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Towards DNA methylation detection using biosensors

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DNA methylation, a stable and heritable covalent modification which mostly occurs in the context of a CpG dinucleotide, has great potential as a biomarker to detect disease, provide prognoses and predict therapeutic responses. It can be detected in a quantitative manner by many different approaches both genome-wide and at specific gene loci, in various biological fluids such as urine, plasma, and serum, which can be obtained without invasive procedures. The current, classical methods are effective in studying DNA methylation patterns, however, for the most part; they have major drawbacks such as expensive instruments, complicated and time consuming protocols as well as relatively low sensitivity, and high false positive rates. To overcome these obstacles, great efforts have been made toward the development of reliable sensor devices to solve these limitations, providing sensitive, fast and cost-effective measurements. The use of biosensors for DNA methylation biomarkers has increased in recent years, because they are portable, simple, rapid, and inexpensive which offers a straightforward way to detect methylated biomarkers. In this review, we give an overview of the conventional techniques for the detection of DNA methylation and then will focus on recent advances in biosensor based methylation detection that eliminate bisulfite conversion and PCR amplification.

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1. Introduction

Epigenetics, which literally means "above genetics" refers to heritable changes in gene expression that arise from changes in chromosomes without alteration of DNA sequence.[1,2] Epigenetic mechanisms of gene regulation include DNA methylation, covalent modifications of histone tails, and nucleosome positioning[3,4] as well as messenger and noncoding RNAs (Fig. 1).[5,6] These modifications can affect chromatin structure and contribute to the major molecular processes including gene and microRNA expression, DNA–protein interactions, cellular differentiation silencing of X chromosome and genomic imprinting.[3,7]

DNA methylation, a covalent modification of cytosines, is one of the most extensively studied modifications in the field

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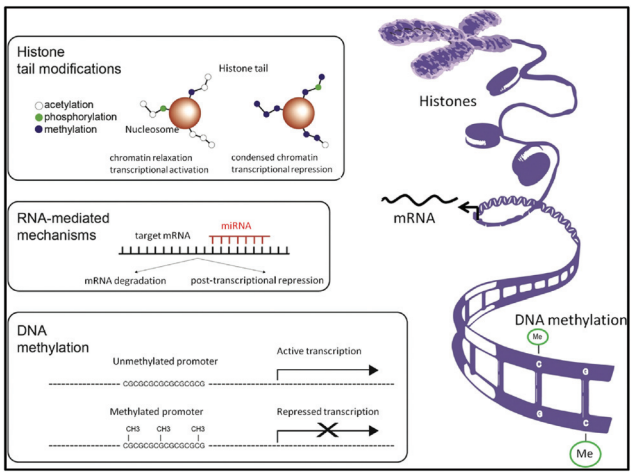

Fig. 1 Epigenetic mechanisms involved in regulation of gene expression. Reprinted with permission from ref. 4.

of epigenetics, and provides a molecular mechanism by which gene expression can be modified. It has been shown to enable transmission and perpetuation of epigenetic information through DNA replication and cell division.[8] DNA methylation is associated with a broad range of biological events including X chromosome inactivation, genomic imprinting,[9] stem cell differentiation,[10] control of tissue-specific gene expression and chromosome stability.[11] More importantly, aberrations in DNA methylation are common contributors to certain genetic diseases and tumors, such as Prader–Willi syndrome,[11] neurological[12] and metabolic disorders,[13] prostate,[14] breast,[15] gastric,[16] and bladder cancers.[17] Alterations in the methylation status of DNA are promising candidates for a new paradigm of disease intervention and treatment. Therefore, analysis and accurate quantification of DNA methylation through profiling could provide one of the most promising tools for the clinical diagnosis and prognosis of human disease and prediction of drug response.[18] Despite the fact that many technologies have been developed in the past decade to detect DNA methylation, these analytical methods present several challenges, including high cost, great technical complexity, time-intensive and laborious sample pretreatment protocols, all of which hinder the clinical translation of conventional methods into routine practice.[19] In recent years, there has been increasing attention on the development of new biodetection methods which will lead to highly sensitive, fast, selective, low power, cost-effective, miniature and portable biosensors with significant advantages over classical assays.[20,21] These biosensor technologies must provide more rapid, easier to use and cost effective diagnostic devices in order to address the market requirements not met by conventional systems.

Several reviews of the applications of conventional methods for DNA methylation analysis have been published,[22–25] but there are no summary reports about biosensors for DNA methylation detection. Therefore, this review provides an overview of the available conventional methods and then focuses on recent progress in biosensors based on nanomaterials for the detection of DNA methylation markers. Finally, future opportunities and challenges in this field will be discussed.

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Dr Michael Norton received his Ph.D. in Solid State Chemistry from Arizona State University in 1982, working in the area of two-dimensional materials. As an NRC postdoctoral fellow, he worked briefly in the area of oxide materials at the Naval Weapons Center at China Lake, CA, USA. In his career at Marshall University, his studies have focused on soft matter structures, including DNA-based nanostructures, with emphasis on the fabrication and characterization of optical nanoarchitectures for sensing applications. He is also a co-founder of Parabon NanoLabs, a DNA technology-based company.

2. Overview of DNA methylation

Methylation of the cytosine base is the best known epigenetic modification. It occurs by enzymatic transfer of a methyl group from the methyl donor S-adenosylmethionine to the carbon-5 position of cytosine with the help of DNA methyltransferases (DNMTs). So far, five kinds of DNMTs including DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L have been identified but only DNMT1, DNMT3a and DNMT3b possess methyltransferase activity.[7] DNMT1 is involved in maintenance methylation during development and cell division, whereas DNMT3a and DNMT3b show *de novo* DNA methylation activity that establish DNA methylation patterns during early development. The DNMTs recognize CpG dinucleotides at specific sites within the genome that often tend to cluster in regions known as CpG islands (CGIs) in the 5′ regulatory regions of genes. The CGIs are defined as regions of more than 200 bases in length with high GC content (>50%) and a ratio of CpG frequencies of at least 0.6 which usually locate in the promoter region of genes. Straussman *et al.* reported that approximately 60% of human gene promoters are linked to CpG islands and are usually unmethylated in normal cells, although 6% of them become methylated during early development or in differentiated tissues.[26]

In general, CpG-island methylation can directly inhibit transcription by blocking the binding of transcription factors, and indirectly by facilitating the binding of methyl-CpG-binding proteins (MBPs), which in turn induce chromatin conformational modifications and inhibit the access of the transcriptional machinery to gene promoter regions, thus altering gene expression levels.[11] Generally, methylated CpGs are associated with gene silencing whereas promoter

demethylation is correlated with gene expression. DNA methylation has received much research attention due to their important roles in various biological processes and health implications.[27] It has been studied extensively in cancer[28–30] and several other diseases such as diabetes,[31] neurodegenerative and autoimmune disorders.[32] Moreover, DNA methylation provides a useful source of biomarkers for risk stratification and disease diagnosis.[33,34]

The following section presents the potential of DNA methylation markers for early diagnosis, prognosis and monitoring of therapy in body fluids for clinical use.

3. Clinical applications of DNA methylation biomarkers

A biomarker is a biological molecule which is typically detected in blood, other body fluids or tissues in order to diagnose disease, predict the outcome and the response to a pharmacological agent.[21,35]

In recent years, abnormal patterns of DNA methylation have been proposed as a new generation of biomarkers and hold great promise in both clinical diagnostics and therapeutics.[7] Methylated DNAs can serve as useful noninvasive biomarkers for routine diagnostics due to the amplifiable and stable nature of bisulfite modified (converted) DNA. Additionally, circulating DNA biomarkers are ideal candidates for detection of various diseases because of their sensitivity, specificity and, reproducibility.[36–39]

To date, the vast majority of DNA methylation biomarkers have been detected and quantified in different types of human cancers such as prostate, breast, colorectal and lung cancers.[33,37,40] In addition to cancer, DNA methylation also appears to be useful for diagnosis of several other diseases, including psychiatric and neurodegenerative disorders,[12] diabetes,[41,42] obesity,[43] cardiovascular disease,[44] and imprinting disorders such as Prader–Willi and Angelman syndrome.[9]

3.1. DNA methylation as a diagnostic tool for cancer diagnosis and other diseases

Association of aberrant DNA methylation with cancer development and progression has been comprehensively reviewed in a number of excellent articles.[45–49]

Global DNA hypomethylation across the genome and site-specific DNA hypermethylation of CpG islands are two important features of cancer cells. Hypomethylation promotes tumorigenesis by aberrant activation of proto-oncogenes, loss of imprinting or genomic instability whereas hypermethylation is associated with inactivation of tumor suppressor genes, cell cycle genes, and DNA repair genes.[40,47] These changes can be used for cancer screening, classification and prognostic purposes as well as therapeutic monitoring. For example, hypermethylation of O^6-methylguanine-DNA methyltransferase (MGMT) in glioma and glutathione S-transferase pi 1 (GSTP1) in prostate cancer have been used for determining an appropriate therapeutic choice in glioma and in the diagnosis of prostate cancer, respectively.[18] According to a meta-analysis study, GSTP1 hypermethylation was found in prostate cancer patients with a sensitivity of 82% and a specificity of 95%.[50] Furthermore, methylation analysis based on multi-marker approaches would improve the diagnostic efficiency over use of a single marker. For instance, methylation of six genes including CYCD2, HIC1, PAX 5, RASSF1A, RB1 and SRBC was able to distinguish colorectal cancer patients from controls with sensitivity and specificity of 84% and 68% respectively.[51] Similarly, methylation analysis of a gene panel containing APC, BIN1, BRCA1, CST6, GSTP1, P16, P21 and TIMP3 showed sensitivity and specificity more than 90% for breast cancer detection.[52] Table 1 presents some of the studies which used circulating cell-free DNA in serum or plasma as a diagnostic biomarker for cancer.

There are also several specific DNA methylation biomarkers for proteins, including vimentin (VIM), septin 9 (SEPT9), syndecan 2 (SDC2), GSTP1 and cyclin-dependent kinase inhibitor 2A (CDKN2A) and short stature homeobox 2 (SHOX2) which have already been translated into commercial clinical assays for the early detection of cancers (Table 2).

