Electrochemical biosensing strategies for DNA methylation analysis

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A B S T R A C T

DNA methylation is one of the key epigenetic modifications of DNA that results from the enzymatic addition of a methyl group at the fifth carbon of the cytosine base. This addition plays a crucial role in cellular development, genomic stability, and gene expression. Aberrant DNA methylation is responsible for the pathogenesis of many diseases including cancers. Over the past several decades, many methodologies have been developed to detect DNA methylation. These methodologies range from classical molecular biology and optical approaches, such as bisulfite sequencing, microarrays, quantitative real-time PCR, colorimetry, Raman spectroscopy to the more recent electrochemical approaches. Among these, electrochemical approaches offer sensitive, simple, specific, rapid, and cost-effective analysis of DNA methylation. Additionally, electrochemical methods are highly amenable to miniaturization and possess the potential to be multiplexed. In recent years, several reviews have provided information on the detection strategies of DNA methylation. However, to date, there is no comprehensive evaluation of electrochemical DNA methylation detection strategies. Herein, we address the recent developments of electrochemical DNA methylation detection approaches. Furthermore, we highlight the major technical and biological challenges involved in these strategies and provide suggestions for the future direction of this important field.

1. Introduction

DNA methylation is the covalent addition of a methyl group to the fifth carbon of the cytosine base in the CpG dinucleotides of DNA (Bird, 2002). This important epigenetic alteration converts cytosine (C) to methylcytosine (5mC) while keeping the original DNA sequences unchanged (Bird, 2002; Tucker, 2001). DNA methylation plays important roles in various cellular regulatory pathways, such as gene expression and regulation, maintaining genomic stability, X chromosome inactivation and mammalian cell development (Constancia et al., 1998; Jones and Gonzalez, 1997; Mohandas et al., 1981; Robertson and Jones, 2000). Thus, abnormal DNA methylation can result in different genetic and physiological anomalies, causing a number of diseases including neurodegenerative disorders, cardiovascular diseases and cancers (Ehrlich, 2002; Esteller, 2005; Jones and Gonzalez, 1997).

An irregular high level of methylated DNA is known as hypermethylation, and in contrast, an unusually low level of methylated DNA is known as hypomethylation. Early studies on DNA methylation have established a hypothesis that aberrant DNA hypermethylation of the promoter region of tumor suppressor genes can lead to gene silencing and cancer initiation (Ehrlich, 2002). Hypermethylation can also deactivate homeobox genes to result in abnormal morphological development and cancerous tumor growth (de Caceres et al., 2004; Jones...
and Gonzalgo, 1997). Likewise, hypomethylation of a large genome portion is also associated with cancer development (Ehrlich, 2009). DNA hypomethylation can lead to abrupt mitotic recombination, reactivation and subsequent integration of DNA at random sites of the genome; leading to mutagenesis and genomic instability in cancer (Ehrlich, 2002; Taleat et al., 2015; Jones and Baylin, 2002). Due to the strong pathological effects of alterations in methylation levels, DNA methylation status has become an important biomarker for the prognosis and diagnosis of cancer and other diseases.

Over the past few decades, considerable attention has been dedicated to the development of effective DNA methylation analysis methodologies for both research and diagnostic purposes (Herman et al., 1996; Singer-Sam et al., 1990; Hernandez et al., 2013; Taleat et al., 2015; Zhang et al., 2015b; Islam et al., 2016). One of the early developed methods is bisulfite sequencing (Frommer et al., 1992), which consists of bisulfite based conversion of methylated cytosines followed by polymerase chain reaction (PCR) amplification and sequencing of the target methylation site. A range of bisulfite conversion-based approaches with modified PCR and sequencing procedures have been adopted for DNA methylation analysis. These approaches include methylation specific PCR (MSPCR) (Herman et al., 1996), quantitative MS-PCR (Hibi et al., 2011), real-time PCR (BonDurant et al., 2011), methylhydth (Eads et al., 2000), methylation-sensitive high resolution melting (MS-HRM) (Wojdacz and Dobrovic, 2007), methylation-sensitive single nucleotide-primer extension (MS-SnuPE) (Gonzalgo and Jones, 1997), next generation sequencing (NGS) (Taylor et al., 2007), reduced representation bisulfite sequencing (RRBS) (Meissner et al., 2005), and methylation analysis by pyrosequencing (Tost and Gut, 2007). Among these approaches, pyrosequencing approach combines a simple reaction protocol with reproducible and accurate measures of the degree of methylation at several CpGs in close proximity with high quantitative resolution, and is considered one of the best bisulfite conversion-based methods of DNA methylation analysis.

Apart from bisulfite conversion-based approaches, conventional analytical methods such as high performance liquid chromatography (HPLC), high performance capillary electrophoresis (HPCE) and mass spectroscopy (MS) have also been used for accurate detection of 5mC (Friso et al., 2002; Tost and Gut, 2006). Yet, despite the detection accuracy of HPLC, HPCE and MS methods, routine clinical application of such techniques is limited by the requirement for large amounts of input DNA, sophisticated instruments, long analysis time and low sensitivity. In recent years, other advanced detection techniques have also been extensively developed. These include combined bisulfite restriction analysis (COBRA) (Xiong and Laird, 1997), microarray-based DNA methylation profiling (Gao et al., 2005; Yan et al., 2001), surface enhanced Raman spectroscopy-based assays (Wang et al., 2015a; Wang et al., 2016a), fluorescence-based biosensors (Duan et al., 2010; Ma et al., 2015; Taleat et al., 2015; Wang et al., 2009; Wu et al., 2011), colorimetric assays (Ge et al., 2012; Geng et al., 2014; Wee et al., 2015a), surface plasmon resonance-based assays (Carrascosa et al., 2014; Sina et al., 2014a) and quartz crystal microbalance (QCM) assay (Taleat et al., 2015). Among these readout methods, colorimetric assays (i.e., naked eye detection) offer relatively simple and inexpensive detection of DNA methylation (Geng et al., 2014). Most of the other methods are commonly disadvantaged by their multi-step and time-consuming sensor fabrication steps, requirement for large amounts of input DNA sample, and/or expensive biological molecules such as antibodies. Hence, the development of a cost-effective, convenient, and accurate method for analyzing DNA methylation status is important for biological and clinical research.

