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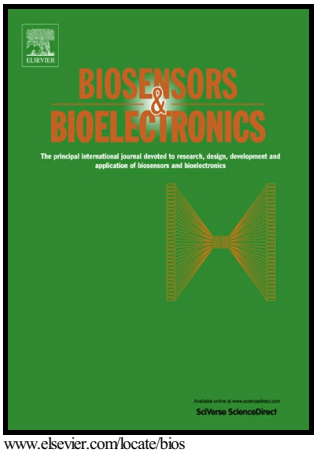

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Current Trends in Electrochemical Sensing and Biosensing of DNA Methylation

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Abstract

DNA methylation plays an important role in physiological and pathological processes. Several genetic diseases and most malignancies tend to be associated with aberrant DNA methylation. Among other analytical methods, electrochemical approaches have been successfully employed for characterization of DNA methylation patterns that are essential for

the diagnosis and treatment of particular diseases. This article discusses current trends in the electrochemical sensing and biosensing of DNA methylation. Particularly, it provides an overview of applied electrode materials, electrode modifications and biorecognition elements applications with an emphasis on strategies that form the core DNA methylation detection approaches. The three main strategies as (i) bisulfite treatment, (ii) cleavage by restriction endonucleases, and (iii) immuno/affinity reaction were described in greater detail. Additionally, the availability of the reviewed platforms for early cancer diagnosis and the approval of methylation inhibitors for anticancer therapy were discussed.

Keywords

Nucleic Acids; DNA methylation; Sensor and Biosensor; Electrochemistry; Cancer Diagnosis;

1. Introduction

DNA methylation is considered to be the most critical mechanism in epigenetics (Choy et al. 2010; Kundakovic and Champagne 2015), when it strongly affects the regulation of gene expression (Herranz and Esteller 2007). Through the DNA methylation, genes are turned on or off (Bergman and Mostoslavsky 1998; Razin and Cedar 1991). Chemical reactions involved in the DNA methylation process are conducted by DNA methyltransferases. In mammals, these enzymes mediate the addition of a methyl group (CH_3-) to the carbon five position of cytosine (C) residues, forming 5-methylcytosine (5-mC) (Bestor 1988; Zhang et al. 2015a), which is also shown in Fig. 1A. Methylated cytosines are located primarily at CpG-rich regions of genes (Conerly and Grady 2010; Holliday and Grigg 1993), whereas unmethylated cysteines can be found in gene promoters (Tucker 2001). The

rearrangement of these patterns has been associated with several genetic diseases (Ciernia and LaSalle 2016; Fries et al. 2016; Salminen et al. 2015) and most malignancies (Goding et al. 2014; Koga et al. 2009; Oakes et al. 2014; Yang et al. 2015a), as illustrated in Fig. 1B.

Based on differences in methylation patterns, cancer cells can be distinguished from healthy tissue (Bass et al. 2014; Plass et al. 2013; Wainfan and Poirier 1992; Zardo et al. 2002). Additionally, the cancer stage and grade can be identified (Catto et al. 2005; Zighelboim et al. 2007) or valuable information on disease prognosis obtained (Figueroa et al. 2010; Gorlia et al. 2008). In addition to 5-mC, another epigenetic modification of C occurs and is known as hydroxymethylcytosine (5-hmC). 5-hmC is an intermediate of 5-mC oxidation performed by the ten-eleven translocase (Tet) protein family (Tahiliani et al. 2009) (Fig. 1A). The level of 5-hmC is significantly reduced in various malignancies (Kudo et al. 2012; Yang et al. 2013), providing undeniable evidence that 5-hmC is associated with tumourigenesis.

Since the aberrant DNA methylation influences tumourigenesis, there is huge potential for the development of approaches to identify cancer states and, thereby, improve treatment prediction and prognosis. To this end, several methods have been widely used, including polymerase chain reaction (PCR), sequencing, high-performance liquid chromatography (HPLC), mass spectrometry (MS) and isotope labelling (see Table 1).

Table 1. Methods for determination of DNA methylation related with cancer disease.

Method	Author and year
PCR	(Belinsky et al. 1998; Cheow et al. 2015; Liu et al. 2016a; Ma et al. 2015)

Sequencing	(Heller et al. 2016; Kern et al. 1991; Long et al. 2016; Miyoshi et al. 2016)
HPLC	(Alonso et al. 2014; De Prins et al. 2013; Head et al. 2014; Cheng et al. 1997)
MS	(Huang et al. 2016; Lin et al. 2016; Okamoto et al. 2016; Tost et al. 2003)
Isotope labelling	(Bachman et al. 2014; Herring et al. 2009; Jones and Taylor 1981)

In recent years, a variety of alternative techniques, such as surface-enhanced Raman spectroscopy (Li et al. 2016b; Wang et al. 2016b), colourimetric and fluorometric assays (Dadmehr et al. 2015; Liu et al. 2015c; Wee et al. 2015b), atomic force spectroscopy (Cassina et al. 2016), fluorescence resonance energy transfer (Song et al. 2016; Yoo et al. 2016), and surface plasmon resonance (Kurita et al. 2015; Poulose et al. 2016), have been employed.

Each of these techniques has its own features and suitable solution for specific issues; all of them also have limitations. Classification and discussion of the appropriateness of these methods for the DNA methylation detection are not the objectives of our article, largely because other authors have already published excellent articles on these topics (Fouse et al. 2010; Fraga and Esteller 2002; Kristensen and Hansen 2009; Plongthongkum et al. 2014; Poh et al. 2016; Syedmoradi et al. 2016).

Electroanalytical methods offer obvious advantages in terms of accuracy, sensitivity, simplicity, and portability (Jelen et al. 1997; Jing et al. 2014; Wang et al. 2013c). Moreover, limits of detection within the attomole range (Sadik et al. 2009; Sage et al. 2014; Suginta et al. 2013), multitarget analyses (Ko et al. 2011; Wang 2003) or biobarcoding (Wang and Ma 2009; Wu et al. 2014) could be achieved by relatively low-cost instrumentation. In the last

two decades, considerable interest has been directed towards the development of electroanalytical applications for the detection of genes or gene mutations associated with human diseases (Drummond et al. 2003; Chikkaveeraiah et al. 2012; Wang 2000).

In this review, the recent trends in electrochemical sensing and biosensing of the DNA methylation, including the strategies that serve as the backbones of these techniques, will be discussed in great details. The potential role of the DNA methylation in early-stage cancer diagnosis will be presented, and using a set of examples, we will outline most of the studied concepts. Because the assessment of DNA methylation is important also for the development of new methylation-targeted anticancer drugs (methylation inhibitors, MIs), determining of their drug efficiency by electrochemical analysis will be discussed as well.

2. Electrochemical sensing and biosensing of DNA methylation

In electrochemical sensors, working electrode (WE) serves as physicochemical detector that provide a rapid response in the presence of a target analyte and convert the recognition process into a measurable electrical signal (Li et al. 2010; Lucarelli et al. 2004). In biosensors, the recognition of a target is mediated by biorecognition element (BE) (Lucarelli et al. 2004). In electrochemical sensing and biosensing, electrode material, electrode modification and optional use of BE are highly relevant. Therefore, the following three subsections focus on those aspects.

2.1. Electrode material

A properly selected WE material should provide low signal-to-noise ratios and reproducible signal response corresponding to the concentration of the target analyte without influence of surrounding environment. The first electrode material, mercury was introduced by Professor Heyrovsky in 1922 (Heyrovsky 1966). Despite the fact that hanging mercury drop electrode (HMDE) still possess unsurpassed properties, it became replaced by another electrode materials because of the trend towards green chemistry and the reduction of toxic substances. The application of different WE materials for the detection of DNA methylation reports Table 2.

Table 2. Electrode materials for DNA methylation detection.

Electrode material	Author and year
Mercury	(Bartosik et al. 2012; Ioannou et al. 2010)
Gold	(Brotons et al. 2016; Ge et al. 2005; Hong et al. 2016; Hou et al. 2003; Ibn Sina et al. 2014; Li et al. 2016a; Shen et al. 2016; Wang et al. 2013a; Yang et al. 2015c; Yin et al. 2015a; Zhang et al. 2015c; Zhou et al. 2014)
Platinum	(Saheb et al. 2014; Wee et al. 2015a)
Carbon	(Baek et al. 2013; Daneshpour et al. 2016; Liu et al. 2015a; Liu et al. 2016a; Milanesio et al. 2008; Wang et al. 2016a; Wang et al. 2013b; Zhou et al. 2015)

The recent development in electrode materials is moving towards electrochemical transducers with enhanced features, such as disposability, miniaturisation, high stability, and surface biocompatibility (Cox and Zhang 2012). As a result, disposable screen-printed electrode (SPE) (Koo et al. 2014a), bioavailable boron-doped diamond electrode (BDDE)

(Sanjuan et al. 2016), and micro electrode (Furst et al. 2014) have been employed for the detection of DNA methylation. Despite the huge number of newly developed electrode materials, the carbon and gold remain the most preferred, even though they require complex surface modifications and polishing before each measurement. The advantages and limitations of each electrode material are summarised in Table 3.

Table 3. Pros and cons of various electrode materials.

Electrode	Potential window	Advantages	Disadvantages
HMDE	+ 0.1 to − 2.0 V	High sensitivity, cleaning/polishing is not needed	Mercury toxicity, no portability
Carbon rod electrode	+ 1.3 to − 1.0 V	Nontoxic, bioavailable, portable, miniaturisable	Cleaning or polishing step required, high price per piece
Gold rod electrode	+ 1.0 to − 0.8 V		
Platinum rod electrode	+ 0.9 to − 0.8 V		
SPE	Depends on WE material	Portable, disposable, low price per piece, cleaning/polishing not needed	Large quantities of waste

2.2. Electrode modification

To enhance the sensitivity and selectivity of target analyte detection, the WE surface can be modified. In electrochemical sensing and biosensing, the main intentions of surface electrode modification include the acceleration of electron transfer (Martinez et al. 2009; Rawson et al. 2013), facilitation of target analyte access to the electrode surface (Ge et al. 2005; Saheb et al. 2014), and prevention of interference. A thin layer of a selected modifier

(nanostructured material, or simple chemical substance) on the electrode surface is intended to confer suitable chemical, electrochemical or transport properties to the WE.

To detect the DNA methylation, gold nanoparticles (AuNPs) have been commonly employed for WE surface modification (Liu et al. 2015a; Yin et al. 2013b; Yin et al. 2015b; Zhou et al. 2015). The AuNPs facilitated the capture of DNA (or DNA probe) *via* the Au–S bond and increased the electrode surface. A typical procedure for AuNP-based DNA immobilisation was described by Yin *et al.* in 2013 (Yin et al. 2013a). Similar to most other authors, they used only primary modification of electrode surface with AuNPs. In 2015, a more sophisticated AuNP-based modification, AuNPs in combination with 4-aminophenylboronic acid, and 11-mercaptoundecanoic acid were assembled on glassy carbon electrode (GCE) to facilitate the recognition of the DNA methylation (Yin et al. 2015b). An another nanomaterial, multiwalled carbon nanotubes (MWCNTs) have been also employed for WE modification. MWCNTs possess large specific surfaces, hydrophobic structures and adsorption of DNA *via* π–π interactions. The modification of GCEs by MWCNTs in combination with β-cyclodextrin (Wang et al. 2016a), over-oxidised polypyrrole (Wang et al. 2013b), or choline (Wang et al. 2014) has been employed to improve the electrochemical signal of the target.