Diseases other than cancer, such as neurodevelopmental, metabolic and cardiovascular disorders reveal altered DNA methylation patterns, which make epigenetic analysis applicable to a broader range of diseases.[65] For example, genome-wide and locus-specific CpG methylation changes have been observed during the onset and progression of human atherosclerotic lesions. As compared to the other blood-related diseases and neoplastic disorders, these lesions displayed different methylation profiles, offering desirable characteristics for atherosclerosis detection.[66]

3.2. DNA methylation as a therapeutic target

There is clear evidence that the dynamic nature of DNA methylation can be targeted for disease treatment.[18] Fortunately, CpG methylation can be reversed by demethylating compounds or certain enzymes such as DNA demethylases. To date, two DNMT inhibitors, 5-azacytidine and 5-aza-2′-deoxycytidine or decitabine, have been approved by the US Food and Drug Administration (FDA) as treatments against myelodysplastic syndrome (MDS).[18,73,74]

Indeed, these inhibitors can be used to restore cell functions including proliferation and invasion which have been disabled by methylation. Furthermore, such therapeutic agents can also be employed against different types of leukemia, including acute myeloid leukemia, chronic myelogenous-leukemia, MDS, and chronic myelomonocyticleukemia. In addition to its therapeutic target potential, methylation profiles can be used to predict the response to certain drug treatments. For example, a positive response to drugs based on carmustine and temozolomide can be anticipated in cases of glioma arising from hypermethylation at the MGMT promoter. Pharmacological research has therefore shown that MTases can act as significant drug targets, enabling a substantial step forward in solid cancer tumor treatment.[18]

Table 1 Detection of cancer in plasma/serum using circulating methylated DNA biomarkers

Cancer	Biomarker	Sample size (cases/controls)	Sensitivity	Specificity	Methods	Ref.
Bladder	TIMP3, APC, RARB, TIG1, GSTP1, p14, p16, PTGS2, RASSF1A	174/53	62%	89%	MSRE-qPCR	53
Breast	ITIH5, DKK3, RASSF1A	250/237	67%	69%	qMSP	54
Colorectal	ALX4, SEPT9, TMEFF2	182/170	81%	90%	MethylLight	55
Gastric	KCNA4, CYP26B1	92/30	91%	92%	MSP	56
Glioma	MGMT, RASSF1A, p15INK4B, p14ARF	33	70%	N/A	MSP	57
Head and neck	CDH1, TIMP3, HIC1, PGP9.5	42/209	81%	43%	qMSP	58
Hepatocellular carcinoma	APC, GSTP1, RASSF1A, SFRP1	109/41	93%	82%	MSRE-qPCR	59
Lung (NSCLC)	APC, AIM1, CDH1, DCC, MGMT, RASSF1A	76/30	84%	57%	qMSP	60
Ovary	RASSF1A, CALCA, EP300	30/30	90%	87%	MethDet test	61
Pancreatic	BNC1, ADAMTS1	42/26	81%	85%	qMSP	62
Prostate	GSTP1, RASSF1, RARβ2	83/40	63%	N/A	MSP	63
Thyroid	CALCA, CDH1, TIMP3, DAPK, RARβ2	96	68%	95%	qMSP	64

Table 2 Commercially available DNA methylation tests in clinical samples. Reprinted with permission from ref. 49

Biomarker	Application	Cancer	Sample	Diagnostic test kit	Ref.
SEPT9	Early detection	Colorectal	Blood	Epi proColon, ColoVantage, RealTime mS9	67
VIM	Early detection	Colorectal	Stool	Cologuard	68
SHOX2	Early detection	Lung	Bronchial fluid	Epi prolong	69
GSTP1	Early detection	Prostate	Urine	Predictive biosciences	70
MGMT	Predictive	Brain	Tumor	PredictMDx for glioblastoma	71
TWIST2+NID2	Predictive	Bladder	Urine	CertNDx™ bladder Cancer assay	72

4. Conventional methods for DNA methylation analysis

In the last two decades, a multitude of techniques have been developed to determine the DNA methylation status in tissues and biological fluids.[75] These various methods have three steps that enable the selection and analysis of methylated DNA from unmethylated DNA of a target genome (Fig. 2). In the first step, methylated and unmethylated fragments can be differentiated by three main classes of pretreatments including bisulfate conversion, digestion with methylation-sensitive restriction enzymes and affinity purification of methylated DNA. Bisulfite treatment has been widely used in many detection platforms, relying on the conversion of unmethylated cytosines to uracils while 5 mC remain intact. In this way, the unmethylated and methylated fragments can be distinguished by their different sequences.[76] Methylation-sensitive and insensitive restriction enzymes such as HpaII and MspI have been employed in gene-specific methylation assays. HpaII is blocked by the presence of 5 mC in the context of CCGG tetranucleotides whereas MspI is not affected by DNA methylation, thus the analysis of digested DNA products can be used for detecting CpG methylation at specific genomic loci.[37]

Physical separation can be performed by employing antibodies and proteins that bind to methylated cytosine.[37] The second step serves to amplify the treated DNA by PCR and in the last step, DNA methylations are detected using any of a number of different technique such as methylation-specific PCR (MS-PCR)[77] combined with bisulfate restriction analysis (COBRA),[78] pyrosequencing,[79] bisulfate sequencing,[80] methylation-specific quantum dot fluorescence resonance energy transfer (MS-qFRET),[81] and microarray analysis.[82] These techniques have been developed to determine the DNA methylation status at the global, gene-specific, or in genome-wide levels and are described in detail in a number of excellent reviews.[22–25,83,84] Initially, analyses focused on locus specific approaches, but now, they can be performed on a genome-wide scale by using new powerful technologies, such as comprehensive DNA methylation microarrays and genome-wide bisulphate sequencing.[85] Several important features of such methods have been summarized and are presented in Table 3.

Although much progress has been made recently in DNA methylation analysis, there are still some drawbacks that limit their application in clinical laboratories. First, significant errors in PCR amplification, DNA extraction and bisulfite conversion steps lead to false results. Second, both bisulfate and differential PCR experiments are time consuming and laborious. Third, the new high-throughput technologies are expensive and/or too complicated to implement in standard laboratories.[86,87] Therefore, designing new diagnostic devices with higher efficacy without the requirement of PCR amplification, bisulfite treatment or sophisticated instrumentation would provide useful tools for direct methylation assays.

4.1. Methods for discovery and validation of DNA methylation biomarkers

The development of accurate and robust DNA methylation biomarkers requires several bioinformatics and statistical

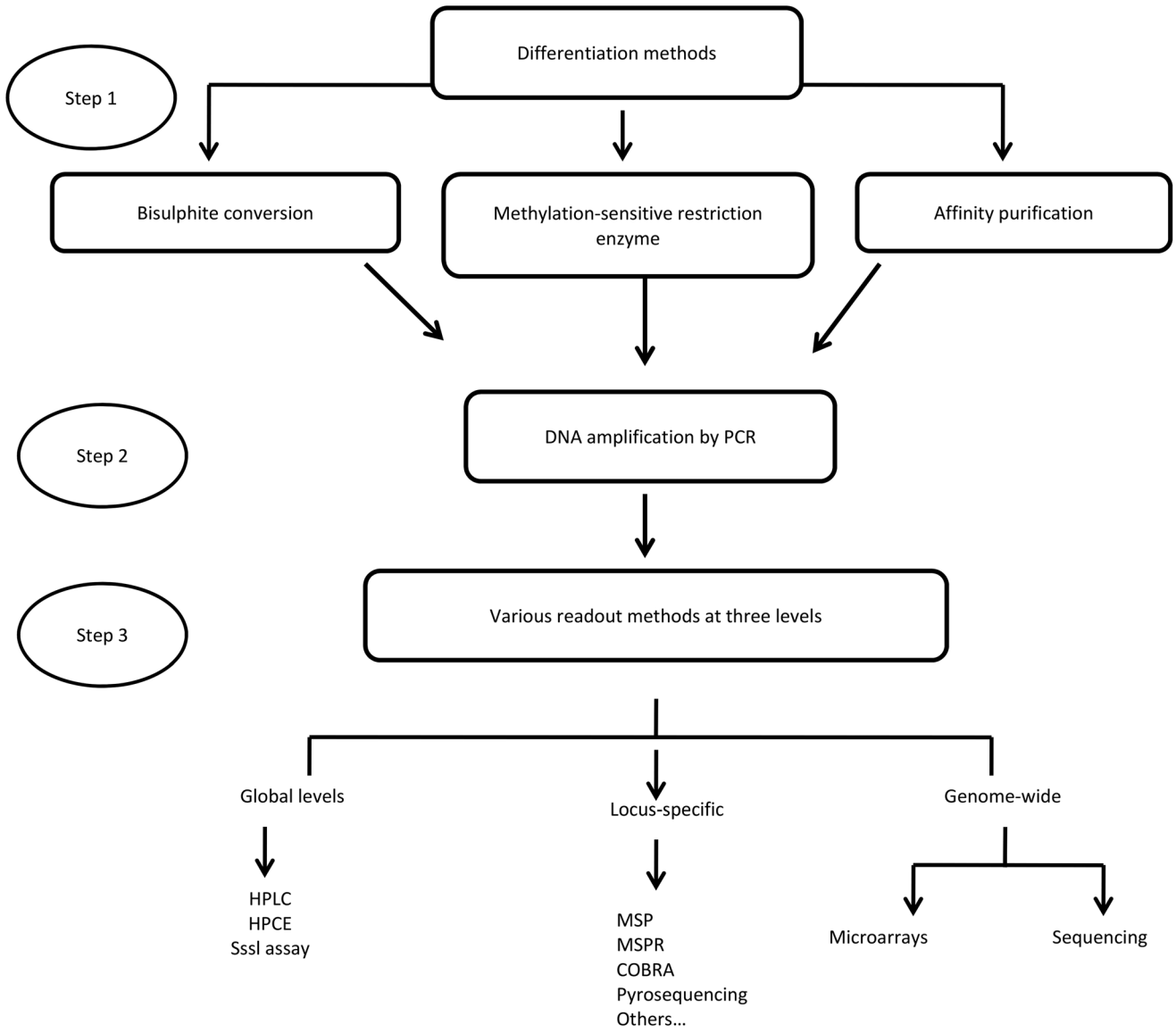

Fig. 2 The steps involved in DNA methylation analysis.

steps. In addition to gene-specific methylation biomarkers, there is interest in global alterations in genomic methylation levels because such dysregulation has been associated with both risk and progression of diseases including cancer, CVD, and diabetes. Global DNA methylation is mainly assessed by methylation of repetitive sequences such as satellite DNAs and long interspersed nucleotide elements (LINE)-1. Methylation plays an important role in the maintenance of genome stability beyond the regulation of specific gene function.[88]