To overcome the major drawbacks of conventional DNA methylation analysis methods, efforts have been put into the development of new strategies based on electrochemistry and photoelectrochemistry (Labib et al., 2016; Lee et al., 2016; Liu et al., 2016; Taleat et al., 2015; Koo et al., 2014a; Sina et al., 2014b; Koo et al., 2014b; Dai et al., 2013; Sato et al., 2010). In comparison to the other detection approaches, electrochemical approaches are relatively inexpensive and rapid (Drummond et al., 2003). Moreover, electrochemical techniques are highly sensitive and only require a low sample amount. The electrochemical detection instrument is also amenable to miniaturization (e.g., a small potentiostat that is only a few millimeters in size is commercially available), and highly suitable for point-of-care settings.

In recent years, DNA methylation detection techniques have been reviewed by various groups. Taleat and colleagues reviewed recent nanotechnology-based strategies (till 2014) (Taleat et al., 2015). Shanmuganathan et al. (2013) and Zhang et al. (2015b) comprehensively discussed conventional DNA methylation techniques, whereas Hernandez et al. (2013) and Islam et al. (2016) focused solely on the PCR-based and optical techniques. To the best of our knowledge, electrochemical techniques for DNA methylation analysis have not been comprehensively reviewed. Herein, we summarize the recent advances in electrochemical methylation detection strategies and discuss the challenges involved in electrochemical methylation sensors along with our perspectives on future progress in this field.

2. Diagnostic and prognostic significance of DNA methylation

DNA methylation is a common epigenetic modification for negative gene regulation, and mainly occurs at cytosine bases (Qureshi et al., 2010). A family of DNA methyl transferases (DNMTs) catalyses the DNA methylation process. The de novo DNMTs, DNMT3a and DNMT3b, are responsible for transferring methyl groups onto unmethylated DNA. During subsequent DNA replications, DNMT1 (also known

Fig. 1. DNA methylation and its association with tumorigenesis. (i) Representation of the transfer of a methyl group to naked DNA by Dnmt3a and Dnmt3b, while Dnmt1 upholds the DNA methylation pattern during semiconservative replication. (ii) Representation of the changes in methylation in early periods of tumorigenesis that can lead to cancer. Reproduced from Moore et al. (2013) and Robertson (2005) with permission from the Nature Publishing Group.
as the maintenance DNMT) copies the existing methylation patterns from parental DNA strands onto newly synthesized daughter strands (Fig. 1) (Moore et al., 2013).

In 1983, Feinberg and Vogelstein first showed the relationship between DNA methylation and cancer through hypomethylation in cancer cells as compared to normal cells (Feinberg and Vogelstein, 1983). Next, Greger et al. (1989) showed that hypermethylation also occurs at the 5’ end of retinoblastoma gene in tumor which is usually unmethylated in normal cells. Later on, Ohtani-Fujita et al. (1993) and Herman et al. (1994) correlated the methylation of the tumor-suppressor genes with their gene silencing in cancer. Fig. 1 represents a region of a normal cell and progression to becoming a tumor cell. In tumor cells, hypomethylation occurs within the heterochromatin structure, and contributes to genomic instability and the de novo methylation in CpG islands. This results in transcriptional silencing of growth-regulatory genes as early events of tumorigenesis (Robertson, 2005).

In mammalian genomes, around 85% CpG dinucleotides are distributed across the genome (scattered) with nearly 40% in repetitive sequences. In a normal cell, these CpGs are largely hypermethylated to reflect a stable genome. On the other hand, these CpGs are hypomethylated in cancer cells with association to higher genomic instability (Bird, 1992; Baylin and Ohm, 2006; Gaudet et al., 2003). The remaining 15% of unscattered CpGs exist in clusters known as “CpG islands”. Around 40–50% of human genes have CpG islands within or near to gene promoter regions, thus indicating that DNA methylation is involved in transcription control (Zhu and Yao, 2009). Moore et al. (2008) showed that DNA hypomethylation in circulating peripheral blood cells is tightly associated with an increased risk of human bladder cancer, and that such cancer cells can be detected early by the global DNA hypomethylation status. Moreover, cancer genes are elevated with the promoter CpG islands containing hypomethylation and it has been associated with inactivation of tumor suppressor genes as well as oncogenic transformation. Thus, aberrant methylation of CpG islands can result in tumor formation and progression; and the detection of such alterations in DNA methylation can aid in the detection of pre-malignant or early stage of cancer (Montavon et al., 2012). Besides, aberrant DNA methylation may serve as a cancer biomarker to indicate presence of tumor cells (Barton et al., 2008).

Caceres et al. (2004) showed that analysis of tumor-specific hypermethylation in serum DNA improves the early detection of ovarian cancer. In this approach, DNA hypermethylation was observed in 41 out of 50 patient serum samples (82% sensitivity), including 13 out of 17 stage I disease cases. In contrast, no hypermethylation was observed in normal control samples collected from 40 healthy women (100% specificity). The study suggested that promoter hypermethylation is a common and early event in ovarian cancer, and can be detected in the serum DNA of patients with stage IA or B tumors (early localized stages of cancer).