Apart from nanomaterials, which usually require time-consuming and material-intensive preparation processes, simple chemical substances have been employed. Zhou *et al*. described the simple fabrication of bismuth (Bi) modified GCE. The unique properties of the resulting electrodes (high sensitivity, excellent peak resolution, and high hydrogen potential) were employed for the DNA demethylase activity detection (Zhou et al. 2014). Hong *et al.* modified a gold electrode (AuE) with benzenedithiol to create an AuNPs-DNA network for the methyltransferase activity quantification (Hong et al. 2016). Zhang *et al.* modified AuE

with 6-hydroxy-1-hexanethiol to provide sufficient space for simple DNA immobilisation (Zhang et al. 2015c). Ge *et al.* employed the sequential modification of AuE with 2-aminoethanethiol and covalently bonded glutaraldehyde, than an amino-modified DNA probe was assembled through the formation of Schiff bases (Ge et al. 2005).

Besides the above-mentioned materials, usage of modifiers with polymeric nature was described. Saheb *et al.* reported the use of pyrrole polymerisation to modify a platinum electrode (PtE) for DNA methylation detection (Saheb et al. 2014). Daneshpour *et al.* modified carbon SPE with polythiophene to improve the stability and sensitivity of their methylation biosensor (Daneshpour et al. 2016).

Despite the unlimited options for electrode surface modification, some authors preferred bare electrodes. The natural affinity of the AuE surface to self-assemble SH-modified DNA *via* Au–S bonds has been leveraged by several researchers (Baek et al. 2013; Furst et al. 2014; Shen et al. 2016; Zhang et al. 2015c). To improve the Au–S binding efficiency, the pretreatment of AuE surface by flame-annealing (Brotons et al. 2016) or AuE surface activation with 0.1 M H_2SO_4 (Wang et al. 2013a) have been applied. The most notable advantages of bare electrode-based biosensing are that no electrode preparation is needed and that the sensitivity remains unchanged (Wang et al. 2013c; Zhang et al. 2015c).

2.3.The use of BE for target immobilization

In general, for biosensors target-specific recognition is mediated by an enzymatic or affinity reaction (Tamayo et al. 2013; Zhu et al. 2015). In the DNA methylation biosensing, the targeted methylated DNA is bounded selectively through a complementary DNA probe (Wang et al. 2013b), methyl-binding domain protein (MBD) (Wee et al. 2015a), or anti-5-mC

(Daneshpour et al. 2016) and anti-5-hmC (Yang et al. 2015b) antibodies. In enzymatic DNA methylation biosensors, the methylated or demethylated DNA is targeted and the amount of resulted product is coupled with the quantification of methyltransferase/demethylase activity (Wang et al. 2012a; Yang et al. 2015b; Yin et al. 2013b).

3. Electrochemical strategies for the detection of DNA methylation

A number of publications aimed at the electrochemical sensing and biosensing of DNA methylation has increased significantly over the past few years, as illustrated in Fig. 2. Electrochemical strategies can be targeted on electrochemical detection of methylation enzymes (methyltransferase, demethylase) or products, resulting from their enzymatic activity. 5-mC, 5-hmC, and C can be detected directly (DNA bases oxidation) or indirectly (electroactive labels). In the quantification of methylation enzymes activity only indirect detection have been described. Most approaches applied to investigate the DNA methylation fall into three main categories: restriction endonuclease (RE)-based, bisulfite treatment (BS)-based, and immuno/affinity-reaction-based biosensing, but the sensing approach cannot be neglected. Basic principles on which RE, BS, immune, and affinity strategies are based illustrates Fig. 3.

3.1. Restriction endonuclease-based methods

Restriction endonucleases (REs) are enzymes that selectively cleave DNA at specific sites (Ferrin and Cameriniotero 1991; Cheng 1995). There are two types of REs: methylation-sensitive REs, which can digest methylated DNA (Baek et al. 2013; Liu et al. 2015a; Wang et

al. 2013c), and methylation-resistant REs, which can cleave only unmethylated DNA (Yin et al. 2015a; Zhou et al. 2014). REs cut the dsDNA at specific recognition sequences *via* the cleavage of blunt or sticky ends (Gronenborn and Messing 1978; Mierzejewska et al. 2016). Differences in cut-offs are widely exploited in molecular biology (Aiba et al. 2011; Mierzejewska et al. 2016; Sistla and Rao 2004), genetic engineering (Chan et al. 2011), and electrochemical biosensing (Li et al. 2016a; Yin et al. 2015a).

In electrochemical RE-based biosensors, dsDNA acts as the substrate for enzymatic reactions involving methyltransferases, and REs perform the specific cleavage of DNA, with DNA methylation pattern playing a crucial role. The workflow of RE-based biosensors is fairly similar across approaches. Firstly, dsDNA is attached to the supporting surface, then the methyltransferase is allowed to work. According to the presence of the enzyme and its quantity, the methylation patterns are changed. Next, the change in DNA methylation status is recognised, and DNA is cleaved by the RE. Finally, the RE-based cleavage product is electrochemically detected. Scheme 1 provides a sketch of all the important criteria within all of RE-based reviewed studies.

Using RE-based biosensors three main approaches of DNA immobilisation were described. The surface of the WE (Li et al. 2015; Wang et al. 2013c; Yang et al. 2015c; Yin et al. 2015a) is employed most frequently. In addition to the WE, MPs (Li et al. 2016a) or non-WE (Yang et al. 2015c; Zhou et al. 2014) have also been used. In these approaches, the DNA immobilisation and treatment are conducted on separate surfaces, and the signal response is detected by the WE.

To improve the efficiency of DNA immobilisation at WEs, the supporting surface has been modified by AuNPs (Liu et al. 2015a; Liu et al. 2016a; Yin et al. 2015a; Zhou et al.

2014). Alternatively, a bare AuE can be employed as well (Baek et al. 2013; Furst et al. 2014; Wang et al. 2013b; Zhang et al. 2015c); Au–S covalent linkages were utilised in both cases. To avoid the direct immobilisation of a DNA probe on the AuE surface, Hong *et al.* used benzenedithiol (Hong et al. 2016).

To obtain the dsDNA, the complementary ssDNA was hybridised with previously immobilised probe (Li et al. 2015; Wang et al. 2013a) or hairpin consisting of a base-paired stem (Liu et al. 2016a; Shen et al. 2016) was employed. Once the dsDNA was immobilised, the target methylation enzyme was allowed to be manifested. Three different methyltransferases DNA adenine MTase (DAM MTase) (Baek et al. 2013; Liu et al. 2016a), M. SssI MTase (Liu et al. 2015a; Muren and Barton 2013), and DNMT1 (Furst et al. 2014) were employed. DAM MTase specifically methylates the adenine of the 5'-GATC-3' sequence of synthetic DNA. Thus, DAM MTase has been mostly employed for the methylation of synthetic oligonucleotides (Baek et al. 2013; Wang et al. 2013c). In addition to the methyltransferase activity quantification, some authors have addressed the DNA demethylase (Shen et al. 2016; Yin et al. 2015a; Zhou et al. 2014).

The presence and quantity of methyltransferase (or DNA demethylase) results in corresponding changes in the DNA methylation pattern. Based on this change, the DNA is cleaved by a particular RE (Sistla and Rao 2004) using DpnI alone (Baek et al. 2013; Liu et al. 2016a; Wang et al. 2013a) or in combination with Exonuclease III (Exo III) digestion (Li et al. 2016a; Yang et al. 2015c). The ExoIII digestion was performed to better differentiate and highlight the signal responses of methylated and unmethylated DNA. To cleave the unmethylated DNA, various methylation-resistant REs, such as MboI (Hong et al. 2016; Zhang et al. 2015c), BstUI (Shen et al. 2016; Zhou et al. 2014), BssHII (Furst et al. 2014; Muren and Barton 2013) and HpaII (Liu et al. 2015a), have been employed. Combined

cleavage and digestion were achieved using BstUI/Exo III (Yin et al. 2015a) and HpaII/ExoIII (Zhang et al. 2015b).

After the successful cleavage of dsDNA, the final product can be determined by electrochemical analysis. Indirect detection coupled with the use of different electroactive labels has been employed. The use of $[Ru(NH_3)_6]^{3+}$ complex (Wang et al. 2013b), $[Fe(CN)_6]^{3-}$ (Yin et al. 2015a), or combination of $[Fe(CN)_6]^{3-}$ and methylene blue (MB) (Furst et al. 2014) was described. As seen from Scheme 1, MB was the most commonly used electroactive label (Furst et al. 2014; Hong et al. 2016; Li et al. 2015; Liu et al. 2015a; Liu et al. 2016a). In the study of Liu *et al.* recognition of non-cleaved MB-labelled probe was targeted (Liu et al. 2016a). Hong *et al.* employed MB to design a DNA–AuNP network; while only the non-methylated (and uncleaved) DNA–AuNP network provide electrochemical signal (Hong et al. 2016). Liu *et al.* introduced the application of nano-mesoporous silica (MSN) that was functionalised by a G-quadruplex with MB (MSN–MB complex) (Liu et al. 2015b). For the quantification of DAM MTase, the use of ferrocene (Fc) was reported by Zhang *et al.*; the signal response was enriched by AuNPs and Fc tag remaining close to the electrode, whereas electron transfer was facilitated (Zhang et al. 2015c). Instead of Fc, Shen *et al.* employed Fc carboxylic acid to facilitate the binding of an NH_2-modified hairpin probe (*via* an NH_2–COOH bond) (Shen et al. 2016). Zhou *et al.* quantified DNA demethylase activity through recognition by alkaline phosphatase (ALP) and the hydrolysate of *p*-nitrophenol (Zhou et al. 2014). The use of quantum dot (QD) for DAM Mtase recognition was reported by Beak *et al.* (Baek et al. 2013).

As a demonstrative example of RE-based biosensor, study of Liu *et al*. will be presented. Firstly, dsDNA was assembled on AuNPs modified GCE, than the DAM MTase allowed to be manifested. In the next step, DpnI digestion of methylated DNA triggered the

formation of G-quadruplex DNAzymes and the DAM MTase activity was detected indirectly through the oxidation of hydroquinone by H_2O_2 (Liu et al. 2015a) (scheme of RE-based biosensor presents Fig. 4).

Table 4. provides sensitivity comparison of all RE-based reviewed studies. The ultrasensitive DNA MTase activity assay (0.004 U/ml), was reported by Li *et al.*. In this study a duplex DNA containing the methylation-responsive sequence was methylated, when the DAM MTase was present. Than, the change of dsDNA methylation pattern was recognized and cleaved by DpnI, which triggers the ExoIII-catalyzed autonomous cycling cleavage processes. Following this, a large amount of MB-labeled mononucleotides were released, generating a significantly amplified electrochemical signal toward the Dam MTase activity assay (Li et al. 2015).

Table 4. Sensitivity of RE-based biosensors.

Author and year	LOD	Methods	Inhibitors	Real sample
(Li et al. 2015)	0.004 U/mL	DPV	Benzylpenicillin sodium, gentamycin sulfate and 5-fluorouracil	-
(Jing et al. 2014)	0.020 U/mL	CV/DPV	5-fluorouracil	-
(Hong et al. 2016)	0.020 U/mL	CV/EIS/DPV	-	-
(Zhang et al. 2015c)	0.024 U/mL	CV/EIS/DPV	-	-
(Li et al. 2016a)	0.031 U/mL	EIS/DPV	5-fluorouracil	-
(Wang et al. 2013a)	0.180 U/mL	DPV	-	-
(Liu et al. 2016a)	0.270 U/mL	DPV	-	-
(Liu et al. 2015b)	0.280 U/mL	CV/EIS/DPV	Zebularine	-
(Yang et al. 2015c)	0.310 U/mL	DPV	Epicatechin	-
(Baek et al. 2013)	0.790 U/mL	SWASV	5-fluorouracil	-

(Liu et al. 2015a)	0.960 U/mL	EIS/DPV	RG 108	-
(Yin et al. 2015a)	0.150 ng/mL	DPV	-	-
(Shen et al. 2016)	0.170 ng/mL	CV/EIS/DPV	-	-
(Zhou et al. 2014)	1.300 ng/mL	EIS/DPV	-	-
(Muren and Barton 2013)	-	CV	-	-
(Furst et al. 2014)	-	CPA	-	Tumour tissue

3.2. Bisulfite treatment-based methods

The goal of DNA BS treatment is to convert non-methylated C to uracil (U) while leaving methylated C unchanged (Frommer et al. 1992). BS treatment was firstly described by Frommer in 1992 (Frommer et al. 1992) and modified by Clark a few years later (Clark et al. 1994). In 1996, methylation-specific PCR (MSP) (Herman et al. 1996) was introduced, which included BS treatment, the amplification of a BS product by methylation-specific primers, and the detection of PCR amplicons by gel electrophoresis. As an alternative to MSP, which is considered the gold standard for DNA methylation detection (Clark et al. 2006), electrochemical BS-based biosensors are presented. An overview of the strategies, which describe workflows from the choice of supporting surfaces, through BS treatment of target sequences, to the choice of electroactive labels and methods for their identification provides Scheme 2. Particular attention will be payed to the BS treatment, forming the core of the strategies described in this chapter.

