

Mini review

Recent Progress in Electrochemical Biosensors for Detection of DNA Methylation and Methyltransferase Activity

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DNA methylation is one of the most studied epigenetic modifications of DNA. The methylation at C-5 position of cytosine has been proved to be related to a wide range of biological phenomena. Therefore, it is very important to develop a simple, sensitive, accurate and reliable method for DNA methylation analysis. In this paper, the electrochemical biosensors for assays of DNA methylation and methyltransferase activity are reviewed.

Keywords: DNA methylation; electrochemical biosensors; methyl binding antibodies; methyl binding proteins; endonuclease

1. INTRODUCTION

DNA methylation is one of the most studied epigenetic modifications. It refers to the transfer of a methyl group from S-adenosylmethionine to a specific base in the DNA strand by DNA methyltransferase (Mtase). DNA methylation can occur at N-6 position of adenine, N-4 position of cytosine, N-7 position of guanine or C-5 position of cytosine [1]. In mammals, DNA methylation occurs mainly on cytosine (C) of the CpG dinucleotide sequence, producing 5-methylcytosine (5-mC). As the fifth base of DNA, 5-mC is a genetic marker, which plays an important role in regulating gene expression, embryonic development, cancer and other major diseases [2]. In some regions of the mammalian genome, CpG sequences have high densities, which can reach more than five times the mean, and become the enrichment areas of guanine and cytosine, forming so-called CpG islands. There

are about 40 thousand CpG islands in the mammalian genome, and only the cytosine of CpG islands can be methylated. About 50% of human genes contain CpG islands, which are normally non-methylated or hypomethylated, while CpG sites outside the CpG islands are usually methylated. This form of methylation is stable in the process of cell division. The demethylation of CpG sequences outside the CpG islands increases when tumorigenesis occurs, while the CpG sequences in the CpG islands show a highly methylated state [3]. Therefore, determination of abnormal DNA methylation level, methylation site and methyltransferase activity are valuable for the early diagnosis of some tumors, the treatment of epigenetic diseases, and the development and screening of new drugs related to tumors [4, 5]. Electrochemical analysis has many advantages, such as short detection time, simple operation, low cost, and easy miniaturization [6-9]. Many investigations have been reported for the analysis of DNA methylation and methyltransferase activity [10-13]. Usually, DNA methylation can facilitate the binding of antibodies or proteins with methyl, and allow for or protect from DNA digestion by the corresponding restriction endonucleases. In this paper, the electrochemical methods for analysis of DNA methylation and methyltransferase activity based on the methyl binding antibodies or proteins and the restriction endonuclease cleavage are reviewed.

2. METHYL BINDING ANTIBODY OR PROTEINS

Electrochemical-affinity biosensors based on the interaction between methyl and antibodies or proteins offer great selectivity and sensitivity for assays of DNA methylation [14]. For example, in 2012, Yin and Ai reported a sensitive and selective electrochemical biosensor for DNA methyltransferase activity analysis through antibody-specific binding and restriction endonuclease HpaII [15]. As shown in Figure 1, the double DNA helix structure was assembled on the surface of gold nanoparticles (AuNPs)-modified electrode. When the electrode was incubated with DNA methyltransferase (M. SssI), the double DNA helix will be methylated in the CpG site. Consequently, anti-5-methylcytosine antibody was specifically conjugated on the CpG methylation site, which allowed for the further attachment of horseradish peroxidase (HRP)-labeled goat antimouse IgG (HRP-IgG). The resulting HRP molecules on electrode surface can catalyze the electrochemical oxidation of hydroquinone to benzoquinone. However, without DNA methylation, the double DNA helix will be digested by HpaII enzyme, resulting in the release of the CpG methylation site sequence. As a result, the anti-5-methylcytosine antibody and HRP-IgG can not be anchored onto the electrode surface. Therefore, once the M. SssI activity was inhibited by the inhibitors such as 5-aza-2'-deoxycytidine, procaine, epicatechin, and caffeic acid, a lower electrochemical signal would be observed. Moreover, Liao and Geng demonstrated that the strategy the methylation site can be recognized the antibody modified with Ag NPs decorated carbon nanocubes (Ag NPs/CNCs) [16]. The electrochemical signal was measured by the stripping of Ag NPs. The method obtained a relatively lower detection limit of 0.03 U/mL for M.SssI.

