Electrochemical evaluation of DNA methylation level based on the stoichiometric relationship between purine and pyrimidine bases

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An efficient electrochemical approach for the evaluation of DNA methylation level was proposed according to the oxidation signal of DNA bases at an oxidized polypyrrole (PPyox) directed multiwalled carbon nanotubes (MWNTs) film modified glassy carbon electrode (GCE). The PPyox/MWNTs/GCE exhibited remarkable electrocatalytic activities towards the oxidation of DNA bases due to the advantages of wide potential window, large effective surface area, and excellent antifouling property. As a result, all purine and pyrimidine bases of guanine (G), adenine (A), thymine (T), cytosine (C) and 5-methylcytosine (5-mC) exhibited well identified oxidation peaks at the PPyox/MWNTs/GCE. The direct potential resolution between 5-mC and C was obtained to be 180 mV, which was large enough for their signal recognition and accurate detection in mixture. In particular, the signal interference from T, a great challenge in exploring DNA methylation, was successfully eliminated by an innovative strategy, which was developed based on the stoichiometric relationship between purine and pyrimidine bases in DNA molecular structure. The proposed method was effectively applied to the rapid detection of DNA methylation status in real sample within 45 min with satisfactory results.

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1. Introduction

DNA methylation is an essential epigenetic mechanism that plays crucial roles in many biological events, including embryogenesis, genetic imprinting, cellular differentiation, and regulation of gene expression (Wolffe and Matzke, 1999; Okamoto et al., 2002; Jones and Baylin, 2007; Bareyt and Carell, 2008). In mammals, DNA methylation occurs almost exclusively in CpG dinucleotides, where DNA methyltransferases catalyze the transfer of a methyl group from S-adenosylmethionine to the position 5 of cytosine (Meissner et al., 2008; Badran et al., 2011). 5-Methylcytosine (5-mC) is an important modified pyrimidine base containing heritable information (Kato et al., 2008a; Kurita et al., 2012; Kurita and Niwa, 2012), which is often associated with transcriptional silence of tumor suppressor genes (Ushijima, 2005; Choy et al., 2010; Cao and Zhang, 2012). It has been demonstrated that aberrant methylation of CpG islands in promoter regions of genes is a new generation of cancer biomarker, and can be regarded as a hallmark of various human cancers (Kristensen et al., 2008; Liu et al., 2011; Su et al., 2012; Yin et al., 2013). The detection of DNA methylation can provide a powerful tool for understanding not only the molecular pathology for early cancer diagnosis, but also the mechanisms for epigenetic regulation of genetic information (Dai et al., 2012).

Traditional methods for assessing genomic methylation status are mainly focused on methylation-specific polymerase chain reaction (PCR) and bisulfite conversion analysis (Herman et al., 1996; Brenna et al., 2006; Clark et al., 2006). The classical methods are effective in mapping DNA methylation patterns, however, the false-positive possibility and significant error often occur at PCR amplification, DNA extraction and bisulfite conversion steps, which result in wide variations in precision (Laird, 2010; Wang et al., 2010; Moskalev et al., 2011). In recent years, a variety of new intriguing techniques were developed to overcome the above limitations, such as fluorescence resonance energy transfer (Feng et al., 2008), surface plasmon resonance (Pan et al., 2010), optofluidic ring resonator (Suter et al., 2010), colorimetric assay (Liu et al., 2010), bipartite split-luciferase sensor (Badran et al., 2011), electrochemiluminescence (Kurita et al., 2012; Li Y. et al., 2012), and surface enhanced Raman spectroscopy (Hu and Zhang, 2012). Although these methods exhibit some advantages and merits, laborious operations, expensive instruments, or time-consuming DNA pretreatments are usually involved (Liu et al., 2011; Dai et al., 2012).

