Electrochemical Strategies for DNA Methylation Detection

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Abstract

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. DNA methylation plays 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, which range from classical molecular biology and optical read-out based approaches such as bisulfite sequencing, microarrays, quantitative real-time PCR, colorimetry, Raman spectroscopy to the more recent electrochemistry-based approaches. Among these, electrochemistry-based approaches offer sensitive, simple, specific, faster, and cost-effective analysis of DNA methylation. Additionally, electrochemistry based methods are highly amiable to miniaturization and have the ability of multiplexing. In recent years, several reviews have become available on DNA methylation strategies. However, to date, no comprehensive review solely discussed electrochemistry based DNA methylation detection strategies. Herein, we review the recent developments of electrochemistry based DNA methylation detection approaches. We also highlight the major technical and biological challenges involved in these strategies.

Keywords: DNA Methylation, Electrochemical Detection, Hypermethylation, Hypomethylation, Global Methylation, Regional Methylation.

1) Introduction

DNA methylation is the covalent addition of a methyl group to the fifth carbon of the cytosine base in the CpG dinucleotide of DNA (Bird, 2002). This important epigenetic alteration converts cytosine (C) to methylcytosine (5mC) keeping the original DNA sequences unchanged (Bird 2002; Tucker 2001). It 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 Gonzalgo, 1997; Mohandas et al., 1981; Robertson and Jones, 2000). Thus alteration in 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 Gonzalgo, 1997).

The high level of DNA methylation compared to its native DNA is known as hypermethylation. Early studies on DNA methylation have established a hypothesis that an abberant DNA hypermethylation of the promoter region of tumor suppressor gene can initiate cancer by silencing the tumor suppressor gene. It could also deactivate homeobox gene which results in abnormal morphological development, causing cancers (Ehrlich, 2002; Jones and Gonzalgo, 1997). However, hypomethylation (i.e. an aberrant methylation where methylation level is decreased compared to the level of methylation in native DNA), spanning over a large portion of the genome, was later found to be actively involved in cancer development (Ehrlich, 2009). DNA hypomethylation leads to abrupt mitotic recombination, reactivation and subsequent integration at random sites of genome leading to mutagenesis and genomic instability resulting in cancer (Ehrlich, 2002; Taleat et al., 2015). Due to these strong pathological and diagnostic role of DNA methylation, it becomes an important biomarker for understanding prognosis and diagnosis of diseases including cancers.

Over the past few decades, considerable amount of attention has been dedicated to develop effective methodologies to analyze DNA methylation for both research and diagnostic purpose (Herman et al., 1996; Singer-Sam et al., 1990; Taleat et al., 2015; Zhang et al., 2015b; Islam et al., 2017). The most widely used DNA methylation detection technique is bisulfite sequencing (Frommer et al., 1992). This techniques consists of bisulfite conversion of DNA followed by PCR amplification and sequencing of the target methylation site. A range of modified PCR and sequencing approaches have been adopted with bisulfite conversion based approaches for DNA methylation analysis, for instance; methylation specific PCR (MSPCR) (Herman et al., 1996), quantitative MS-PCR (Hibi et al., 2011), real-time PCR (BonDurant et al., , 2011), methylight (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) and reduced representation bisulfite sequencing (RRBS) (Meissner et al., 2005). 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). Despite high accurate detection of HPLC, HPCE and MS methods, large input DNA, longer analysis time, low sensitivity and sophisticated instruments used in HPLC, HPCE and MS methods limit their routine clinical uses. In recent years, other advanced detection technique 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., 2015c, 2016b), fluorescence based biosensor (Duan et al., 2010; Ma et al., 2015; Taleat et al., 2015; Wang et al., 2009), colorimetric assays (Ge et al., 2012; Wee et al., 2015a), surface plsmon resonance based assays (Carrascosa et al., 2014; Sina et al., 2014a) and quartz crystal

microbalance (QCM) assay (Taleat et al., 2015). All of these methods have their own advantages and disadvantages. In most cases, the inconvenience arises due to the multi-step time consuming analytical procedures, use of large input DNA, hazardous radiolabeling, expensive biological molecules (*e.g.*, antibodies) and other numerous reasons.

To overcome these major drawbacks of conventional methods for DNA methylation analysis, much attention has been given on developing new strategies based on electrochemistry and photoelectrochemistry (Labib et al., 2016; Taleat et al., 2015; Koo et al., 2014a; Sina et al., 2014b; Koo et al., 2014b; Dai et al., 2013; Dai et al., 2012; Sato et al., 2010). In comparison to the other detection approaches, electrochemistry based techniques are relatively simple, inexpensive, rapid, independent of radioactive substrates, selective and sensitive. Moreover, these techniques require low volume of sample. The electrochemical detection instrument is amenable with miniaturization *(e.g.,* a small potentiostat of few milimetres in size is commercially available), which is highly suitable for clinical 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). Shanmuganathanet et al. (2013) and Zhang et al. (2015b) comprehensively discussed conventional DNA methylation techniques, whereas Hernándezet al., (2013) and Islam et al., (2017) focused solely on the PCR based and optical readout based techniques. To the best of our knowledge, electrochemistry based techniques for DNA methylation analysis have not been comprehensively reviewed. Herein, we summarise the recent advances in cutting edge electrochemistry based methylation detection strategies and discuss the challenges involved in electrochemical methylation sensor along with our perspective on future progress in this field.