Many strategies have been applied for the discovery and validation of methylated biomarkers and each strategy has its own advantages and disadvantages.[36,88,89] Generally, candidate biomarkers are founded by genome-wide DNA methylation methods such as sequencing and microarray approaches. After discovery, the accuracy, sensitivity, and specificity of biomarkers is confirmed in a large cohorts of patient using locus-specific methods.[89,90] For example, bisulfate sequencing has been mostly used for identify a variety of candidate biomarkers including MLH1, CDKN2A, and MGMT. In another study, Campan *et al.* using a bead array procedure (Infinium 27 K), developed the IFFO1 biomarker for detection of ovarian cancer. Following the identification of this biomarker, the MethyLight assay was used as a gene specific method for confirmation of the initial result obtained using the microarray platform.[91]

5. Alternative types of biosensors for detection of DNA methylation

A biosensor is made up of two components: a recognition element (protein, DNA, or RNA) that provides the selective

Table 3 Overview of methods for DNA methylation analysis at different levels

Methodology	Pre-treatment	Principle	Advantages	Disadvantages	Ref.
Global					
High-performance liquid chromatography (HPLC)	Enzymatic/ chemical hydrolysis	Digestion/hydrolysis of genomic DNA to nucleotides or bases and fractionation on HPLC	Highly quantitative, accurate, and reproducible	Requires large amounts of DNA and costly instrumentation	92
High-performance capillary electrophoresis (HPCE)	Enzymatic/ chemical hydrolysis	Digestion/hydrolysis of genomic DNA to nucleotides or bases, then separation and quantification by SDS micelle system	More specific, faster and cheaper than HPLC	Requires large amounts of DNA and sophisticated instrumentation	93
Sssl assay	Enzymatic	Evaluating genomic DNA methylation levels by SssI methylase	Simplicity	Low sensitivity, imprecise, low reproducibility	94
Gene specific					
Methylation-specific PCR (MSP-PCR)	Bisulfite conversion	After sodium bisulfite treatment, CpG site at or near the 3′ primer allow amplification of only methylated DNA and stringent annealing temperatures limits amplification of unconverted DNA	Very sensitive, simple method, cost-effective	Not quantitative, risk for false positive & negative results, requires two primer sets for methylated and unmethylated DNA	77
Methyl-sensitive restriction enzyme PCR (MSRE-PCR)	Enzyme digestion	Detection methylation status at specific restriction sites using southern blotting	Standard techniques	Limited to specific restriction targets, semi-quantitative	95
MethyLight	Bisulfite conversion	Detection of methylation by combination the basis of MSP with the real-time detection	Very high sensitivity closed tube assay low false positive rates	Detection of only methylated DNA Only semi-quantitative in a sample with heterogeneous methylation	96
Pyrosequencing	Bisulfite conversion	Real-time DNA sequencing based on the luminometric detection of pyrophosphate release following nucleotide incorporation.	Highly quantitative, single-nucleotide resolution, low amount of DNA required, commercial service available	Requires extensive optimization for assay-set-up, high cost, short reads (~100 bp)	79
Methylation-sensitive single nucleotide Primer extension (MS-SNuPE)	Bisulfite conversion	The region of interest is amplified with non-methylation-specific primers and the primer is then extended by the addition of labeled dNTPs. Subsequently, the products are visualized by gel or capillary electrophoresis.	Requires small amounts of DNA, rapid and quantitative, accurate, the simultaneous quantification of multiple CpG sites by using multiplex reactions.	Quite labor-intensive, require two reactions for quantification, requires a capillary electrophoresis instrument	97
Combined bisulfite restriction analysis (COBRA)	Bisulfite conversion	PCR products amplified from bisulfate modified DNA is digested with restriction enzymes that distinguish methylated from unmethylated sequences.	Rapid, quantitative, sensitive, reproducible	Limited to specific restriction targets, relatively time-consuming	78
Methylation sensitive-high resolution melting (MS-HRM)	Bisulfite conversion	PCR products amplified from bisulfite modified DNA have different melting profiles and are then analyzed by high-resolution melting curve.	High throughput screening, commercial kit available (Qiagen), inexpensive, rapid	No information on specific sites of methylation, estimation of methylation levels in a semi-quantitative manner in the presence heterogeneous DNA methylation	98
Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA)	Enzyme digestion	MS-MLPA is based on MLPA probe and a digestion step with the HhaI enzyme prior to PCR amplification to distinguish methylated from unmethylated fragments.	High-throughput screening, relatively cost-effective not requiring bisulfite conversion step	Limited to specific restriction targets	99

Table 3 *(Contd.)*

Methodology	Pre-treatment	Principle	Advantages	Disadvantages	Ref.
Mass ARRAY EpiTYPER	Bisulfite conversion	PCR products amplified from BS modified DNA followed by *in vitro* transcription, base specific RNA cleavage and MALDI-TOF analysis	Quantitative, high-throughput, detection of heterogeneous DNA methylation patterns, commercial service available	Expensive instrument	100
Genome-wide *Gel & microarrays*					
Restriction landmark genomic scanning (RLGS)	Enzyme digestion	Digested DNA is radioactively labeled at methylation-specific cleavage sites and then separated by 2D gel electrophoresis.	Sensitive, the simultaneous quantification of multiple CpG sites	Low resolution, semi-quantitative very time consuming, labor intensive, require very large amount of DNA for profiling	101
Differential methylation hybridization (DMH)	Enzyme digestion	Digested DNA is amplified by primers complementary to the linker sequence and the products are labeled and hybridized to arrays containing of thousands CpG islands	Quantitative, relatively simple, requires low amount of DNA	Limited to specific restriction targets incomplete digestion lead to false positive results	102
Methylated DNA immunoprecipitation and microarray chip (MeDIP-chip)	5-Methylcytosine specific antibody	Immunoprecipitation of methylated DNA with a specific antibody, followed by microarray chip hybridization	Independent of the specific methylation-sensitive restriction sites, identification of a large number of genes with methylated CpG islands	Low resolution, semi-quantitative, high cost of specific antibody and array instrumentation, biased towards hypermethylated regions, requires bioinformatics knowledge	103
Bead arrays (Illumina)	Bisulfite conversion	Bisulfite-converted DNA is amplified with fluorescent primers and then ratio of the methylated and unmethylated PCR products is determined by Illumina's bead array platform	Single-base resolution, quantitative, detecting simultaneously of up to 1536 CpG sites in 96 samples.	Cost, require design of a large set of selective primers,	104
Sequencing					
Whole-genome bisulfite sequencing	Bisulfite conversion	PCR products amplified from bisulfite modified DNA followed by sequencing	Single-base resolution, gold-standard technology for detecting DNA methylation patterns of individual DNA molecule	Labor intensive, time-consuming, requires a large amount of sequencing for accurate quantification, high cost of sequencing	80
Single molecule real time (SMRT) sequencing	Direct detection	Incorporation of fluorescently labeled nucleotides into complementary nucleic acid strands by DNA polymerase and then real-time detection of fluorescence pulses	Direct quantification, no need of bisulfite conversion	High costs, requires bioinformatics knowledge	105
MethylCap sequencing	5-methylcytosine specific methyl binding proteins	Immuno precipitation of DNA using the methyl DNA binding domain proteins, followed by sequencing	Regional quantification, moderate resolution, independent of restriction site limitations	Cost with specific protein and sequencing, requires bioinformatics knowledge	106

binding with a particular analyte and a transducer that converts this binding event into quantifiable electronic signals. Transducers can be divided into four main classes on the basis of detection signal: electrochemical (*i.e.*, amperometric and potentiometric), optical (*i.e.*, colorimetric, fluorescent, luminescent, and interferometric), mass based (*i.e.*, piezoelectric and acoustic wave), and calorimetric (Fig. 3).[21,107]

Biosensors for DNA methylation monitoring have been reported which can detect DNA methylation biomarkers at pico[108] and femtomolar levels,[109] levels which could be beneficial for early diagnosis and treatment of diseases.

Depending on the type of transducer, biosensor platforms for DNA methylation detection can be classified into electrochemical, electrical, optical, and mass methods (Fig. 4). Apart from classification based on the type of transducer, these biosensors are also classified into labeled and label-free detection schemes based on the sensing strategy. Enzymes, radionuclides, nanoparticles, fluorescent and electrochemiluminescent probes are widely used as labels. However, label-free detection methods have a number of advantages for use in biomedical assays, such as elimination of the laborious and/or error prone/inefficient labeling processes, and raising the

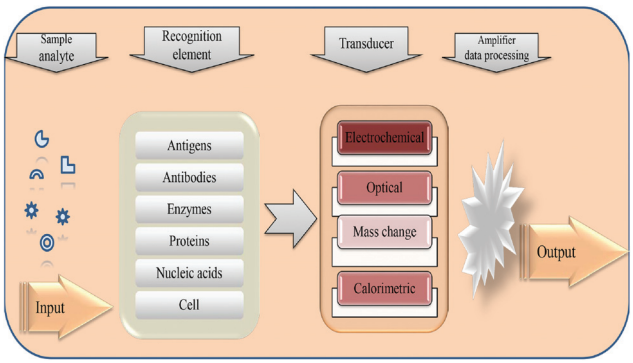

Fig. 3 Schematic representation of biosensors sensing principle.

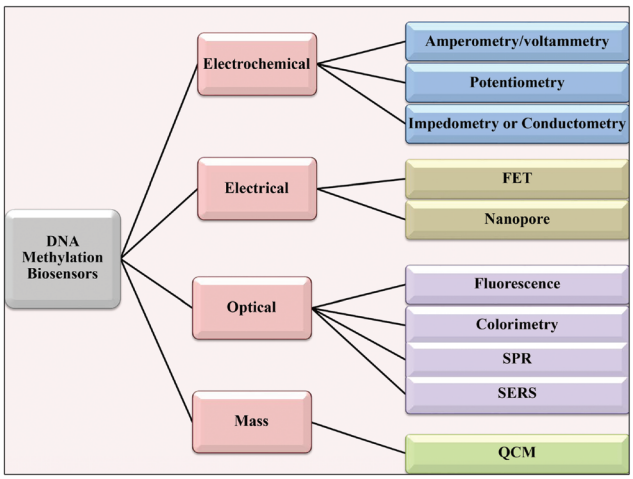

Fig. 4 Overview of different types of biosensors for DNA methylation detection.

possibility of the real-time monitoring of binding interactions, which is generally not possible with label based biosensors.