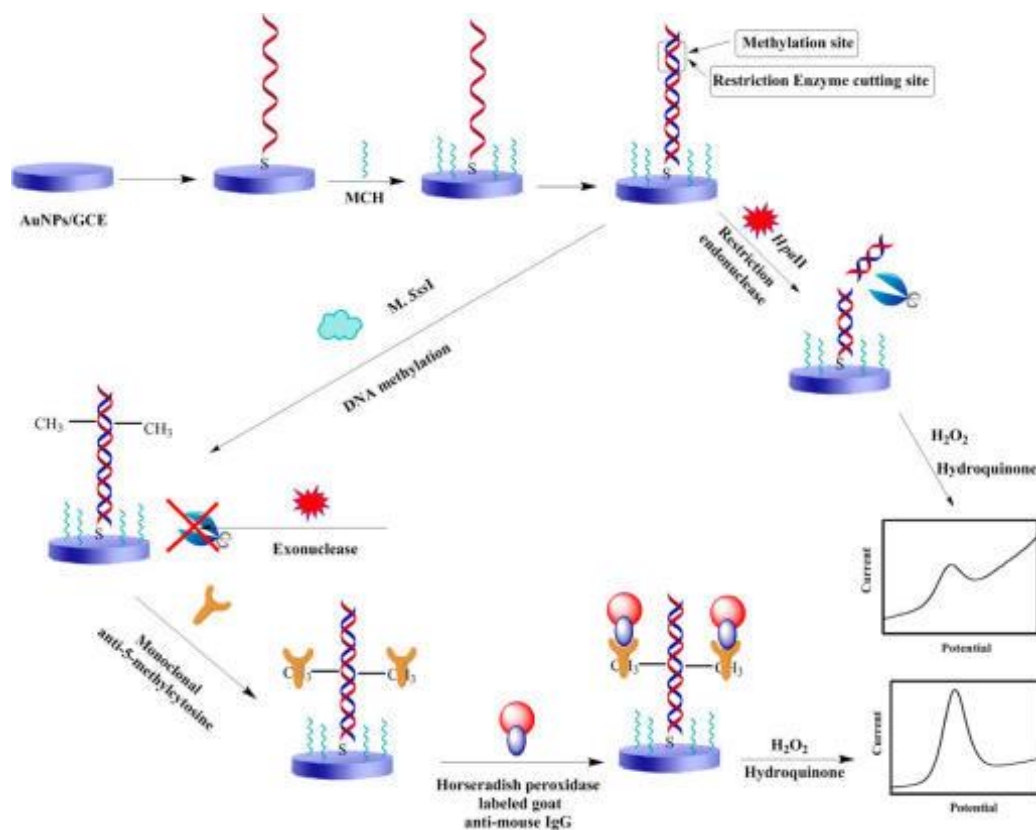


Figure 1. Schematic representation of the developed method for detection of DNA methylation and assay of *M. SssI* MTase activity. Reprinted with permission from reference [15]. Copyright 2012 American Chemical Society.

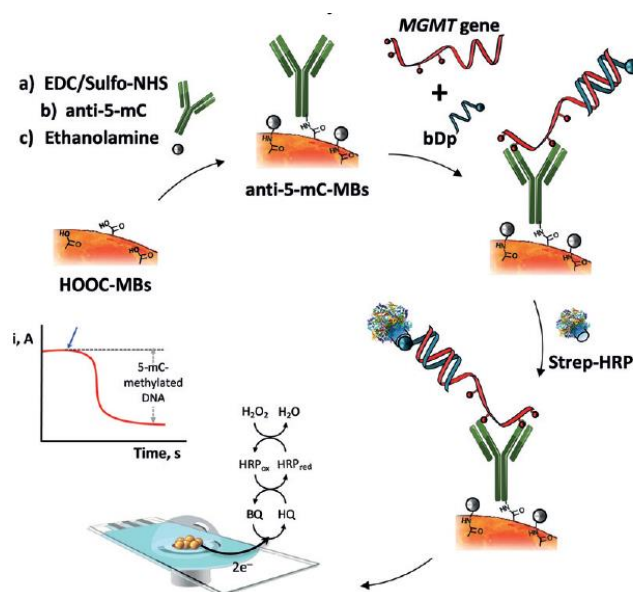


Figure 2. Schematic of the method developed for the determination of specific 5-mC methylations at MGMT tumor suppressor gene promoter. Reprinted with permission from reference [17]. Copyright 2018 John Wiley and Sons.

Differing from the above methods, a novel detection strategy based on the use of magnetic beads (MBs) modified with antibodies have been proposed by Campuzano and Pingarrln [17, 18]. The

designed principle is presented in Figure 2. Antibody-modified MBs were used to capture 5-mC-methylated ssDNA sequences. The captured ssDNA in the non-methylated region was then hybridized with a biotinylated DNA probe. This allowed the conjugation of Strep-HRP through the biotin-streptavidin interaction. After magnetic capture of the resulting MBs on the electrode surface, the attached HRP allowed for the amperometric measurement with the H₂O₂/HQ system. This method exhibited a linear range of 3.9 ~ 500 pM and a detection limit of 1.2 pM for the synthetic methylated DNA sequence. It can be used for the real sample assays without PCR amplification, bisulfite, or labeling processes.

