Ultrasensitive electrochemical immunoassay for DNA methyltransferase activity and inhibitor screening based on methyl binding domain protein of MeCP2 and enzymatic signal amplification

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\textbf{A R T I C L E  I N F O}

Article history:
Received 1 February 2013
Received in revised form 20 April 2013
Accepted 22 April 2013
Available online 3 May 2013

Keywords:
DNA methylation
Methyl binding domain protein
Anti-histidine tag antibody
Horseradish peroxidase labeled immunoglobulin G
Electrochemical detection

\textbf{A B S T R A C T}

In this work, we fabricated a novel electrochemical immunosensor for detection of DNA methylation, analysis of DNA MTase activity and screening of MTase inhibitor. The immunosensor was on the basis of methyl binding domain protein of MeCP2 as DNA CpG methylation recognition unit, anti-His tag antibody as “immuno-bridge” and horseradish peroxidase labeled immunoglobulin G functionalized gold nanoparticles (AuNPs–IgG–HRP) as signal amplification unit. In the presence of M. SsI MTase, the symmetrical sequence of 5′-CCGG-3′ was methylated and then recognized by MeCP2 protein. By the immunoreactions, anti-His tag antibody and AuNPs–IgG–HRP was captured on the electrode surface successively. Under the catalysis effect of HRP towards hydroquinone oxidized by \textit{H}_2\textit{O}_2, the electrochemical reduction signal of benzoquinone was used to analyze M. SsI MTase activity. The electrochemical reduction signal demonstrated a wide linear relationship with M. SsI concentration ranging from 0.05 unit/mL to 90 unit/mL, achieving a detection limit of 0.017 unit/mL (S/N=3). The most important advantages of this method were its high sensitivity and good selectivity, which enabled the detection of even one-base mismatched sequence. In addition, we also verified that the developed method could be applied for screening the inhibitors of DNA MTase and for developing new anticancer drugs.

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1. Introduction

DNA methylation is a kind of epigenetics event, and plays crucial roles in regulation of gene expression, genomic stability, and cell development (Chen et al., 1998; Reik et al., 2001). Recently, it has been reported that aberrant DNA methylation is always associated with various diseases, especially cancers (Esteller and Herman, 2002; Momparler and Bovenzi, 2000). However, DNA methylation is catalyzed by DNA methyltransferase (MTase), which can transfer a methyl group from methyl donor of 5-adenosylmethylione to 5′ position of cytosine to form 5-methylcytosine in the CpG dinucleotide (Momparler and Bovenzi, 2000). The aberrant DNA methylation has been found to be related to the aberrant DNA MTase activity, and it has also been reported that the activity of DNA MTase increased significantly in progression of colon cancer (Issa et al., 1993) and lung cancer (Belinsky et al., 1996). Therefore, aberrant DNA methylation and DNA MTase activity can be regarded as a biomarker in the early diagnosis of cancer. It is crucial to develop sensitive and selective methods for quantitative detection of DNA methylation and analysis of DNA MTase activity.

Up to now, a few methods have been developed for the detection of DNA methylation and assay of DNA MTase activity, such as radioisotope labeled substrate (Bergerat et al., 1991), polymerase chain reaction (PCR) (Eads et al., 1999), western blotting (Boyce et al., 1992), bisulfite treatment (Wan et al., 2007), gas chromatography/mass spectrometry (Tang et al., 2012), colorimetric approaches (Li et al., 2010) and fluorescence method (Li et al., 2007). Though these techniques have their own advantages, most of them are time-consuming, requiring expensive and sophisticated large-scale instrument, demanding tedious treatment process. Especially, some of them require poisonous reagent or radioactive isotope labeled substrate, which are harmful to human health.