Electrochemical techniques have the promising ability to evaluate DNA methylation status in a rapid, convenient and accurate way...
Can an efficient electrochemical biosensor (Barroso et al., 2011a,b; Barroso 2008a). Barroso et al. realized the quantification of purine bases using chemical oxidation of DNA bases is the simplest approach (Kato et al., 2008a, selective labeling and electrochemical indicator, the direct electrochemical oxidation of DNA bases is the simplest approach (Kato et al., 2008a, b; He et al., 2011). Tanaka et al. realized the ep 53 tumors suppressor gene was developed using peptide nucleic acid (PNA) capture probe and [Ru(NH)₃]Cl₂⁺ indicator in our group (Wang et al., 2012). Compared with the detection methods of selective labeling and electrochemical indicator, the direct electrochemical oxidation of DNA bases is the simplest approach (Kato et al., 2008a). Barroso et al. realized the quantification of purine bases using an efficient electrochemical biosensor (Barroso et al., 2011a,b; Barroso et al., 2012). Kato et al. created an excellent strategy for exploring DNA methylation based on the electrochemical oxidation of DNA bases at a nanocarbon film electrode (Kato et al., 2008a; Kato et al., 2011). The direct oxidation method was rapid, convenient and straightforward, while a remarkable challenge was encountered due to the interference from thymine (T), which had almost the same oxidation potential as that of 5-mC, and greatly disturbed the signal recognition of 5-mC (Kato et al., 2008a; Wang et al., 2010; Kato et al., 2011). In order to eliminate the interference of T, we developed a unique subtraction method according to the principle of complementary base pairing (Wang et al., 2010). The overlapped oxidation current of T was subtracted by considering the same contents of adenine (A) and T in DNA sample (Wang et al., 2010). However, the contribution of T was about 20 times larger than that of 5-mC, resulting in significant inaccuracy in the subtraction process.

Herein, an innovative approach for eliminating the interference of T is presented based on the stoichiometric relationship between purine and pyrimidine bases in DNA molecular structure. It has been proved that the complementary base of both 5-mC and C is G (Goto et al., 2010; Kato et al., 2011), suggesting that the molar composition of G is equal to the sum total of C and 5-mC. Although the oxidation signals of 5-mC and T were completely overlapped at the sensing interface, the electrochemical responses of G and C were clearly distinguished. Therefore, the contents of G and C can be detected accurately from their well-separated oxidation peaks. As a result, the content of 5-mC was calculated according to the stoichiometric relationship between G, C and 5-mC. The proposed method was successfully applied to the evaluation of C methylation level in salmon sperm DNA sample without the subtraction of contribution of any DNA base.

2. Experimental

2.1. Chemicals and reagents

G, A, T, C, 5-mC and salmon sperm DNA were purchased from Sigma (USA). Pyrrole was obtained from Aldrich and purified twice by distillation under the protection of high purity nitrogen and then kept in refrigerator before use. Sodium dodecyl sulfate (SDS) was purchased from Shanghai Chemical Co. Ltd. (Shanghai, China). Multiwalled carbon nanotubes (MWNTs; purity > 95%) were obtained 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. 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 Na₂HPO₄ and KH₂PO₄ and adjusted by H₃PO₄ or NaOH (Beijing Chemical Reagent Company, Beijing, 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.

2.2. Apparatus and measurements

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 system was used for all electrochemical experiments, which consisted of a working electrode, a platinum wire counter electrode and an Ag/AgCl reference electrode. The electrochemical solutions were thoroughly deoxygenated by nitrogen for 15 min before sampling and nitrogen 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). X-ray photoelectron spectroscopy (XPS) was performed on an ESCALAB spectrometer (VG Co., UK) equipped with the Mg Kα X-ray radiation as the source for excitation at a pressure of less than 10⁻⁹ Torr in the chamber.

2.3. Electrode preparation and modification

Prior to use, glassy carbon electrode (GCE) was sequentially polished with 1.0, 0.3 and 0.05 μm alumina powder and ultrasonically washed in doubly distilled water and ethanol for 10 min, respectively. The cleaned GCE was dried with nitrogen steam for the next modification. The MWNTs were dispersed in 0.05 M SDS aqueous solution and sonicated for 1 h. Then, pyrrole monomer was dissolved in this emulsion solution under ultrasonic stirring for 30 min at room temperature. The PPy/MWNTs composites modified GCE was prepared by immersing the GCE in 0.05 M SDS aqueous solution and sonicated for 1 h. The obtained PPy/MWNTs/GCE was gently rinsed with distilled water, and then transferred into 0.1 M pH 7.0 PBS for 2 h aging to remove co-deposited species. Afterward, the electrochemical oxidation of PPy was performed at +1.8 V for 4 min. The resulting electrode was denoted as PPyox/MWNTs/GCE. For comparison, a PPyox/GCE was also prepared under the similar conditions.