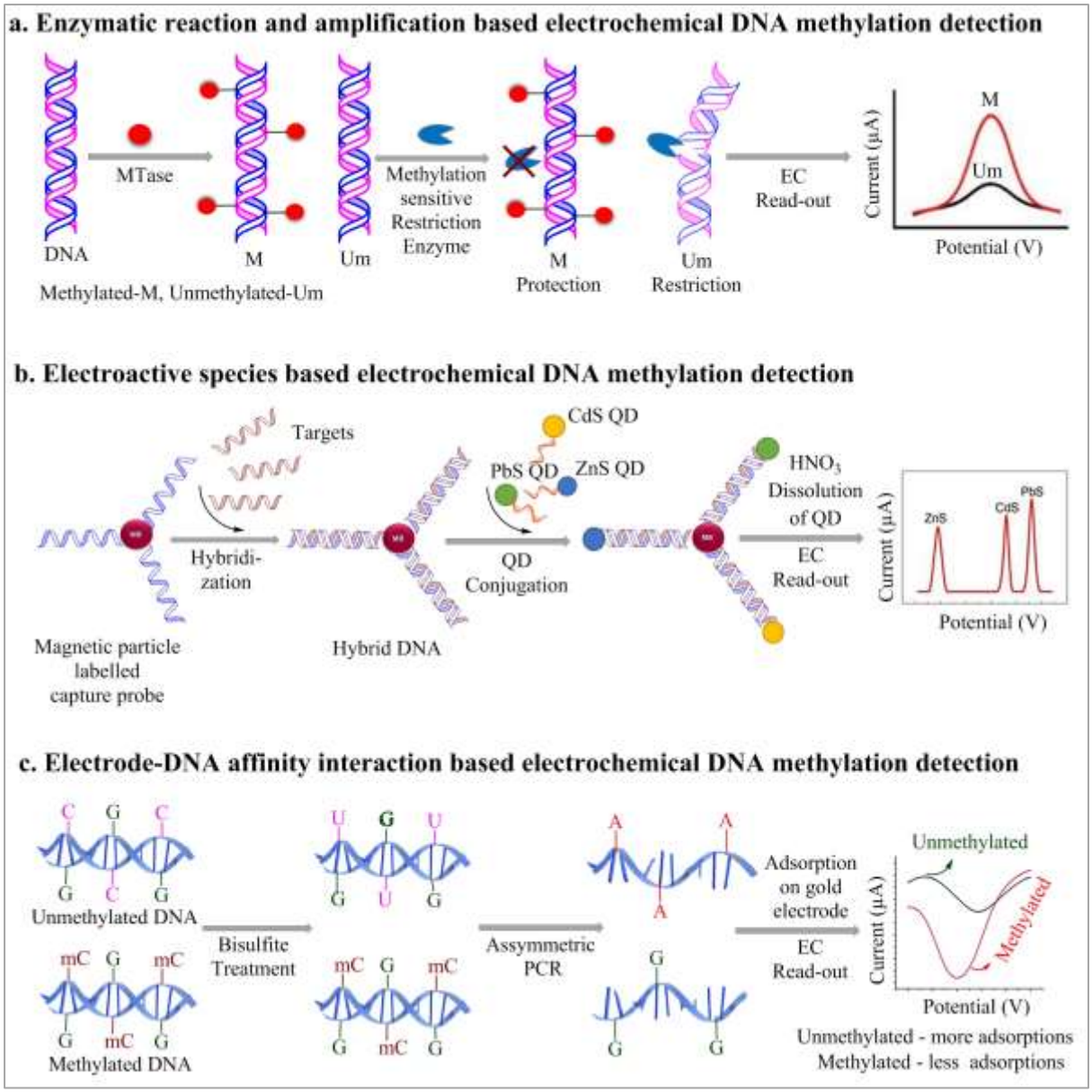

Fig. 1. *Schematic representation of different electrochemical stratagies for the detection of DNA methylation pattern.*

2) Diagnostic and prognostic significance of DNA methylation

As one of the most common epigenetic signalling tools, DNA methylation mainly occurs at the cytosine bases that have been used for regulating gene expression negatively (Phillips, 2008; 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 group in unmodified DNA, whereas during replication, Dnmt1 known as the

maintenance Dnmt, copy the methylation pattern onto the newly synthesized daughter strand from parental DNA (Fig. 1) (Moore et al., 2013).

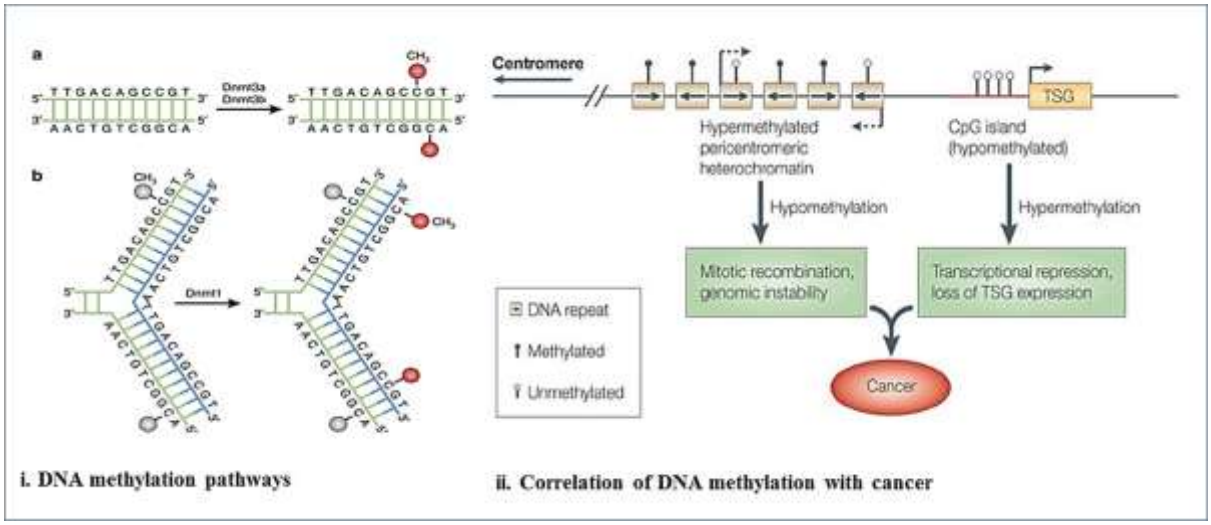

Fig. 2. *DNA methylation and its association with tumorgenesis. i) represent the transfer methyl group to naked DNA by Dnmt3a and Dnmt3b, and Dnmt1 uphold DNA methylation pattern during semiconservative replication (Moore et al., 2013). ii) represent the changes in methylation in early periods of tumorgenesis (Robertson, 2005)*

In 1983, Feinberg and Vogenstein, first showed the relationship between DNA methylation and cancer that the cancer cell genomes are hypomethylated compared to their respective normal cells (Feinberg and Vogelstein, 1983). Later on, Greger et al., (1989) showed that hypermethylation also occured at the 5' end of retinoblastoma gene in tumor which is usually unmethylated in normal cells. Further, Ohtani-Fujita et al., (1993) and Herman et al., (1994) correlated the methylation of the tumour-suppressor genes with their actual silencing in cancer. Fig. 2 represents a region of a normal cell and progression to becoming a tumor cell. In tumor cell, hypomethylation occurs at heterochromatin structure, contributes to genomic instability and the de novo methylation in CpG islands, resulting transcriptional silencing of growth-regulatory genes, which are very common early events in tumorgenesis (Robertson, 2005).