BS treatment alone or combined with other techniques, such as PCR and RE-based cleavage, have been applied. The use of all of three methods (BS/PCR/RE) was described by Hou *et al.*, they employed the combination of double consecutive restriction (MseI and

BstUI), BS treatment, and subsequent MSP for the detection of target gene methylation (Hou et al. 2003). Yanagisawa *et al.* used the same combination of procedures, but in another consequence. BS-treated target DNA was amplified by PCR and then cleaved by HpyCH4IV (Yanagisawa et al. 2015). Combination of BS treatment with MSP PCR was employed by Sato et al. (Sato et al. 2006) and Liu *et al.* (Liu et al. 2016b). Koo *et al.* also combined BS treatment with PCR, but instead of MSP PCR, ligase chain reaction (LCR) was involved (Koo et al. 2014a). Contrary to wide range of combinations, even simple BS treatment without help of any other method was employed (Saheb et al. 2014; Wang et al. 2012b; Xu et al. 2016). Table 5 presents the BS-based methods according to their sensitivity.

Table 5. Sensitivity of BS-based biosensors.

Author and year	LOD	Method	Real sample
(Koo et al. 2014a)	0.04 pM of targeted promoter	DPV	Breast cancer cell line (DNA)
(Wang et al. 2012b)	18 pM methylated p53 gene	CV/DPV	-
(Yanagisawa et al. 2015)	0.5 nM (PCR product)	CV/ASV	Genomic DNA
(Xu et al. 2016)	1.3 nM loc. A, 0.38-nM loc. B	SWV	-
(Saheb et al. 2014)	0.1 μM GSTP1 gene	CV	-
(Liu et al. 2016b)	0.01% methylation of SFRP2 gene	EIS/DPV	Breast tumour tissue (DNA)
(Hou et al. 2003)	-	CV/SWV	Gastric tumour tissue (DNA)

(Sato et al. 2006)	-	DPV	-

The most sensitive (required 0.04 pM of starting material, sensitive to 10–15% methylation change) method was described by Koo *et al.* This unique approach in which BS treatment and LCR were employed to amplify methylation events schematicaly presents Fig. 5. After bisulfite conversion of target sequences, duplex LCR recognized and amplified a C to T base change at a CpG site, while two different species of LCR products representing methylated or unmethylated events were generated. In the next step, the relative proportion of two LCR products was detected *via* DNAzyme-mediated electrocatalytic reduction of hydrogen peroxide (H_2O_2) (Koo et al. 2014a).

An another interesting strategy, where ferrocenyl naphthalene diimide (FND)-based hybridisation was applied for the $p16^{Ink4a}$ gene methylation detection presents Fig. 6. After treatment of DNA sample with BS, target region was amplified by the PCR (MSP PCR respectively) with the methylation-specific primers. Subsequently, the PCR product was hybridized with two DNA probes for unmethylated and methylated specific sequences. The FND assisted probe-target base pairing was characterised by an electrochemical array chip, peak current of FND before and after hybridization was detected (Sato et al. 2006).

BS has been widely applied for the detection of DNA methylation, but when 5-hmC as the 6^{th} DNA bases was described (Tahiliani et al. 2009), one drawback arose. In BS conversion, 5-hmC has been shown to exhibit the same behaviour as 5-mC (Huang et al. 2010). Simple BS-based methods cannot distinguish 5-hmC and 5-mC. Recently, oxidative BS treatment (Field et al. 2015; Houseman et al. 2016; Stewart et al. 2015) and Tet-1 protein-assisted BS sequencing (Yu et al. 2012) have been described.

3.3. Immuno/affinity-based methods

The two previously described methods have several disadvantages, including the loss of samples (e.g., from cut-off because of cleavage in RE-based methods) or irreversible changes of the samples (e.g., C to U conversion in BS treatment-based methods). The strategies described in this chapter overcome these disadvantages, and methylated DNA residue recognition is mediated by antibodies (i.e., immunoreaction) (Wang et al. 2012a; Yin et al. 2015b) or by the MBD protein family (i.e., affinity reaction) (Wee et al. 2015a). In immuno-based recognition, some problems relating to the antibody specificity (i.e., a cross reaction) and quantity (appropriate dilution) can arise. The use of MBD family proteins (i.e., MBD1, MBD2, MBD3, MBD4 and MeCP2) avoids these problems (Pan et al. 2010; Yin et al. 2013a). The disadvantage is that neither immuno- not affinity-based techniques provide information about distinct patterns in DNA sequences, and DNA methylation can be affected by surrounding impurities (Syedmoradi et al. 2016). Which is at least partially offset by the advantage that the 5-mC and 5-hmC can be distinguished based on the specificity of MBD, anti-5-mC and anti-5-hmC (Yang et al. 2015b). The following two subchapters will focus on the application of immuno/affinity approaches in biosensing of DNA methylation.

3.3.1 Affinity-based methods

The affinity-based approach is based on MBD specificity for 5-mC in a target DNA sequence. In context with this the involvement of MBD (or other member of MBD protein family) will be discussed in detail. Scheme 3 summarises the application of MBD, MBD1 and MeCP2 in overviewed affinity-based DNA methylation biosensors.

Yin *et al.* described a biosensor for M. SssI MTase activity detection. In this approach, only the methylated targeted DNA remains uncleaved by HpaII and available for interaction with MBD and Coomassie brilliant blue (CBB), while the CBB signal was detected by chronopotentiometry (CA) (Yin et al. 2013a). Combination of MeCP2 (in the role of BE) with AuNPs labelled by horseradish peroxidase (HRP) as signal amplification unit for the DNA methylation detection was described by Yin *et al.* Under the HRP catalysis, hydroquinone was oxidized by H_2O_2, then the electrochemical reduction of benzoquinone was detected by differential pulse voltammetry (DPV) (Yin et al. 2013b). Wee *et al.* described an assay in which MBD-modified MPs were applied for the rapid evaluation of genomic and gene-specific methylation. An HRP/H_2O_2 system coupled with chromogen (3,3′,5,5′-tetramethylbenzidine) was utilised, the signal of oxidised chromogen was measured by amperometry (Wee et al. 2015a). Fig.7 presents study, in which MBD1 and hyperbranched rolling circle amplification (RCA) were applied to recognise M. SssI MTase activity. In the first step, the MBD1 protein was applied to recognize methylated cytosine in target dsDNA, then anti-His tag antibody and biotinylated IgG was specifically bound. In the next step, biotinylated oligonucleotide was captured. Subsequently, the RCA reaction was initiated and ALP catalysed the hydrolysis of 1-naphthyl phosphate to form 1-naphthol (Xu et al. 2014b).

Table 6. presents a comparison of methods in terms of sensitivity. The most sensitive method was described by Xu *et al.* In this study MBD1 was employed for specific recognition of methylated DNA and anti-his-tag antibody tagged AuNPs and the ALP labeled IgG tagged AuNPs were involved for dual signal amplification. The ALP converted 1-naphthyl phosphate into 1-naphthol and the electrochemical signal was detected by DPV (Xu et al. 2014a).

Table 6. Affinity-based biosensors

Author and year	LOD	Method	Inhibitor	Real sample
(Xu et al. 2014a)	0.01 U/mL M. SssI MTase	EIS/DPV	Chlorpyrifos and methyl parathion	-
(Xu et al. 2014b)	0.03 U/mL M. SssI MTase	DPV	Paclitaxel	-
(Yin et al. 2013a)	0.04 U/mL M. SssI MTase	EIS/CA	Fisetin, chlorogenic acid	-
(Yin et al. 2013b)	0.17 U/mL M. SssI MTase	EIS/CV/DPV	5-fluorouracil and epicatechin	-
(Wee et al. 2015a)	37.5 pg of methylated DNA	Amperometry	-	Human urine DNA

3.3.2 Immuno-based methods

In immuno-based approaches, anti-5-mC and anti-5-hmC antibodies have been primarily employed for the recognition of the DNA methylation, 5-mC and 5-hmC respectively. Scheme 4 sumerises immuno-based approaches, each of strategies will be individually described in more detail.

Wang *et al.* reported a method for the M. SssI MTase activity quantification (Fig. 8, in which HpaII was used for the selective recognition of methylated/unmethylated DNA sequences. Only the methylated sequence remains uncleaved and specifically conjugates with the anti-5-mC antibody and secondary antibody labelled by HRP. Signal of benzoquinone, the result of a catalytic reaction of HRP was detected by DPV (Wang et al. 2012a). Yang *et al.* employed the anti-5-hmC antibody in order to distinguish healthy and tumour tissues based on the hydroxymethylation of DNA . GCE was modified by perylene-3,4,9,10-tetracarboxylic acid (PTCA). Then, the presence of 5-hmC was recognised by the anti-5-hmC antibody, followed by catalysis of *p*-nitrophenyl phosphate by ALP (Yang et al. 2015b). The use of the

anti-6-mA antibody was described by Yin *et al.* GCE modified by AuNPs and 4-aminophenylboronic acid was assembled through the interaction between the boric acid and glycosyl groups of anti-6-mA antibodies. Then, the methylated RNA was captured (based on the presence of 6-mC), and the decrease in the $[Fe(CN)_6]^{3-}$ signal was quantified by DPV (Yin et al. 2015b). Finally, an excellent study described by Daneshpour *et al*. will be introduced (Fig. 9). In that study, target DNA hybridisation was performed to capture probes immobilised on MPs modified by N-trimethyl chitosan and AuNPs (MPS/TMC/AuNPs). The target methylated DNA/probe/MPs complex was specifically bound to an anti-5-mC antibody-modified carbon SPE. The signal was mediated by AuNPs, and the pre-oxidation of AuNPs (in the Fe_3O_4/TMC/Au nanocomposites) and reduction of Au(III) to Au^0 mediated the electrochemical detection (Daneshpour et al. 2016). Table 7 presents a comparison of methods in terms of sensitivity.

Table 7. Immuno-based biosensors.

Author and year	LOD	Method	Inhibitor	Real sample
(Daneshpour et al. 2016)	$2 \cdot 10^{-15}$ M methylated DNA	CV/DPV	-	Human blood plasma DNA
(Yin et al. 2015b)	2.57 pM methylated RNA	DPV	-	Rice seedlings (RNA)
(Wang et al. 2012a)	0.1 U/mL M. SssI MTase	DPV	5-aza-2′-deoxycytidine, procaine, epicatechin, caffeic acid	-
(Yang et al. 2015b)	-	DPV	-	Breast cancer cell line (DNA)

3.4. Other biosensors

Biosensors that cannot be classified using the previously described strategies, will be briefly introduced in this section and summarised in Scheme 5 and Table 8.