Besides antibody, it has been reported that DNA methylation site can be specifically recognized by a family of protein factors that contain conserved methyl-CpG binding domains (MBDs). To date, at least five MBD family members have been characterized in mammals (MeCP2, MBD1, MBD2, MBD3, and MBD4). Among them, MBD1, MBD2 and MBD4 have been shown to bind with the methylated CpG motif. With these proteins as the recognition elements, Ai's group have developed several electrochemical biosensors for the detection of DNA methylation and methyltransferase activity [19-21]. For example, they demonstrated that the MBD protein conjugated to the methylation site of CpG can be stained with brilliant blue G250 [20]. The electrochemical signal can be measured by the oxidation of CBB-G250. The method allowed the detection of M. SssI MTase concentration in the range of 0.1 ~ 40 unit/mL with a detection limit of 0.04 unit/mL.

3. RESTRICTION ENDONUCLEASE

Methylation of a specific DNA sequence can allow for or protect from digestion by the corresponding restriction enzymes, including BssHII, BstUI, Dpn I, HpaII, Mbo I, and so on. In this part, the electrochemical biosensors for the detection of DNA methylation and methyltransferase activity were summarized respectively according to the types of endonucleases.

3.1. *BstUI and BssHII endonucleases*

BstUI and BssHII restriction endonucleases can cut the unmethylated DNA in the dsDNA duplex. Based on the digestion of DNA by BstUI and BssHII restriction endonucleases, Barton's group reported the detection of both bacterial and human methyltransferase activity using unmethylated or hemethylated DNA-covered electrodes [22]. As shown in Figure 3, methylene blue (MB)-labeled DNA substrates with a human methylation site (5'-CG-3') in the recognition site of a methylation-sensitive restriction endonuclease showed a strong electrochemical signal. The 5'-CG-3' site can be methylated when the electrode was treated with methyltransferase. In this case, digestion of methylated DNA strands by the restriction endonuclease was protected and the electrochemical signal of MB was retained. Without treatment of electrode by methyltransferase, the unmethylated DNA would be readily cut by the restriction endonuclease, thus resulting in a decrease or even disappearance of the electrochemical signal. This method allowed for the detection of methyltransferase down to nanomolar concentration. Based on the same detection procedure, Barton's group also reported a novel strategy to detect human

DNA(cytosine-5)-methyltransferase1 (DNMT1) methyltransferase activity, in which MB tag acts as a diffusing electrocatalyst for the redox of $\text{Fe}(\text{CN})_6^{4-}/\text{Fe}(\text{CN})_6^{3-}$ (Figure 4) [23].

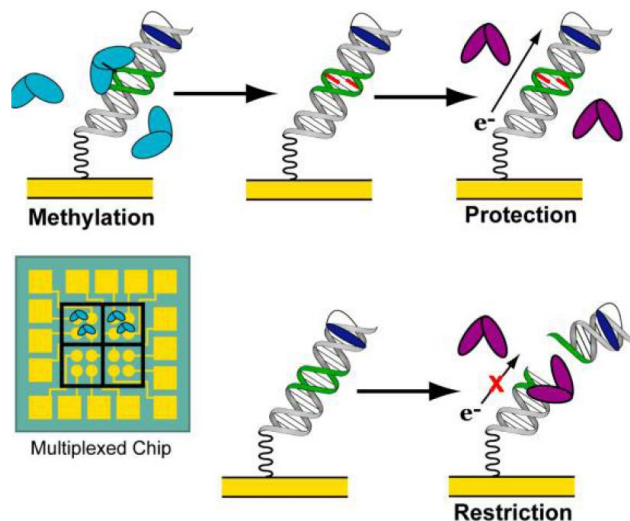


Figure 3. Assay for the electrochemical detection of methyltransferase activity. Reprinted with permission from reference [22]. Copyright 2013 American Chemical Society.