In the mammalian genomes, around 85% CpG dinucleotides are scattered, nearly 40% are in repetitive sequences. In a normal cell, these CpGs are largely hypermethylated reflecting that the genome is stable. On the other hand, these CpGs are hypomethylated in cancer cells, indicating a higher level genomic instability (Bird, 1992; Baylin and Ohm, 2006; Gaudet et al., 2003). The remaining 15% CpGs exist as clustered and form "CpG island". Around 40–50% of human genes have CpG islands, within or near to the promoter region which reflects that DNA methylation is involved in the controlling of the transcription of these gene (Zhu and Yao, 2009). Moore et al., (2008) showed that the hypomethylation state of circulating peripheral blood cells is tightly associated with an increase in risk of human bladder cancer, providing that such cancer can be early detected by using global hypomethylation state. Moreover, cancer genes are elevated with the promoter CpG islands containing genes and it has been associated with inactivation of tumor suppressor genes as well as oncogenic transformation. Thus, aberrant methylation of CpG islands of genes can play roles in tumor formation and progression and detection of such alternation in DNA methylation play significant roles in the detection of pre-malignant or early stage of disease (Montavon et al., 2012). Besides, aberrant DNA methylation provides a binary signal, where the presence of methylation indicates the presence of tumor cells (Barton et al., 2008). Caceres et al. (2004) showed that analysis of tumor-specific hypermethylation in serum DNA enhances the early detection of ovarian cancer. In this approach, hypermethylation is observed in the matched serum DNA of 41 of 50 patients (with 82% sensitivity) where 13 of 17 cases of stage I disease. In contrast, no hypermethylation was observed in normal samples derived from 40 control women (100% specificity). The study suggested that in ovarian cancer, promoter hypermethylation is a common and early event that can be detected in the serum DNA from patients with stage IA or B tumors (at early localized stage of cancer).

Apart from its diagnostics roles, DNA methylation has been reported to be a good indicator to track tumor prognosis. Milani et al., (2010) first reported the prognostic roles of DNA methylation profiling for childhood acute lymphoblastic leukaemia samples. In this study, the measurement of DNA methylation level of 401 patients and 1320 CpG sites helped to classify the patients into acute lymphoblastic leukaemia sub-types. In 2015, Chan et al., (2005) reported the positive prognostic potential of DNA methylation in cancer where it was shown that hypermethylation of 18S and 28S rDNA methylation levels were higher in patients with long progression-free survival versus patients with short survival. Thus, DNA methytlation has the potential to be used as therapeutic biomarker as well. For instance, Teodoridis et al., (2005) showed that methylation of the *BRCA1*, *GSTP1* and *MGMT* gene responsible for DNA repair/drug detoxification is associated with improved response to chemotherapy in late stage cancer patients with epithelial tumor.

3) Electrochemical detection of DNA methylation based on enzymatic reaction and amplification

3.1) Restriction enzyme digestion based assays

Recent demonstrations have shown that restriction digestion can be used in DNA methylation analysis by two ways. First, using methylation sensitive restriction enezymes (*e.g.*, HpaII and Not I) to selectively isolate 5mC followed by quantification *via* electrochemical readout (Dai et al., 2012). Second, using restriction enzymes to directly oxidize the target which can cut the single base allowing the detection of 5mC (Kato et al., 2011). In the first format, methylation sensitive restriction enezymes, as their name implies, only cleave the phosphate backbone at the second cytosine of unmethylated 5′-CCGG-3′ sequence (*i.e.*, methylation sensitive restriction enzyme cannot cleave 5mC residues) (Quint and Cedar, 1981). This was experimentally demonstrated by Hou et al. (2013), where regional methylation at *p16Ink4a*

tumor suppressor gene was quantified. In this method, genomic DNA was first treated with restriction enzyme MseI, which resulted in DNA fragments with cleaved end. These cleaved ends were then ligated with unphosphorlylated linkers (which act as universal PCR primers). The ligated DNA was further digested with methylation sensitive restriction endonuclease BstUI. The digested products were amplified by PCR and put on the working gold electrode (modified with oligonucleotide capture probe complementary to the target sequence). A fast readout for methylation was observed from the cyclic voltammogram (CV) and square wave voltammogram (SWV) readings in the presence of a $[Co(phen)_3](CIO_4)_3$ intercalators.

Later, an enhanced digestion based electrochemical method for detecting methylation was proposed which avoided cumbersome bisuflite sequencing and PCR amplification (Dai et al., 2012). In this assay, gold electrode was fabricated with oligonucleotide (ssDNA) capture probe and an electrochemically active indicator methylene blue (MB) was used to enhance electrochemical response. Following the addition of target DNA sample on the electrode, both methylated and unmethylated DNA were hybridized with the capture probe forming dsDNA. In MB solution, these dsDNA produced less current compared to that of the ssDNA modified electrode (as less MB was adsorbed in dsDNA due to the less interaction between guanine residues and MB compared to that of ssDNA). Next, a restriction enzyme HpaII (methylation sensitive endonuclease which only cleave unmethylated region leaving 5mC strand unaffected) was added which precisely cleaved the unmethylated DNA. This resulted in a partial loss of intercalated MB present in the unmethylated dsDNA sample which results in a reduction of the current in CV compared to that of methylated counterpart (Fig. 3). By measuring the relative current changes, the degree of DNA methylation was thus obtained.

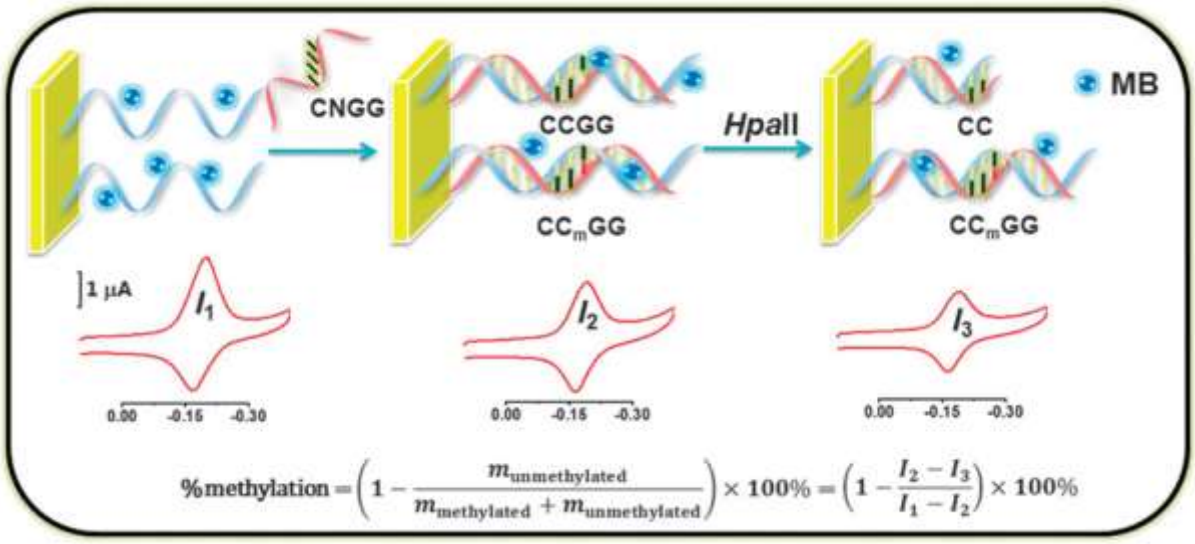

Fig 3. *Restriction enzyme based (HpaII endonuclease) gene specific methylation detection (Dai et al. 2012)*