Ge *et al.* employed a probe immobilised on glutaraldehyde-modified AuE for target sequence hybridisation. The single mismatch (C versus 5-mC) in the PCR-amplified target sequence was distinguished based on the signal of $[Co(phen)_3](ClO_4)_3$ (Ge et al. 2005). Wee *et al.* reported a study in which LCR amplification and Au microelectrodes with pre-immobilised capture probes were used. The resulting product (i.e., the LCR-amplified target sequence) was detected by the electrocatalytic reduction of hydrogen peroxide (H_2O_2) (Wee et al. 2013). Zhou *et al.* described a biosensor for 5-hmC detection in which a GCE modified by AuNPs and the ssDNA probe was used for target sequence hybridisation. The target DNA was glycosylated *via* the enzymatic reaction of T4 β-glucosyltransferase. As a result, the glucosyl group was covalently modified on the hydroxymethyl of target DNA. Phenyldiboronic acid as bridging agent for ALP capturing reagent was selected. The immobilized ALP catalyses the hydrolysis of *p*-nitrophenyl phosphate disodium salt and resulted *p*-nitrophenol was detected by DPV (Zhou et al. 2015) (Fig. 10).

Table 8. Other biosensors.

Author and year	LOD	Method	Real sample
(Zhou et al. 2015)	1.43 pM hydroxymethylated DNA	EIS/DPV	-
(Ge et al. 2005)	Single mismatch detection	CV/SWV	-
(Wee et al. 2013)	-	CV	Breast cancer cell line (DNA), serum derived DNA

3.5. Sensors

In sensors, no biomolecule bridges the space between the target and the WE surface. The target (i.e., a DNA sequence or free DNA bases) in which the methylation is to be examined, comes into close contact with the WE surface. The direct (DNA bases moieties) and indirect (electrochemical indicators) signals have been employed to quantify DNA methylation. Scheme 6 provides an overview of all the important criteria within reviewed studies dedicated to sensing of DNA methylation.

Sina *et al.* combined the DNA base-dependent affinity of the AuE surface, BS treatment and asymmetric PCR. In their study, the unmethylated DNA sequence was adsorbed to a lesser extent on the AuE surface (than the methylated), and a higher signal response of $[Fe(CN)_6]^{3-}$ was obtained (Ibn Sina et al. 2014). Another BS treatment-based sensor was described by Bartosik *et al.*, who reported the use of an HMDE and a solid amalgam electrode to distinguish between methylated and unmethylated DNA. Based on the reduction peaks, the sequences did not differ significantly. Therefore, BS treatment was employed, and the reducible C was transformed into non-reducible U. Using this approach, the peak of C decreased strongly, whereas for BS-resistant 5-mC, the C reduction peak remained unchanged (Bartosik et al. 2012). An HMDE was also employed by Ioannou *et al*., instead of BS, SYBR Green I and ethidium bromide were used to distinguish between methylated and unmethylated DNA amplicons (Ioannou et al. 2010).

In addition to HMDEs and AuE, various carbon-based electrodes (e.g., GCE, BDDE, and nanocarbon film electrode) have been used to detect DNA methylation. The use of GCE modified by MWCNTs and polypyrrole was described by Wang *et al.* Thanks to the modification of GCE, all bases (G, A, T, C, and 5-mC) exhibited well-defined oxidation

peaks; however, the thymine (T) interference had to be eliminated to better differentiate between C and 5-mC (Wang et al. 2013b). The oxidation of DNA bases at GCE modified by MWCNTs and β-cyclodextrin was reported by Wang *et al.* (Wang et al. 2016a). Well-defined peaks were obtained *via* electro-oxidation of G, A, T, C, and 5-mC, and elimination of the T signal was not required, unlike in the previous study (Wang et al. 2016a). Unmodified GCE and BDDE were employed by Sanjuan *et al.* for the determination of another marker of DNA methylation: N7-methylguanine (7-mG). Good resolution of anodic peaks of 7-mG, A, G, and 8-oxoguanine (8-oxoG) was reported (Sanjuan et al. 2016). Two other studies were dedicated to use of nanocarbon film electrodes, which were able to distinguish 5-mC and C bases (Goto et al. 2010; Kato et al. 2011). Goto *et al.* observed single C methylation, regardless of the methylation position in the targeted gene sequence. For better resolution of the C and 5-mC oxidation peaks, the pH of the supporting electrolyte was optimised (Goto et al. 2010). Kato *et al.* used a nanocarbon film electrode for the direct oxidation of 5-mC and C in CpG oligonucleotides. Then, P1 endonuclease was employed to digest the target CpG into mononucleotides, and the resulting sensitivity was 4.4 times higher than that achieved without P1 treatment (Kato et al. 2011). Table 9 presents a comparison of DNA methylation sensors in terms of sensitivity.

Table 9. Sensors.

Author and year	LOD	Method	Real sample
(Bartosik et al. 2012)	Nanomolar/ subnanomolar levels of ODN	SWV	-
(Ioannou et al. 2010)	0.06 μM (amplicons)	DPV (CPE), ACV (HMDE)	-
(Wang et al. 2013b)	0.1 μM (G), 0.3 μM (C)	CV/DPV	Salmon sperm DNA sample

(Wang et al. 2016a)	0.2 μM (G), 0.6 μM (C)	EIS/DPV	Salmon sperm DNA sample
(Sanjuan et al. 2016)	1.2 μM (7-mG)	CV/SWV	Human urine DNA
(Goto et al. 2010)	Methylation ratio 30%	CV/SWV	-
(Kato et al. 2011)	-	SWV	-
(Brotons et al. 2016)	-	voltammetry	-

4. Methyltransferase inhibitors

Aberrant DNA methylation leading to altered gene expression can induce several genetic diseases, including cancer (Esteller 2008; Feinberg 2007). Disease-causing aberrant DNA methylation is associated with the malfunction of methyltransferases, which are the enzymes that drive the under- or over-expression of certain genes (Esteller 2008). In cases of the hypermethylation of tumour suppressor genes, the upregulation of methyltransferases plays a critical role. Thus, inhibiting these enzymes constitutes an interesting therapeutic strategy (Hanahan and Weinberg 2011; Patch et al. 2015; Vogelstein and Kinzler 1993). To date, several MIs have been approved by the Food and Drug Administration for cancer treatment (Blagosklonny 2004; Bonate et al. 2006; Hadaczek et al. 2013). The most successful epigenetic drug to date 5-azacytidine is currently recommended as the first-line treatment for high-risk myelodysplastic syndromes. In recent decades, an increasing number of drugs targeting DNA methylation have been developed (Yang et al. 2010).

To further extend the biosensing strategies for methyltransferase activity quantification, some authors have monitored the inhibitory effects of select therapeutics. RE-based strategies have been applied to the screening of MI efficiency, and the MTase inhibition effects of 5-fluorouracil (Baek et al. 2013; Li et al. 2016a), epicatechin (Yang et al. 2015c),

RG108 (Liu et al. 2015a), and zebularine (Liu et al. 2015a) were investigated. Liu *et al.* tested the inhibition activity of zebularine using M. SssI MTase in the inhibition assay, and the 50% inhibitory concentration (IC_{50}) value of zebularine was found to be 6 μM (Liu et al. 2015a). Beak *et al*. compared the inhibition activities of three potential MIs (gentamycin, benzyl penicillin, and 5-fluorouracil). However, only the most effective (5-fluorouracil) was analysed further, and its IC_{50} value was estimated to be 0.96 μM (Baek et al. 2013). This result was in good agreement with the value determined previously by a standard inhibition experiment (Li et al. 2007; Liu et al. 2010).

In addition to RE-based strategies, immuno-based strategies using anti-5-mC antibodies have been developed. Wang *et al.* monitored the inhibition of M. SssI activity using 5-aza-2'-deoxycytidine (5-aza), procaine, epicatechin, and caffeic acid. The maximum inhibition efficiency of 5-aza was approximately 75.15%, much higher than those of the other inhibitors studied (Wang et al. 2012a). Yin *et al.* reported the down-regulation of the methylation level of mRNA using 6-benzylaminopurine (Yin et al. 2015b). Additionally, the MBD1 protein was used to create another immunoassay in which M. SssI MTase was inhibited by paclitaxel, as described by Xu *et al.* The inhibition efficiency increased as the paclitaxel concentration increased from 10 to 300 μM (Xu et al. 2014b). Thus, the evaluation of MI efficiency is one promising application of strategies for the electrochemical detection of DNA methylation.

5. Current stage and future perspectives

Translating the knowledge accumulated during the past few decades into strategies for the early detection, prevention and treatment of cancer has been extremely difficult

(Widschwendter and Jones 2002). The role of DNA methylation in tumourigenesis is not exceptional. Although one might assume that the aim of studies investigating early cancer diagnosis would be the verification of proposed methods using real samples, this was not the case. In fact, most of the reviewed studies focused on demonstrating the feasibility of the proposed techniques using synthetic DNA oligonucleotides instead of real samples. However, finding excellent studies that meet the requirements for real sample testing is not impossible.

In 2003, Hou *et al.* proposed a BS-based strategy for the quantification of p16 ^{Ink4A} gene methylation of whole blood cells in healthy and gastric tumour tissues. This procedure was based on the coupling of DNA electrochemical sensing with the PCR amplification of DNA from human gastric tumour tissue and whole blood cells from healthy humans (Hou et al. 2003). More than ten years later, in 2014, Koo *et al.* reported a method for quantifying locus-specific methylation in breast cancer cell lines. The accuracy of this method was found to be comparable to those of a fluorescence-based method and next-generation sequencing (Koo et al. 2014b). In 2015, Wee *et al.* demonstrated the feasibility of their method in different complex biological samples. First, two cancer cell lines were assayed and their methylation levels compared. Then, the methylation status of urine DNA derived from prostate cancer patients was characterised, and high methylation at the GSTP1 gene promoter was found in three of the four patients (Wee et al. 2015a). In 2015, Yang *et al.* used an electrochemical immunosensor for quantitative 5-hmC detection in the genomic DNA of breast cancer tissue, in which substantially reduced 5-hmC levels were observed (Yang et al. 2015b). In 2015, Yanagisawa *et al.* verified their BS-based method for the detection of DNA methylation. Their results were based on blending methylated and unmethylated lambda phage DNA to form model genomic DNAs containing 0, 25, 75 and 100 % 5-mC (Yanagisawa et al. 2015).

Proof of concept in drug development is somewhat different. In pharmaceutical research, directly measuring whether a drug is effective for treating the desired disease is not appropriate, and instead, the prospective curative effect is used to determine whether or not to proceed with further testing (Nadler and Downing 2010). In that context, two studies targeted the characterisation of the 5-aza demethylation effect. Wee *et al.* demonstrated the feasibility of their method for DNA methylation detection by tracking the response of a cancer cell line before and after 5-aza treatment. Based on the methylation status of two gene promoters (GSTP1 and ESR1), the effects of demethylation therapy were evaluated (Wee et al. 2015a). In 2016, Liu *et al.* suggested that the SFRP2 promotor demethylation in breast cancer cell lines was a function of the DNA demethylation response to 5-aza (Liu et al. 2016b).

6. Conclusion

Since methylated cytosine came into the spotlight in 1948 (Hotchkiss 1948) and an association between aberrant DNA methylation and cancer development was hypothesised in 1964 (Srinivasan and Borek 1964), DNA methylation has been the subject of investigations. Conventional assays, such as MSP, MS, HPLC and radiolabelling, represent the standard approaches for detecting DNA methylation, but they have limitations. Thus, researchers are interested in discovering new methods that can overcome these limitations. Electrochemical analysis coupled with sensing and biosensing strategies offers many attractive features that could be relevant for future DNA methylation research. The current state of research into the electrochemical sensing and biosensing of DNA methylation was summarised in this article. Additionally, studies focusing on the use of methyltransferase inhibitors in cancer diagnosis and therapy were discussed.

