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Enzyme-based electrochemical biosensor for sensitive detection of DNA demethylation and the activity of DNA demethylase

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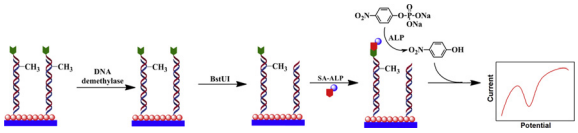
HIGHLIGHTS

- DNA demethylase activity was assayed by electrochemical method.
- Double enzyme system of DNA demethylase and BstUI endonuclease was applied.
- The detection is based on alkaline phosphatase catalytic signal amplification.
- This method showed high detection selectivity and good sensitivity.

GRAPHICAL ABSTRACT



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ABSTRACT

A novel electrochemical method is developed for detection of DNA demethylation and assay of DNA demethylase activity. This method is constructed by hybridizing the probe with biotin tagged hemi-methylated complementary DNA and further capturing streptavidin tagged alkaline phosphatase (SA-ALP) to catalyze the hydrolysis reaction of *p*-nitrophenyl phosphate. The hydrolysate of *p*-nitrophenol (PNP) is then used as electrochemical probe for detecting DNA demethylation and assaying the activity of DNA demethylase. Demethylation of target DNA initiates a degradation reaction of the double-stranded DNA (dsDNA) by restriction endonuclease of BstUI. It makes the failed immobilization of ALP, resulting in a decreased electrochemical oxidation signal of PNP. Through the change of this electrochemical signal, the DNA demethylation is identified and the activity of DNA demethylase is analyzed with low detection limit of 1.3 ng mL^{-1}. This method shows the advantages of simple operation, cheap and miniaturized instrument, high selectivity. Thus, it provides a useful platform for detecting DNA demethylation, analyzing demethylase activity and screening inhibited drug.

1. Introduction

DNA methylation is a kind of importantly epigenetic mark, which plays important role in mammalian development and genomic imprinting [1]. Aberrant DNA methylation is conducive to cancer occurrence and development by causing genomic instability and inappropriate silencing of tumor suppressor genes [2]. Although DNA methylation has been viewed as a stable epigenetic mark, researches in the past decade have revealed that this modification is not as static as once thought because DNA demethylation pattern has been observed in specific contexts [3,4]. DNA demethylation is a reverse of DNA methylation that methyl group is removed from the C-5 position of cytosine in the dinucleotide sequence CpG by DNA demethylase [5]. It has been reported that DNA demethylation plays crucial role in the epigenetic regulation of genes, and dysfunction of DNA demethylase has been involved in many important diseases such as cancers, imprinting-related diseases, and psychiatric disorders [6,7]. Therefore, it is important to develop a simple,

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sensitive and selective method for analyzing the activity of DNA demethylase.

The available methods for the activity assay of DNA methyltransferase and demethylase were mainly depended on the detection of methylation state of DNA sequence, such as bisulfite treatment followed by PCR [8], fluorescence method [9], colorimetric approach [10], high-performance liquid chromatography [11], surface enhanced Raman scattering [12], and electrochemiluminescence [13]. However, most of these methods suffer from expensive and large-volume instruments, multiple separation and wash steps, which might limit their applicability for routine assays. Recently, electrochemical methods have been attracted many attentions on detection of methylation state of DNA sequence and assay of DNA methyltransferase activity [14–21]. Moreover, the electrochemical techniques have the advantages of instrument miniaturization, operation simplification, low consumption of reagent and high sensitivity, which make electrochemical technique as a suitable platform for DNA demethylase activity assay.

In this work, we developed a sensitive electrochemical method for analysis of DNA demethylation and demethylase activity based on DNA hybridization and endonuclease-assistant alkaline phosphatase (ALP) catalysis signal amplification. As presented in Scheme 1, the complementary DNA was designed to have a hemi-methylated site of 5′-C^{m}GCG-3′ with a biotin at its 5′-terminal. After hybridization, the formed double-strand DNA could be served as the substrate of DNA demethylase. It is well known that the 5′-CGCG-3′ site can also be recognized and cleaved by restriction endonuclease of BstUI, and the digestion can be blocked by hemi-methylation of this site. However, after demethylation, the barrier was eliminated and the hybridized DNA could be cleaved by BstUI, yielding to two double-stranded DNA fragments and the fragment containing labeled biotin was released into the buffer from the electrode surface, which resulted in the failed immobilization of streptavidin tagged alkaline phosphatase (SA-ALP). As a result, the hydrolysis level of *p*-nitrophenyl phosphate (PNPP) decreased, which made the reduction of the electrochemically active hydrolysis product of *p*-nitrophenol (PNP). Base on the change of the electrochemical oxidation signal of PNP, the demethylation event was detected and DNA demethylase activity was assayed.