One considerable drawback of digesting large CpG oligonucleotides by restriction enzymes for methylation analysis is the resultant false positive oxidative response. Because, in longer DNA sequence, the electrochemical reaction suffers from hindrances due to the relatively large distance between each base and electrode surface and hence sensitivity decreases many fold (Ivandini et al., 2003; Kato et al., 2011). This issue can be avoided by another type of restriction digestion based approach developed by Kato et al., (2011), where individual bases of a target DNA is cut by restriction enzyme and the direct oxidation of each base was measured to quantify 5mC. In this method, a long CpG oligonucleotide was treated with restriction endonuclease P1, which cut the single base and formed an identical mononucleotide 50-dNMP (20-deoxy-ribonucleoside-50-monophosphate). The direct oxidation of both 5-mC and C of this mononucleotide was measured on a sputtered nanocarbon film electrode from their SWVs. In comparison to the other P1 digestion free sample, this method showed 4.4 times better result in terms of sensitivity and wider concentration range. This label free technique has the potential in point of care applications, because it only requires

simple digestion of sample DNA followed by direct electrochemical detection on a device that can easily be manufactured at low cost.

3.2) DNA MTase activity based assays

Besides the direct detection of 5mC, measuring the activity of DNA methyltransferase enzyme (DNA MTase, catalyzed the mammalian genomic DNA methylation) could provide an indirect assessment of methylation pattern in a target DNA. This is because, DNA MTase activity is diretly related to DNA methylation pattern. An over-expression of DNA MTase triggers aberrant DNA methylation, that facilitates the tumor progression through the inactivation of CpG island methylation mediated gene (J.Issa et al., 1993; Vertino et al., 1996; Robertson, 2001). For instance, MTase level is higher in tumorigenic cell line rather than that of non-tumorgenic cell lines and colon tumorgenesis is also accompanied by the increased expression of this enzyme (J.Issa et al., 1993). Therefore, DNA methylation can be analyzed via assaying DNA MTase activity. In recent years, such assays have been studied by several groups. Liu et al., (2011) developed an electrochemical assay for the detection of DNA methylation and the activity of MTase enzyme at specific CpG methylation sites. In this method, an electroactive labeller ferrocene acetic acid (FcA) was conjugated to hybrid DNA. The labeled DNA was then methylated by methyltransferase M.*Ss*I. After that, the restriction enzyme HpaII endonuclease cleaved the portion of unmethylated double stranded DNA (the endonuclease identifies symmetrical sequence 5´-CCGG-3´ sites) between adjacent cytosines. Hence, the voltammetric signal of FcA disappeared (or decreased). On the other hand, CpG methylation sites block this cleavage of endonuclease (i.e., the endonuclease did not act on the methylated sequence) and thereby retain the signal of FcA, which indicates the level of methylation and MTase activity.This method is helpful for genomic DNA methylation analysis but sensitivity

was low compared to other reported methyltransferase assays which is also limited by the use of FcA labeling.

Muren et al., (2013) developed an innovative assay based on signal-on strategy with a multiplexed platform for detecting both human (Dnmt 1) and bacterial (Sssl) methyltransferase activity. In this assay, a multiplexed chip consisting of sixteen electrodes was taken and divided into four quadrates. Each quadrate was modified with different DNA substrates (containing methylation site 5′-CG-3′ of humans) and treated with a unique MTase enzyme. And each electrode was also combined with a covalent redox probe. The treatment of electrodes with MTase (active form) made the sites (5′-CG-3′) methylated. The methylated CpG sites protect the DNA from cutting by following restriction enzyme treatment. Hence the redox signal from the probe (as DNA was intact and contained redox probe) was retained that is signal-on. While without the activity of MTase (due to the presence of inactive form of MTase), unmethylated DNA was easily cut down by restriction enzyme and separated the redox probe, resulting in the signal-off state by the near complete disappearance of redox signal. This method is able to compare up to four types of different DNA methylations side by side and four MTase activities on the same surface (Fig. 4. i).

Over the last few years additional analysis has also been done for designing a potent and powerful tool for the detection of DNA methylation and activity of MTase assay, which is suitable in clinical diagnosis, disease prognosis and drug development. For instance, He at al., (2011) developed a label-free signal-on assay using gold nanoparticle amplification (AuNPs). Wang et el. (2012) developed a novel method based on HRP-IgG conjugated catalysis. Baek et al., (2013) developed a one-step assay based on the transport of a CdS/ZnS-Qd signaling tracer. Xu et al., (2013) developed a method based on methylation sensitive endonucleases, DNA-Au bio bar code and enzymatic signal amplification. Wang et al., (2015b) developed a homogenous assay based on methylation responsive exonuclease III-assisted signal

amplification. In order to enhance sensitivity and clinical application, recently scientists employed a mimic-hybridization chain reaction (mimic-HCR) for sensitive electrochemical detection of MTase activity to determine the level of DNA methylation. In general, a mimic-HCR is a room temperature, enzyme free amplification approach. For example, a mimic-HCR method has been reported using spherical nucleic acid gold nanoparticles as both primary amplification units and inhibitors of HCR (Wang et al., 2015c). Later, a more sensitive assay combining mimic-HCR strategy with sandwich-typed (DNA-AuNPs on the electrode surface) assay using RuHex as an indicator was also developed (Zhang et al., 2016). Very recently, Hong et al., (2016) developed an (signal-on) assay with easy, self assembling of DNA–AuNPs with two complementary AuNPs modified single-stranded DNA by the linkage of benzanedithiol bridge. Due to the thiol bridge no incubation was required for hybridization. Here, after the treatment of MTase, the methylated DNA-AUNPs were methylated and became restricted to digestion (by restriction endonucleases MboI). Thus, it provided inserting sites of a large amount of MB (phenothiazine dye) molecules, hence a high electrochemical signal was achieved. While without MTase treatment, DNA hybrid was unmethylated and was digested by MboI, allowed adsorption of very few MB molecules on electrode surface. This gave a comparatively low electrochemical signal.