For circumventing those limitations mentioned above, electrochemical methods have attracted some attentions due to the advantages of inexpensive instrument, easy operation, simple
treatment process, low reagent-consuming and high sensitivity (Wu et al., 2012). For instances, Liu et al. (2011b) developed an electrochemical approach for the assay of MTase activity and the detection of DNA methylation based on the voltammetric response of ferrocene acetic acid, which was conjugated to the terminus of the target DNA, after the DNA hybrid was methylated by M. Sssl methyltransferase and further cleaved by HpaII endonucleases. For further increasing the detection sensitivity, Li et al. (2012) improved the detection strategy, and employed another signal amplification unit of Thiounine/graphene oxide to enhance the assay sensitivity. Using this new amplification unit, the detection limit for DNA MTase decreased from 0.1 unit/mL to 0.05 unit/mL. In our previous work, we also fabricated an electrochemical biosensor for the activity assay of DNA MTase and direct detection of DNA methylation based on the methylation site recognition unit of anti-5-methylcytosine antibody and signal amplification unit of horseradish peroxidase labeled IgG (IgG–HRP) (Wang et al., 2012). However, the detection limit of this method towards DNA MTase is 0.1 unit/mL, which needs further improvement.

In order to develop a convenient electrochemical method for DNA methylation analysis with high sensitivity, methyl binding domain (MBD) proteins caught our attentions because they can bind specifically to symmetrically methylated CpG motifs in double stranded DNA (Buschdorf and Strätling, 2004; Rauch et al., 2006). The MBD-based assay does not require bisulfite treatment and PCR amplification, and this assay can target all genomic regions (Hiraoka et al., 2012). The family of MBD protein for binding CpG islands contains four proteins, MBD1, MBD2, MBD4, MeCP2 (Yu et al., 2010). For instance, Suter et al. performed label-free DNA methylation analysis by opto-fluidic ring resonators using the MBD2 protein as the capture molecule (Suter et al., 2010). Recently, our group developed a convenient electrochemical method for activity assay of DNA MTase and detection of DNA methylation based on MBD1 protein. After the protein was stained by coomassie brilliant blue G250 (CBB-G250), the electrochemical signal of CBB-G250 was used to monitor the methylation event (Yin et al., 2013b).

We reported here an ultrasensitive electrochemical immunoassay method for DNA methylation and DNA MTase activity using MeCP2 protein as methyl recognition unit and IgG–HRP functionalized gold nanoparticles (AuNPs–IgG–HRP) as signal amplification unit (Scheme 1). The C-terminal of MeCP2 protein was labeled with 6 × His tag, which could be immunologically recognized by anti-His tag antibody. After the hybridized DNA was methylated by M. Sssl MTase, MeCP2 protein was immobilized on the electrode through the specific interaction towards the methylated sites. Then, through the first immunoreaction between His tag at the C-terminal of MeCP2 and anti-His tag antibody, the antibody was modified on the electrode. Subsequently, as goat anti-mouse IgG can specifically bind with murine monoclonal antibody, the signal amplification unit of AuNPs–IgG–HRP was captured by the second immunoreaction between anti-His tag antibody (extracted from mouse) and IgG in AuNPs–IgG–HRP. The AuNPs possess high specific surface area, which allows AuNPs to load a large amount of IgG–HRP molecules. HRP molecules on AuNP–IgG–HRP can catalytically oxidize hydroquinone in the presence of H2O2. And the amplified electrochemical reduction signal of the oxidized product, p-benzoquinone, is related to the activity of DNA MTase. According to our knowledge, this is the first report for using MeCP2 protein, anti-His tag antibody and goat anti-mouse IgG–HRP functionalized AuNPs simultaneously as biosensors in one immunoassay for detection of DNA methylation and assay of DNA MTase activity. Furthermore, our developed method has the potential ability for screening inhibitor towards DNA MTase.