2. Experimental

2.1. Materials

DNA demethylase (methyl-CpG-binding domain protein 2, MBD2) and MBD4 were purchased from Epigentek Group Inc. (Farmingdale, NY, USA). Restriction endonuclease, BstUI, was obtained from New England Biolabs (USA). PNPP, chloroauric acid ($HAuCl_4 \cdot 3H_2O$), tris(hydroxymethyl) aminomethane (tris) and tris (2-carboxyethyl) phosphine hydrochloride (TCEP) were supplied by Aladdin (Shanghai, China). Mercaptopropionic acid (MPA) was purchased from Alfa Aesar (USA). SA-ALP and all DNA sequences were provided by Sangon Biotech Co., Ltd. (Shanghai, China). The oligonucleotides sequences were shown as follows. DNA probe S1, 5′-SH-$(CH_2)_6$-TTC ACC TGT GTT TTT TCA **CGC GTG**-3′, hemi-methylated complementary DNA (DNA S2), 5′-biotin-CA**C^{m} GCG** TGA AAA AAC ACA GGT GAA-3′. The CGCG sequence highlighted in bold is the recognition site for BstUI, and hemi-methylation of this site can block the cleavage reaction.

The buffer solutions employed in this work are as follows. 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 7.0), electrochemistry determination buffer, 10 mM Tris–HCl (pH 9.8) containing 1 mM $Mg(NO_3)_2$, washing buffer: 10 mM Tris–HCl buffer (pH 7.4).

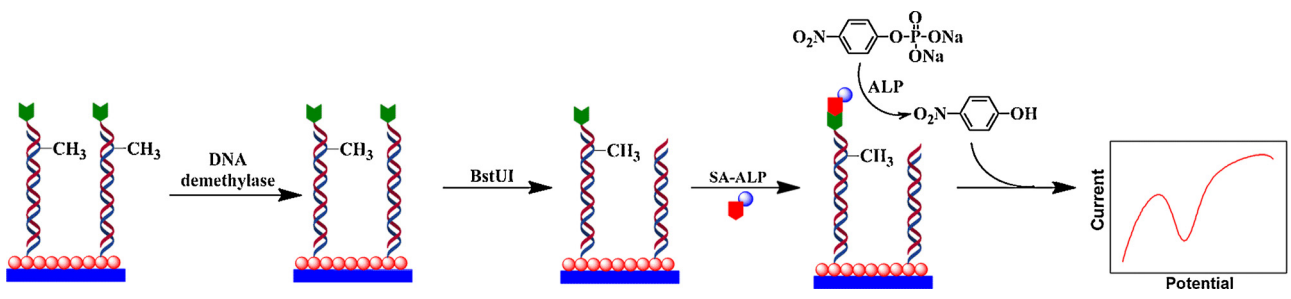
2.2. Apparatus

Electrochemical measurements are performed on CHI660C electrochemical workstation (CHI Instruments, USA). A three-electrode system is used in the experiment with bismuth film modified glassy carbon electrode (Bi/GCE, $d = 3$ mm) as the working electrode, a saturated calomel electrode (SCE) and a Pt wire electrode as reference electrode and counter electrode, respectively.

2.3. Biosensor preparation

Prior to modification, a gold electrode (2 mm in diameter) was polished to a mirror-like surface using 0.3 and 0.05 μm alumina slurry. After ultrasonicating in ethanol and double distilled deionized water successively, the electrode was rinsed thoroughly with double distilled deionized water and dried under nitrogen flow.