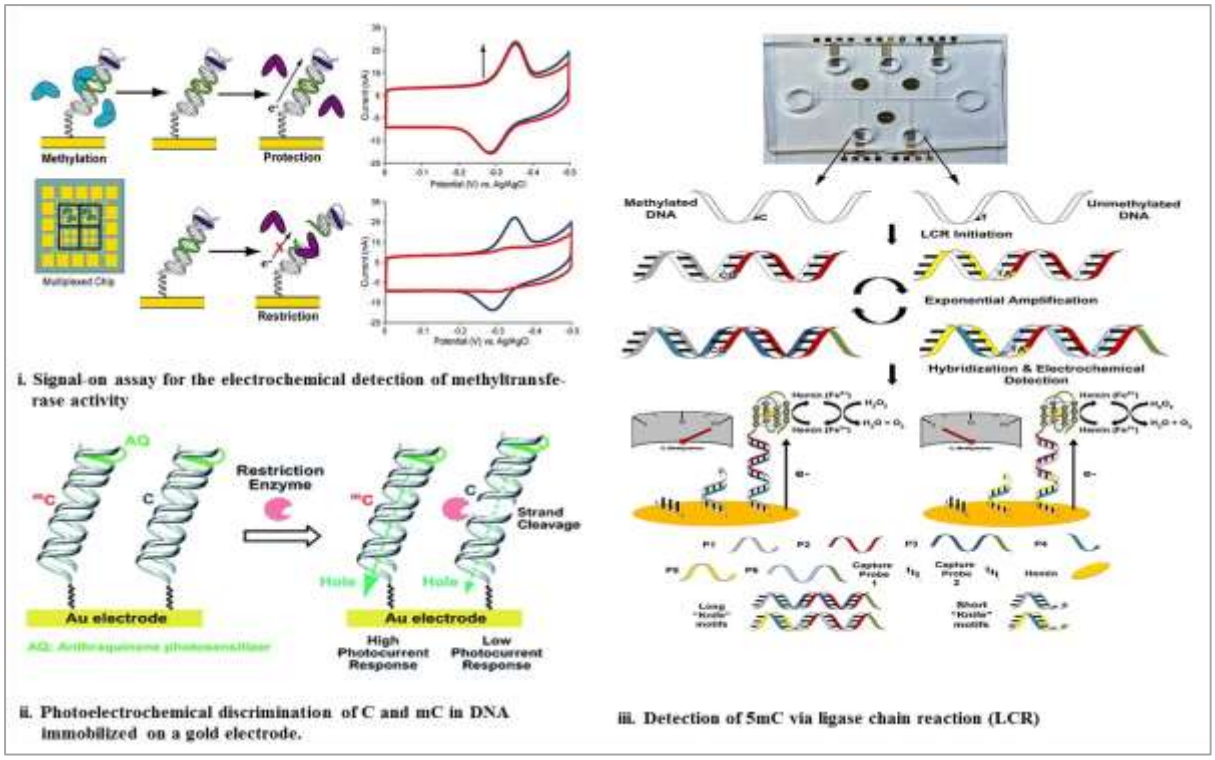

Fig. 4. *Enzymatic and amplification based electrochemical DNA methylation detection; i. Signal-on assay, where unmethylated DNA and methylated DNA produced two distinct redox signals in CV resulting in the activity analysis of MTase (as the different pattern of MTase activity is directly related to the distinct current changes in CV) (Muren et al., 2013); ii. Representation of photoelectrochemical discrimination of C and mC in DNA immobilized on a gold electrode (Yamada et al., 2008); and iii. representation of a Biosensor for detection of 5mC via ligase chain reaction followed by electrochemical readout (Kevin et al., 2014).*

3.3) Ligase chain reaction (LCR) based strategies

In LCR based electrochemical detection of DNA methylation, the LCR amplification adopted two adjacent probes positioned in such a way, that it can discriminate the methyl cytosine at single base. Generally, the readout part of target LCR products have been obtained by optical methods or mass spectrometry which are limited by high running cost and complex instrumentation of the sensing method. In 2012, for the first time, Wee and colleagues coupled

LCR with simpler and inexpensive electrochemical readout method (referred to as eLCR) to detect single base mismatch (Wee et al., 2012). Later, Koo et al., (2014) extended this method to detect 5mC in a microdevice based electrochemical assay (Fig. 4. iii). In this method, bisulfite converted and PCR amplified DNA target (where, C: methylated, T: unmethylated) was further amplified by LCR with six probes to analyze the methylated DNA. This exponential LCR amplification produced two sets of ligated long knife motifs (One representing 5mC and the other represents the unmethylated DNA). In contrast, the unligated probes produced short knife motifs. Next, DNAzyme-mediated electrocatalytic reduction of hydrogen peroxide (H_2O_2) at electrodes surfaces resulted in the relative level of methylation at a particular CpG site. The applicability of this assay was tested in breast cancer cell lines and serum sample.

Similarly, in 2015, Wee et al., (2015b) developed a LCR based electrochemical method referred as 'epiQ' which could quantify heterogeneous methylation of epialleles DNA strands (epialleles are the DNA strands containing a mixture of methylated, unmethylated and partially methylated regions). Previously, NGS and digital PCR were commonly used to quantify this epialleles which are limited by the high running cost and several multistep procedures. However, epiQ uniquely offers an alternative to NGS and digital PCR to quantify heterogenous methylation of genomic DNA. In this approach, electrochemical microdevice was fabricated using photolithography. Gold electrodes surface of the microdevice was functionalized with capture probe via gold thiol self-assembling monolayer formation. Following LCR, biotin labelled oligonucleotides (specific for respective epiallele sequence) were added with the LCR products in different wells of the microdevice. After the addition of horse radish peroxidase (HRP) in each well, electrocatalaytic reduction of H_2O_2 on the modified gold microelectrode resulted in different CV response for different epialleles enabling the quantification of heterogeneous DNA methylation.

3.4) Photoelectrochemical-biosensing of DNA methylation

Generally, in photochemical approach, the sensor consists of excitation source (irradiation light, a monochromator, and a chopper), a cell, and an electrochemical station. Upon illumination, charge transfer of the photoactive species resulted in electric signal between the working electrode and the counter electrode in electrolyte solution (Wu et al., 2013; Zhao et al., 2014). Due to the direct electrical readings in photoelectrochemical reactions, sensitive detection of DNA methylation can be obtained without the requirement of an expensive signal transduction instrument. In 2008, a novel photoelectrochemical based strategy was developed to differentiate between cytosine and 5mC using enzymatic digestion. In this method, first a photosensitizer-linked DNA duplex carrying the target region was immobilized on the gold electrodes. This duplex DNA was then treated with specific restriction enzyme capable of cleaving cytosine region (i.e., will not cleave mC region) of the target. Following restriction digestion, the methylated duplex showed increased response (as duplex DNA is unaffected by restriction enzyme) whereas unmethylated DNA resulted in reduced photocurrent density (due to the restriction digestion of the duplex DNA strands) (Fig. 7) (Yamada et al., 2008).