2.4. DNA sample preparation

DNA sample was hydrolyzed as follows for the evaluation of C methylation level. In brief, 10.0 mg salmon sperm DNA was hydrolyzed in 600 μL of 88% (w/w) formic acid at 170 °C for 30 min in a sealed glass tube according to the procedure reported in literature (Ivandini et al., 2007; Wang et al., 2010). The hydrolysate was then adjusted to neutrality with NaOH. Afterward, the obtained sample solution was diluted with doubly distilled water, and added into 0.1 M pH 7.0 PBS for detection.

3. Results and discussion

3.1. Characterizations of modified electrode

The interfacial morphology of the constructed PPyox/MWNTs/GCE was characterized by FE-SEM. As shown in Fig. 1A, many one-dimensional nanostructures that stood almost vertically on the
electrode surface could be observed. They were oriented towards outside of the deposited film. Such nanostructures were of uniform size with average diameter of about 200 nm. Compared with the diameter of MWNTs, the size of the obtained nanostructures was much larger. Therefore, the one-dimensional nanostructures were MWNTs enwrapped within polypyrrole, not MWNTs themselves. These oriented nanostructures could extremely enhance the active surface area of GCE for promoting interfacial electron transfer.

XPS was employed to investigate the elemental distribution and chemical composition of the surface-modified electrode. As shown in Fig. 1B, the N1s signal was clearly observed at 399.8 eV, indicating that pyrrole monomer (C₅H₅N) was successfully electropolymerized onto the surface of GCE (Chen et al., 2012). Moreover, the S2p peak at 168.9 eV confirmed the presence of SDS molecules in the PPyox/MWNTs composite (Zhu and Tour, 2010). The formation mechanism of PPyox/MWNTs nanocomposite film can be explained as follows: in the mixed system of SDS and MWNTs, SDS could be strongly adsorbed onto the surface of MWNTs through van der Waals’ force, which was favorable for the stable dispersion of MWNTs in solution (Islam et al., 2003; Kang and Taton, 2003; Hilmer et al., 2012). After the addition of pyrrole monomers into the SDS-encapsulated MWNTs solution, they could enter the interiors of micelle-encapsulated MWNTs and locate at the interfaces between MWNTs and surfactants (Zhang et al., 2004; Li et al., 2007). In the presence of electric field, the negatively charged SDS-MWNTs nanocomposite would orient towards the surface of electrode through electrostatic interaction (Kamat et al., 2004). As a result, the SDS-encapsulated MWNTs nanostructures were uniformly assembled onto GCE. When the electrochemical cell was performed with a high potential, the pyrrole monomers were electropolymerized at the surfaces of both MWNTs and GCE (Zhang et al., 2004; Li et al., 2007), and MWNTs were synchronously mingled in the nanocomposite.

3.2. Electrocatalytic oxidation of DNA bases

The electrochemical oxidation of all DNA bases including G, A, 5-mC, T and C at bare GCE was shown in Fig. 2A. As can be seen, the DNA bases exhibited very weak and irreversible oxidation peaks due to the slow electron transfer kinetics of bare GCE. However, well-defined catalytic oxidation peaks with obvious reduction of overpotentials were observed at PPyox/GCE (Fig. 2B). The peak currents were about three times higher than those obtained at the bare GCE, suggesting that PPyox film could efficiently facilitate the interfacial electron transfer between GCE and biomolecules in solution. Moreover, significant enhancements of peak currents as well as further decreased overpotentials were observed at PPyox/MWNTs/GCE (Fig. 2C), indicating that the catalytic capability of PPyox/MWNTs/GCE was higher than that of PPyox/GCE. Such an increased catalytic performance was attributed to the fast electron transfer kinetics and excellent antifouling property of PPyox/MWNTs/GCE, which may result from the large effective surface area of PPyox/MWNTs nanocomposite film. The catalytic mechanism of the PPyox/MWNTs/GCE was attributed to the fact that the oriented nanostructured composite film could remarkably increase the density of edge-plane-like active sites of GCE for effective promotion of the electron transfer involved in the oxidation of DNA bases (Wang et al., 2011).

The direct potential resolution between 5-mC and C was investigated at bare GCE, PPyox/GCE and PPyox/MWNTs/GCE in pH 7.0 PBS. As shown in Fig. 2D, the oxidation currents of 5-mC and C were completely overlapped at about 1.28 V at bare GCE (curve a). Therefore, it was difficult to acquire the individual current signal of 5-mC and C from their broad and indistinguishable oxidation peak. However, in the case of PPyox/MWNTs/GCE (curve c), the peak potentials of 5-mC and C were separated efficiently and discriminated clearly at 1.13 and 1.31 V, respectively. The peak separation between 5-mC and C was 180 mV, indicating the reliability of simultaneous detection of 5-mC and C with satisfactory potential resolution. Although PPyox/GCE can also be used for the recognition of the oxidation signals of 5-mC and C (curve b), the PPyox/MWNTs/GCE exhibited better potential resolution and higher current sensitivity for DNA methylation analysis.