The pretreated gold electrode was then immersed into 3 mM $HAuCl_4$ solution containing 0.1 M KNO_3, and the gold nanopartilces (AuNPs) were electrochemically deposited on the gold electrode surface using single-potential mode at −0.2 V for 250 s. The obtained AuNPs modified gold electrode (noted as AuNPs/Au) was washed with double distilled deionized water and dried under nitrogen flow. Then, 5 μL of probe immobilization buffer solution containing 5.0×10^{-6} M probe DNA S1 was dripped on AuNPs/Au surface and incubated for 12 h under humid conditions, and then rinsed with washing buffer. The obtained electrode was noted as S1/AuNPs/Au. Afterwards, S1/AuNPs/Au was further incubated with 5 μL of 3.3×10^{-5} M MPA to obtain a well-aligned probe monolayer and remove the unspecific adsorbed probe. After rinsed three times with washing buffer, the modified electrode was further incubated with 5 μL of hybridization buffer containing 5.0×10^{-6} M complementary DNA S2 for 2 h at 37 °C in a humidified chamber to complete the hybridization event. After that, the electrode was rinsed three times with washing buffer to remove the un-hybridized target DNA S2. The obtained electrode was noted as S2-S1/AuNPs/Au. Finally, the electrode was incubated



Scheme 1. Schematic illustration of electrochemical assay for the DNA demethylase activity.

with 5 μL of 10 mM PBS (pH 7.4) containing 0.01 mg/mL SA-ALP for 1 h in a humidified chamber at ambient conditions [22]. After rinsed with washing buffer for three times, the obtained electrode (noted as ALP/S2-S1/AuNPs/Au) was dried under nitrogen flow. The electrode was stored in 10 mM Tris–HCl (pH 7.4) at 4 °C in a refrigerator when not in use.

2.4. Demethylation

The demethylation of S2–S1 hybrid was performed at 37 °C for 70 min by incubating S2-S1/AuNPs/Au with 5 μL of 50 mM Tris–HCl buffer (pH 8.0) containing various concentration of demethylase (from 0 to 100 ng mL^{-1}).

2.5. Cleavage of BstUI endonuclease

BstUI endonuclease cleavage was performed according to previous report with some modifications [12]. In brief, the electrode was incubated with 10 mM Tris–HCl buffer (pH 8.5) containing 10 mM $MgCl_2$, 100 mM KCl, 0.1 mg mL^{-1} BSA and 20 unit mL^{-1} BstUI at 37 °C for 1 h. After cleavage, the electrode was thoroughly rinsed with washing buffer and dried with nitrogen.

2.6. Electrochemical detection

Electrochemical impedance spectroscopy (EIS) was carried out in 5 mM $[Fe(CN)_6]^{3-/4-}$ (1:1) solution containing 0.1 M KCl as the supporting electrolyte. The frequency was ranged from 10^{-1} to 10^5 Hz. Differential pulse voltammetry (DPV) was performed in 10 mL of 0.1 M Tris–HCl (pH 9.8) containing 3.5 mM PNPP and 1 mM $Mg(NO_3)_2$. 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. All DPV curves were recorded by Bi/GCE after the detection buffer was incubated with different electrode prepared in Section 2.2. For preparation Bi/GCE, the well polished GCE was immersed into a 0.1 M acetate buffer solution (pH 4.5) containing 40 ug L^{-1} bismuth nitrate. The deposition of the bismuth film proceeded for 200 s while holding the electrode at −1.2 V and stirring the solution. The Bi/GCE was then washed carefully with double distilled water.

3. Results and discussion

3.1. Biosensor characterization

The biosensor preparation process was monitored by EIS. As shown in Fig. 1, the bare gold electrode presented a well semi-circle structure in high frequency region (curve a), indicating an interface electron transfer resistance (R_{et}). Then the R_{et} decreased significantly and only a straight line was observed in the whole frequency region after AuNPs were deposited on gold electrode surface (curve b) due to the good conductivity and large specific surface area of AuNPs. However, the R_{et} increased successively with the immobilization of probe DNA S1 (curve c) and hybridization with complementary DNA S2 (curve d), which could be explained as that the negatively charged phosphoric acid backbone of DNA repelled the negatively charged $Fe(CN)_6^{3-/4-}$ and inhibit the electron transfer. After capturing the huge volume protein of SA-ALP on the electrode surface, the R_{et} further increased (curve e). It could be explained as the steric hindrance effect of enzyme on the diffusion of redox probe. The EIS was also used to characterize the endonuclease digestion. After the S2–S1 hybrid was demethylated by DNA demethylase and digested by BstUI endonuclease, the R_{et} decreased (curve f) compared with that obtained at S2-S1/AuNPs/Au (curve d). After demethylation, the S2–S1 hybrid could be cleaved by BstUI, which decreased the electron transfer resistance caused by negatively charged phosphoric acid backbone of DNA towards redox probe. It also proved the successful demethylation and endonuclease digestion.