Apart from diagnostics roles, DNA methylation has been reported to be a good indicator for tracking tumor prognosis. Milani et al. (2010) first reported the prognostic roles of DNA methylation profiling for childhood acute lymphoblastic leukaemia samples. In this study, they measured DNA methylation levels of 401 patients across 1320 CpG sites to classify the patients into acute lymphoblastic leukaemia subtypes. In 2005, Chan et al. (2005) reported the positive prognostic potential of DNA methylation in ovarian cancer where it was shown that 18S and 28S rDNA hypermethylation levels were higher in patients with long progression-free survival versus patients with short survival. In a recent study, serum RASSF1A and APC promoter 1 A hypermethylation were found to be frequent epigenetic events in early operable gastric cancer patients (Balgkouranidou et al., 2015). In this study, 73 gastric cancer samples were examined and the APC promoters for 61 patients and RASSF1A promoters for 50 patients were found to be 93.6% and 68.5% methylated respectively. Tang et al. (2011) also reported on a strong evidence of promoter hypermethyla-

tion in the tissue, fecal and serum samples of patients with colorectal carcinoma, which was significantly linked with poor differentiation grade, lymphnode metastasis status, TNM stage and shorter overall survival. In addition, methylation of SHOX2 in lung cancers (Dietrich et al., 2012), FHIT promoter methylation in ESCC (Lee et al., 2006), as well as PITX2 promoter methylation in prostate (Dietrich et al., 2013) and breast carcinomas (Nimmrich et al., 2008) have been correlated with worse patient prognosis.

Lastly, DNA methylation also has the potential to be used as a predictive biomarker for therapeutics. For instance, Teodoridis et al. (2005) showed that methylation of the BRCA1, GSTP1 and MGMT genes responsible for DNA repair/detoxification is associated with improved response to chemotherapy in late stage cancer patients with epithelial tumor.

3. Electrochemical techniques for DNA methylation detection

In electrochemical detection of DNA methylation, a recognition element (e.g. antibody, enzymes, oligonucleotide probe etc.) generally interacts with the target sequence to selectively recognise the methylated region present in that sequence. Then, an electroactive signal transducer is incorporated to obtain measurable electrochemical signal to quantify the level of DNA methylation (Labib et al., 2016). The electrochemical readout is usually performed via voltametric techniques (i.e., cyclic voltammetry (CV), linear sweep voltammetry, differential pulse voltammetry (DPV), square wave voltammetry (SWV) and stripping voltammetry), amperometry and impedimetric methods. Among these readouts, voltametric techniques, especially CV and DPV, are generally preferred (Topkaya et al., 2016). Due to the ability of electrochemical sensors to detect DNA methylation with high sensitivity, a number of such electrochemical assays have been developed over the past several years. Based on their working principles, these assays can be broadly categorized into three main types using: (i) enzymatic reaction and amplification, (ii) electroactive species, and (iii) DNA nucleobases affinity interactions. These assays are discussed in the remaining sections of this review.

3.1. Electrochemical DNA methylation assays based on enzymatic reaction and amplifications

3.1.1. Direct electrochemical oxidation of 5mC

The electrochemical activity (i.e., oxidation) of 5mC and other nucleobases can be used to quantify the presence of 5mC within a target DNA sequence. Since each base possesses a defined oxidation potential with most conventional electrodes (i.e., carbon, gold or indium tin oxide (ITO)), the resultant magnitude of the 5mC oxidation current after electrochemical oxidation of the entire target sequence can allow for 5mC quantification (Kato et al., 2013; Brotons et al., 2016a; Brotons et al., 2016b). However, this direct oxidation-based 5mC quantification technique is limited by two issues. First, the oxidation peak potential of C and 5mC are extremely different in comparison to that of 5mC with most unmodified electrode materials, thus resulting in significant interference for the recognition and detection of 5mC (Wang et al., 2010). One of the best ways to avoid these challenges is to develop new electrode materials with a wider potential window and higher electrode activity (Ivandini et al., 2007; Wang et al., 2013; March et al., 2015). In 2007, Einaga and colleagues reported an electrochemical method based on a boron-doped diamond (BDD) thin-film electrode which showed a wider potential window in acid medium (i.e., low pH). They successfully detected 5mC in fish sperm DNA samples by incorporating a reverse phase HPLC separation with BDD-based electrochemical detection to achieve a highly-resolved 5mC peak (Ivandini et al., 2007).
To achieve electrode materials with a wider potential window and higher electrode activity, Kato et al. (2008) have developed a nanocarbon film electrode where electron cyclotron resonance (ECR) was used to deposit sp2 and sp3 carbon mixture on a sputtered nanocarbon film to obtain high electrode stability. In this method, a significant potential difference (130–150 mV) between 5mC and C oxidation peaks was observed from the background-subtracted square wave voltammetric data. The authors also demonstrated that despite having large potential window, BDD film based electrodes displayed relatively lower current responses for 5mC and C (i.e., low electrode activity). This is due to the excess amount of sp3 carbon content being present on the diamond surface of BDD (DNA bases prefer to adsorb more on sp2 or sp2-sp3 hybridized carbon surface due to Π-Π interactions). However, this method was only tested with short synthetic DNA sequences and one obvious disadvantage is the associated false background signals resulting from the bases hidden inside the helical structure of DNA.