5.1. Electrochemical

Electrochemical methods are valuable analysis tools that combine the sensitivity of electrochemical transducers with the high specificity of biomolecules as recognition elements for the development of high-performance biosensors. They have been employed widely in biosensing due to their high sensitivity, low cost, simplicity of construction, low power consumption and compatibility with miniaturization/microfabrication technologies.[110]

Electrochemical biosensors can be divided into three main classes on the basis of signal detection: amperometry/voltammetry, potentiometry, and impedometry or conductometry.[110] There are two formats of electrochemical methods including labeled or label-free detection that have been used for monitoring methylated DNA and MTase activity.

5.1.1. Labeled detection. Labels based on nanomaterial such as gold nanoparticles,[111] and graphene[112] as well as electroactive label (ferrocene acetic acid and methylene blue)[113,114] have been employed in DNA methylation detection researches.

For example, Wei *et al.* designed a simple, portable and parallel biosensor using methylene blue-modified molecular beacon DNA on an indium tin oxide (ITO) microelectrode. An electrochemical response was produced after endonuclease cleavage (Dpn I) in the presence of methylated DNA and accumulation of MB-labeled fragments on a negatively charged ITO microelectrode. The suggested procedure has great potential for use in clinical DNA methylation analysis as well as for drug screening for MTase inhibitors.[114]

Two types of DNA probe using two different labels, ferrocene acetic acid and graphene oxide (GO) were described by Cai's group, for the determination of levels of DNA methylation and methyltransferase activity, also enabling methyltransferase inhibitor screening.[112,113] The principles governing these biosensors are that a label-conjugated probe is hybridized with target molecule and methylated by methyltransferase (M.SssI), and then subjected to enzymatic digestion by HpaII endonuclease which is sensitive to the symmetrical unmethylated dsDNA molecules. Finally, the discrimination of the methylated and unmethylated DNA is determined by measurement of the electrochemical signal of the label before and after digestion. In the GO-based platform, an electrochemical reporter (thionine) has been utilized to construct a rapid, highly selective and real-time biosensor which is able to distinguish single-base mismatches with a detection limit of 0.05 units per ml (U mL^{-1}).[112]

In another study, based on gold nanoparticles as an effective signal amplifier and MB as an electrochemical indicator, a sensitive and selective biosensor has been constructed for detection of methylated DNA, MTase activity as well as its inhibitors. In this study, after hybridization of DNA-modified gold nanoparticles with captured probe sequences, selective digestion of unmethylated DNA by endonuclease is performed after methylation using Adenine MTase. The current response of MB was reduced due to a decreased MB population on the electrode. In view of the observed advantage of relatively low detection limit of 0.02 U mL^{-1}, this biosensor is capable of cancer diagnosis and drug screening.[111]

5.1.2. Label-free detection. Using similar approaches, two studies have been performed using "signal on" and "signal off" strategies to assess MTase activity and DNA methylation with MTase activity respectively.[115,116] These biosensors employ probe immobilization, hybridization with complementary DNA, methylation by Dam MTase and then digestion with Dpn I. In the "signal on" strategy, SWCNTs were applied to ssDNA which remains on the electrode after melting the dsDNA fragments. Thus, SWCNTs provided a very effective signal transduction approach involving electron transfer between the electrode and redox species, resulting in the relatively low detection limit of 0.04 U mL^{-1}.[116] In the "signal off" approach, the electroactive complex $[Ru(NH_3)_6]^{3+}$ acting as a signal molecule was utilized to assemble a rapid and highly sensitive biosensor with a 0.18 U mL^{-1} detection limit.[115]

Recently, Dai and Xu also reported label-free electrochemical platforms, based on a similar principal, using the electrochemical indicator MB for quantification of DNA methylation and detection of MTase activity respectively.[117,118] In the former study, MB current peaks were used to identify the degree of DNA methylation for concentrations higher than 10 nM.[118]

In another electrochemical approach to screening for the occurrence of methylation, in place of enzymatic reactions MBD proteins were exploited for specifically recognizing methylated regions. These proteins could then be stained by coomassie brilliant blue G250 (CBB-G250) which then acts as an electroactive molecule.[119]

Carbon based electrodes, including carbon film-modified electrode [overoxidized polypyrrole/MWNT],[87] electron cyclotron resonance nanocarbon,[120,121] MWCNT-β-cyclodextrin[122] have several advantages including wide potential window, reduced electrode fouling, large active surface area, and low background currents with high electrocatalytic activity. In optimal cases, the oxidation current of bases can be used for the direct detection of a single methylated base in a 24 base sequence at a concentration of 5 μM. This type of biosensor will be suitable, for example, for integration into a micro-total analysis system/lab-on-a-chip due to the potential for nanocarbon film deposition at low temperatures and compatibility with microfabrication techniques.[120]

In another study, an ultrasensitive biosensor was developed for fully methylated p53 gene analysis using PNA as a capture probe, an electroactive indicator ($[Ru(NH3)_6]^{3+}$), and a AuNP film on an electrode as an enhancer of electron transfer. After bisulfite treatment methylated DNA was captured by the complementary PNA probe; while in the presence of unmethylated DNA the hybridization reaction did not occur. Therefore, the methylation state of the p53 gene was transduced by measuring the current signal mediated by $[Ru(NH_3)_6]^{3+}$, which was modulated by its electrostatic interaction with the hybridized PNA probe. A lower limit of detection of 18 pM was obtained in this assay and it was possible to distinguish the methylation state of multiple CpG sites with complete generality to any DNA sequence.[108]

Screen-printed gold electrodes (SPE-Au) are a worthy candidate for making point-of-care evaluation devices owing to their simplicity, low cost and potential for single-use application. eMethylsorb is a platform based on SPE-Au, and was designed to determine the level of DNA methylation based on DNA–gold affinity. In this approach, the bisulfite treatment and asymmetric PCR are first performed in order to produce unmethylated and methylated ssDNA species containing adenine and guanine respectively. Based on the higher affinity of adenine to the SPE-Au surface, greater amounts of unmethylated adenine accumulate on the electrode surface, which in turn leads to a lower current response due to increasing columbic repulsion with bulk ferricyanide ($Fe(CN)_6^{3-}$) ions. This sensitive biosensor could measure a 10% methylation level in 10 min.[123]

5.1.3. Other electrochemical methods. Much attention has been devoted to combining recognition elements with electronic elements to develop different types of electrochemical biosensors.[124] Incorporation of both chemiluminescence and photo irradiation techniques to electrochemical detection has created electrochemiluminescence (ECL) and photoelectrochemical (PEC) sensors respectively.[125] The ECL (or electrogenerated chemiluminescence) process is based on converting electrochemical energy into radiative energy *via* an applied potential. ECL has attracted abundant interest from many researchers due to its simplicity, low-cost, easy controllability, low background signal and high sensitivity. Three categories of materials, including (a) transition metal complexes and (b) organic molecules and (c) nanomaterials have been surveyed for their ECL effects.[126] Among these materials, the compound [tris(2,2′-bipyridyl)ruthenium(II)] or $[Ru(bpy)_3^{2+}]$ is widely used as the ECL luminophore because of its superior properties, which include strong luminescence, good solubility in many kind of solvents and the fact that it is capable of regeneration after light emission.[127] Applications of ECL which have been reported include immunoassays, DNA hybridization detection and enzymatic biosensors; however, there are a few studies that have been reported to monitor methylated DNA and activity of MTase.[128–130] For example, Li *et al.* developed the biosensing technique based on enzyme-linked reactions which exploit as sDNA–ruthenium ECL tag and a coreactant (ethylenediamine) on the surface of gold electrodes. The ECL intensity decreased after the hybridization and MTase/endonuclease coupling reaction due to a decrease in the concentration of unmethylated DNA–ECL tags on the electrode surface. This highly sensitive ECL biosensor could detect methylated DNA with a detection limit of 0.02 U mL^{-1}.[130]

It is worthy to note that ECL emission detection from a single nucleotide is impossible using conventional hybridization techniques. Toward this end, Kurita *et al.* could determine single methylated cytosines through an enzyme linked immunosorbent assay (ELISA) with ECL reporting in an extended real genomic sample. In this study, using an acetylcholine esterase-labeled anti methyl cytosine antibody, thiocholine was accumulated on a gold electrode surface which was generated by enzymatic reaction. Application of a potential to the gold electrode containing thiocholine and a ruthenium complex created bright and distinctive emission in the presence of the methylated site on DNA. This immunosensor displayed high sensitivity and selectivity and could detect mC in the presence of other, non-methylated bases, with a lower detection limit of 0.18 pmol.[128]

Recently, signal amplification has been demonstrated in a label-free ECL procedure based on a supersandwich DNA construct, providing highly sensitive detection of DNA methylation and methyltransferase activity. In the supersandwich structure, instead of single reporters, multiple signaling molecules can be incorporated through DNA hybridization combined with $Ru(phen)_3^{2+}$ luminophore intercalation. The approach uses three ssDNA sequences, S1 was immobilized on the electrode surface, then after partial hybridization of S2 and S3, long concatemers were generated. Following intercalation of the ECL tag into these concatemers, methylation by MTase and HpaII digestion, the response of $Ru(phen)_3^{2+}$ decreased in a response

that was proportional to the concentration of methylated DNA. This method, without any sophisticated tools, displayed a detection limit of 3×10^{-6} U mL^{-1} which makes it a promising tool to assess and test inhibitors of methyltransferase.[129]

The PEC-based biosensors are operate through a combination of both optical and electrochemical principles. In contrast to ECL, the PEC-based biosensors utilize light to excite a photoactive species and the generated electrical signal is used for detection. Due to the use of different mechanisms for excitation and detection, these methods provide for high sensitivity, low background current and superior detection properties. Indeed, the photoelectrochemical sensors have the benefits of both optical and electrochemical sensors.[131] The PEC biosensors have been used in the detection of DNA, proteins as well as DNA methylation and MTase activity assay. For example, Zhou *et al.* designed a novel visible light-activated PEC biosensor based on using a bismuth oxyiodide (BiOI) nanoflake-modified ITO electrode as the photoactive in an MTase assay. In this approach, after dsDNA immobilization and methylation, MBD's, acting as recognition proteins for the identification of methylated cytosine, were captured on the AuNPs/BiOI electrode surface. The photocurrent signal of the biosensor was reduced due to steric interference of the surface caused by the MBD protein, which disrupts electron transfer from the ascorbic acid donor to the electrode surface. Because this decrease is related to the activity of DNA MTase, these sensors can be used to screen for inhibitors screening in addition to their application in MTase assays. This biosensor presented good reproducibility and was found to have a limit of detection of 0.035 unit per mL.[132]