3.2. Detection of DNA demethylation

For proving the feasibility on DNA demethylation assay using our developed strategy, the detection buffer (0.1 M Tris–HCl, pH 9.8) containing 3.5 mM PNPP and 1 mM $Mg(NO_3)_2$ was first treated with different electrodes, then the differential pulse voltammograms were recorded using Bi/GCE as working electrode with potential scan from 0.5 to 1.1 V (Fig. 2). Curve a presented the DPV response of the detection solution treated with S2-S1/AuNPs/Au. It was clear that no oxidation peak was observed in the scan region, indicating that PNPP was non-electrochemical active molecule and no factor of hydrolysis was introduced into the detection system. However, a well-defined oxidation peak was obtained after SA-ALP was immobilized on

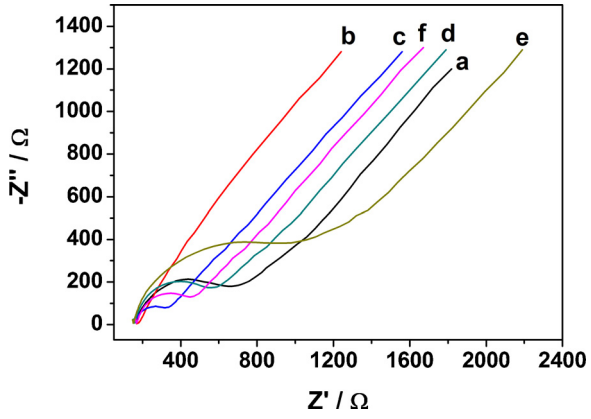


Fig. 1. Nyquist plots of the bare Au electrode with different assembly processes. (a) Au electrode. (b) AuNPs/Au. (c) S1/AuNPs/Au. (d) S2-S1/AuNPs/Au. (e) ALP/S2-S1/AuNPs/Au. (f) S2-S1/AuNPs/Au after the S2–S1 hybrids were treated with DNA demethylase and BstUI.

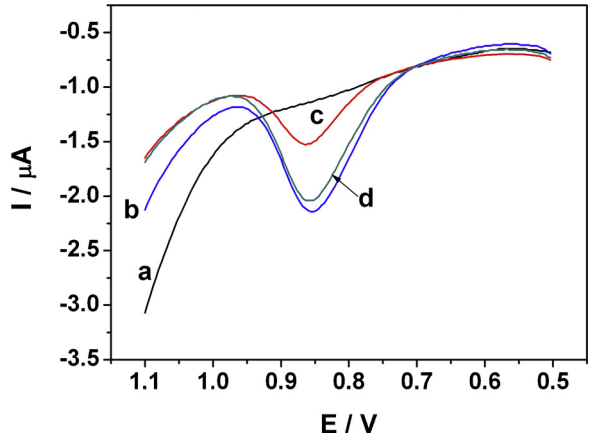


Fig. 2. Differential pulse voltammograms of Bi/GCE in 10 mL Tris–HCl (pH 9.8) containing 3.5 mM PNPP and 1 mM $Mg(NO_3)_2$, where the detection solution was treated with different electrodes. (a) S2-S1/AuNPs/Au, (b) ALP/S2-S1/AuNPs/Au, (c) the hybrid of S2–S1 on AuNPs/Au surface was first incubated with DNA demethylase and then with BstUI endonuclease and SA-ALP successively, and (d) the hybrid of S2–S1 was incubated with BstUI and SA-ALP, successively.