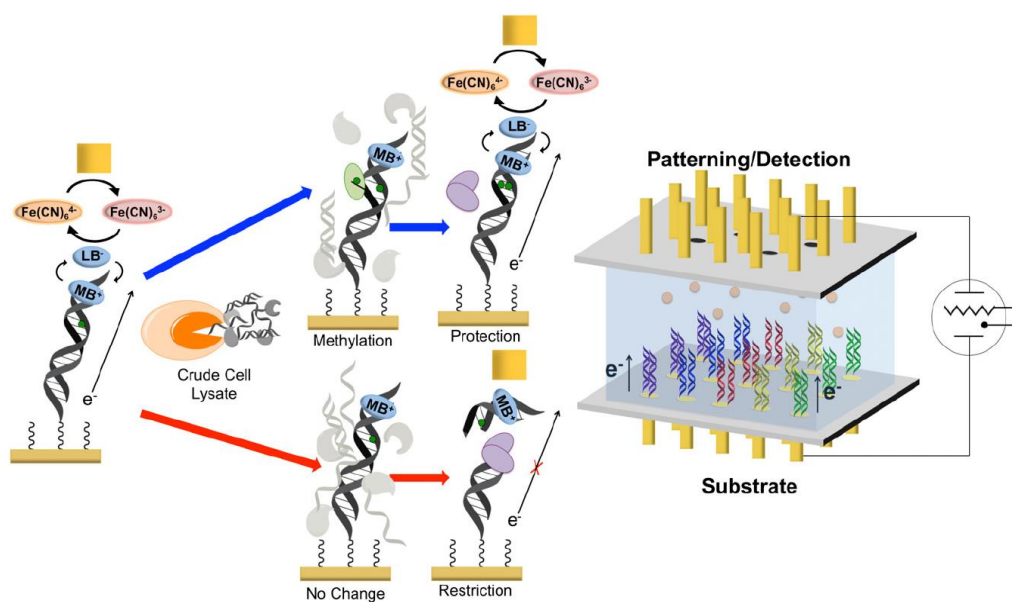


Figure 4. Electrochemical platform (Right) and scheme (Left) for the detection of human methyltransferase activity from crude cell lysates. Reprinted with permission from reference [23]. Copyright 2014 National Academy of Sciences.

Recently, Zhang's group developed an AuNPs-based dual signal amplification strategy for DNMT1 detection (Figure 5) [24]. In this work, AuNPs were modified with two different DNA sequences: one for hybridization with the DNA on electrode surface, and the other for the adsorption of $\text{Ru}(\text{NH}_3)_6^{3+}$ through the electrostatic interaction. When the activity of DNMT1 in the cell lysate was inhibited, the dsDNA conjugated with Au NPs would be digested by BssHIII, thus leading to the release of the DNA-modified Au NPs from the electrode surface. In this case, less amount of $\text{Ru}(\text{NH}_3)_6^{3+}$

molecules were attached onto the electrode surface. However, once the dsDNA duplex was methylated by active DNMT1, the restriction enzyme cleavage would be protected. The DNA-modified Au NPs remaining on the electrode surface would enhance the electrostatic attraction of $\text{Ru}(\text{NH}_3)_6^{3+}$ to the negatively charged DNA-AuNPs complexes. The resulting $\text{Ru}(\text{NH}_3)_6^{2+}$ can be cycled by $\text{Fe}(\text{CN})_6^{3-}$ after the electro-reduction of $\text{Ru}(\text{NH}_3)_6^{3+}$, thus leading to the recycling of Ru(III)/Ru(II) during the electrochemical scanning and the further increasing in the cathodic current. With this method, a detection limit down to 0.3 U/mL for pure DNMT1 and 8 MCF-7 cells was achieved.