Based on a similar strategy, Bi_2S_3 nanorods were used instead of BiOI nanoflakes as the photoelectric conversion substance in a novel PEC immunosensor for DNA methylation assay. In this biosensor, the electrode blocking effects of an MBD protein recognition event is amplified by using an anti-his-tag antibody (Fig. 5) which binds through immunoreaction with the his-tag modified MBD protein. This multilayered antibody/MBD1/AuNPs/Bi2S3/ITO electrode immune-based sensor attained the relatively low detection limit of 3.5×10^{-14} M with excellent photoelectronic properties and high detection specificity.[86]

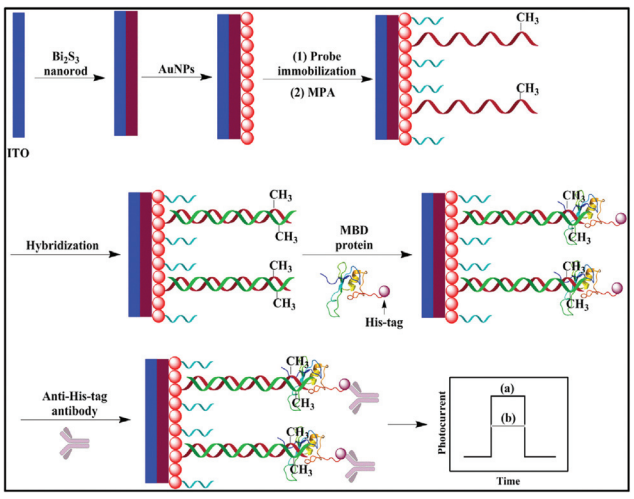

Fig. 5 Schematic diagram of the photoelectrochemical immunosensor construction process. Reprinted with permission from ref. 86.

A point to note here is that two types of current responses can be generated in the PEC DNA biosensors: "signal on" and "signal off". A photocurrent is boosted in the "signal on" strategy, while the signal is decreased after the detection of biological molecules in the "signal off" mode. Generally, the "signal-off" sensor configuration possess considerable background responses and is more susceptible to false positive responses, hence the "signal-on" biosensor is considered to be more useful.[133] For instance, Yang *et al.* have fabricated a novel signal-on photoelectrochemical biosensor for detecting hydroxymethylcytosine (hmC) based on an *in situ* electron donor generating approach under white light irradiation. In this device construction, the ITO electrode contains CdS quantum dots which act as the working electrode for PEC measurement. Using PEC response change, the concentration of hmC was analyzed with this biosensor yielding a detection limit of 0.167 nM.[134]

5.2. Electrical

Electrical biosensors present another approach for label-free, sensitive, and rapid monitoring of biomolecules such as proteins[135] and oligonucleotides.[136] This method is based on measuring changes of conductance/resistance, capacitance, or impedance across a channel arising from analyte binding. In contrast to the electrochemical technique, electrical sensing allows direct detection of biomarkers without any electroactive mediators.[135,137]

Since these devices are typically fabricated using standard lithography process, they can easily be integrated into microfluidic platforms to develop lab-on-a-chip biosensors for POC testing.[137]

There are two distinct forms of electrical sensing including field effect transistors (FETs)[138] and nanopore assays[139] that have been used to detect methylated DNA.

5.2.1. FET-based biosensors. A FET is a type of transistor that is composed of a gate and a semiconducting channel which has source and drain electrodes. The function of a conventional FET is to change the measured current, or the conductivity of the channel as the applied gate voltage is varied, leading to gain or amplification. FET biosensors, by using this transistor functionality, convert the interactions between receptors and targets on the surface of the FET gate into electrical signals which are related, linearly or nonlinearly, to the analyte concentration.[140–142]

As compared to other electrochemical biosensors, FET-based biosensors exhibit better performance in terms of sensitivity, selectivity, response time, ease of nanoscale fabrication, and device dimensions. In recent years, a number of nanoscale FET devices based on semi-conducting silicon nanowires,[143,144] carbon nanotubes[145] or graphene[146] have been

used for the label free and sensitive detection of protein and DNA biomarkers.

The report of DNA methylation detection based on a nano-FET is highlighted here. In this study, Maki *et al.* fabricated a highly sensitive sensor by assembling silicon nanowires on an insulating SiO_2 substrate layer. When methylated $p16^{INK}$ molecules were captured by anti-5-methylcytosin antibodies which had been immobilized onto the surface of the nanowires, a detectable electronic signal was generated (Fig. 6). This platform was capable of detecting 2.5×10^{-19} M methylated DNA with no false positives.[138]

5.2.2. Nanopore. Single nanoscale channels (nanopores) embedded within an insulating membrane are a new class of nanosensors for label-free and rapid electrical detection of charged biomolecules, including DNAs, RNAs, proteins, ions and drug molecules.[147–149] Based on the pore material, the nanopore technologies can be divided into two types: biological and solid-state. In biological nanopores, protein channels such as a-Hemolysin (α-HL), mycobacterium smegmatisporin A (MspA), and bacteriophage phi29 (phi29) are embedded in lipid bilayer or solid state membranes while in artificial solid-state, nanopores, the pore is simply a through hole, fabricated in the membrane materials, which consist of silicon nitride (SiN_x) SiO_2, SiC, Al_2O_3 or graphene.[147,150]

In both types, the membrane separates two chambers containing conductive electrolytes (*e.g.*, KCl or NaCl electrolyte) into *cis* and transcompartments. By applying an electrical potential *via* electrodes immersed in each chamber, an ionic current signal is produced *via* the motion of ions moving from one side of the membrane to the other side. This small current is measured as a function of time.

When charged molecules such as DNA are electrophoretically driven into the nanopore the current signal is blocked. This mode of sensing is known as "ionic-current blockade" which reveals useful information about physical and chemical properties of the target molecules. Generally, various analytes ranging from small molecules to nucleic acids and proteins can be differentiated by the four main factors including the amplitude and duration of the blockade current, the capture rate, and the time between translocation events.[147,150] All of these factors can be influenced by the bias voltage and nanopore geometry.[150]

This approach has been successfully employed for the detection and quantification of methylated DNA at single base resolution without the need for labels or amplification.[139,151,152] For example, by using both biological (aHL, MspA)[153,154] and solid-state nanopores (SiN)[155] researchers have developed sensing platforms capable of even discriminating methylcytosine (mC) from hydroxymethylcytosine (hmC). Under a biased voltage, three types of DNA molecules, (C, mC, and hmC) were driven into the pores electrophoretically, which generated unique current signatures corresponding to each individual DNA molecules (Fig. 7).

Shim *et al.* fabricated a solid-state nanopore for DNA methylation detection based on the MBD protein's high affinity for methylation sites. When MBD-bound methylated DNA traversed through the pore, the electrical current was

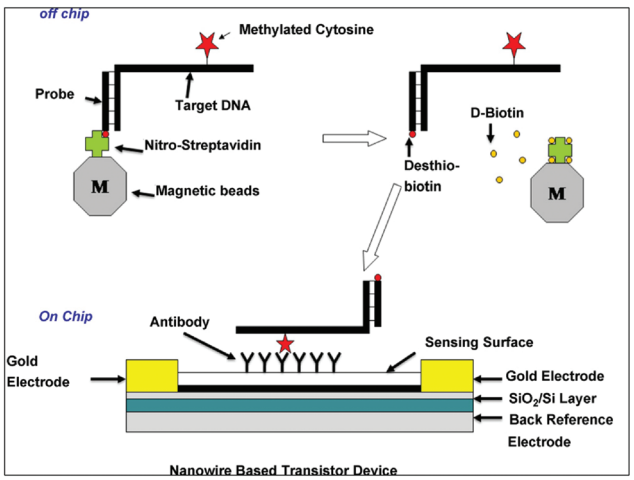

Fig. 6 An overview of detection procedure; an "off chip" procedure was designed for capturing and concentrating of target DNA molecules. Hybrids of target DNA and its sequence specific probes were captured on magnetic beads through affinity binding of desthio-biotin to the nitro streptavidin. Captured hybrids were released from the beads in the presence of a high concentration of D-biotin. "On chip" detection was preformed when methylated hybrids interacted with anti-5-methylcytosinantibody on the sensing surface. Reprinted with permission from ref. 138.

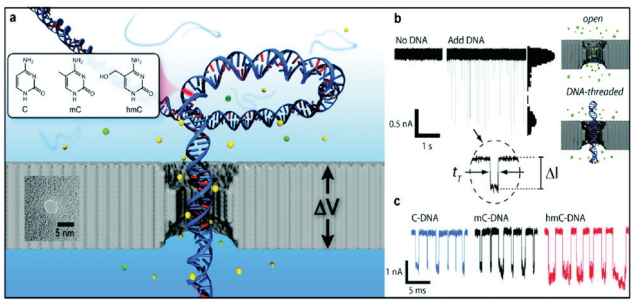

Fig. 7 Nanopore detection of modified cytosines. (a) Scheme of a nanopore in a thin silicon nitride membrane, showing a DNA molecule being driven through it by an applied voltage ΔV. A TEM image of a 4 nm diameter pore in a 20 nm thick SiN membrane is also shown. The measured signal is the ion current of an electrolyte driven through the pore (*e.g.*, KCl), represented by the yellow and green spheres. Three types of DNA molecules are driven through the pore, with the only difference between them being the chemical structure of cytosine residues in their sequence, shown in the inset. (b) Ion current traces of a 4 nm pore at 21 °C under 300 mV applied voltage, before and after the addition of 3 kbp DNA to the analyte chamber (*i.e.*, the chamber with the negative electrode). The deep spikes after the addition of DNA correspond to transport of DNA across the pore. The all-point histogram to the right of the current trace shows characteristic peaks for the open and DNA-occluded pores. A magnified event is shown in which ΔI and t_T are defined. (c) Typical events that correspond to transport of C-DNA, mC-DNA, and hmC-DNA through the pore in (a). Transport of hmC-DNA exhibits larger ΔI and t_T values than that of C-DNA or mC-DNA. Reprinted with permission from ref. 155.

blocked more than it was when in the presence of the smaller diameter unmethylated DNA.[139]

Later, the same group advanced this system for differentiating hypermethylated and unmethylated DNA of controlled 30, 60, 90 bp DNA fragment lengths using sub 10 nm nanopores. Hypermethylated DNA fragment–MBD complexes increased the amplitude and duration of the blockade current by 2.1 to 6.5-fold and 4.5 to 23.3 fold, respectively.[156]

Recently, Ahmed *et al.* using molecular dynamics (MD) simulation showed that the transport of cytosine and methylated cytosine through a graphene based nanopore resulted in a distinct current signature for each molecule.[157]

Nanopore sensing, as a fourth-generation tool, provides an entirely new approach for high throughput DNA sequencing. The major advantages of nanopores compared with previous generations of sensors include the simplicity of sample preparation and data processing, low cost, ultralong read lengths, label-free, very low consumption of materials and rapid sequencing of DNA. Particularly, the nanopore approach can be used to differentiate unmethylated from methylated DNA without the need for fluorescent labeling, PCR amplification or bisulphite conversion.[147,149]

5.3. Optical

Optical biosensors represent a powerful collection of detection devices which measure a change in the optical properties, such as absorption, reflectance and emission of specific wavelengths of light in response to the binding event between the receptor and target molecules.[158,159] These biosensors, based on the nature of the transduction mode, can be classified into five distinct classes: colorimetry, luminescence, fluorescence, surface plasmon resonance (SPR), and surface-enhanced Raman scattering (SERS).[159,160] While there are many advantages to employing an optical transducer, they usually require expensive devices for signal readout.[160]

The following subsections focuses on optical biosensors in the recent literature, based on use of different transduction events to detect DNA methylation.