In a follow-up report which aimed to reduce background signals from other nucleobases, Kato et al. (2011) also proposed a unique assay for quantifying 5mC in which DNA sequences were cut into single bases by restriction endonuclease prior to electrochemical oxidation. In this assay, a long CpG oligonucleotide was treated with restriction endonuclease P1 to form an identical mononucleotide 50-dNMP (20-deoxyribonucleoside-50-monophosphate) (Fig. 2(i)). The direct oxidation of both 5-mC and C was measured on a sputtered nanocarbon film electrode using square wave voltammetry. In comparison to the control sample without P1 digestion treatment, a several-fold improvement was observed in terms of sensitivity and dynamic concentration range. Due to the simple digestion of DNA sample and direct 5mC electrochemical oxidation on a device that can easily be manufactured at low cost, this method has significant potential in real biological and clinical applications.

Although direct oxidation-based detection of 5mC with modified electrodes is inherently sensitive, they are limited by the need for background subtraction of voltammetric signal of known DNA sequences (i.e., bases) to discriminate 5mC peaks from that of T. This could create a significant challenge for an unknown sample (if the DNA sequence is unknown, the quantification of 5mC could be influenced by overlapping T current signals). Wang et al. (2010) addressed this challenge by using an innovative subtraction formula based on the principle of complementary base pairing in DNA sequence. They developed a simple method for DNA methylation detection by using direct electrochemical oxidation with a multiwalled carbon nanotubes- and enzyme- modified glassy carbon electrode. This method also relies on the background subtraction method. As the molar concentrations of A and T are equal (complementary base pairing) in an unknown DNA sequence, subtracting the voltammetric signal of A (equivalent to T signal) from overlapped 5mC and T signals allowed direct quantifica-

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**Fig. 2.** Direct electrochemical oxidation of 5mC. (i) Individual nucleobases and 5mC were cut by restriction endonuclease followed by their oxidation which produced distinct oxidation peak for 5mC and C. (ii) Background-subtracted DPV responses from the direct oxidation of bases where 50 mM 5mC (A) and 50 mM C (B) 50 mM 5mC and C (C) 25 mM G and A, and 50 mM 5mC and C (D) were used to differentiate 5mC from C. (a)-(d) represents the fabrication steps of the electrode where bare (a) GCE, (b) Ch/GCE, (c) MWNTs/GCE and (d) MWNTs/Ch/GCE were used. Reproduced from Kato et al. (2011) and Wang et al. (2010) with permission from The American Chemical Society and The Royal Society of Chemistry, respectively.
tion of 5mC level in samples of unknown DNA sequences (Fig. 2(ii)). They also extended this work to detect DNA methylation using over-oxidized polypyrrole (PPyox)-directed multiwalled carbon nanotubes-based electrodes (Wang et al., 2013). In recent years, in order to achieve good resolution between oxidation peaks of 5mC and C, several other direct electrochemical approaches based on graphene, graphite, and carbon nanotube-modified electrodes have also been reported (Zhang et al., 2013; Wang et al., 2015c; Wang et al., 2016b; Meng et al., 2013; Brotons et al., 2013; Park et al., 2014).

3.1.2. Restriction enzyme digestion based assays

Recent demonstrations have shown that restriction enzyme digestion can be used in electrochemical detection of DNA methylation by two ways. First, direct oxidation of restriction enzyme-digested DNA bases at modified or unmodified electrodes (e.g., 5mC and other bases at nanocarbon film electrode (Kato et al., 2011), as discussed above). Second, selective recognition of 5mC using methylation sensitive restriction enzymes such as HpaII and Not I (Dai et al., 2012). In these assays, as their name implies, methylation-sensitive restriction enzymes cannot cleave the methylated DNA sequences but can fully digest the unmethylated DNA sequences (Cedar et al., 1979; Quint and Cedar, 1981). This was experimentally demonstrated by Hou et al. (2003), where regional methylation of p16ink4a tumour suppressor gene was electrochemically quantified. In this method, genomic DNA was first treated with MseI restriction enzyme to get DNA fragments with cleaved ends, which were then ligated with unphosphorylated linkers (in this case, they functioned as universal PCR primers). The ligated DNA was further digested with the methylation-sensitive restriction endonuclease BstUI followed by PCR amplification. This resulted in two sets of products: (i) unmethylated DNA which was not amplified due to BstUI digestion (ii) amplified methylated DNA, as methylated DNA was unaffected by BstUI. The amplified products were then detected by using conventional hybridization technique. CV and SWV voltammetric techniques were used to read the methylation information in the presence of a [Co(phen)2][ClO4]2 intercalator.

One of the major complications of this method is the use of PCR amplification. In order to avoid PCR, Dai et al. (2013) have proposed an enhanced digestion-based electrochemical assay, whereby oligonucleotide capture probes-functionalized gold electrodes were used to specifically hybridize both methylated and unmethylated DNA targets. Methylene blue (MB), an electrochemically active dye, was then intercalated into the surface-bound dsDNAs due to the interaction between guanine residues and MB. These electrodes were then treated with the methylation-sensitive restriction enzyme HpaII, which precisely cleaved the unmethylated DNA leaving 5mC-containing dsDNA unaffected. This resulted in a partial loss of intercalated MB present in the unmethylated dsDNA sample and offered a significant reduction in the voltammetric current as compared to that of the methylated counterpart. Finally, by measuring the relative current changes for methylated and unmethylated samples, the degree of DNA methylation was calculated.