2. Experimental

2.1. Reagents and instruments

Chloroauric acid (HAuCl4), trisodium citrate, tris(hydroxymethyl)aminomethane (Tris), triglycerol and phosphate hydrochloride (TCEP, 98%), epicatechin and 5-fluorouracil were purchased from Aladdin (Shanghai, China). 3-Mercaptopropionic acid was obtained from Alfa Aesar (Heysham, Lancashire, UK). Catechol-O-methyltransferase (COMT) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, USA). 0.5% (w/v) BSA was prepared with 10 mM phosphate buffered solution (PBS). M. Sssl MTase and restriction endonuclease HpaII were supplied by New England BioLabs (Ipswich, MA) and Fermentas (Maryland, USA), respectively. Anti-His tag antibody (extracted from mouse) was obtained from MBL (Nagoya, Japan). HRP modified goat anti-mouse IgG (IgG–HRP) and the synthetic oligonucleotides were purchased from Shanghai Sangon Biotechnology Co. (Shanghai, China) and used without further purification. 1 mg/mL IgG–HRP was prepared with 10 mM PBS (pH 7.4). The base sequences of oligonucleotides are as follows: thiol-capped probe DNA (DNA S1), 5′-SH-(CH2)6-TAG TGT GTC ACC TAG TTG ACC TT-3′; target DNA (DNA S2), 5′-ATC CGG AAG GTC AAC TAG GTG ACA TCA CAC TA-3′; one-base mismatched DNA (DNA S3), 5′-ATC CTG AAG GTC AAC TAG GTG ACA TCA CAC TA-3′. The synthesized oligonucleotides were diluted in TE buffer (Containing 10 mM Tris–HCl and 1 mM EDTA, pH 8.0) to desired stock concentrations and stored at −20 °C according to the manufacturer’s instructions. AuNPs and AuNPs–IgG–HRP were synthesized according to previous reports (see Supporting information) (Jia et al., 2009; Liu and Lu, 2006; Zhou et al., 2011).

The buffer solutions employed in this study are as follows. M. Sssl stock buffer: 10 mM Tris–HCl, 50 mM NaCl, 1 mM dithiothreitol and 10 mM MgCl2 (pH 7.4). HpaII stock buffer: 10 mM Tris–HCl, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.2 mg/ml BSA and 50% glycerol (pH 7.4). Probe immobilization buffer: 10 mM Tris–HCl, 1.0 mM EDTA, 1.0 M NaCl, and 1.0 mM TCEP (pH 7.4). DNA hybridization buffer: 10 mM Tris–HCl, 1.0 mM EDTA, and 1.0 M NaCl (pH 7.4). Electrochemistry determination buffer: 0.1 M phosphate buffered saline (PBS, pH 7.4). All reagents were analytically pure grade. All of the solution and redistilled deionized water used were autoclaved.

Electrochemical experiments are performed at CHI600C electrochemical workstation (USA) with a conventional three-electrode cell. A bare GCE or modified GCE is used as working electrode. A saturated calomel electrode (SCE) and a platinum wire are used as the reference electrode and auxiliary electrode, respectively. The parameters for electrochemical detection were located in Supporting information. Transmission electron microscopy (TEM) image was taken with JEOL-1200EX instrument (Japan).

2.2. MeCP2 protein expression and purification

Recombinant plasmid pHET30b-Mecp2-77–167 was received from prof. Adrian Bird’s lab (Edinburgh, UK). The pET30b construct encoding the 6X His-tagged methyl-CpG-binding domain (MBD) of human MeCP2, residues 77–167, was overexpressed in Escherichia coli BL21 (DE3). The expression and purification was done as previously described (Free et al., 2001; Hendrich and Bird, 1998; Nan et al., 1993; Valinluck et al., 2004).

2.3. Methylation and cleavage

After probe DNA S1 immobilization and hybridization with DNA S2 (see Supporting information), the methylation and cleavage were performed. Firstly, a series of M. Sssl MTase solutions
were prepared from 0.05 unit/mL to 150 unit/mL. To protect the activity of M. SssI MTase, all the solutions were prepared under 4°C in clean bench and stored under −20°C. The methylation of S1/S2 hybrid was performed at 37°C for 2 h in 10 mM Tris–HCl buffer (pH 7.4) containing 160 μM SAM, 50 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol (DTT), and various concentration of M. Sssl (from 0 unit/mL to 150 unit/mL). Then, the treated electrode was rinsed with 10 mM Tris–HCl (pH 7.4) for three times.

Restriction endonuclease HpaII digestion was performed at 37°C in 10 mM Tris–HCl buffer (pH 7.4) containing 50 unit/mL HpaII, 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 200 μg/mL bovine serum albumin (BSA) and 50% glycerol for 2 h. After digestion, the electrode was thoroughly washed with 10 mM Tris–HCl (pH 7.4) for three times.