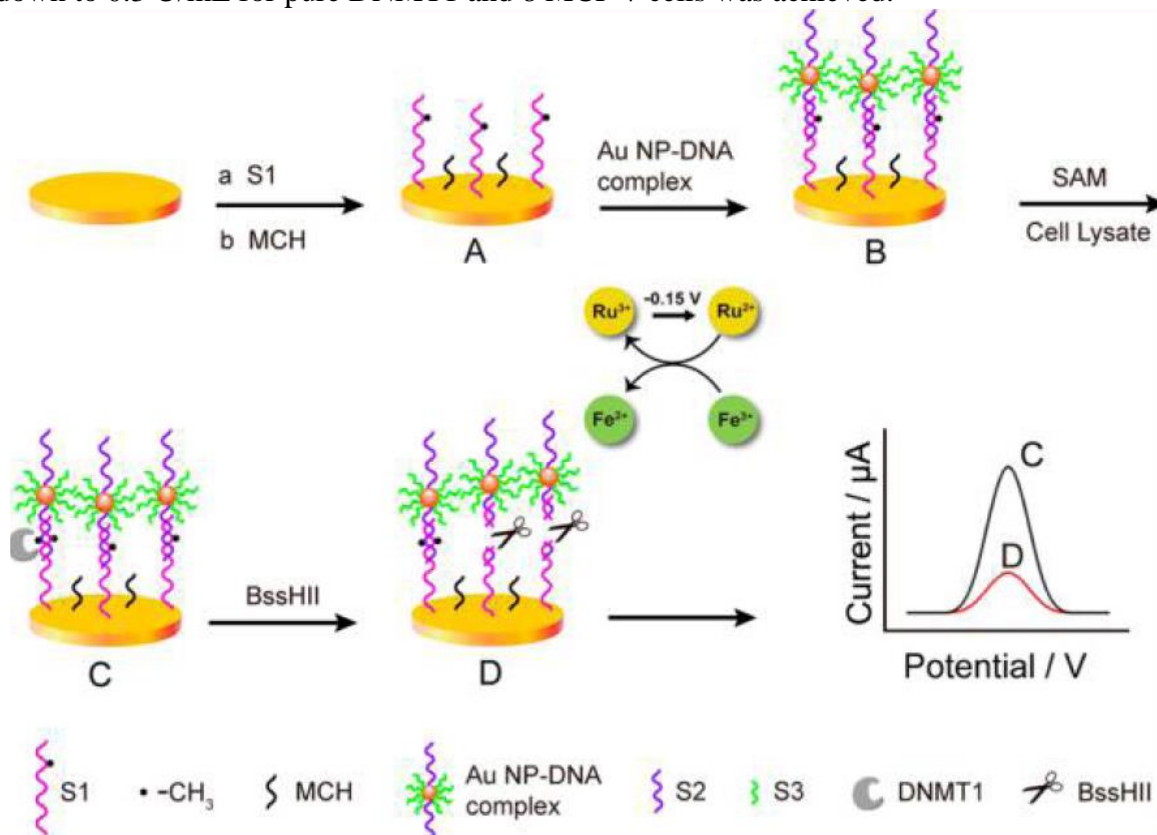


Figure 5. Schematic illustration of Au NP–DNA complexes and the Ru(III) redox recycling dual signal amplification electrochemical assay for DNMT1 activity detection. Reprinted with permission from reference [24]. Copyright 2014 American Chemical Society.

3.2. HpaII endonuclease

The restriction endonuclease HpaII can recognize the 5'-CCGG-3' sequence in dsDNA, and methyltransferase can catalyze DNA methylation with the CpG site in the 5'-CCGG-3'. Based on these results, electrochemical biosensors have been constructed for detection of DNA methylation. The simplest method is based on the redox labeled DNA. When the dsDNA duplexes were methylated by methyltransferase and follow-up cut by HpaII between the cytosines, the electrochemical signal of electroactive label in terminal of DNA decreased greatly or even disappeared [25]. Furthermore, the signal have been readily amplified by graphene [26-28], magnetic beads [29, 30], AuNPs [31, 32], or exonuclease III and mimic-hybridization chain reaction [33, 34]. For example, in 2012, Cai's group demonstrated that the dsDNA strands assembled on the AuNPs-modified glass carbon electrode can be

labeled with thionine-loading GO (Figure 6) [26]. The detection sensitivity was enhanced by AuNPs and thionine conjugated to probe DNA by GO.

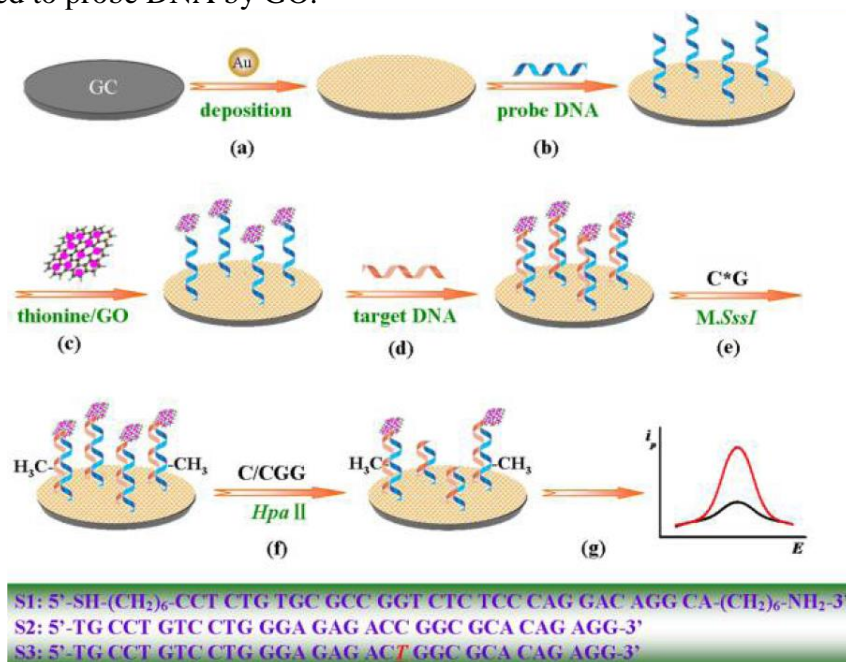


Figure 6. Schematic illustration of the procedures of the gene-specific DNA methylation detection and MTase activity assay based on the electrochemical signal amplification of GO and restriction endonuclease. Reprinted with permission from reference [26]. Copyright 2012 American Chemical Society.