Another recently reported immunosensor offer the quantification of DNA methylation *via* measuring photoelectrochemical current where DNA methylation recognition protein MBD1 was tagged with anti-his-tag antibody to detect targeted CpG region. Bi_2S_3 nanorods and AuNPs modified electrodes were used as photoelectric conversion material. MBD attached to the CpG region of the sample for their strong affinity towards CpG. Then anti-his antibody was added, which attached with 'anti-his-tag antibody' tagged MBD1. When methylation is present, anti-his-MBD immunocomplex produced decreased current on the electrode due to blocking of the surface. On the contrary, if the sample is unmethlated, MBD1 does not bind to the CpG, therefore, cannot form any immunocomplex giving negative response. This sensitive

method has high detection specificity and it could even distinguish a single-base mismatched sequence (Yin et al., 2014).

4. Electrochemical detection of DNA methylation based on electroactive species

4.1) Methods based on semiconductors quantum dots (QDs)

QD is an inorganic, semiconductor nanocrystal, obtained from the periodic groups II–VI, III–V, or IV–VI, having a typical size of 1 to 20nm. The opto-electrical characteristics, biocompatibility, sharp and well-resolvevoltametric signals, make QD's as a potential candidate for electrochemical labelling for getting amplified signal of DNA methylation (Cui et al., 2007; Amelia et al., 2012). Moreover, the well separated, intense strpping peaks of QD core ions (e.g. Cd, Zn, Pb ion) specify the existence of one target and render the opportunity of multiplexing (Zhang and Wang, 2012). DNA methylation detection assays based on QD are generally carried out by using 5mC specific capture probe modified with QDs. This QD-containing probes hybridize with methylated DNA whereas unmethylated DNA do not hybridize with the capture probe. Upon addition of nitric acid in the reaction mixture, dissolved QDs from hybridized target release the metallic core which produce distinct electrochemical readout. In absence of DNA methylation, QD is not present in the target analyte thereby a sharp fall in the electrochemical signal is observed (Xu et al., 2016).

For instance, Dai et al., (2013) developed QD based electrochemical readout method referred to as *EmsLDR* (methylation-specific ligation-detection reaction) for the simultaneous and multiplexed quantification of DNA methylation. In *EmsLDR*, fragments of p53 gene with two 5mC loci were selected as target, and 5mC specific four probes were designed and functionalized with QDs (i.e., probe 1 and probe 2 were labeled with PbS and CdS QDs to form PbS–P1 and CdS–P2 conjugates, respectively, and probe 3 and probe 4 were functionalized on

magnetic beads to produce the magnetic bead–P3P4 conjugate). First, bisulfite converted target DNA (p53 gene fragments) was treated with the magnetic bead–P3P4 conjugate which resulted in the hybridization of targets with magnetic beads. After another incubation of magnetic beads with the PbS–P1 and the CdS–P2 probes, magnetic beads were added to the E. coli DNA ligase to initiate the LDR (ligation detection reaction) followed by a heating steps. If the target contains 5mC, the probes covalently interlink to the target forming a stable duplex and ligase chain reaction occurs. Upon heating only the ligated probes with their QD tags remain on the magnetic beads. When sample is unmethylated, the probes show single-base mismatch and cannot be ligated. By perfoming voltametric analysis of dissolved QDs, the level of methylation can be quantified from square wave voltamogram (Fig. 6). Alhough EmsLDR avoids PCR amplification or restriction enzyme digestion, it is limited by several hybridization and heating steps along with longer assay time. To avoid these drawbacks, more recently, Xu et al., (2016) developed a method for quantifying methylation using QD barcodes. In this method, the targets were designed in a similar way of *EmsLDR;* however, hairpin probes (HP) were used instead of linear probes. Due to the use of HP, 5mC region of the target selectively matched the loop of the corresponding HP resulting in the exposure of loops of HP which bound to the target. After that, the HP with its QDs tag was selectively hybridized onto magnetic bead-capture probe conjugate. On the other hand, when target was unmethylated, HP's loop could not open causing no hybridization among target and magnetic bead-capture probe conjugates. This binding event resulted in SWV responses and thus the methylation level of the target p53 gene fragment was quantified.