2.4. MeCP2 protein, anti-His tag antibody and AuNPs–IgG–HRP immobilization

After methylation and digestion, the electrode was incubated with 10 μL of 0.1 M PBS containing 0.5% (w/v) BSA for 30 min to prevent any possible nonspecific binding. Following that, the electrode was rinsed three times with 10 mM Tris–HCl (pH 7.4) for 15 min. Afterwards, 10 μL MeCP2 protein (0.6 mg/mL) was dropped on the electrode surface and incubated for 1 h in humid conditions at 37°C. The obtained electrode (noted as MeCP2/dsDNA/AuNPs/GCE) was rinsed with 10 mM Tris–HCl (pH 7.4) for 15 min. Subsequently, 10 μL anti-His tag antibody (12 μg/mL) was dropped on the electrode surface and incubated for 60 min at 37°C under humid conditions. The electrode (noted as Antibody/MeCP2/dsDNA/AuNPs/GCE) was then rinsed three times with 10 mM Tris–HCl (pH 7.4) and dried at room temperature.

To perform the immunoreaction between AuNPs–IgG–HRP and anti-His tag antibody, the fabricated anti-His tag antibody modified electrode was incubated with 10 μL AuNPs–IgG–HRP at 37°C under humid conditions for 50 min. After rinsed with 10 mM Tris–HCl (pH 7.4) for three times, the obtained electrode (noted as AuNPs–IgG–HRP/Antibody/MeCP2/dsDNA/AuNPs/GCE) was stored at 4°C in a refrigerator before use.

For comparison, IgG–HRP/Antibody/MeCP2/dsDNA/AuNPs/GCE was fabricated using the same process.

2.5. Inhibition of M. Sssl MTase activity

The inhibition effect could be quantitatively analyzed using the reduction peak current change of enzymatic product, p-benzoquinone. For investigating the inhibition effects of 5-fluourouracil and epicatechin on the activity of M. Sssl MTaseity, the methylation of S1/S2 hybrid was performed at 37°C in 10 mM Tris–HCl buffer (pH 7.4) containing 160 μM SAM, 50 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol (DTT), 90 unit/mL M. Sssl, and various concentration of the inhibitors. For epicatechin, COMT was also added into the inhibitory system. The inhibition efficiency (%) is estimated as follows:

\[
\text{Inhibition (\%)} = \frac{I_2-I_3}{I_2} \times 100\%
\]

where \(I_1\) is the peak current of H\(_2\)O\(_2\)–hydroquinone system obtained after the S1/S2 hybrids successively treated with M. Sssl, HpaII and anti-His tag antibody, \(I_2\) is the peak current of H\(_2\)O\(_2\)–hydroquinone system obtained after the S1/S2 hybrids successively treated with M. Sssl, HpaII, anti-His tag antibody and AuNPs–IgG–HRP, and \(I_3\) is the inhibited peak current of H\(_2\)O\(_2\)–hydroquinone system.

3. Results and discussion

3.1. Characterization of the biosensor

The electrode fabrication process was characterized by cyclic voltammetry (Fig. 1A) and electrochemical impedance spectroscopy (Fig. 1B) using Fe(CN)\(_6\)\(^{3-/-1}\) as redox probe (see Supporting information).
3.2. Electrochemical activity of the fabricated biosensor