3.1.3. DNA methyltransferase (MTase) activity-based assays

As discussed in Section 2, the relative level of methylation within the whole genome or a specific gene is regulated by the DNA methyltransferase (Mtase) enzyme. An over-expression of DNA MTase triggers aberrant DNA methylation and facilitates tumor progression (Issa et al., 1993; Vertino et al., 1996; Robertson, 2001). For instance, in comparison to non-tumorgenic cell lines, MTase level was reported to be higher in tumorgenic cell lines. Additionally, Issa et al. (1993) reported that colon tumorigenesis is also accompanied by the increased expression of MTase. Therefore, DNA methylation can be indirectly analyzed via assaying DNA MTase activity. In recent years, much attention has been focused on developing electrochemical methods for measuring MTase activity (Li et al., 2015; Liu et al., 2015; Liu et al., 2016b). For example, Liu et al. (2011) reported an electrochemical assay for DNA methylation detection and MTase activity at specific CpG methylation sites. In this method, dsDNA was labeled with ferrocene acetic acid (FcA) by conjugation. This labeled DNA was then treated with Mtase M.SssI enzyme followed by a methylation-sensitive restriction enzyme HpaII endonuclease. The enzyme treatments cleaved the unmethylated dsDNA portion between adjacent cytosines, causing the loss of FcA labels and leading to a reduced voltammometric signal of FcA. On the other hand, since HpaII cannot exert its activity in presence of 5mC, FcA-labeled methylated dsDNA was not cleaved, thereby producing a higher voltammometric signal. The generated voltammetric current level is directly related to the methylation level and MTase activity. Although this method is helpful for genomic DNA methylation analysis, the sensitivity is relatively lower as compared to other reported MTase assays. Furthermore, it is also limited by the need for FcA labeling.

In another assay, Muren and Barton (2013) used an innovative strategy with a multiplexed platform for detecting both human (Dnmt1) and bacterial (SssI) Mtase activity. In this assay, a multiplexed chip consisting of sixteen electrodes were divided into four quadrates. Each quadrate was modified with a DNA substrate (each contains the human methylation site (5’-CG-3’) within the recognition site of a methylation-sensitive restriction enzyme). Upon treatment of the quadrates with active Mtase, these sites become methylated, and thereby protecting the DNA from restriction during subsequent restriction enzyme treatment. The intact methylated DNA which still retained the redox probe was therefore able to offer positive redox signal. In contrast, in absence of active MTase, the DNA remains unmethylated. These unmethylated DNA was digested by restriction enzyme, resulting in complete disappearance of redox signal. This method is able to compare up to four types of different DNA methylation targets and four Mtase activities on a single platform (Fig. 3(iii)).

Due to the ability for detecting aberrant DNA methylation, MTase activity-based assays have been the subject of many significant studies in disease diagnosis and prognosis. Most of these methods were largely reliant on physical (i.e., nanomaterial based), enzymatic, and non-enzymatic amplification processes (He et al., 2011; Wang et al., 2012; Baek et al., 2013; Xu et al., 2013; Wang et al., 2015d; Zhang et al., 2015a, 2016; Hong et al., 2016). For example, Zhang et al. (2016) have developed a method for DNA methylation detection via measuring M.SssI MTase activity. The detection was performed by combining AuNPs based signal amplification, non-enzymatic hybridization chain reaction (HCR), and methylation sensitive restriction enzyme digestion steps. In this method, AuNPs and gold electrodes were initially modified with P3 and C1 probes respectively. A linker probe (L2) was assembled onto the electrode surfaces by hybridization between C1 and L2 probes, resulting in dsDNA containing the 5’ -CCGG-3’ sequences. These ds-DNA sequences were then methylated via treating with M.SssI Mtase enzyme. The resultant methylated ds-DNA is prone to restriction enzyme treatment. Then, P3 probes-modified AuNPs were attached to the electrode surface via sandwich hybridization (L2 contains two complementary regions of C1 and P3) prior to the addition of two HCR hairpin probes. This formed a large amount of dsDNA on the electrode surface. In the subsequent step, RuHex redox molecules were used to neutralize the electrode surface (this resulted in large amount of RuHex tags on the electrode surface, resulting a large amount of DPV current). Under the unmethylated condition (i.e., without M.SssI Mtase treatment step), 5’-CCGG-3’ can be recognized and cleaved by endonuclease HapII, and further digested by exonuclease EcoIII enzymes. This results in no DNA-AuNPs on the electrode surface, and hence no DPV current was observed. Using the similar working principle, Jing et al. (2014) have developed another method where they have used MB as electrochemical signal indicator and DNA-modified AuNPs as signal amplification units for the detection of DNA methylation, determination of DNA MTase activity and screening of MTase inhibitor.
3.1.4. Ligase chain reaction (LCR)-based strategies

LCR is an exponential amplification system and uses DNA ligases which are sensitive to mismatches on the 3' end of a ligation DNA probe (Barany et al., 1991). Generally, the readout of LCR products is obtained by optical methods or mass spectrometry which are limited by high running cost and complex instrumentation (Wee et al., 2013; Wiedmann et al., 1994).

In 2012, Wee et al. developed a method called eLCR which coupled LCR with a simple and inexpensive electrochemical readout to detect single base mismatches (Wee et al., 2012). By using single base changes that arise from bisulfite treatment of methylated and unmethylated DNA, Koo et al. (2014b) extended this eLCR method for DNA methylation detection in a microdevice-based electrochemical assay (Fig. 3(iii)). In this method, bisulfite-treated initial DNA targets with base changes at a single CpG site (C for methylated, T for unmethylated) were amplified by LCR with six probes (P1-P6). In the first LCR cycle, probes complementary to C (P1) and T (P5) ligated with P2 probes. These P1/P5-P2 ligated probes then served as templates for further LCR amplification by P3, P4 and P6 probes. The exponential LCR amplification produced two types of ligated long knife motifs (representing initial methylated and unmethylated targets) which carried electrocatalytic DNAzyme sequences. In contrast, the excess unligated probes produced short knife motifs without electrocatalytic moieties. These long and short knife motifs were then hybridized with P1 and P2 specific capture probes that were pre-immobilized onto separate gold electrode surfaces. P1 and P2 capture probes hybridized to LCR products of initial methylated and unmethylated targets respectively. Lastly, DNAzyme-mediated electrocatalytic reduction of hydrogen peroxide ($\text{H}_2\text{O}_2$) enabled quantitative detection of DNA methylation levels in initial targets. The applicability of this assay was successfully tested in breast cancer cell lines and a patient serum sample.