5.3.1. Fluorescence. Assays employing fluorescence dominate some areas of biosensing because this optical method has very high intrinsic sensitivity and can be integrated with receptors with high selectivity. In fluorescence detection, fluorophore molecules are excited by a specific wavelength of electromagnetic radiation and then the intensity of wavelength shifted emitted light is detected by an optical transducer.[159,161] There are three different approaches to biosensing utilizing fluorescence. The first is direct detection of targets before and after the binding events. The second form is indirect detection of target analytes by adding fluorescent labeling reagents including organic dyes, and nanoparticles (quantum dots, dye-doped silica nanoparticles). Organic dyes can be used for multiplexed assays and also have the potential to be applied in studying dynamic processes of molecular events within living cells.[159] However, organic dyes have several drawbacks including limited extinction coefficients and/or quantum yields, and low photostability. To overcome these problems, fluorescent nanoparticles, including quantum dots (QDs), dye-doped nanoparticles, carbon nanodots, metal and rare earth nanoparticles have been developed as alternatives to traditional fluorophores.[162,163] For instance, Dadmehr *et al.* constructed a fluorimetric nanobiosensor based on gold coated magnetic nanoparticles for simple and rapid DNA methylation detection. In this approach, dipyridamole as a fluorescence probe was introduced into the double stranded DNA which distinguished the unmethylated *vs.* methylated fragments with a very low detection limit of 1.2×10^{-16} and 3.1×10^{-16} M for unmethylated and methylated DNAs respectively (Fig. 8).[164]

In another study, a label-free and highly sensitive fluorescent strategy for DNA methylation sensing as well as Dam MTase activity monitoring has been developed based on carbon nanoparticles (CNPs), SYBR Green as a dye reporter and employing a methylation sensitive restriction reaction. The CNPs, act as both a nanoscaffold and a nanoquencher for fluorophore-tagged oligonucleotides, providing improved sensitivity compared to the conventional methods for the detection of methylation.[165] In a similar approach, MTase activity and inhibition in homogeneous solution has been detected by using multiwalled carbon nanotubes (MWCNT) as a signal amplifier. High specificity, wide dynamic range, and a sensitivity two orders of magnitude higher than those in previous reports make this a suitable candidate for the detection of various DNA MTases and for drug screening.[166]

The third category of fluorescence assay is fluorescence resonance energy transfer (FRET). FRET is a nonradiative energy transfer process which usually occurs when two fluorophores are in close proximity (10–100 Å), to each other and the emission spectrum of the donor overlaps with the excitation spectrum of the acceptor. The rate of energy transfer is highly dependent on the distance of the donor and acceptor pairs as well as the extent of spectral overlap, the dipole angular orientation of FRET pairs and the quantum yield of the donor.[167,168]

FRET, as a spectroscopic technique, is widely employed in various areas of biology and medicine including probing conformational changes of biomolecules, monitoring intermolecular interactions, nucleic acid analysis and assaying

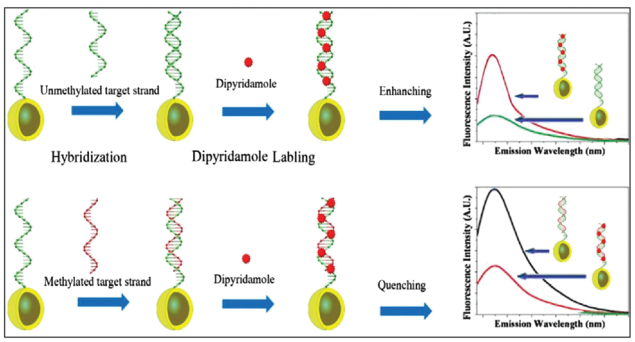

Fig. 8 Schematic representation of the dipyridamole probe based assay for DNA methylation detection. Reprinted with permission from ref. 164.

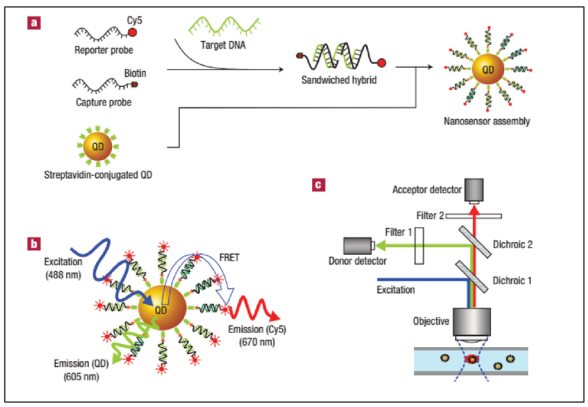

Fig. 9 Schematic of single-QD-based DNA nanosensors. (a) Conceptual scheme showing the formation of a nanosensor assembly in the presence of targets. (b) Fluorescence emission from Cy5 on illumination on QD caused by FRET between Cy5 acceptors and a QD donor in a nanosensor assembly. (c) Experimental setup. Reprinted with permission from ref. 170.

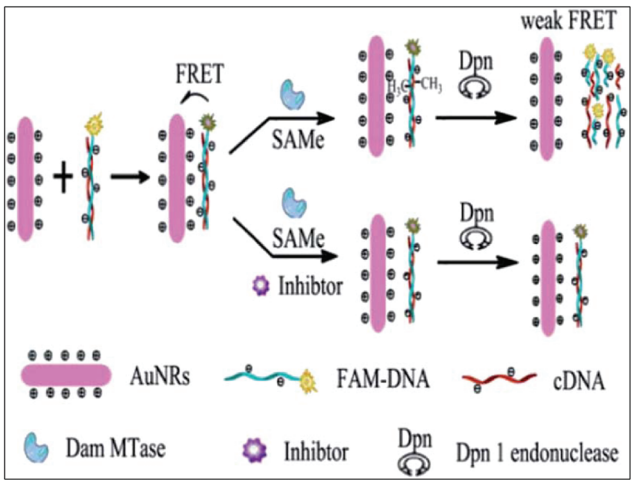

Fig. 10 Schematic illustration of the ultrasensitive detection of DNA methylation *via* a gold nanorod-based fluorescence resonance energy transfer (FRET) assay. Reproduced from ref. 171 with the permission of the Royal Society of Chemistry.

enzyme activity.[167] Several combinations of nanomaterials employing the FRET mechanism have been fabricated for use as nanosensors, with one example provided in Fig. 9.[169,170] A series of nano-FRET systems based on gold nanorods,[171] QDs[172] and polymers have recently been developed for detection of DNA methylation and MTase activity.[173]

In another example, MS-qFRET, a nanotechnology variant of MSP, combines the high specificity of MSP with the high sensitivity and simplicity of QD-FRET technology to offer a valuable analytical approach for the sensitive, quantitative detection of DNA methylation.

In this method, streptavidin conjugated QDs serve as both a scaffold to capture the labeled PCR products and a donor for transferring energy to the Cy5 acceptor that is incorporated into the amplicons during PCR. Using the MS-qFRET method, direct detection DNA methylation in real samples with high (tumors and cell lines) or low concentrations (serum and sputum) of target DNA is possible. This approach provides an effective platform for use in early cancer diagnosis, prognosis as well as monitoring therapeutic efficacy.[172] Later, the same group advanced this system for detecting DNA methylation of five tumor-suppressor genes ($p15^{INK4b}$, $p16^{INK4a}$, RASSF1A, TMS1, and CDH13) in cancer patients.[81]

Recently, a novel gold nanorods-based FRET assay was reported by Wang *et al.* for ultrasensitive detection of DNA methylation and use as an assay of methyltransferase activity. This method exhibits a detection limit of 0.25 U mL^{-1} for methyltransferase (Fig. 10).[171]

In addition to nanoparticles, fluorescent cationic conjugated polymer (CCP) has also been used for the fabrication of FRET biosensors for the detection of DNA methylation.[173–175] For example, the CCP-based FRET technique has been employed for the detection of methylated biomarkers including RASSF1A, OPCML, and HOXA9 in ovarian cancer. This fluorescence assay can be applied for diagnostic and screening of cancer due to its demonstrated high sensitivity (85.7%) even for the low methylation levels in real samples.[175]

5.3.2. Colorimetry. Colorimetric biosensing makes use of one of the simpler optical processes, which can lead to low-cost devices because it takes advantage of changes in the intensity of the color signal accompanying the detection event and can even be read with the naked eye for semi-quantitative measurements. Unlike other optical transducers, sensing devices based on this principle can minimize or eliminate the requirement of using expensive and complex tools for detection.[176] The colorimetric-based detection not only can be designed with chromogenic tags as labels, but also can be constructed label free for DNA methylation detection.[177,178]

A colorimetric assay has been designed based on the use of magnetic microspheres to provide an enrichment factor and gold nanoparticle aggregation to produce a red-to-blue color change which could detect methylated DNA with a limit of detection 80 fmol.[179]

A similar strategy, which employed DNA functionalized AuNPs along with methylation and endonuclease digestion was exploited to determining MTase activity also based on the color change accompanying aggregation of AuNPs.[180]

Recently, Geng *et al.* developed a sensitive colorimetric platform using isothermal hyperbranched rolling circle amplification (HRCA) as a signal enhancement mechanism. Using the methyl sensitive HpaII endonuclease as a discriminator, only the methylated strands were amplified. Addition of biotinylated probe strands, followed by the addition of a streptavidin-HRP enzyme and a chromogenic substrate (TMB) enabled direct monitoring of methylated DNA with a reported detection limit of 93 fM during short test time (3 h).[181] In another study, discrimination between methylated and unmethylated samples was provided by a DNA sequence which contained a loop which could nucleate the growth of a templated fluorescent silver nanocluster.