For proving that the developed method could be used to analysis DNA MTase activity, we investigate the electrochemical behavior of different electrodes in 0.1 M PBS (pH 7.4) containing 0.1 mM H2O2 and 0.1 mM hydroquinone. As shown in Fig. 2, after dsDNA/AuNPs/GCE was methylated by M. Sssl MTase, a well defined reduction peak was obtained at −0.080 V with the peak current of 0.415 μA (curve a). This reduction peak should be attributed to the reduction of p-benzoquinone, which was produced from the oxidation of hydroquinone by H2O2 in the detection system. However, the reduction peak current of p-benzoquinone decreased (0.271 μA, curve b) after the cytosine (marked with underline) in the sequence of symmetric tetranucleotide 5′-CGCG-3′ was methylated by M. Sssl MTase and further specifically conjugated with MeCP2 protein. This decreased current could be ascribed to the immobilization of non-conductivity MeCP2 protein, which decreased the electron transfer rate and blocked the reduction of p-benzoquinone. Subsequently, the reduction peak current further decreased to 0.142 μA (curve c) after the specific immunoreaction between the His tag at the C-end of MeCP2 and anti-His tag antibody. As expected, the reduction peak current of p-benzoquinone increased significantly (3.61 μA, curve e) when AuNPs–IgG–HRP was captured on the electrode surface through the secondary immunoreaction between anti-His tag antibody and IgG–HRP modified on AuNPs. It was well known that HRP, a kind of catalase, could catalyze the oxidation reaction between H2O2 and hydroquinone (Bonel et al., 2011; Park et al., 2012). Therefore, in the presence of HRP, the yield of p-benzoquinone could be improved, which could result in a higher reduction peak current. Based on the change of this reduction peak current, the activity of M. Sssl MTase could be investigated because the immobilization amount of AuNPs–IgG–HRP was relative to the methylation level. To further prove that AuNPs–IgG–HRP conjugates could amplify the electrochemical detection signal, the electrochemical behavior of IgG–HRP/Antibody/MeCP2/dsDNA/AuNPs/GCE was also investigated under the same conditions. As seen in Fig. 2, the reduction peak current of p-benzoquinone was 1.296 μA at IgG–HRP/Antibody/MeCP2/dsDNA/AuNPs/GCE (curve d), which was much lower than that obtained at AuNPs–IgG–HRP/Antibody/MeCP2/dsDNA/AuNPs/GCE (curve e). In fact, the electrochemical signal increased by 1.79 times as compared with that only IgG–HRP using as signal amplifier. From this comparison, one can conclude that the detection sensitivity of the developed method could be effectively improved when AuNPs–IgG–HRP was used as signal amplification unit.

3.3. M. Sssl MTase detection

The content of DNA MTase has been found to be increased in human breast and colon cancer cells (el-Deiry et al., 1991). In leukemic cells, two kinds of DNA MTase, DNMT1 and DNMT3A, are recruited by the oncogenic transcription factor promyelocytic leukemia-retinoic acid receptor α oncoprotein to hypermethylate gene promoters (Godley et al., 2011). Therefore, the overexpression of DNA MTase might cause cancer. In addition, it has been reported that rate of DNA methylation is directly related to the amount of DNA MTase (Liu et al., 2009). Therefore, it is important to assay the activity of DNA MTase and detect its content. In this work, according to the relationship between the reduction current and M. Sssl MTase concentration, the quantitative detection of M. Sssl MTase could be achieved. To achieve this, the hybridized S1/S2 DNA was methylated by various concentrations of M. Sssl MTase (0–150 unit/mL) for 100 min and digested by HpaII for 2 h successively. Then the methylated DNA was further treated successively with MeCP2, anti-His tag antibody and AuNPs–IgG–HRP. Finally, the differential pulse voltammograms were recorded in 0.1 M PBS containing 0.1 mM H2O2 and 0.1 mM hydroquinone. As shown in Fig. 3, under optimized conditions (The detection parameter optimization was shown in Supporting information), the electrochemical reduction peak increased linearly with M. Sssl MTase concentration from 0.05 unit/mL to 90 unit/mL and then leveled off at high concentration. Because almost all of the hybridized DNA S1/S2 have been methylated by high concentration of M. Sssl MTase, no extra AuNPs–IgG–HRP could be captured on the electrode surface to amplify the electrochemical signal. The linear regression equation can be expressed as I_pc = 0.21c + 0.7 (μA, unit/mL, R = 0.9993, 0.05–2 unit/mL) and I_pc = 0.027c + 1.22 (μA, unit/mL, R = 0.9987, 2–90 unit/mL). The detection range of the developed method (0.05–90 unit/mL) is much more wider than some of previous reports based on electrochemical and colorimetric approaches, such as 1.0–10 unit/mL (Liu et al., 2009), 0.1–50 unit/mL (Su et al., 2012), 0.8–40 unit/mL (Li et al., 2007), 0.1–20 unit/mL.
biosensor was investigated by detecting 40 unit/mL of M. SsSI MTAse using five independent fabricated biosensors. The RSD was about 5.97%, revealing an acceptable repeatability of the developed method. In addition, the interference measurement of this method was also evaluated by two models. On the one hand, Dam MTAse was selected as an interference enzyme, which could recognize and methylate adenine in the sequence of symmetric tetranucleotide 5′-G-A-T-C-3′. As shown in Fig 5-S3-A, the result demonstrated that this sensor exhibited good selectivity for M. SsSI MTAse. On the other hand, we selected one-base mismatched synthetic DNA (DNA S4) to evaluate the selectivity of the fabricated biosensor. After hybridization, the methylation site could not be recognized because the base of “G” was replaced by “T”. As seen in Fig 5-S3-B, the electrochemical signal of the S1/S2 (curve a) hybrids was much higher than that at S1/S3 (curve b), indicating that the developed method can distinguish even one-base mismatched DNA, and as a result, it can be used for highly selective determination of DNA methylation at the site of 5′-C-C-G-G-3′.