the S2-S1/AuNPs/Au surface (curve b) through the specific recognition reaction between biotin (at the 5'-end of DNA S2) and streptavidin. This oxidation peak could be attributed to the biocatalytic reaction of ALP, which triggered the hydrolysis of PNPP to produce PNP, a kind of electrochemical active molecule. When the hemi-methylated S2–S1 hybrids were first incubated with DNA demethylase, then with BstUI endonuclease and SA-ALP successively, the oxidation peak current for PNP decreased greatly (curve c). After the S2-S1/AuNPs/Au was incubate with DNA demethylase, the methyl in DNA S2 was eliminated, which resulted in the cleavage of the hybrids of S2–S1 by BstUI endonuclease and released the biotin from the 5′-end of DNA S2. Consequently, only a few SA-ALP can be captured on the electrode surface to catalyze the hydrolysis reaction of PNPP, which led to the decrease of the oxidation peak current of PNP. For further confirming the demethylation effect of DNA demethylase on the capture of SA-ALP and PNPP hydrolysis, we performed control experiment by successively incubating the hemi-methylated S2-S1/AuNPs/Au only with BstUI endonuclease and SA-ALP (curve d). As anticipated, the oxidation peak current was enhanced and closed to that obtained at ALP/S2-S1/AuNPs/Au. This result demonstrated that the cleavage of S2–S1 hybrids by BstUI endonuclease was blocked due to the hemi-methylated site and SA-ALP was successfully captured on the electrode surface to catalyze the hydrolysis of PNPP. This finding also implied that the developed assay strategy could be used to monitor DNA demethylation event and analyze DNA demethylase activity.

3.3. DNA demethylase activity analysis

The ability of the developed biosensing strategy for quantitative activity assay of DNA demethylase was investigated. For achieving this aim, the S2-S1/AuNPs/Au was first incubated with different concentration of demethylase for 70 min, and then incubated successively with BstUI endonuclease and SA-ALP, respectively. Then, the prepared electrode was immersed into 10 mL 0.1 M Tris–HCl (pH 9.8) containing 3.5 mM PNPP and 1 mM $Mg(NO_3)_2$ for 60 min with stir. Finally, the differential pulse voltammogram was recorded by Bi/GCE. As depicted in Fig. 3A, the oxidation peak current of PNP decreased with increasing demethylase concentration, suggesting a decreased yield of PNP at higher demethylase concentration. With increasing demethylase concentration, more and more hemi-methylated 5′-CGCG-3′ site at complementary DNA S2 was demethylated and the S2–S1 hybrids were cleaved into two fragments by BstUI endonuclease, which made the immobilization amount of SA-ALP decreased because more biotin at the 5'-end of DNA S2 was released from electrode surface. As a result, the concentration of PNP decreased gradually with increasing demethylase concentration. As shown in the insert of Fig. 3A, the oxidation peak current of PNP decreased linearly with the logarithm value of demethylase concentration in the range from 4 to 100 ng mL^{-1} with a correlation coefficient of 0.996. The detection limit was 1.3 ng mL^{-1} (S/N = 3), which was higher than that obtained by enzymatic control of plasmonic coupling as a surface enhanced Raman scattering nanosensor (0.1 ng mL^{-1}) [12].

The effect of incubation time on demethylase activity was also investigated by first incubating the S2-S1/AuNPs/Au with 10 ng mL^{-1} demethylase for different time, and then treating with 20 unit mL^{-1} BstUI and 0.01 mg mL^{-1} SA-ALP successively. Afterwards, the prepared electrode was immersed into 10 mL 0.1 M Tris–HCl (pH 9.8) containing 3.5 mM PNPP and 1 mM $Mg(NO_3)_2$ for 60 min with stir. Finally, DPV response was recorded using Bi/GCE as working electrode. As seen in Fig. 3B, the oxidation peak current decreased quickly and linearly with extending demethylation time from 0 to 70 min, then the rate for the current decrease slowed. With prolonging incubation time, the demethylation degree increased gradually, resulting in an increased cleavage level for 5′-CGCG-3′ site and a decreased SA-ALP immobilization.

The developed analytical method showed very desirable reproducibility with the relative standard deviations (RSDs) of 5.69%, 4.87% and 2.21% for five repetitive assays of 5, 10 and 50 ng mL^{-1} of demethylase. These results suggested that the fabricated biosensor held potential for quantitative activity assay of DNA demethylase with desirable sensitivity and reproducibility.

The selectivity of this assay for DNA demethylase activity was also investigated using some proteins possibly coexisting with DNA demethylase, human serum and cancer cell protein extracts as interferents. For achieving this work, the S2-S1/AuNPs/Au was first incubated with DNA demethylase (10 ng mL^{-1}) and different interferents for 70 min at 37 °C respectively, then it was further treated as descried process in Section 2.2. For comparison, the oxidation peak current of PNP in detection buffer treating with ALP/S2-S1/AuNPs/Au was used as control. The current changes were compared. As presented in Fig. 4, the coexisting protein, such as MBD1 protein (50 ng mL^{-1}), MBD4 (50 ng mL^{-1}), MeCP2 protein (50 ng mL^{-1}), uracil-DNA glycosylase (UDG, 50 unit mL^{-1}), RNase H (50 unit mL^{-1}), histone H2A (50 ng mL^{-1}) and HeLa cell protein extracts showed little interference with this biosensor. For MBD1, MBD4 and MeCP2 proteins, they are methyl-CpG binding domain protein without demethylation activity, so they could not remove the methyl from DNA S2, and the cleavage of BstUI endonuclease is still blocked, presenting no interference on