By means of a colorimetric and fluorimetric assay, the presence of unmethylated targets was detected with the relatively low detection limit of 9.4×10^{-10} M.[182]

Although many researchers have focused on using biosensor technology for locus-specific analysis, two reports have applied this approach for the detection of global gene methylation.[177,183]

In the first study, both global and gene specific methylations were detected *via* similar strategies, by using a colorimetric assay. The process consists of four steps. First, the genomic DNA was digested by using a restriction enzyme in order to fragment the genomic DNA. Second, this digested DNA was enzymatically labelled with biotin to produce biotinylated DNA sequences. Third, paramagnetic particles conjugated with MBD protein were added to the solution in order to select methylated sequences from the solution *via* magnetic separation. Finally, methylation levels were determined by the addition of a streptavidin-HRP enzyme and TMB, followed by measuring the absorbance. The intensity of the resulting color was proportional to the level of methylation. For gene-specific applications, methylated segments of the restriction enzyme selected genomic DNA are selectively magnetically separated, followed by an isothermal amplification/biotinylation reaction of the targeted region in order to identify highly differentially methylated regions (HDMRs) (Fig. 11).[177]

In another study, Badran *et al.* developed a split-luciferase biosensor for the direct, *i.e.* unamplified, detection of the global or locus specific CpG methylation status of DNA. In this method, MBD attached to the N-terminal and zinc finger (ZF) domain is attached to the C-terminal of two fragments of luciferase were used as two components of a nanosensor for the site specific detection of methylation. Symmetric methylation of the CpG sequence could be accomplished by attaching MBD to both fragments when DNA targets were present, a considerable signal was generated.[183]

5.3.3. Surface plasmon resonance (SPR). SPR detection is based on monitoring changes in the collective oscillation of the conduction electrons that occur at the interface of metallic structures, most commonly gold or silver and a dielectric material, when excited by incident light.[158,159] In SPR biosensors, biorecognition molecules, such as antibodies, oligonucleotides, or aptamers, are immobilized on the metal surface without the use of labels. When target analytes bind to the immobilized probes *via* affinity interaction, the resulting change in refractive index (RI) at the SPR sensor surface can be measured optically. The magnitude of RI change is proportional to the concentration of analyte or surface density.[158] SPR based biosensing can be used to monitor the biomolecular interactions in a label-free, highly sensitive, and real time manner.[184] This method has been successfully applied for nucleic acid,[185,186] protein[184,187] as well as epigenetic biomarker[188] detection with high sensitivity.

For instance, an SPR sensor chip has been applied for direct detection of methylated biomarkers such as APC [189] and MGMT.[190] To perform the detection, biotinylated probes were first conjugated onto a streptavidin-modified planar SPR sensor chip followed by hybridization with complementary target DNA. After that, the introduction of MBD onto the chip and its interaction with the methylated DNA increased the RI which in turn reflected the presence of the target analytes (Fig. 12).[189,190]

Recently, more complex gold nanostructures, including nanostars and nanorods, have been employed as signal ampli-

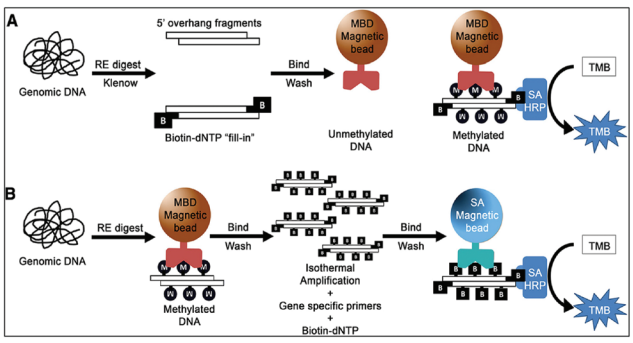

Fig. 11 Assay schemes. (A) Strategy for total genomic methylation. Genomic DNA is restriction enzyme (RE) digested, enzymatically biotinylated *via* a fill-in reaction with Klenow polymerase and biotin-dNTPs. MBD magnetic beads are then used to select for methylated DNA. Colorimetric evaluation is mediated by SA-HRP which recognizes the biotin on enriched methylated DNA. (B) Strategy for gene-specific methylation. Genomic DNA is RE digested and methylated DNA is selected *via* MBD enrichment. Gene-specific isothermal amplification is then performed with biotin-dNTP to generate biotin-DNA polymers which are in turn selected for with SA magnetic beads and SA-HRP for colorimetric evaluation. Reprinted with permission from ref. 177.

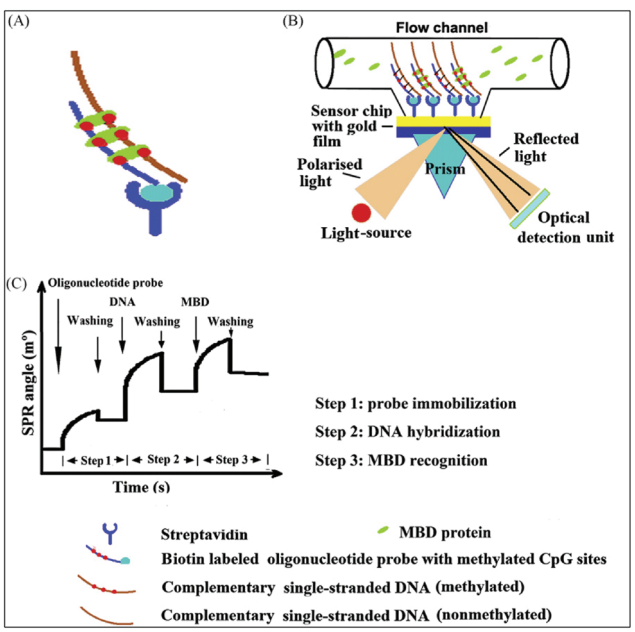

Fig. 12 Schematic diagram for double recognition of oligonucleotide and protein. (A) Double recognition strategy; (B) DNA methylation detection with SPR biosensor; (C) SPR curve of three steps. Reprinted with permission from ref. 189.

fiers in SPR assays for the sensitive and direct detection of methylated DNA and MTase activity.[188,191] For example, the assembly of nanorod send-to-end produced an enhanced SPR platform which provided a sensitive system with a wide linear range (0.5 to 120 U mL^{-1}) and a low detection limit (0.2 U mL^{-1}) for MTase activity. The excellent detection capability and simplicity of this approach offers a potentially convenient method for early clinical diagnosis.[191]

In another report, the coupling of SPR readout with molecular inversion probe (MIP) generated nanocircle templates for amplification and "reading" *via* probe strands presented a rapid, label-free and high resolution method for detection of local DNA methylation without the need of large amounts of bisulfate modified DNA.[192]

In addition to the SPR method, silicon microring resonators (SMR) have also been used to detect, in a label free, fast, and highly sensitive manner, three methylated biomarkers of bladder cancer (DAPK, E-cadherin and RARβ).[193]

5.3.4. Surface-enhanced Raman scattering. Raman spectroscopy is based on the detection of wavelength shifted light resulting from the inelastic scattering of light from a material. In the case of biomolecules a complex fingerprint spectrum results from the combination of vibrational bands exclusive to the functional group composition of the molecule. Standard Raman spectroscopy as a label-free technique has several drawbacks, particularly including the weak signal and the very intense luminescence which may be displayed by some types of biomolecules under laser excitation. To overcome these drawbacks and increase the sensitivity, SERS has been offered as an effective way to significantly enhance the intensity of the Raman scattering signal.[194] By employing Raman-active substrates including metals, semiconductor or metallic nanoparticles, usually silver, gold or copper, this phenomenon can improve the Raman scattering intensity by factors ranging from 10^6 to 10^{14}.[194,195] Several groups have reported applications of SERS spectroscopy for detection of DNA,[196] protein,[197] bacteria,[198,199] and cells;[200] however, there are two studies that have reported the monitoring of methylated DNA.

Hu *et al.* developed a SERS-single base extension reaction for the highly sensitive and selective detection of methylated DNA. After sodium bisulfite treatment, sample DNA was extended by addition of cyanine 5-deoxyribonucleoside triphosphate (cy5-dGTP). Hybridization of the target strand with a probe modified with a AuNP generated an enhanced SERS signal in the case of a methylated substrate, while in the presence of unmethylated DNA the signal was very weak due to poor hybridization with a mismatched target. The detection limit of this assay was 3 pM. The technique was also capable of distinguishing 1% methylated DNA in a mixture of methylated and unmethylated DNA.[201]

In order to increase sensitivity, an accurate and multiplexed platform using gold nanoparticles as SERS nanotags and Raman reporters was developed as a DNA methylation assay. By employing ligase chain reaction (LCR), this method exhibited single DNA base resolution with a detection limit at 0.5 pM which means that it could be expanded to serve as a diagnostic for epigenetic biomarkers.[202]

5.4. Mass

Mass based mechanical transducers include micro and nanocantilevers as well as acoustic wave (quartz crystal microbalance and surface acoustic wave) sensors.[203] Quartz crystal microbalances (QCM) are piezoelectric devices fabricated from a quartz-crystal plate coated with gold electrodes. QCM are ultrasensitive systems which measure changes in the resonant frequency of the quartz crystal resonator as a function of the mass loaded onto its surface.[204,205] The thickness of the crystal is an important factor, determining the resonant frequency, and thus is critical to the mass sensitivity of the QCM. However, the low crystal stability of thinner QCM limits their operation generally to frequencies lower than 50 MHz.[205]

Nevertheless, the unique properties of QCM, including high sensitivity, fast response, low cost, and label-free detection make it a suitable candidate technology for sensing a wide range of bioanalytes.[204,206]

So far, only one article reports experiments designed using QCM sensing to detect methylated DNA biomarkers. This system involves QCM and HpaII-PCR method to assay the genes p16 and GALR2 in two cell lines. The process consists of three steps. First, genomic DNA was digested by HpaII, a methylation-sensitive restriction enzyme, in order to distinguish methylated from unmethylated DNA. Second, DNA resulting from the PCR amplification was delivered to the QCM chip, where it was hybridized with thiolated-DNA probe molecules immobilized at the surface of the QCM electrode. Finally, a significant QCM frequency shift was generated by adsorption of the amplicons resulting from the uncut, methylated DNA while the cleaved unmethylated DNA showed negligible adsorption associated changes. The quantitative nature and reproducibility of QCM-based methods suggest this method for early cancer diagnosis and screening of DNA markers. Moreover, it can be applied for monitoring methyltransferase inhibitors which are useful for cancer treatment.[207]