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Figure 1 (**A**) The scheme of DNA methylation and hydroxymethylation. (**B**) Rearrangement of the DNA methylation patterns associated with tumourigenesis.

Figure 2 (**A**) The schematic layout of the subchapters dedicated to strategies for the electrochemical detection of DNA methylation. (**B**) Trend in the number of publications on the electrochemical detection of DNA methylation per year. The pie chart describes the distribution of the number of publications across types of strategies: sensing (S), biosensing (B), bisulfite treatment based (BS-based), restriction endonuclease based (RE-based), immuno/affinity based (I/A-based).

Figure 3 Scheme of basic principles on which using of bisulfite treatment (BS treatment), restriction endonuclease (RE), and immuno/affinity strategies are based.

Figure 4 A typical RE-based biosensor. A sensitive RE (DpnI) and the formation of G-quadruplex DNAzymes were employed for the electrochemical evaluation of DAM MTase activity. Reproduced from Liu *et al.* (Liu et al. 2015a) with permission from Elsevier.

Figure 5 An example of a BS-based biosensor. The experimental work was based on locus-specific DNA methylation and the electrocatalytic reduction of hydrogen peroxide. Reproduced from Koo *et al.* (Koo et al. 2014a) with permission from Elsevier.

Figure 6 An excellent example of a BS-based biosensor, where in addition to BS treatment Ferrocenyl naphthalene diimide (FND)-based hybridisation was used for the $p16^{Ink4a}$ gene methylation detection. Reproduced from Sato *et al.* (Sato et al. 2006) with permission from Elsevier.

Figure 7 A very sophisticated example of an affinity-based biosensor. Rolling circle amplification was utilised for the detection of DNA methyltransferase activity. Reproduced from Xu *et al.* (Xu et al. 2014b) with permission from Elsevier.

Figure 8 An example of immuno-based biosensor, where combination of immune-reaction with restriction endonuclaese (HpaII) cleavage were employed for the M. SssI MTase activity quantification. HpaII was used for the selective recognition of methylated/unmethylated DNA sequences. Reprinted with permission by American Chemical Society from Wang *et al.* (Wang et al. 2012a).

Figure 9 An immuno-based biosensor in which a Fe_3O_4 nanocomposite and polythiophene-modified electrode were employed to evaluate RASSF1A tumour suppressor gene methylation. Reproduced from Daneshpour *et al.* (Daneshpour et al. 2016) with permission from Elsevier.

Figure 10 An outstanding example of biosensor for detection of target DNA sequence hydroxymethylation. 5-hmC in target DNA was chemically modified with glucose, then with the bridge connection of 1,4-phenyldiboronic acid, alkaline phosphatase was further captured to produce p-nitrophenol. Reproduced from Zhou *et al.* (Zhou et al. 2015) with permission from Elsevier.

Scheme 1 Different approaches using RE-based biosensors.

Scheme 2 Different approaches using BS-based biosensors.

Scheme 3 Different approaches using affinity-based methods.

Scheme 4 Different approaches using immuno-based methods.

Scheme 5 Different approaches using other biosensors.

Scheme 6 Different approaches using sensors.

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Highlights

- Current trends in the electrochemical sensing and biosensing of the DNA methylation are reviewed.
- Main strategies are mentioned.
- Their advantages and disadvantages are discussed.

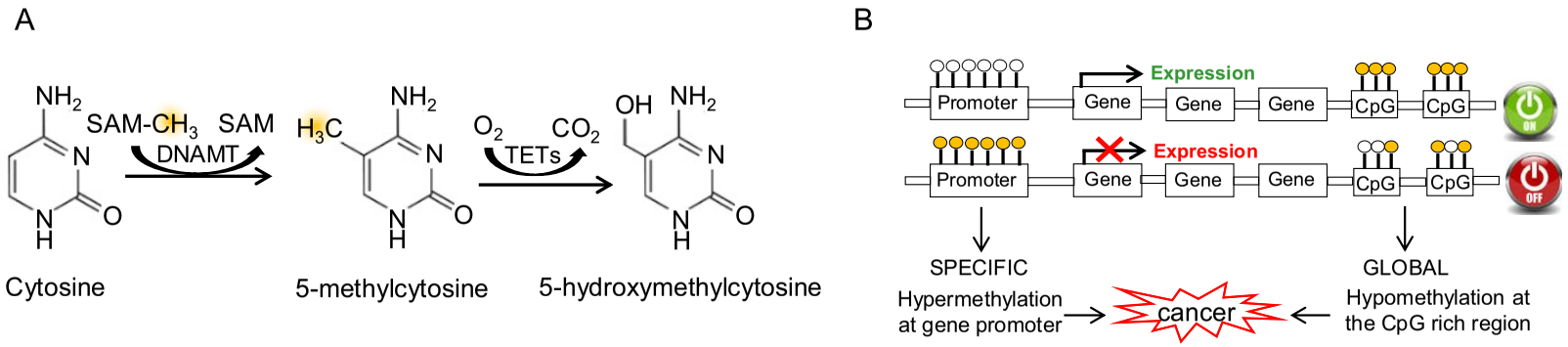
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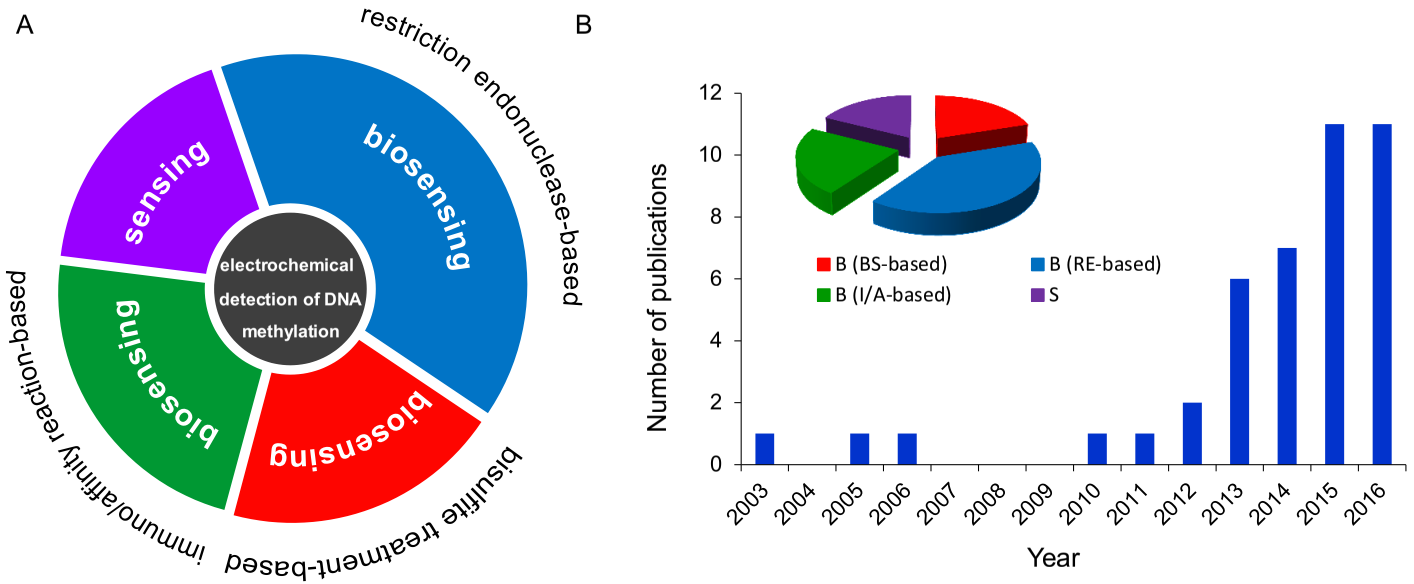
Figure 1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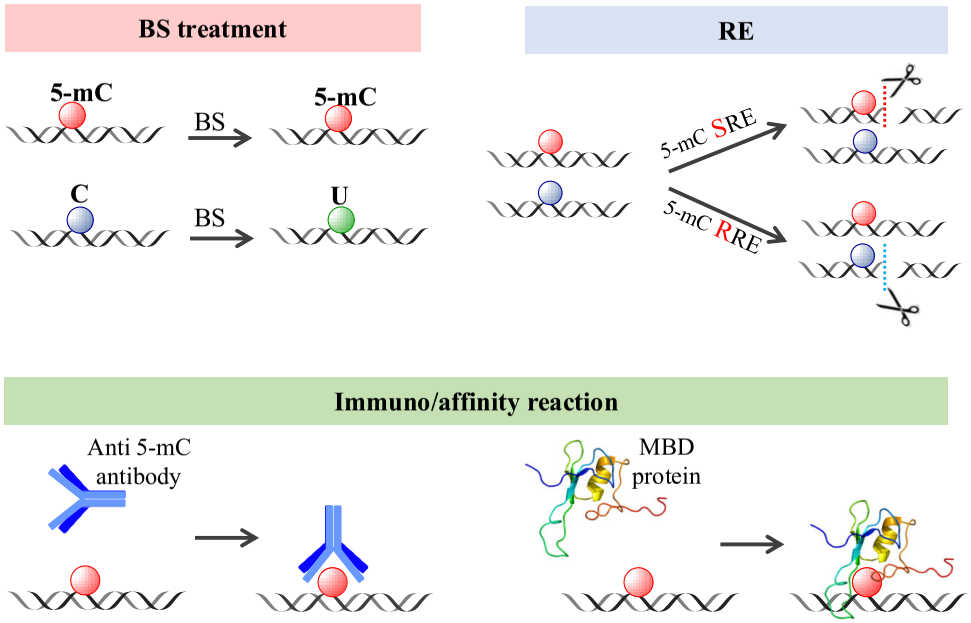
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BS
U
5-mC SRE
5-mC RRE
Immuno/affinity reaction
Anti 5-mC
antibody
MBD
protein

Figure 3

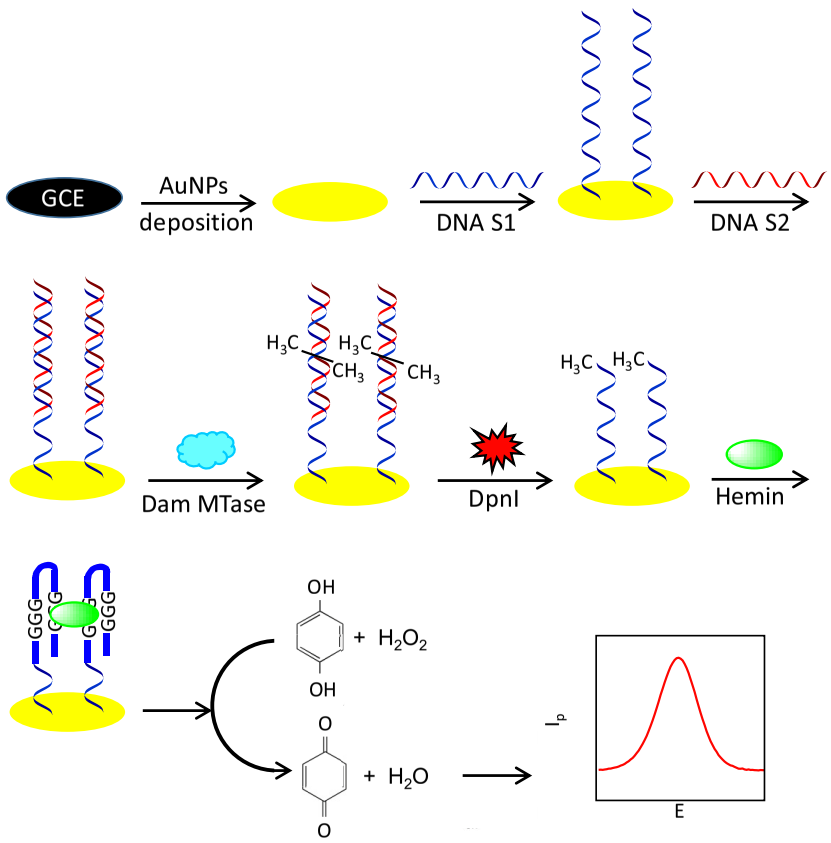

GCE
AuNPs
deposition
DNA S1
DNA S2
Dam MTase
DpnI
Hemin
OH
OH
+ H_2O_2
O
O
+ H_2O
I_p
E

