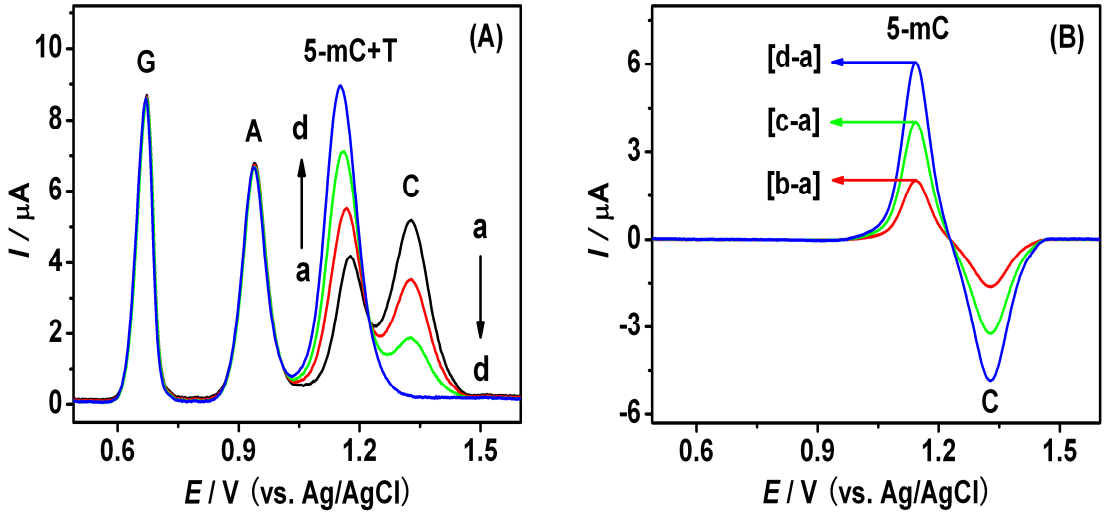

Fig. S6 (A) Background-subtracted DPVs of 10 μM wild type p53 tumor suppressor gene (a: 5'-ATG GCC ATC-3') and methylated p53 tumor suppressor genes (from b to d: 5'-ATG GmCC

ATC-3', 5'-ATG GmCmC ATC-3' and 5'-ATG GmCmC ATmC-3') at MWNTs/Ch/GCE in 0.1 M pH 7.0 PBS. (B) Subtracted curves of methylated and wild type p53 tumor suppressor genes.

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