6. Impact of nanomaterials in biosensor platforms

Nanotechnology-based sensing methods show great promise for meeting the demands of clinical diagnostics in terms of sensitivity, selectivity and cost-effectiveness. A variety of nanomaterials including such substances as metal nanoparticles, carbon-based nanostructures, semiconducting and magnetic nanoparticles have been integrated into biosensor applications, as transducers. These inorganic materials are able to enhance the stability and signal strength of detection systems, leading also to improvements in reproducibility. The precision of molecular discrimination remains at this time the domain of organic molecular species. Properties such as high surface-to-volume ratio and chemical stability improve detection reliability.[208,209] Moreover, this integration of nanomaterials

into biosensor devices, by increasing the amplitude of the measured signal, offers a pathway toward realizing portable, small and inexpensive sensors systems, due to the ease of miniaturization of both the sensor materials and the signal transduction system.[210,211] Another important aspect recommending the use of some nanostructure enabled detection methods is the removal of the necessity for labeling. Finally, because many of these approaches have a surface bound/immobilized component, these biosensors can also be integrated with microfluidic platforms to develop point-of-care (POC) medical diagnostics tests that can be employed near the patient[204,212] and eventually at home or in environments lacking significant health care infrastructure. As reported above, considerable effort has been made to design novel and unique approaches for the fast, sensitive, low-cost and easy-to-use detection of methylated DNAs, and this research has resulted in a wide array of potential DNA biosensors which are combined with different signal transducers. In the following section, we describe the use of biosensors, integrated with microfluidic systems in order to generate diagnostic tools for DNA methylation detection.

6.1. Microfluidic based biosensors

In order for the devices described above to be transformed into useful hand held biosensor systems, these devices will need to be incorporated into microfluidic systems. Microfluidic devices offer several advantages compared to conventional methods including "native" laminar flow, the precise control of fluid volume and flow parameters, low consumption of test samples and reagents, high surface to volume ratio for efficient fluid interaction with devices and reduced reaction time.[204,213] Integrated microfluidics is an essential requirement for the development of practical platforms which provide miniaturized, and multiplexed POC diagnostics which are sufficiently automated that they eliminate the need for laboratory facilities and skilled operators.[203,214]

At this time, many factors favor the integration of optical biosensors into microfluidic systems, both generally and for the detection of DNA methylation. Two reports of studies using fluidic systems that employed MBD protein as there cognition agent and fluorescence as the signal to be transduced are reviewed next.[215,216]

In the first example, Okamoto *et al.* fabricated a rapid and simple microfluidic device using a QD-MBD fluorescence assay for DNA methylation detection with single molecule sensitivity. In this method, the biotin labeled methylated DNA was immobilized then stretched on a streptavidin coated microchannel and then MBD conjugated QDs were delivered into the microchannel, attaching to methylation sites. Finally, the methylation sites were localized using a total internal reflection fluorescence microscope.[215]

In the second study, a rapid microfluidic solid-phase extraction platform was developed for the enrichment and detection of low concentrations of hyper-methylated DNA (<1 ng ml^{-1}). The biotin-MBD protein coated microchannel exhibited capture efficiency more than 90% for methylated DNA which was comparable to kit-based extraction and enrichment. Real-time SPR monitoring and fluorescence spectroscopy was used for qualitative and quantitative characterization of each assay step. This approach offers several advantages over the conventional methylated DNA enrichment techniques including small sample volume, simplicity of operation and rapid, high throughput analysis.[216]

Recently, a much more complex, fully integrated, miniaturized "lab on a chip" system has been reported for bisulfite conversion and DNA methylation analysis. As shown schematically in Fig. 13, the methylation status of the RARβ biomarker extracted from the MCF-7 cell line were analyzed using a process beginning with bisulfite conversion pretreatment and then using isothermal DNA amplification as a signal amplification/detection method. The microfluidic chip significantly reduced sensing time compared to the conventional MS-PCR. Moreover, this approach enables one to distinguish methylated DNA in a label-free, real time, and highly sensitive (1% methylated DNA in a cell mixture) manner. These distinct features

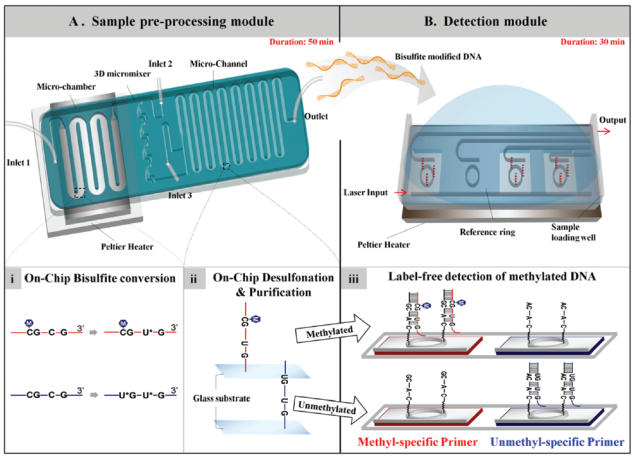

Fig. 13 Schematic of Lab-On-a-Chip system for DNA methylation analysis based on bisulfite conversion (LoMA-B). LoMA-B combines a sample pre-processing module and a detection module for analysis of DNA methylation. (A) A sample pre-processing module for on-chip DNA bisulfite conversion consists of a microchamber, a 3D micromixer, and a microchannel. A Peltier heater is incorporated into the microfluidic device to maintain the temperature for the on-chip bisulfite reaction. (i) Human gDNA with bisulfite solution is loaded into the module using Inlet 1, and incubated at 70 °C for 20 min in continuous flow passing through the microchamber region. (ii) Then, the bisulfite converted DNA was mixed with chaotropic buffer through a 3D micromixer, and bound onto the surface of the microchannel for a desulfonation and purification step. Unmethylated cytosines are converted to uracils while methylated cytosines remain intact. (B) A detection module employs the isothermal solid-phase amplification/detection (ISAD) technique after the immobilization with either methyl or unmethyl-specific primers for the analysis of DNA methylation status. The modified DNA mixed with the RPA solution is loaded onto a sensing window of the SMR sensor and resonant wavelength shifts were observed during the reaction. (iii) Amplification of methylated DNA occurs on the SMR sensor functionalized with the methyl-specific primer while no amplification occurs on the chip with the unmethyl-specific primer or *vice versa*. Reproduced from ref. 217 with the permission of the Royal Society of Chemistry.

Table 4 Comparison of conventional techniques and different types of biosensors for DNA methylation assay

Method	Nanostructure	Detection limit	Analysis time	Quantitative	Ref.
MSP-PCR	—	0.1%	<2 h/96	No	77
COBRA	—	3%	5 h/80–160	No/yes	78
Microarray	—	Single base resolution	3 days/96	Yes	104
Bisulfite sequencing	—	>2%	>4 h/96	Yes	219
Electrochemical					
Cyclic voltammetry	Fe_3O_4/trimethyl chitosan/gold nanocomposite	2×10^{-15} M	<3 h	Yes	109
Differential pulse voltammetry (DPV)	Carbon nanotube/β-cyclodextrin	0.6 μM	<1 h	Yes	122
Photoelectrochemical	Bi_2S_3 nanorod	3.5×10^{-14} M	<1 day	Yes	86
Electrical					
FET	Silicon nanowire	2.5×10^{-19} M		Yes	138
Nanopore	SiN nanopore	Single base resolution		Yes	139
Optical					
Surface plasmon resonance (SPR)	Gold thin film	5 pM	1 h	Yes	189
Colorimetric	Gold nanoparticles	80 fM	N/A	Yes	179
Colorimetric/fluorimetric	Silver nanocluster	9.4×10^{-10} M	1 day	Yes	
Surface-enhanced Raman Scattering (SERS)	Gold nanoparticles	0.5 pM	N/A	Yes	202
Mass					
Quartz crystal microbalance (QCM)	—	20 nM	N/A	Yes	207
Microfluidic	Quantum dot	Single molecule	N/A	Yes	215

make this device as viable, powerful tool for DNA methylation analysis in clinical diagnosis.[217]

Electrochemical detection has great potential cost advantages over optical approaches. A simple microdevice has been developed which couples the ligase chain reaction (LCR) amplification with a DNAzyme-based electrochemical assay on a microfluidic chip for the detection of locus-specific DNA methylation. This device, using only a few (0.04 pM) of starting DNA sample, provided an accurate quantification of methylated DNA in both breast cancer cell lines and serum samples. Moreover, because its accuracy was similar to that obtained with fluorescence and next generation sequencing approaches, it should be considered a competitive technological alternative to conventional, available technologies.[218]

Table 4 summarizes the various methods discussed in this review for assessing DNA methylation.

7. Conclusion and prospective

DNA methylation biomarkers have received increasing attention in over the past decade due to their applications in early cancer diagnosis, along with monitoring disease progression during or after treatment.

Today, PCR, microarrays and DNA sequencing are the major tools employed for determining and for discovery of methylation biomarkers. However, these procedures are lab based, complex, time consuming, and require expensive instruments and skilled operators both for analysis and interpretation. Furthermore, clinical applications of these methods would benefit from advances in accuracy.

Therefore, for broader applicability, the development of simple to operate and sensitive techniques for the detection of multiple methylated biomarkers at very low concentration in biological fluids is essential. Recent advances in nanobiosensors have provided many potential pathways ready for development. Despite the large body of research which has been carried out to produce new sensor designs for DNA methylation detection, there are still several challenges and limitations which need to be addressed. First, the development of efficient sensors to detect target analytes in complex biological fluids including urine, serum, and blood remains a challenge. Second, the integration of nanomaterials into true lab-on-chip systems for the construction of ideal POC biosensor systems for DNA methylation detection requires not only advanced technology, but also collaborative groups enabling the pooling of the required multidisciplinary knowledge in electronics, chemistry, biology and material sciences. Finally, translation of DNA methylation assays based on sensor devices into real clinical mass produced devices will require the development of complex fluidic processing systems in order to provide a user-friendly system by automating the sample preparation process such that the typical steps of extraction, purification and amplification are invisible to the user. Such systems would usher in not only POC but also enabling home diagnostics for very early disease detection.

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