Figure 4

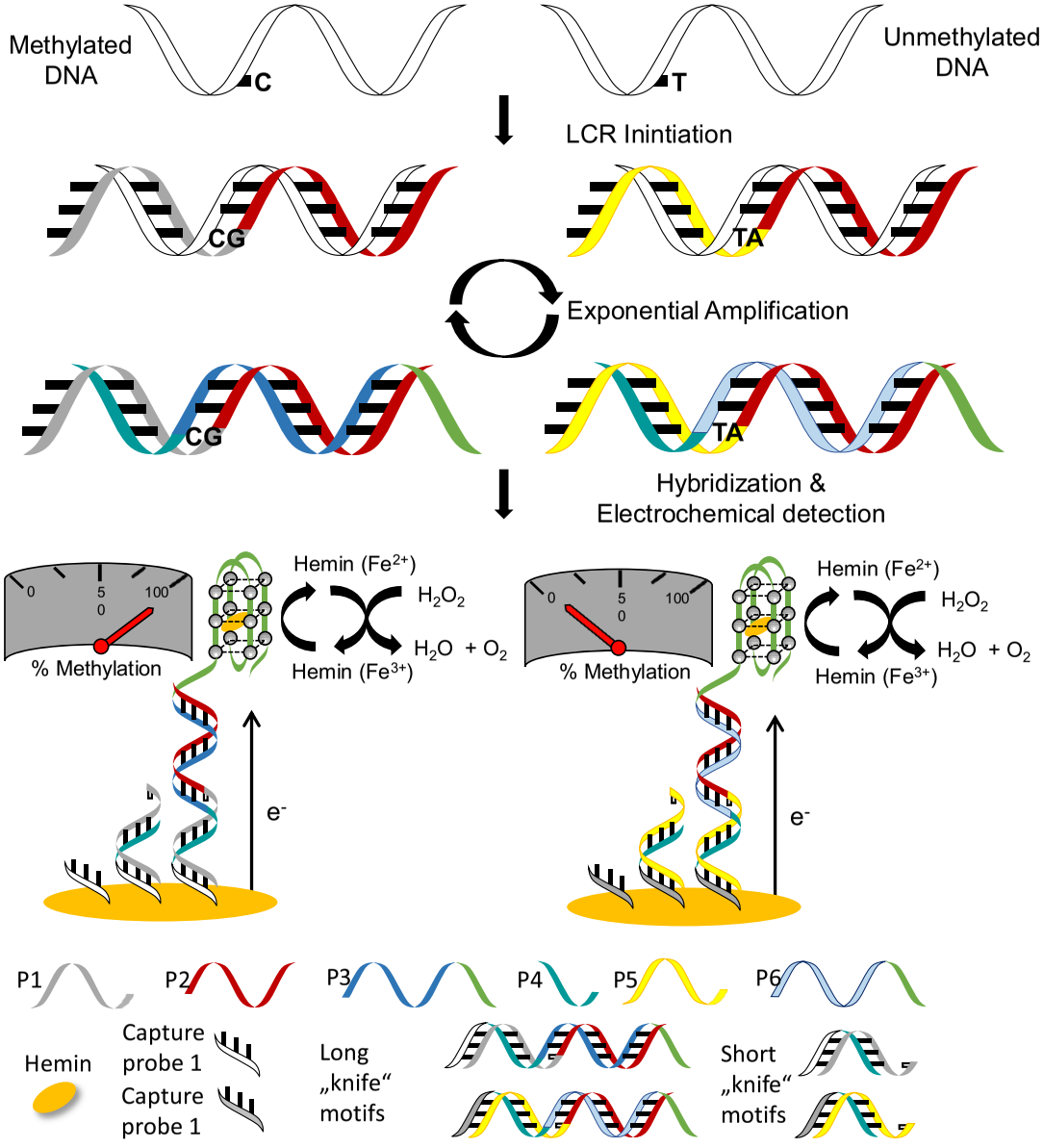

Methylated DNA
C
T
Unmethylated DNA
LCR Inintiation
CG
TA
Exponential Amplification
CG
TA
Hybridization & Electrochemical detection
0
5
0
100
% Methylation
Hemin (Fe2+)
H2O2
H2O + O2
Hemin (Fe3+)
e-
P1
P2
P3
P4
P5
P6
Hemin
Capture probe 1
Capture probe 1
Long „knife“ motifs
Short „knife“ motifs

Figure 5

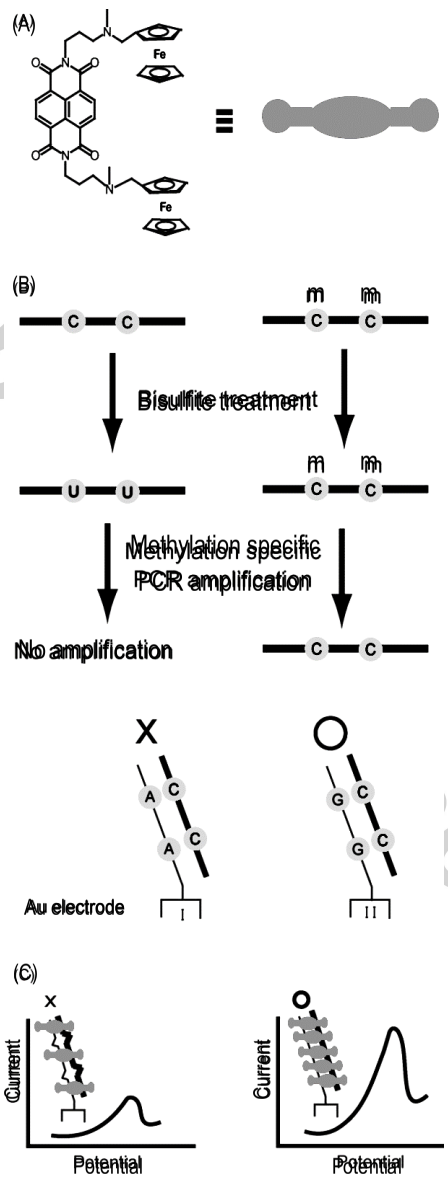


Figure 6

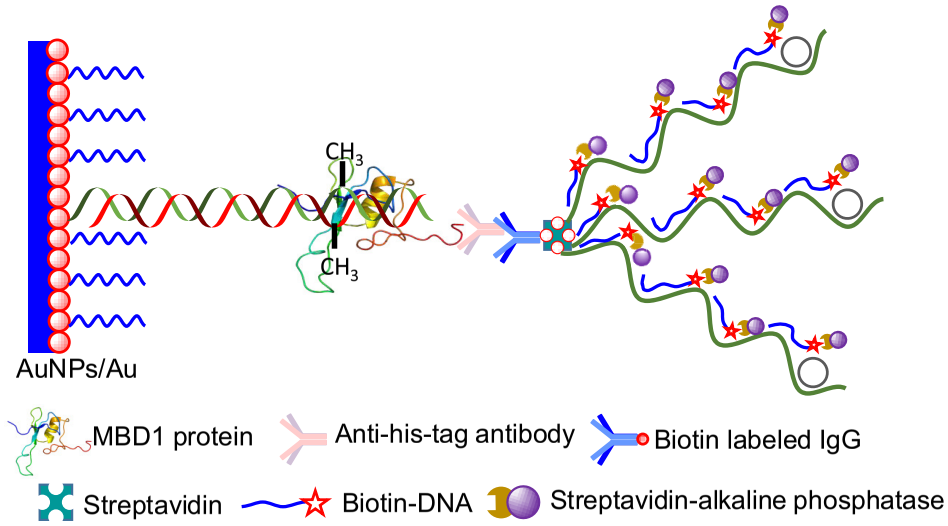

CH3
CH3
AuNPs/Au
MBD1 protein
Anti-his-tag antibody
Biotin labeled IgG
Streptavidin
Biotin-DNA
Streptavidin-alkaline phosphatase