3.3. Simultaneous detection of G and C

The simultaneous detection of G and C was performed under coexistence of A and 5-mC in pH 7.0 PBS. As shown in Fig. 3, with the increase in the concentration of G and C, the oxidation potentials of A and 5-mC were always located at 0.92 and 1.13 V, respectively, suggesting that the electrochemical oxidation processes of purine and pyrimidine bases were relatively independent at the PPyox/MWNTs/GCE. The oxidation peak currents of G and C were proportional to their concentrations in wide ranges of 0.3–500 and 1.0–750 μM, respectively. The linear regression equations for differential pulse voltammetry (DPV) detection of G and C were obtained to be $I_{\text{pa,G}} (\mu A) = 0.29c (\mu M) + 0.11 (n=10, R=0.9994)$ and $I_{\text{pa,C}} (\mu A) = 0.16c (\mu M) + 0.40 (n=10, R=0.9991)$, with detection limits of 0.1 and 0.3 μM ($S/N=3$), respectively. The relative standard deviations (R.S.D.) for ten successive determinations of 40 μM G and C were 2.9% and 3.6%, respectively, demonstrating an excellent detection reproducibility.

![Fig. 1.](image-url) (A) FE-SEM image of the PPyox/MWNTs nanocomposite film modified GCE, and (B) XPS spectrum of the PPyox/MWNTs/GCE.
3.4. Evaluation of C methylation level in salmon sperm DNA sample

As for the direct electrochemical investigation of DNA methylation, a great challenge in recognition and detection of 5-mC is the interference from T, which has almost the same oxidation potential as that of 5-mC (Kato et al., 2008a; Wang et al., 2010; Kato et al., 2011). In order to overcome this difficulty, the proposed approach was applied to the assessment of DNA methylation level in salmon sperm DNA sample. Fig. 4 shows the background-subtracted DPV of DNA sample in 0.1 M PBS. The oxidation peaks of G, A and C were clearly identified at 0.65, 0.92 and 1.31 V, respectively, while the voltammetric responses of 5-mC and T were fully overlapped. As a result, the contents of G, A and C can be detected directly from their current signals, while the detection of 5-mC is a considerable challenge. Fortunately, the complementary base of both 5-mC and C is G (Goto et al., 2010; Kato et al., 2011), indicating that the molar composition of G is equal to the sum total of 5-mC and C in DNA molecular structure. Therefore, the content of 5-mC can be calculated according to the stoichiometric relationship between G, C and 5-mC. Based on the peak currents of DPV curve, the contents of G and C were obtained to be 428.6 and 399.5 µmol g⁻¹, respectively. Consequently, the content of 5-mC was calculated to be 29.1 µmol g⁻¹, and the methylation level of C was obtained to be 6.79%, which was in agreement with reference value (Fraga et al., 2002; Ivandini et al., 2007). Applying Student t-test to compare the detection results of ten-time parallel measurements, there was no significant statistical difference at 95% confidence level, suggesting satisfactory reliability and precision of this method. Finally, standard addition method was used for testing recovery. As shown in Table 1, the recoveries for detections of G and C in salmon sperm DNA sample were obtained to be 102.2% and 98.3%, and the R.S.D. for ten-time successive determinations were calculated to be 3.1% and 4.0%, respectively, demonstrating satisfactory accuracy and precision of this method.
effectively prevented the desorption of MWNTs from GCE surface. This was attributed to the protection effect of PPyox film, which allowed the current signal to decrease about 5% after two weeks, indicating a stable response was observed in the first 7 days (signal decline by 3–7% in the next 7 days). The continuous detection of 40 μmol g⁻¹ G and 39.9 μmol g⁻¹ C for 45 min, which is remarkably faster than literature reports. The analysis approaches of Hu and Zhang (2012), Li et al. (2012), and Ouyang et al. (2012) required 4, 7, and 48 h, respectively. The present work implemented the analysis of DNA methylation in 45 min without the need for bisulfite conversion, enzyme digestion or labeling procedures.