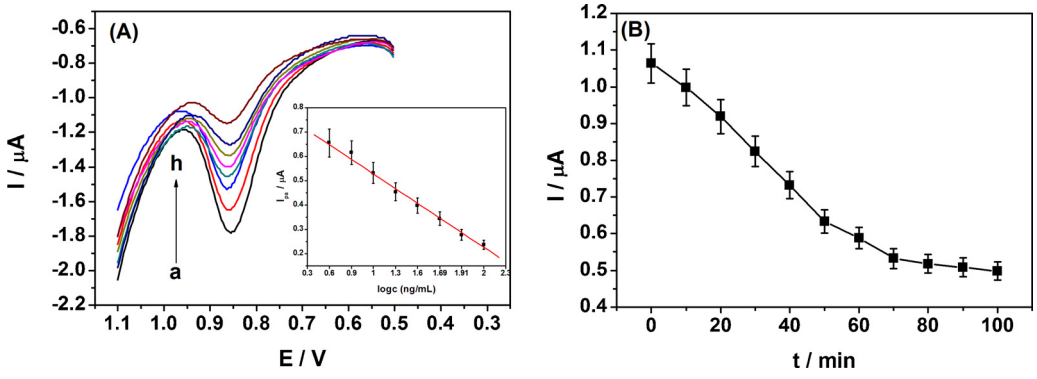


Fig. 3. (A) Differential pulse voltammograms of the biosensor incubated with different concentration of DNA demethylase. a–h: 4, 8, 10, 20, 40, 50, 80, 100 ng mL^{-1}. Inset: the plot of the oxidation peak current of PNP vs. the logarithm value of DNA demethylase concentration. (B) The relationship between the oxidation peak current of PNP and the incubation time of DNA demethylase.

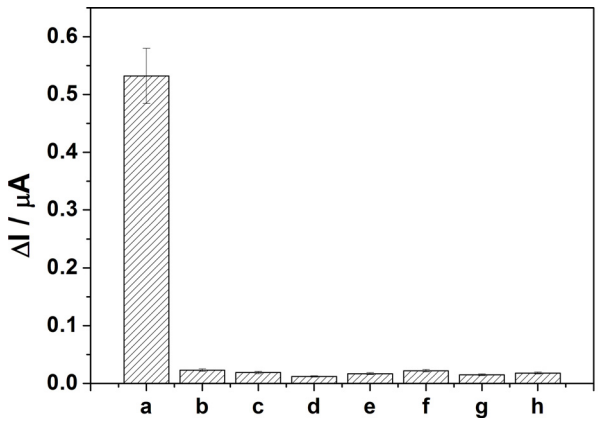


Fig. 4. The histogram for the change of the oxidation peak current of PNP obtained by the different targets possibly coexistence with DNA demethylase. (a) DNA demethylase, (b) MBD1 protein, (c) MBD4 protein, (d) MeCP2 protein, (e) UDG, (f) RNase H, (g) Histone H2A, and (h) Hela cell protein extracts.

DNA demethylase activity assay. Moreover, MBD1, MBD4 and MeCP2 are symmetrical cytosine methylation recognition protein, which can not bind with the hemi-methylated DNA S2 sequence. These results indicated that the proposed strategy could be used for quantifying DNA demethylase in complicated system.

4. Conclusions

In summary, we have developed an electrochemical method for DNA demethylation detection and DNA methylase activity assay based on enzymatic signal amplification. Demethylation of complementary DNA triggered the cleavage of dsDNA by restriction endonuclease of BstUI, which made the labeled biotin at the 5′-end of target DNA released from electrode surface and resulted in a decreased current signal. This biosensor showed a low detection limit of 1.3 ng mL^{-1} for DNA demethylase and high detection selectivity with the aid of restriction endonuclease of BstUI. This assay might provide a useful electrochemical platform for assay of relative-enzyme activity and screening of relative drug for inhibition or promotion DNA demethylase activity.

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