In order to detect heterogeneous methylation of epialleles (epialleles are the DNA strands containing a mixture of methylated, unmethylated and partially methylated regions), Wee et al. (2015b) developed an LCR-based electrochemical method that is referred to as ‘epiQ’. It has some unique advantages over existing methodologies such as (i) multiplex DNA methylation analysis, (ii) quantification of heterogeneous epiallele methylation. Previously, NGS and digital PCR were commonly used to quantify the epialleles and are limited by the high running cost and several multistep procedures. However, epiQ offers an alternative to NGS and digital PCR for quantifying heterogeneous methylation of genomic DNA. In this approach, capture sequences complementary to the reporter probe were first immobilized on electrode surfaces via gold/thiol self-assembling monolayers. Following the ligation step, biotin-labeled DNA oligonucleotide was then added and allowed to hybridize to ligation reaction products. Only biotin-labeled probes of complementary sequence were added to their respective epiallele reactions. Each epiallele reaction was added to separate wells of the microdevice and allowed to incubate. After the addition of horseradish peroxidase (HRP) in each well, electrocatalytic reduction of $\text{H}_2\text{O}_2$ on the modified gold microelectrode resulted in different CV responses for different epialleles, thus enabling the quantification of heterogeneous DNA methylation. The applicability of the epiQ was successfully tested by profiling eight possible epialleles from $\text{CDKN}2\text{B}$ (p15) tumor suppressor gene. The results showed that epiQ can successfully distinguish 5% and 10% of differences in epiallele levels.

3.1.5. Photoelectrochemical-biosensing of DNA methylation

Generally, in photoelectrochemical biosensor, the formation of DNA duplex or other DNA–target complexes could be monitored by the changed electrical signal of the employed photoactive species (Wu et al., 2013; Zhao et al., 2014). As compared to conventional electro-
chemical readout methods, photoelectrochemical approaches may also offer higher sensitivity due to the reduced background noise (Zhao et al., 2014). In 2008, a photoelectrochemical-based strategy was developed to differentiate between C and 5mC by using the photosensitizer-injected hole transfer properties of DNA on gold electrodes (Yamada et al., 2008). In this method, anthraquinone photosensitizer-dioxygen nucleotide duplexes (AQ-ODN) containing 5mC or C were first immobilized on gold electrodes. The AQ-ODN duplexes were then treated with methylation-sensitive restriction enzymes HapII or Hhal which cleaved unmethylated DNA strands. This led to the removal of photosensitizer units from the gold electrode, thereby reducing photocurrent density. In case of methylated DNA (5mC)-containing duplexes, there was no restriction enzyme activity, thus leading to increased photocurrent density (Fig. 3(ii)).

Another photoelectrochemical method has recently been reported for the analysis of DNA methylation using Bi2S3 nanorods as photoelectric conversion material, and 6× His and anti-his tag antibody-labeled recombinant methyl binding domain 1 (MBD1) proteins as DNA methylation recognizing units (Yin et al., 2014). In this method, a methylated capture probe was hybridized with its complementary and symmetrical cytosine methylation in CpG region of ds-electrode surface through the specific interaction between MBD1 protein and symmetrical cytosine methylation in CpG region of ds-DNA. Then, through the immunoreaction between His tag at the end of MBD1 protein and anti-his tag antibody, anti-his tag antibody was further captured on the electrode surface. Based on the immobilization of MBD1 protein and antibody, the photoelectrochemical response decreased significantly, which was used to detect methylated DNA. However, this method is limited by the use of antibodies to achieve enhanced photoelectrochemical responses.

3.2. Electrochemical DNA methylation assays based on electroactive species

3.2.1. Semiconductor quantum dots (QDs) based assays

QDs have shown great potential in bioassays because of their unique optical and electrical characteristics, biocompatibility and versatility in surface modification. In photoelectrochemical bioassays, biomolecules are often detected using QDs as signal amplification labels (Cui et al., 2007; Resch-Genger et al., 2008; Amelia et al., 2012; Wagner et al., 2010). Moreover, the use of anodic stripping voltammetry offers sharp stripping signals in QD-based assays, thus allowing sensitive target detection (Zhang and Wang, 2012). For example, Dai et al. (2013) developed a QD-based electrochemical readout method referred to as E-msLDR (methylation-specific ligation-detection reaction) for the simultaneous and multiplex quantification of DNA methylation. In E-msLDR, two gene-specific methylation loci of p53 gene were selected as targets and four locus-specific sequence probes were designed. Probes 1 and 2 were labeled with QDss whilst probes 3 and 4 were co-immobilized with magnetic beads. Bismuth-converted gene targets were magnetically isolated from the sample mixture using probes 3 or 4, and further hybridized to QDs-modified probes 1 or 2. Then, E. coli DNA ligase was added to initiate LDR (ligation detection reaction). In the case of methylated targets, the QD-modified probes matched the pre-treated p53 gene perfectly and covalently interlined to form a stable duplexes. In contrast, unmethylated targets resulted in single-base mismatches with probes and were unligated. By performing voltammetric analysis of attached QDs on methylated targets, the methylation levels of p53 gene fragment were quantified (Fig. 4).