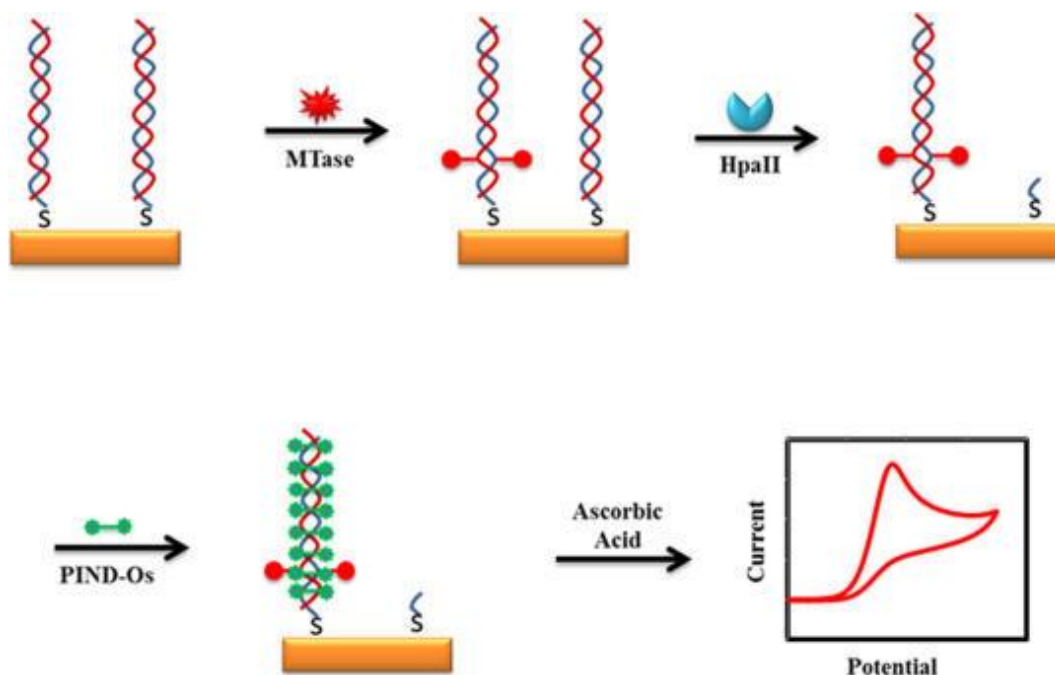


Figure 7. Schematic illustration of the mechanism to develop a simple and generalized electrochemical method for detection methylation and assay of methyltransferase activity. Reprinted with permission from reference [35]. Copyright 2014 American Chemical Society.

The method exhibits a linear range of 0.1 ~ 450 U/mL for M.SssI detection. The detection limit was found to be (0.05 ± 0.02) U/mL, which is lower than that of the ferrocene-labeled method (0.1 ± 0.02) U/mL [25]. Moreover, Gao's group demonstrated that methyltransferase can induce more CpG sites being methylated, thus impeding more digestion process by HpaII (Figure 7) [35]. The remained dsDNA on the electrode surface facilitated the insertion of $\text{Os}(\text{bpy})_2\text{Cl}^+$ ions to the base pairs of dsDNA. The inserted $\text{Os}(\text{bpy})_2\text{Cl}^+$ ions on electrode surface catalyzed the electrooxidation of ascorbic acid (AA) to produce an enhanced current.

3.3. Mbo I endonuclease

Mbo I is another endonuclease to cleave the unmethylated DNA. Xie's group and Wang's group have reported the Mbo I-based detection of DNA methylation based on the signal amplification of single AuNP and AuNPs network, respectively [36-38]. Controlling the conformation, orientation and spatial distribution of the immobilized DNA is an important challenge in DNA assay. To make DNA probe more uniform for enzyme digestion, Xie's group also reported a probe-dragging strategy to improve the sensitivity via AuNPs-assisted signal enrichment [38]. As shown in Figure 8, the dsDNA with a biotin tag can be cleaved by Mbo I, leading to the release of the biotin group from the electrode surface. However, the methylated dsDNA can not be cleaved by Mbo I, which facilitated the attachment of DNA S1-captured AuNPs on the electrode surface through the biotin-streptavidin interaction. To decrease the electrostatic repulsion between signal probe and DNA-covered electrode, the signal probe was endowed to hybridize with DNA S1 residue on the electrode surface. The electrochemical signal change was monitored by measuring the oxidation current of ferrocene tag labeled on the signal probe. With this method, active MTase in the concentration range of 0.05~ 50 U/mL was readily determined with a detection limit of 0.024 U/mL.

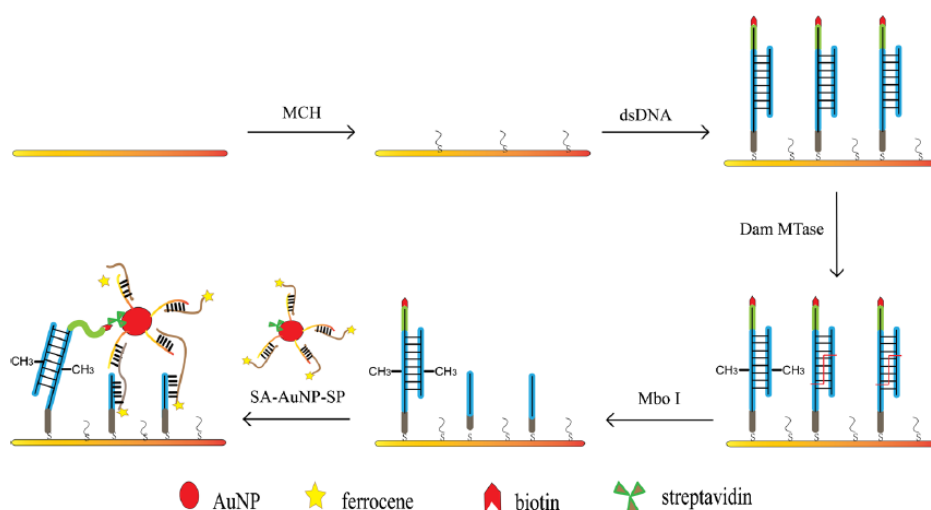


Figure 8. Schematic illustration of sensing system for the detection of Dam MTase activity by using methylation-resistant cleavage coupled with proximity-based cooperative amplification. The washing buffer was used between the main steps of the fabrication process. Reprinted with permission from reference [38]. Copyright 2014 Springer Nature.