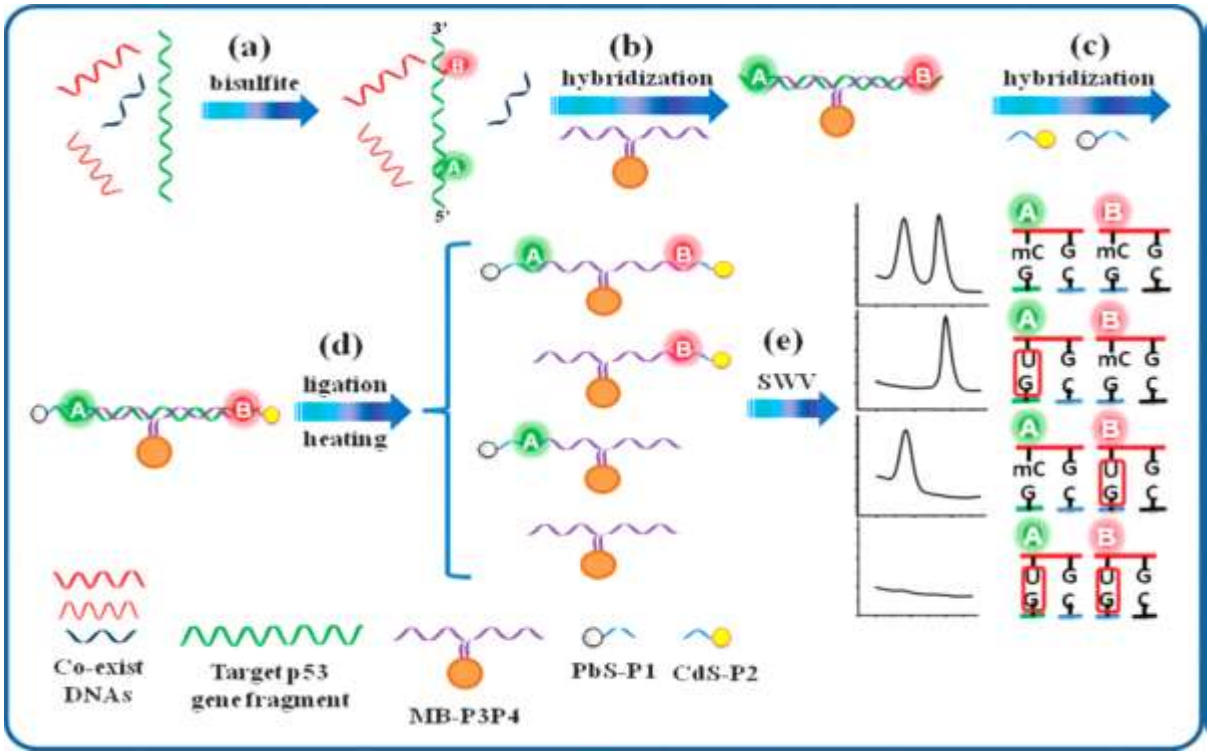

Fig. 5. *Schematic diagram of EmsLDR (Dai et al., 2013)*

4.2) Electrochemically active ligands based methylation analysis

Electrochemically active ligands/species (e.g., MB, F4ND etc.) form a stable non-covalent complex with methylated dsDNA and enhances the electrochemical signal. Over the last few years, a number of electrochemically active ligands (e.g, methylene blue, triphenylmethane, etc.) have been used for targetting DNA in various detection assays, which offer better stability and sensitivity (Dai et al., 2012; Sato et al., 2010). For example, ferrocenyl-naphthalene-diimide (F4ND) has been used to assess DNA methylation in an electrochemical assay that intercalates the double stranded DNA(dsDNA) targets (Sato et al., 2010). Adaptation of FND (contains 4 ferrocene moieties) in this assay improves the electrochemical response by stabilizing the dsDNA. First, the electrode surface was fabricated with a methylated specific probe. Bisulfite treated target DNA was then amplified by asymmetric PCR. Amplified products were put on a gold electrode modified with 5mC specific probe. Methylated DNA

thus formed a strong double stranded complex on the surface of the electrode and due to the F4ND ligand it resulted in higher current in SWV. On the contrary, unmethylated DNA could not hybridize with capture probe resulting in less electrochemical readings. This allows the precise discrimination of DNA methylation. This method was successfully trialed to analyze methylation level of *CDH4* gene (a marker gene responsible for progression of colon cancer).

Barton and co-workers have extensively used this indicator (e,g. methylene blue) for the analysis of various types of DNA targets including DNA point mutation, DNA lesion etc. (Boon et al., 2000; Kelley et al., 1999). Generally, MB attaches either intercalatively to the hybridized dsDNA or covalently to the reporter probes. Using this uniqe ability of MB, in recent years, several electrochemical methods have been reported that use methylene blue as a redox indicator for quantifying DNA methylation (Dai et al., 2012; Hou et al., 2003; Jing et al., 2014). For example, in a recent method developed by Jing et al., 2014, both 5mC and methyltransferase were analyzed. First, the thiolated single-stranded DNA S1 was assembled on gold electrode. Then lower part of DNA S1 was hybridized with DNA S2 (complementary to DNA S1) to form the double-stranded target. On the other hand, upper part of DNA S1 was hybridized with its complementary DNA S3-modified gold nanoparticles (DNA S3-AuNPs) which formed DNA S1/S2/S3- AuNP conjugate. This conjugate contains huge number of DNA molecules which can adsorb ample amount of methylene blue. After that, methylation-resistant endonuclease Mbo I was introduced in the method (Mbo I cannot exert action in the presence of 5mC). Thus, if the target sequence is methylated, Mbo I cannot cleave S1/S2/S3-AuNP conjugate causing high methylene blue adsorption. On the contrary, unmethylated DNA could easily be cleaved by Mbo I resulting in decreased DPV response allowing quantification of DNA methylation.

5. Electrochemical detection of DNA methylation based on unmodified and modified electrodes

5.1) Gold-DNA affinity interaction based method (eMethylsorb)

The adsorption of native DNA sequences on an unmodified gold surface has been regarded as 'complex', 'non-specific' and 'difficult to control'. Mirkin and Rothberg groups have extensively studied this adsorption pattern (Demers et al., 2000; Demers et al., 2002; Kimura-Suda et al., 2003; Ostblom et al., 2005; Li and Rothberg, 2004) and reported that adsorption affinity of DNA bases towards unmodified gold surface shows a definite trend (A > C > G >T). Though, only a few number of methylation detection assays using gold-DNA affinity interaction have been reported (Brotons et al., 2016; Brotons et al., 2015; Koo et al., 2015, Sina et al., , 2014), only Sina et al., (2014) reported the 5mC quanitfication assay which is based on this recognised nucleobases adsorption affinity trend of A > C > G >T towards unmodified gold surface. In this method referred to as *eMethylsorb*, (Fig. 8) DNA was amplified via asymmetric PCR after bisulfite treatment of extracted DNA, . Since DNA–gold affinity interaction follows the trend A > C> G > T, the adenine-enriched unmethylated DNA leads to a larger level of adsorbed DNA on the electrode surface in comparison to the guanine-enriched methylated DNA (Fig. 5. i). The adsorbed bases showed correlation with the Faradaic signal generated by the $[Fe(CN)_6]^{3-/4-}$ redox system . This rapid and sensitive detection technique can detect methylation at a single CpG resolution. Unlike many other lab based conventional assays, *eMethylsorb* avoid typical recognition and transduction layers inside the chip, which extensively simplified the method.

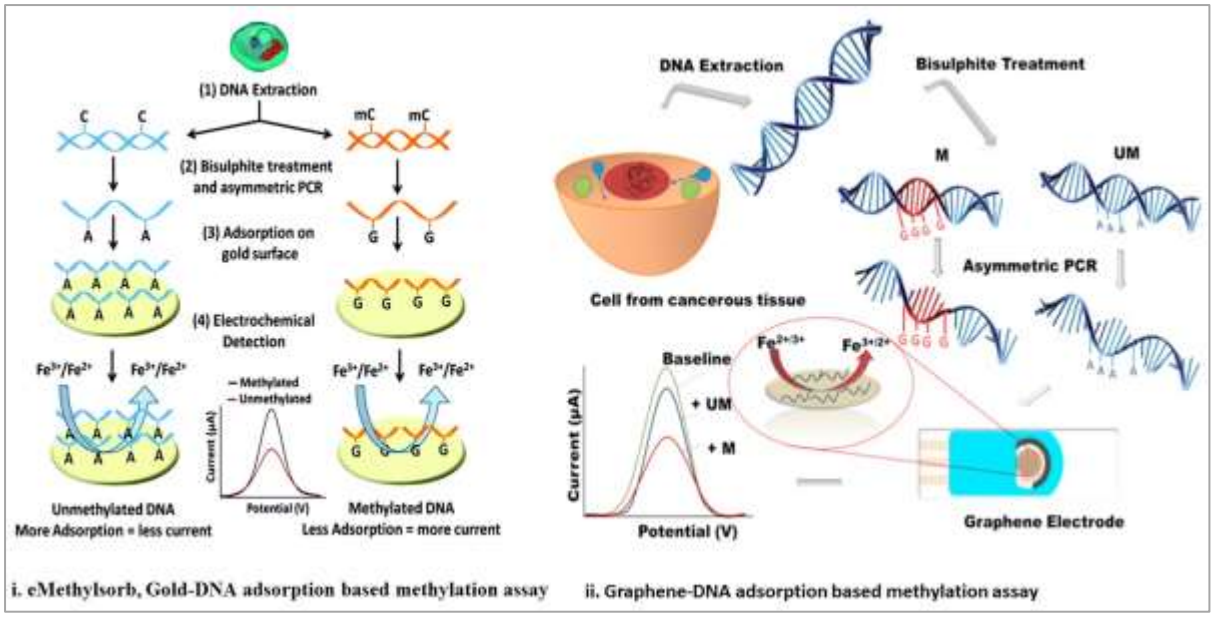

Fig. 6. Detection of DNA methylation by direct adsorption on electrode surface; i) *eMethylsorb, unmethylated portion produced less faradaic current due to more adsorption on the electrode surface compared to methylated counterpart (Sina et al.); ii) Schematic representation of graphene-DNA adsorption based methylation assay. Distinct pulse voltammetric signals showing the guanine-enriched methylated DNA that produces lower DPV currents in comparison to the adenine-enriched unmethylated DNA (Haque et al., 2016).*