### 4. Conclusions

In summary, the present work implements the analysis of DNA methylation in a convenient, rapid and accurate way. Taking advantage of the extraordinary catalytic property of PPyox/MWNTs/GCE, the peak potential of 5-mC was clearly distinguished from that of C. Moreover, the signal interference of T, which markedly blocked the electrochemical exploration of DNA methylation, was successfully eliminated by an innovative strategy based on the stoichiometric relationship between purine and pyrimidine bases in DNA molecular structure. The proposed method realized the rapid detection of DNA methylation information within 45 min without the need for bisulfite conversion, enzyme digestion or labeling procedures.

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### References


### Table 1

<table>
<thead>
<tr>
<th>DNA bases</th>
<th>Detected (μmol g⁻¹)</th>
<th>Added (μmol g⁻¹)</th>
<th>Found (μmol g⁻¹)</th>
<th>Recovery (%)</th>
<th>R.S.D. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>428.6</td>
<td>400.0</td>
<td>837.4</td>
<td>102.2</td>
<td>3.1</td>
</tr>
<tr>
<td>C</td>
<td>399.5</td>
<td>400.0</td>
<td>792.7</td>
<td>98.3</td>
<td>4.0</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Detection method</th>
<th>Analysis time</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bipartite split-luciferase sensor</td>
<td>&gt;12 h</td>
<td>Badran et al. (2011)</td>
</tr>
<tr>
<td>Rolling circle amplification</td>
<td>&gt;21 h</td>
<td>Cao and Zhang (2012)</td>
</tr>
<tr>
<td>Hybridization indicator</td>
<td>&gt;11 h</td>
<td>Dai et al. (2012)</td>
</tr>
<tr>
<td>Fluorescence resonance energy transfer</td>
<td>&gt;15 h</td>
<td>Feng et al. (2008)</td>
</tr>
<tr>
<td>Surface enhanced Raman spectroscopy</td>
<td>&gt;17 h</td>
<td>Hu and Zhang (2012)</td>
</tr>
<tr>
<td>Electrochemiluminescence</td>
<td>&gt;4 h</td>
<td>Li Y. et al. (2012)</td>
</tr>
<tr>
<td>HhaI restriction enzyme digestion</td>
<td>&gt;14 h</td>
<td>Nonura and Barbas (2007)</td>
</tr>
<tr>
<td>Label-free fluorescent assay</td>
<td>&gt;7 h</td>
<td>Ouyang et al. (2012)</td>
</tr>
<tr>
<td>mCGc-sequence enabled reassembly</td>
<td>&gt;48 h</td>
<td>Stains et al. (2006)</td>
</tr>
<tr>
<td>Bisulfite conversion analysis</td>
<td>&gt;19 h</td>
<td>Topkaya et al. (2012)</td>
</tr>
<tr>
<td>Reversed phase HPLC</td>
<td>&gt;16 h</td>
<td>Torres et al. (2011)</td>
</tr>
<tr>
<td>Electrochemical oxidation</td>
<td>45 min</td>
<td>This work</td>
</tr>
</tbody>
</table>

### 3.5. Analysis speed of the proposed approach

The analysis time of the proposed approach was compared with a variety of literature methods. As shown in Table 2, most of the current detection methods require more than 10 h to accomplish the analysis task, except electrochemiluminescence and label-free fluorescent assay, which need 4 and 7 h, respectively (Li Y. et al., 2012; Ouyang et al., 2012). However, the present work realized the rapid detection of DNA methylation information within 45 min without the need for bisulfite conversion, enzyme digestion or labeling procedures, which greatly reduced the analysis time. With regard to the analysis of clinical samples, routine sample preparation procedures are required prior to electrochemical detection, such as sample collection, DNA extraction, and PCR amplification. Although the involvement of PCR amplification in sample preparation process would evidently prolong the total analysis time, the analysis speed of the proposed system is still faster than many literature reports.

### 3.6. Stability of the PPyox/MWNTs/GCE for DNA detection

The stability of the present system was investigated by continuous detection of 40 μM G and C using a same PPyox/MWNTs/GCE. It was found that no apparent decrease in current response was observed in the first 7 days (signal decline < 2%). The current signal decreased about 5% after two weeks, indicating that the stability of the biosensing system was good enough for continual operation. The excellent stability of PPyox/MWNTs/GCE was attributed to the protection effect of PPyox film, which effectively prevented the desorption of MWNTs from GCE surface.