Figure 7

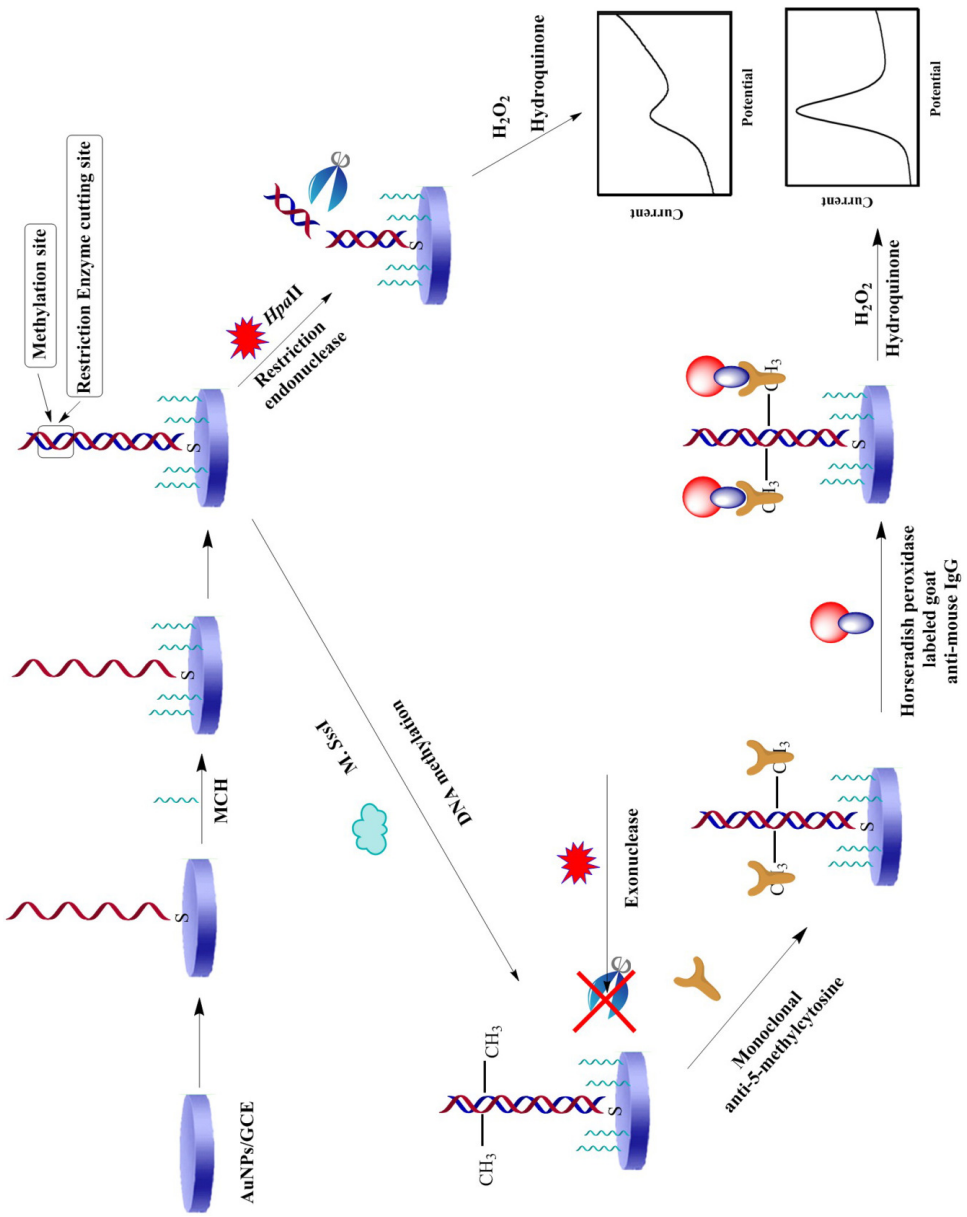


Figure 8

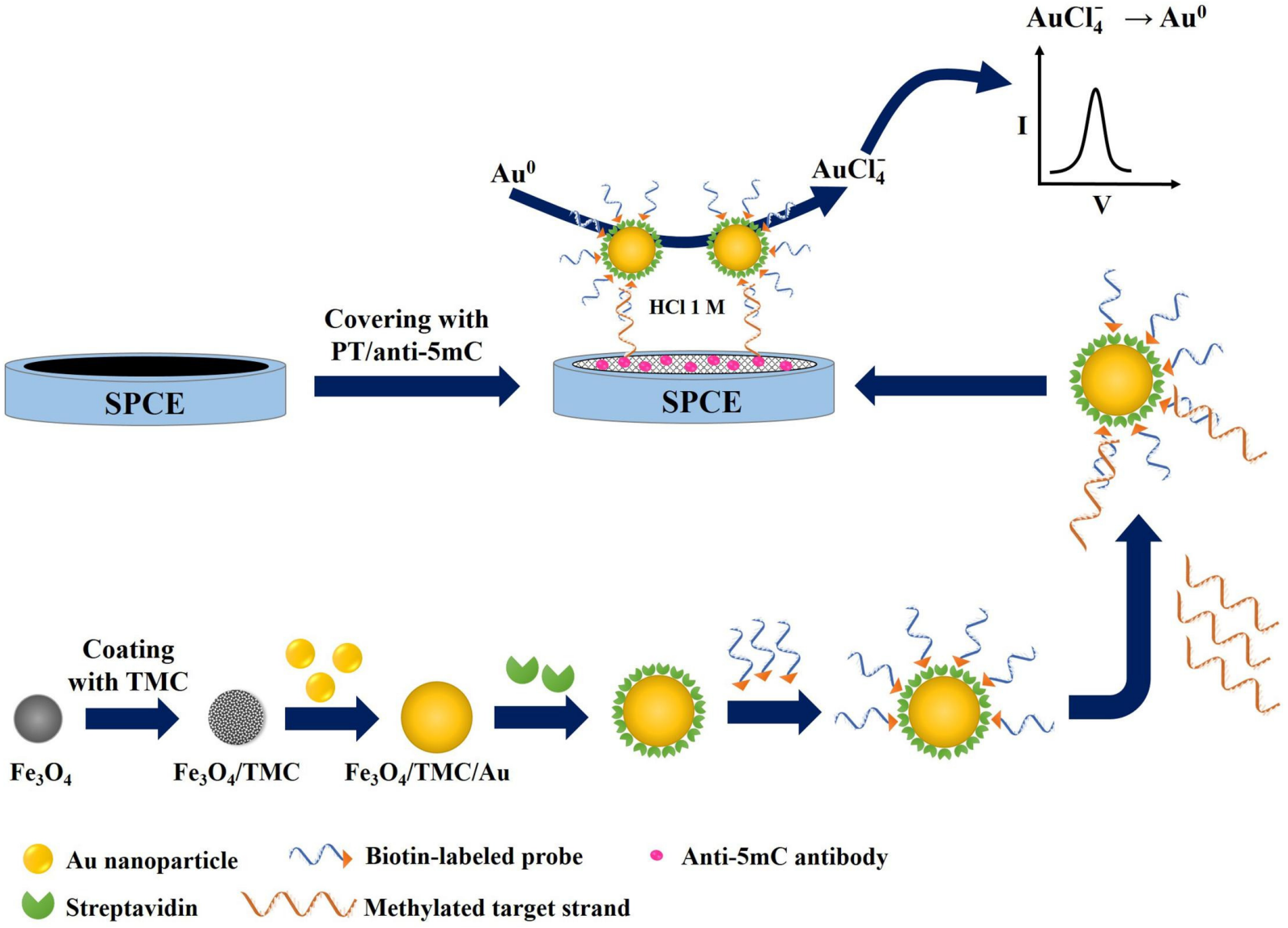


Figure 9

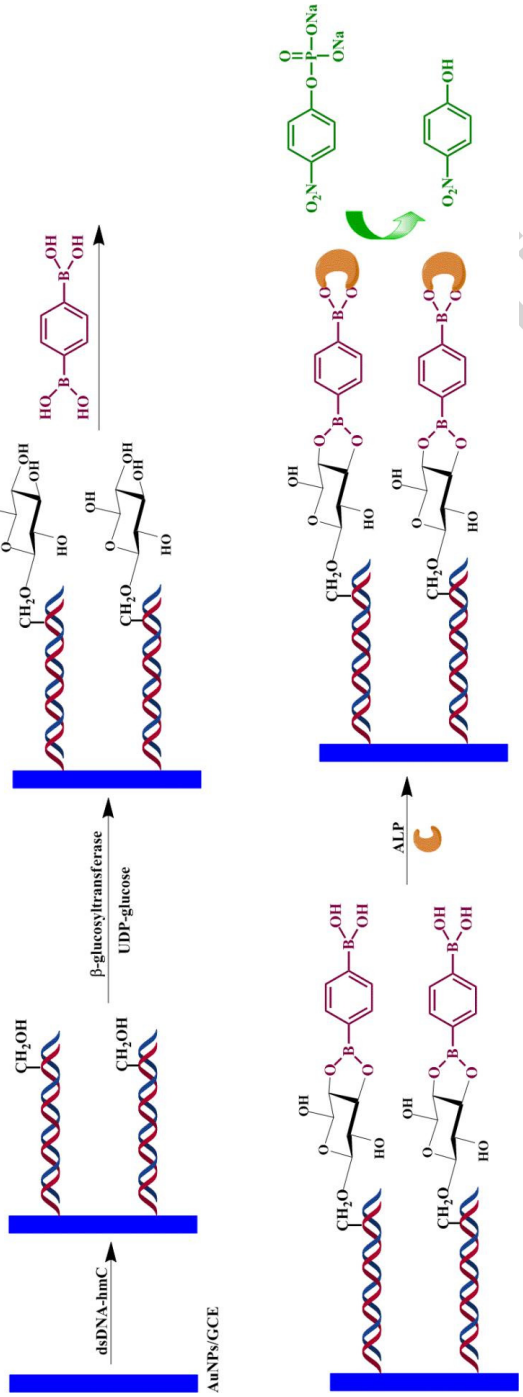
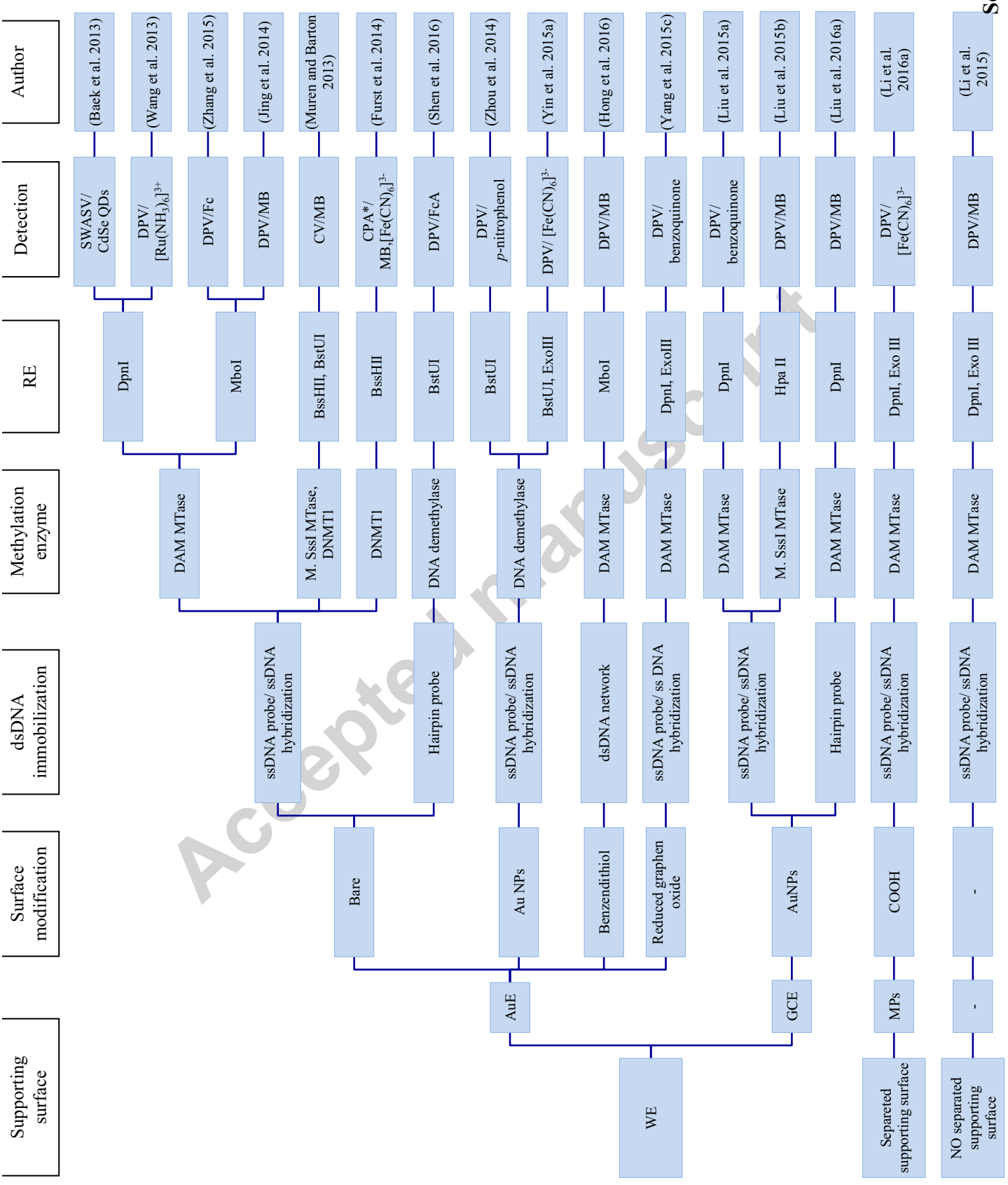
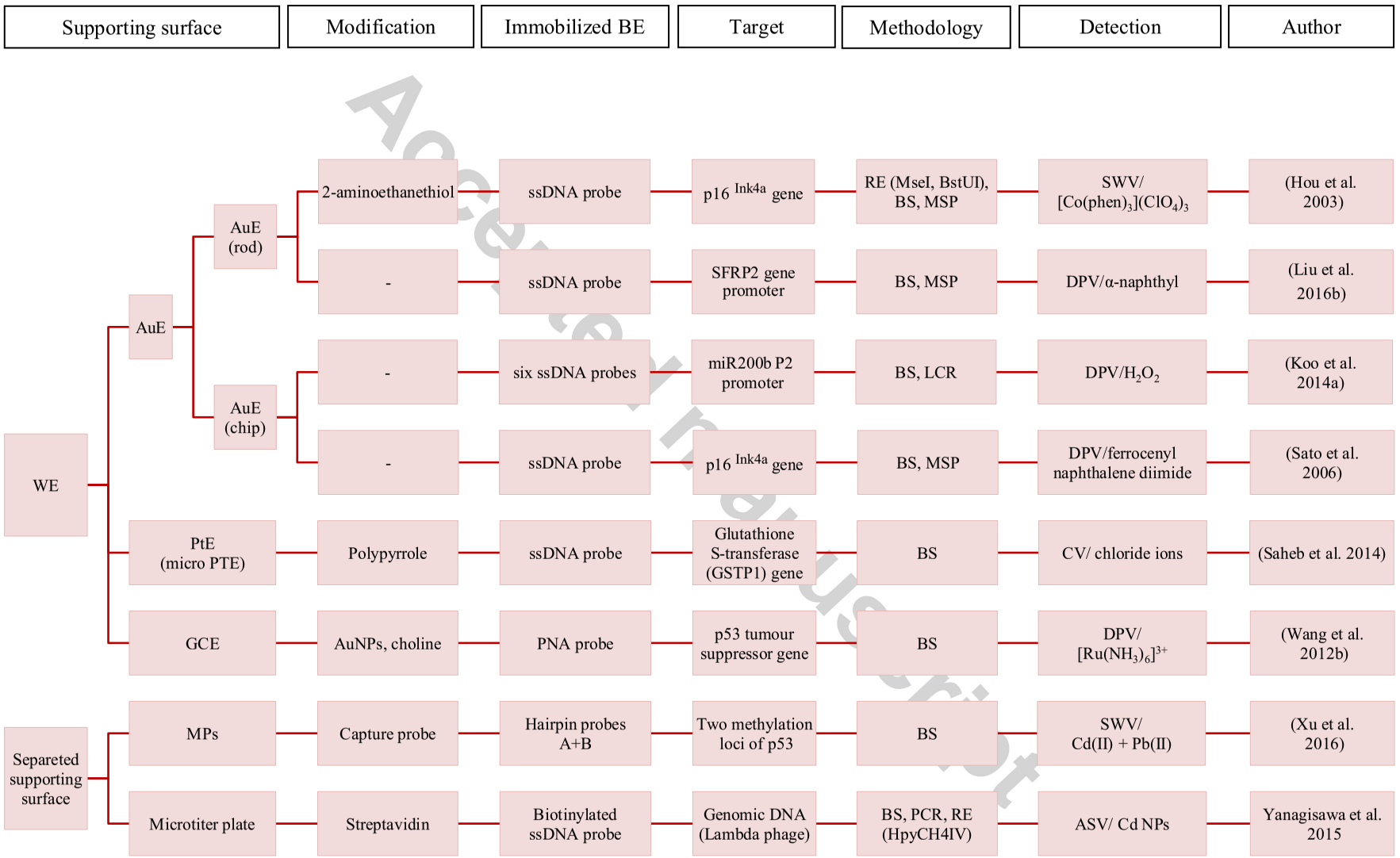