5.2) Graphene-DNA affinity interaction based assays

Similar to the affinity of DNA towards gold surface, the DNA bases show a great affinity towards the graphene and graphene oxide surface (Wu et al., , 2011; Park et al., , 2014). Recent studies showed that this affinity is due to the physisorption between the individual nucleobases and graphene surface (controlled by the polarizabilities of each nucleobases which is directly proportional to van-der-Walls force). Gowtham et al. (2007) and Varghese et al. (2009) reported that nucleobases of DNA follows the adsorption trend as G > A > T > C towards graphene surface. Very recently for the first time, this definite trend of nucleobases towards graphene has been used to quantify 5mC in a simple, inexpensive and direct electrochemical method developed by Haque et al. (2016). In this method, bisulfite treated and PCR amplified DNA sequences are directly adsorbed on the graphene modified screen printed electrode (g-SPCE). Bisulfite-treated guanine-enriched methylated DNA leads to a larger amount of the adsorption on to the g-SPCE in comparison to the adenine-enriched unmethylated DNA. Therefore, methylated DNA will give less electrochemical readout (due to more adsorption of guanine on graphene modified electrode) compared to unmethylated DNA. The level of the methylation is thus quantified by monitoring the DPV current as a function of the adsorbed DNA in presence of $[Fe(CN)6]^{-3/-4}$ redox system (Fig. 6. ii). This highly sensitive approach could distinguish as low as 5% of methylation changes at single CpG resolution. This method is also successfully tested in a panel of cancer cell lines and patient samples derived from oesophageal squamous cell carcinoma to detect *FAM134B* promoter gene methylation.

5.3) Methylation assay using different modified electrodes

More recently, there has been an increasing interest on using carbon based working electrodes in the electrochemical assay for detecting DNA methylation. Several studies have been carried out to detect single 5mC on nanocarbon-film electrode by optimizing the assay condition and

surface modification of the electrode (Goto et al., 2010; Kato et al., 2008; Yanagisawa et al., 2015). Assay performance of nanocarbon-film electrode based strategies highly rely on the physical properties of the electrodes and their functionalization. A number of electrochemical assays for methylation analysis also used conventional glassy carbon electrodes (GCEs)(Wang et al., 2016a; Zhu et al., 2015). Despite having lower oxidation rate, GCEs are chemically inert because of their high density and low porosity. Very recently, Wang et al., (2016) developed a DNA methylation assay using direct oxidation of DNA bases. In this method, GCE was modified with a multiwalled carbon nanotube-β-cyclodextrin (MWCNT-β-CD) composite which provided larger surface area, wide potential window and high stability, thereby resulted in increased oxidation current (Wang et al., 2016a). Upon addition of the DNA sample, the oxidation behavior of individual bases on the electrode surface were measured from the DPV peak, the potential difference between cytosine and 5mC was found to be 130 mV which was large enough for simultaneous detection of 5mC. This method was tested in fish sperm to detect DNA methylation where it simultaneously detected 47 percent of cytosine methylation in less than one hour using direct oxidation of DNA bases on glassy carbon electrodes. For methylation detection, glassy carbon electrodes were also modified with polypyrrole (PPyox) directed multiwalled carbon nanotubes (MWNTs) (Wang et al., 2013) and electro-copolymerisation (ECP) (Zhu et al., 2015).

In 2016, another sensitive electrochemical nanobiosensor for quantifying 5mC of RASSF1A tumor suppressor gene has been developed (Daneshpour et al., 2016). Different types of polymers were incorporated in this electrochemical sensor to modify the electrode which significantly improved the methylation detection limit (femtomolar level). Screen printed carbon electrodes (SPCE) were modified with Fe_3O_4/N-trimethyl chitosan/gold (Fe_3O_4/TMC/Au) nanocomposite and polythiophene (PT). PT is a polymer which helps immobilization of biomolecules resulting in high sensitivity of the assay whereas

Fe_3O_4/TMC/Au nanocomposite work as a tracing label of the DNA probe, which increases the electrochemical readout by several fold due to the presence of AuNPs. Besides, anti 5mC antibody was attached with the PT film which allowed the differentiation of 5mC from cytosine.

Another electrochemical method based on direct oxidation of DNA bases for simultaneous detection of cytosine and 5-mC were reported recently (Wang et al., 2015a). This electrochemical sensor has a polypyrrole functionalized graphene nanowall as the working surface. As mass transport properties of the cytosine and 5mC which changed between planar diffusion and thin layer diffusion resulting in oxidation peaks, 5mC and cytosine showed a large potential difference (*i.e.*, 184mV).

6) Conclusion and perspective

We have thoroughly reviewed here the available electrochemistry based techniques for DNA methylation analysis. We have also pointed out the methodological shortcomings of these strategies. Though electrochemical sensors have a huge potential to be used as a point of care tools in clinics for DNA methylation analysis, significant improvements are still required before they can be considered for routine applications in clinics. For instance, 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 methylation marker resulting in a false negative result).

Most of the methylation detection methods described here also have been developed as a proof of concept study. It is yet to be seen how they perform in real and heterogeneous clinical samples which are far more complex and unpredictable. This is why, electrochemical sensors with simultaneous multiplexing ability and high selectivity are highly amenable. Extensive focus should be given to avoid any possible cross reaction that might occur between target and ligand while simultaneously detecting DNA methylation in a multiplexed platform. We believe

that a fully automated electrochemical sensor that can work with no human intervention is required for their routine methylation analysis studies in clinics. The advent of recent breakthroughs in electrochemical detection approaches for DNA methylation is a reflection of intense focus and endeavor of the researchers in this field. Though there are major challenges which need to be addressed, we foresee that in near future these proof of concept studies of electrochemical sensors will be translated in both clinical and research platforms for analyzing DNA methylation.

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