3.4. Dpn I endonuclease

Dpn I is a restriction endonuclease that can specifically cut methylated DNA with the recognition site of 5'-GATC-3'. Based on the Dpn I induced cleavage of methylated DNA, many groups have developed sensitive electrochemical biosensors for assays of DNA methylation and methyltransferase activity, which include DNA immobilization [39-44] and immobilization-free methods [45-47]. In the DNA immobilization strategy, the thiolated single strand DNA-1 was firstly assembled onto electrode surface, and then hybridized with DNA-2. The dsDNA duplex containing a specific recognition sequence of 5'-GATC-3' can be methylated by adenine methylation MTase. The resulting methylated dsDNA duplex can be digested by methylation-responsive restriction endonuclease Dpn I, thus leading to the signal change. The signal can be readout and amplified by $[\text{Ru}(\text{NH}_3)_6]^{3+}$ [41], quantum dot [39], single wall carbon nanotubes [42], DNA-AuNPs and enzyme labels [43, 44]. For example, Ai's group developed an enzymatic amplification method for the detection of methyltransferase based on the hemin/G-quadruplex system (Figure 9) [44]. After methylation of the dsDNA containing a G-quadruplex, the methylated DNA can be cut by DpnI and follow-up digested by ExoIII. This induced the generation of the hemin/G-quadruplex in presence of K^+ . The resulting hemin/G-quadruplex can catalyze the oxidization of hydroquinone to benzoquinone by H_2O_2 . The produced benzoquinone can be determined by the reduced graphene oxide-modified gold electrode. Without DNA methylation, the digestion of dsDNA did not occur and thus no hemin/G-quadruplex was generated. This method exhibits a detection limit of $0.31 \text{ unit/mL}^{-1}$ for DNA adenine methyltransferase (DAM).

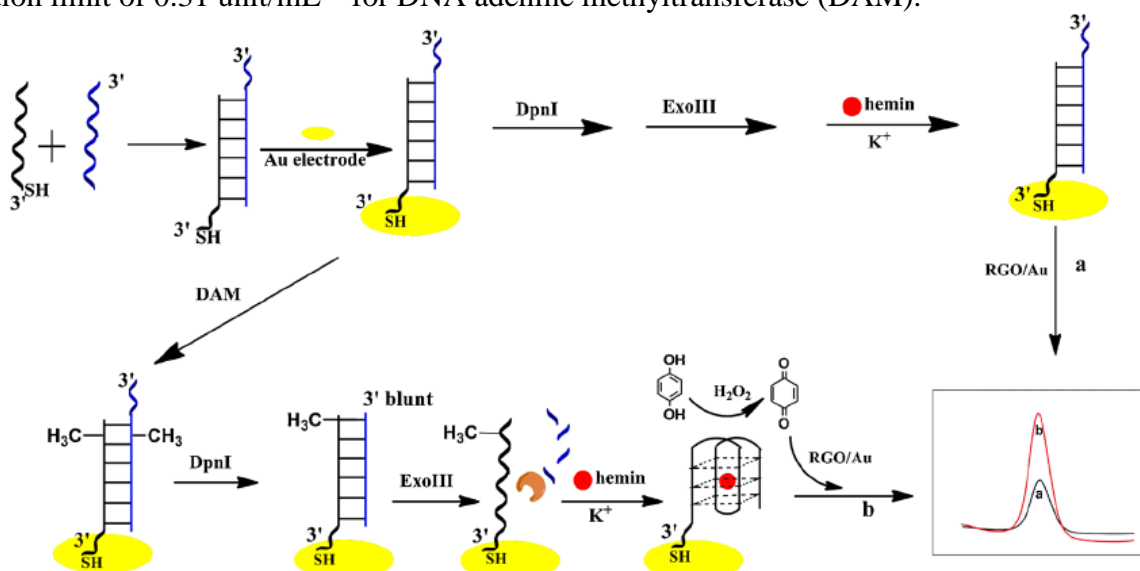


Figure 9. Schematic representation of the developed method for detection of DNA methylation and assay of DAM activity. Reprinted with permission from reference [44]. Copyright 2015 Springer Nature.