Although E-msLDR avoids PCR amplification or restriction enzyme digestion, it is limited by the need for several hybridization and heating steps along with longer assay time. To avoid these drawbacks, the same group has developed a modified method for quantifying methylation level in p53 gene fragment using QD barcodes (Xu et al., 2016). In this method, two dual-functional hairpin probes (HP) were designed and tagged with QDs. When 5mC target regions reacted with QDs-tagged HP, HP loops were exposed to allow hybridization to targets. The targets-attached HPs were then magnetically isolated with magnetic beads-functionalized capture probes. Stripping voltammetric quantification of the QDs on isolated targets was performed to quantify the methylation levels in the target DNA. On the other hand, for unmethylated DNA targets, HP loops remain closed and resulted in no current signals.

3.2.2. Electrochemically active ligands-based methods

In recent years, a number of electroactive ligands or molecules (e.g., MB, triphenylmethane, etc.) have been used in developing various electrochemical methylation assays (Dai et al., 2012; Sato et al., 2010; Tanaka et al., 2007). In 2007, Okamoto and colleagues reported a bisulphite-free and PCR-free method for DNA methylation detection using tag-attachable bipyridine electroactive ligands. Bipyridine derivatives with a tag-attachable amino linkers at C4 positions were initially synthesized and then directly complexed with 5mC residues in a reaction mixture containing potassium osmate and potassium hexacyanoferrate (III). The succinimidyl esters of functional labeling units were then attached to the bipyridine ligands on the 5mC residues. This allowed the electrochemical detection of 5mC targets and analysis of methylation levels via SWV. As the ligand-attached 5mC targets showed higher interfacial electron-transfer resistance as compared to unmethylated samples, Faradic impedance spectroscopic readout in the presence of [Fe(CN)6]3-/4- was also used in this system to detect 5mC.

Sato and colleagues explored the use of ferrocenylphenanthrene diimide (FND) derivatives in detecting 5mC (Sato et al., 2006, 2010, 2012, 2014). These derivatives can intercalate into dsDNA by a threading intercalation mode at every two base pairs, resulting in the arrangement of many ferrocene molecules in the major and minor grooves of dsDNA. This would offer a significant enhancement in electrochemical signal (Sato et al., 2010, 2012). In 2006, they demonstrated that the use of FND in 5mC detection improves the electrochemical response by stabilizing dsDNA and producing more charge transfer through dsDNA (i.e., duplex-mediated charge transport). In this method, a 20 bp-long methylated promoter region of p16inkr4a gene containing two 5mC at the 3’ end of the sequence was selected as the target. Bisulphite treated target region of gene was amplified by the PCR with the methylation-specific primers. The PCR-amplified samples were then hybridized to two DNA probes that had been pre-immobilized on to a chip array containing 25 gold electrodes. These two DNA probes were designed for unmethylation and methylation-specific sequences, where one can detect whether sample is methylated or not by the pattern of signal intensities for DNA probes. During hybridization, methylated DNA-specific probes were fully matched with methylated sequences unlike other mismatched target sequences. Under optimal conditions, both probes formed duplexes with target DNA sequence. However, mismatched duplex would be less stable compared to the matched duplex containing 5mC. This was detected by measuring the voltammetric currents in the presence of FND complexes, where matched duplex DNA produced significantly larger current in comparison to that of the mismatched duplex. This method was further extended for methylation detection of CDH4 and hTERT genes (Sato et al., 2010, 2014). Although, these FND-based electrochemical hybridization methods showed their significant potential in detecting 5mC, they need prior knowledge of the target DNA sequences, and is therefore unsuitable for the analysis of samples containing unknown sequences.

As discussed briefly above, MB is one of the widely-used electroactive indicators for analyzing various DNA targets such as DNA methylation, point mutations and DNA lesions (Boon et al., 2000; Kelley et al., 1999; Jing et al., 2014). Generally, in methylation assays, MB are either intercalated or covalently attached to DNA targets (Dai et al., 2012; Hou et al., 2003; Jing et al., 2014). Among other
The adsorption between gold and ‘native’ DNA has been regarded as ‘complex’, ‘non-specific’ and ‘difficult to control’. Mirkin and Rothberg groups have performed several key studies which have indicated that DNA adsorption on gold surfaces is sequence dependent and follows a definite adsorption trend of adenine (A) > cytosine (C) > guanine (G) > thymine (T) (Demers et al., 2000; Demers et al., 2002; Kimura-Suda et al., 2003; Li and Rothberg, 2004; Ostblom et al., 2005; Zhang et al., 2012; Koo et al., 2015). Since then base-dependent DNA adsorption emerged as one of the most promising solutions to achieve controlled immobilization of unmodified DNA probes onto gold surfaces. Since this adsorption is highly sequence (base)-dependent, it can also be used to distinguish two different DNA sequences (e.g., bisulfite-treated methylated and unmethylated DNA sequences). However, until 2014, this phenomenon has not been applied for DNA methylation analysis. In 2014, Sina et al. (2014b) reported an electrochemical method (referred to as eMethylsorb) that uses the different affinity interactions between DNA bases and unmodified gold electrodes for 5mC quantification (Fig. 5(i)). In eMethylsorb, after bisulfite treatment and asymmetric PCR amplification steps, target methylated DNAs become guanine-enriched, while unmethylated samples become adenine-enriched. Since DNA–gold affinity interaction follows the trend, A > C > G > T, the adenine-enriched unmethylated DNA leads to a higher level of adsorbed DNA on the electrode in comparison to the guanine-enriched methylated DNA. Hence, this generates a lower Faradaic current due to the strong coulombic repulsion between [Fe(CN)₆]³⁻ ions and negatively-charged adenine-enriched DNA strands. This method has been used to distinguish methylated and unmethylated epigenotypes at single CpG resolution.