3.5. Assay of the inhibition of M. SsSI MTAse activity

DNA cytosine methylation catalyzed by M. SsSI MTAse is an important epigenetic method of gene expression regulation and development. Moreover, changes in methylation level could lead to carcinogenesis (Baylin et al., 2001; Cowher and Jeltsch, 2004; Laird and Jaenisch, 1996). Therefore, inhibition of M. SsSI MTAse activity could be a good strategy for safe and efficient cancer therapy. It has been reported that many nucleoside homologs and tea polyphenols have potential in inhibition DNA MTAse activity and some of them have been applied in cancer clinical treatment (Martinet et al., 2012). Therefore, for further demonstrating the potential applicability of our developed method in screening inhibitor of DNA MTAse, 5-fluorouracil and epicatechin were selected as two model inhibitors of M. SsSI MTAse. As shown in Fig 4, the activity of the M. SsSI MTAse decreased with the increasing concentration of 5-fluorouracil and epicatechin, showing significant dose-dependent inhibition effect. The activity of M. SsSI MTAse could be decreased ca. 66.81% and 59.19% by 5-fluorouracil and epicatechin, respectively. These results indicate that the above two compounds are effective inhibitor on M. SsSI MTAse. The IC50 value, the inhibitor concentration required to reduce the enzyme activity by 50%, is acquired from the plots of the relative activity of M. SsSI MTAse versus the inhibitors concentration. They are ca. 94.11 and 193.02 μM for 5-fluorouracil and epicatechin, respectively. These results also demonstrate that the developed method is suitable to investigate the inhibitory effects of anticancer drugs on DNA MTAse and screening DNA MTAse inhibitors.

4. Conclusion

In this work, we developed a novel enhanced electrochemical immunoassay method for detection of DNA methylation, analysis of M. SsSI MTAse and screening of inhibitors based on MeCP2 protein, anti-His tag antibody and AuNPs–IgG–HRP. The MeCP2 protein could improve the detection selectivity for DNA methylation due to specifically recognize the methylated CpG region in hybridized DNA sequence, and the AuNPs–IgG–HRP conjugate could effectively increase the detection sensitivity for DNA MTAse activity due to its catalyzed signal amplification. Compared with previous reports, our developed method achieved a lower the detection limit of 0.017 unit/mL. The results of the influences of drugs on the activity of M. SsSI MTAse indicated that this method could be applied to screen suitable anticancer drugs for inhibiting the activity of DNA MTAse.
Fig. 4. Differential pulse voltammograms of the fabricated biosensor after inhibition by 5-fluorouracil (A) and epicatechin (C). (B) and (D) were the inhibition effect of 5-fluorouracil (B) and epicatechin (D) on M. SssI MTase activity.

Acknowledgments

All the authors thank Prof. A. Bird for kindly providing the pET30b-Mecp2-77–167 plasmid. This work was supported by the National Natural Science Foundation of China (Nos. 21075078, 21105056) and the Natural Science Foundation of Shandong Province, China (Nos. ZR2010BM005, ZR2011BQ001).

Appendix A. Supporting information

Supplementary information associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.04.040.

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