However, the electrode modification process makes the adsorptive electrochemical biosensor encounter a series of problems in practical applications. For example, some components in the biological matrix are easily adsorbed on the electrode surface, which will interfere with the electrochemical determination and reduces the accuracy of the sensor. In addition, modification of the DNA probes on

one-dimensional electrode surface may affect their interaction with the target molecules, thus reducing the detection efficiency of the sensor. The modification step often requires a long time and complex procedure, thus increasing the detection time and analysis cost. For these views, several groups reported immobilization-free methods for assays of methyltransferase activity [45-48]. Typically, Lin's group designed a novel strategy for the detection of DNA methylation and methyltransferase activity based on the fact that DNA sequences with different length show different diffusivity and electrostatic repulsion on the negatively charged indium tin oxide (ITO) electrode [48]. As shown in Figure 10, without DNA methylation, the MB-labeled DNA can not be cut by Dpn I, and the ITO surface with negative charge prevents the approach of the negatively charged long DNA on the ITO surface, thus resulting in a weak electrochemical signal. Contrarily, methylation of DNA allows for the cleavage of long DNA strand and the follow-up generation of small MB-labeled electroactive DNA fragments. The fragments can readily aggregate on the ITO surface, thus producing a strong electrochemical response. Moreover, Yin and Ai designed an electrochemical strategy based on Dpn I-triggered digestion of poly adenine (polyA) DNA using AuNPs-modified glassy carbon electrode. In the presence of Dam MTase, the hairpin probe can be methylated and follow-up cleaved by Dpn I [49]. The resulting MB-labeled polyA signal DNA strand can be assembled on the electrode surface through the polyA-gold interaction.

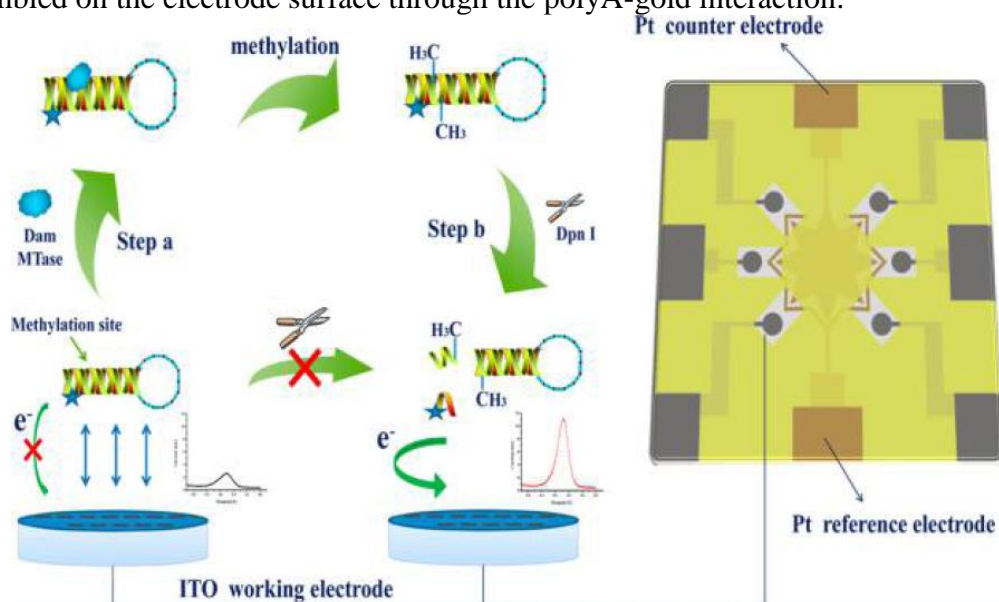


Figure 10. Mechanism of the proposed solution-phase electrochemical molecular beacon biosensor based on an ITO microelectrode chip for DNA methylation detection. Inset: ITO microelectrode chip with six ITO working electrode spots (diameter 1 mm), a Pt counter electrode, and a Pt pseudoreference electrode. Reprinted with permission from reference [48]. Copyright 2014 American Chemical Society.

ExoIII can catalyze the stepwise removal of mononucleotides from 3'-hydroxyl termini of double-stranded DNA. The exonuclease shows no activity toward 3'-overhang end of dsDNA or ssDNA. Thus, ExoIII is also a candidate for the detection of DNA methylation and methyltransferase activity with the aid of Dpn I [45, 47, 50, 51]. Moreover, the terminal transferase (TdTase)-mediated base extension technique can be applied to modify the 3'-OH terminal of DNA with biotinylated dUTP. Using the TdTase-mediated extension technique, Yu's group reported the detection of MTase activity by the

cleavage of methylation-sensitive Dpn I. The signal was amplified by the streptavidin-alkaline phosphatase (SA-ALP) [52].

4. CONCLUSION

Electrochemical biosensors have attracted considerable attention as the point-of-care clinical tools for DNA methylation analysis. However, there are still some challenges to be overcome for the clinical applications. For example, automated electrochemical biosensors without human intervention are highly desired for routine methylation analysis. Additionally, innovative and real-time detection procedures are required to decrease false negative bias. Although the challenges remain to be resolved, we believe that the proposed investigations will be translated into clinical and research platforms in the future.

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