3.3.2. Graphene-DNA affinity interaction based method

More recently, Haque et al. (2017) developed a unique electrochemical method for methylation quantification in oesophageal cancer tissue samples. The underlying principle of this method depends on the different affinity interaction between DNA bases and the graphene electrode surface. The different affinity interaction can be explained by considering the polarizabilities of individual nucleobases. Among all nucleobases, G and A with their five- and six-membered rings possess the largest polarizabilities, whereas other bases with only six-membered rings exhibit lower polarizabilities. Additionally, G with its double-bonded oxygen atom possess a larger polarizability than A. Since the van der Waals (vdW) energy is directly proportional to the interacting nucleobases, Govtham et al. (2007) and Varghese et al. (2009) proposed that vdW interaction is indeed the main driving force for the adsorption of nucleobases onto the graphene and follows the adsorption trend as G > A > T > C. Bisulfite-converted and G-enriched sequences are directly adsorbed on the graphene modified electrode, leading to a larger amount of the adsorption on the graphene electrode surface as compared to the A-enriched unmethylated DNA. Therefore, methylated DNA generates a lower current signal (due to higher adsorption of guanine on a graphene electrode surface) as compared to unmethylated DNA. They successfully tested the method in a panel of cancer cell lines and patient samples derived from oesophageal squamous cell carcinoma to detect FAM134B promoter gene methylation.

The key to the functionality and simplicity of the affinity interaction-based method lies in the use of direct adsorption of DNA samples (i.e., different adsorption affinity of DNA nucleotides) on an unmodified gold or graphene-modified electrodes (Sina et al., 2014b; Haque et al., 2017). The use of direct adsorption of target samples on an unmodified electrode (Sina et al., 2014b) rather than the conventional
biosensing approach of using recognition and transduction layers, simplifies the detection method by avoiding the complicated chemistries underlying each step of the sensor fabrication. It is important to note that the DPV signals obtained by the direct adsorption based methods were unaffected by the nonspecific adsorption of nontarget biomolecules. This is because highly purified DNA samples, diluted in high salt-buffer were studied.

3.4. Methylation assay using carbon-based electrodes

Carbon-based electrodes have also been used for the development of electrochemical DNA methylation assays. Among many carbon based materials, nanocarbon-film consisting of nanocrystalline sp² and sp³ mixed bonds have been used to develop several electrochemical sensors for the detection of DNA methylation (Goto et al., 2010; Kato et al., 2008; Yanagisawa et al., 2015). These films were formed on silicon or boron-doped silicon substrates by using the electron cyclotron resonance (Goto et al., 2010; Kato et al., 2008) and unbalanced magnetron sputtering methods (Yanagisawa et al., 2015). It has been shown that the performance of these sensors highly relies on the assay conditions and effective working areas of the nanocarbon-film-modified electrodes. More recently, GCE and screen-printed carbon electrodes (SPCEs) have also been successfully modified with various conducting materials such as conducting polymer, carbon nanotubes, nanocomposites and nanostructured gold for DNA methylation detection (Wang et al., 2016b; Zhu et al., 2015; Wang et al., 2013; Serpi et al., 2013; Daneshpour et al., 2016; He et al., 2011; Wang et al., 2015b). Daneshpour et al. (2016) used the modified-SPCEs to detect the level of 5mC in a RASSFIA tumor suppressor gene. In this method, SPCEs were modified with Fe₃O₄/N-trimethyl chitosan/gold nanocomposite (Fe₃O₄/TMC/Au), which was used as a tracing tag to label DNA probe. These Fe₃O₄/TMC/AuNPs particles with permanent positive charge could significantly absorbed the negatively-charged AuNPs via the electrostatic forces. The composite film made of conducting polythiophene and 5-mC-specific antibody was electrochemically polymerized on the SPCEs. The target sequence was then captured by hybridization with the Fe₃O₄/TMC/AuNPs-labeled DNA probe, and electrochemical detection was then carried out when methylated DNA interacted with anti-5mC on the electrode surface.

4. Conclusions and perspectives

We have reviewed the current electrochemical strategies for DNA methylation analysis. We have also pointed out the methodological shortcomings of these strategies. The diagnostic and prognostic significance of 5mC have also been discussed. We also discussed the direct detection of 5mC by either nucleobase oxidization or direct adsorption of targets onto the gold or graphene electrodes that could significantly simplify the assay protocol by avoiding the multistep surface functionalization process. It is now apparent that conventional electrode materials could be modified with various nanomaterials to achieve improved analytical performance of the electrochemical DNA methylation assays. It is also apparent that most methylation detection methods described in this review have been developed as proof-of-concept studies and are yet to be tested on real, heterogeneous clinical samples which are far more complex and difficult to analyse. However, the advent of the recent breakthroughs in electrochemical detection approaches for DNA methylation is a reflection of intense focus and endeavor of the researchers in this field.

Although electrochemical sensors could potentially be used as a point-of-care clinical tools for DNA methylation analysis, there are still significant challenges to be addressed before clinical translation. We believe that a fully automated electrochemical sensor that can work with no human intervention will be required for routine methylation analysis in clinics. Furthermore, innovative and real-time sampling strategies are needed to avoid false negative bias (i.e., small volume of blood taken from a cancer patient may not carry the necessary methylation markers and will result in a false negative result). Despite the major challenges which remain to be addressed, we foresee that these proof-of-concept studies will be translated into both clinical and research platforms for analyzing DNA methylation in the near future.

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