Figure 10

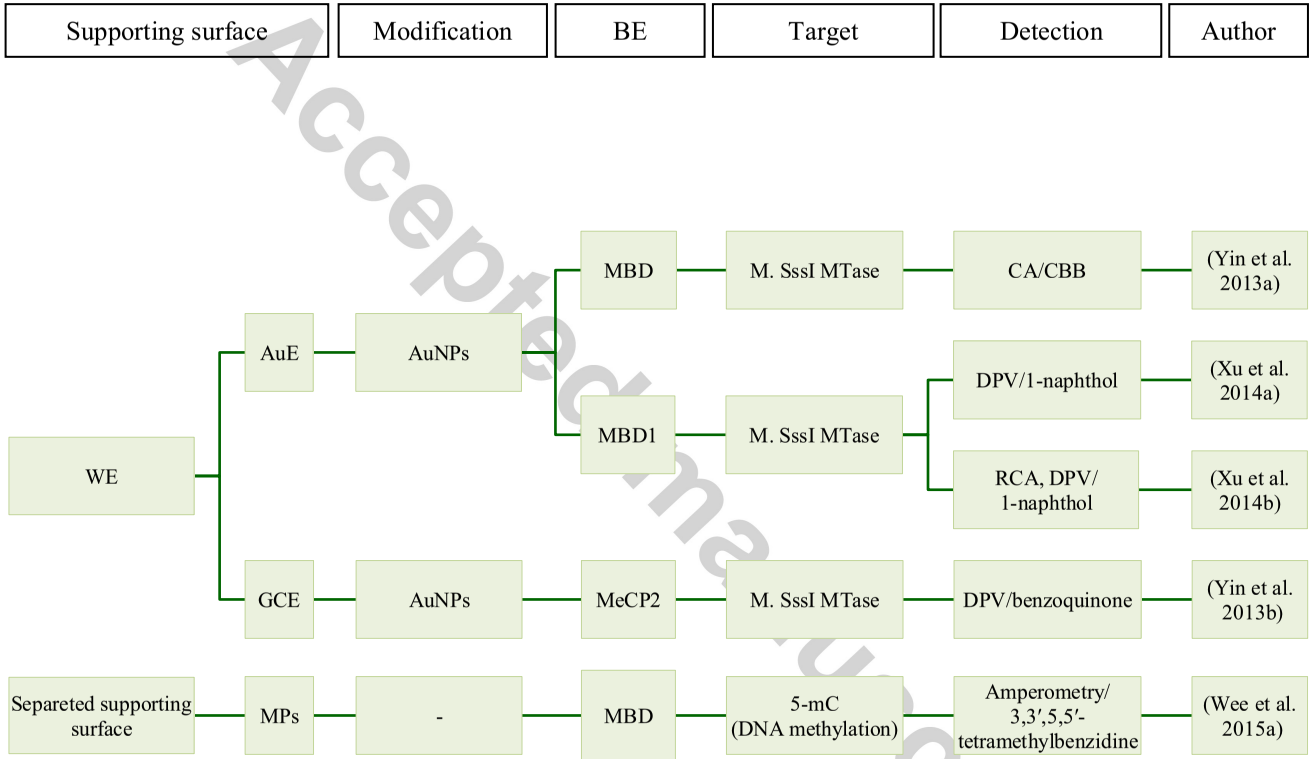


CPA* = constant potential amperometry

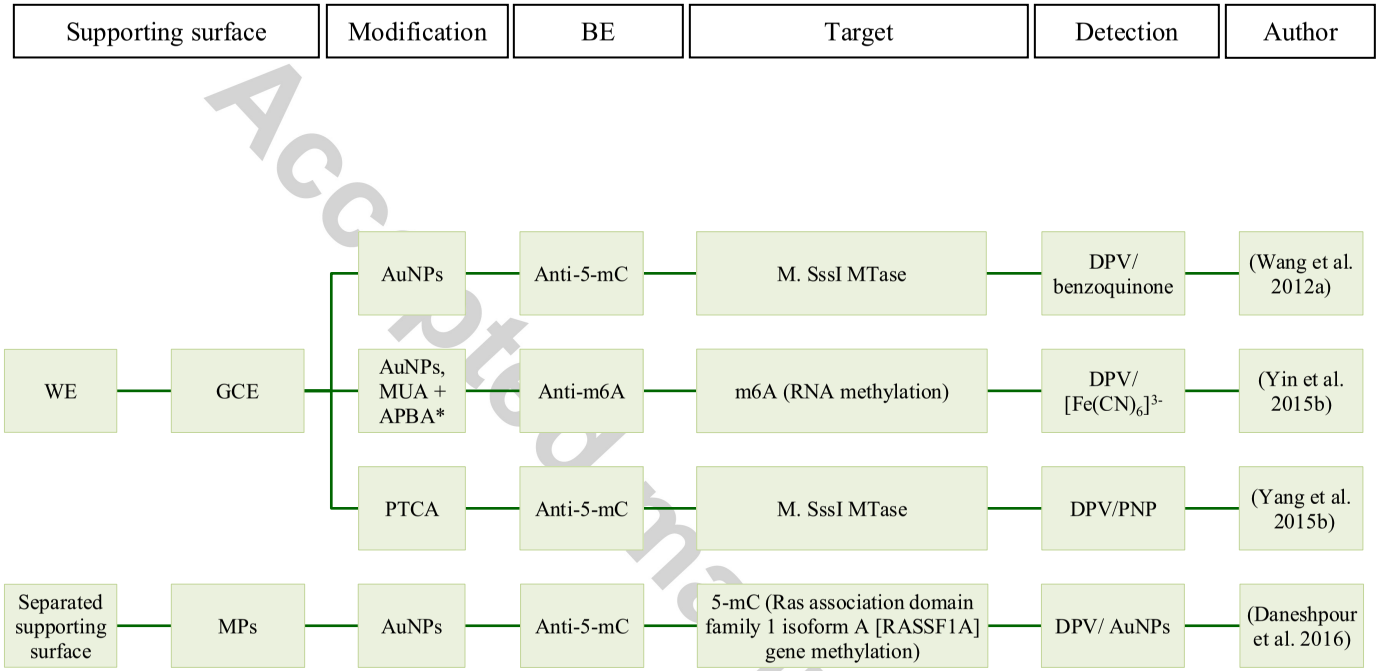
Scheme 1



Scheme 2

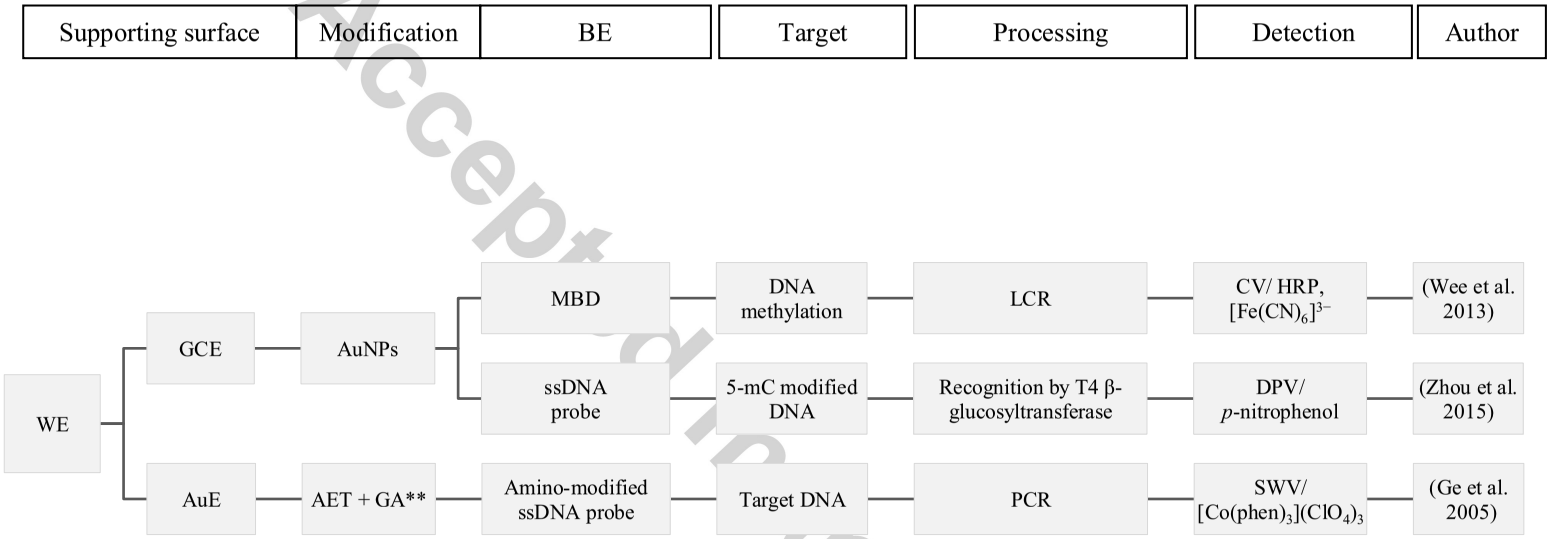


Scheme 3



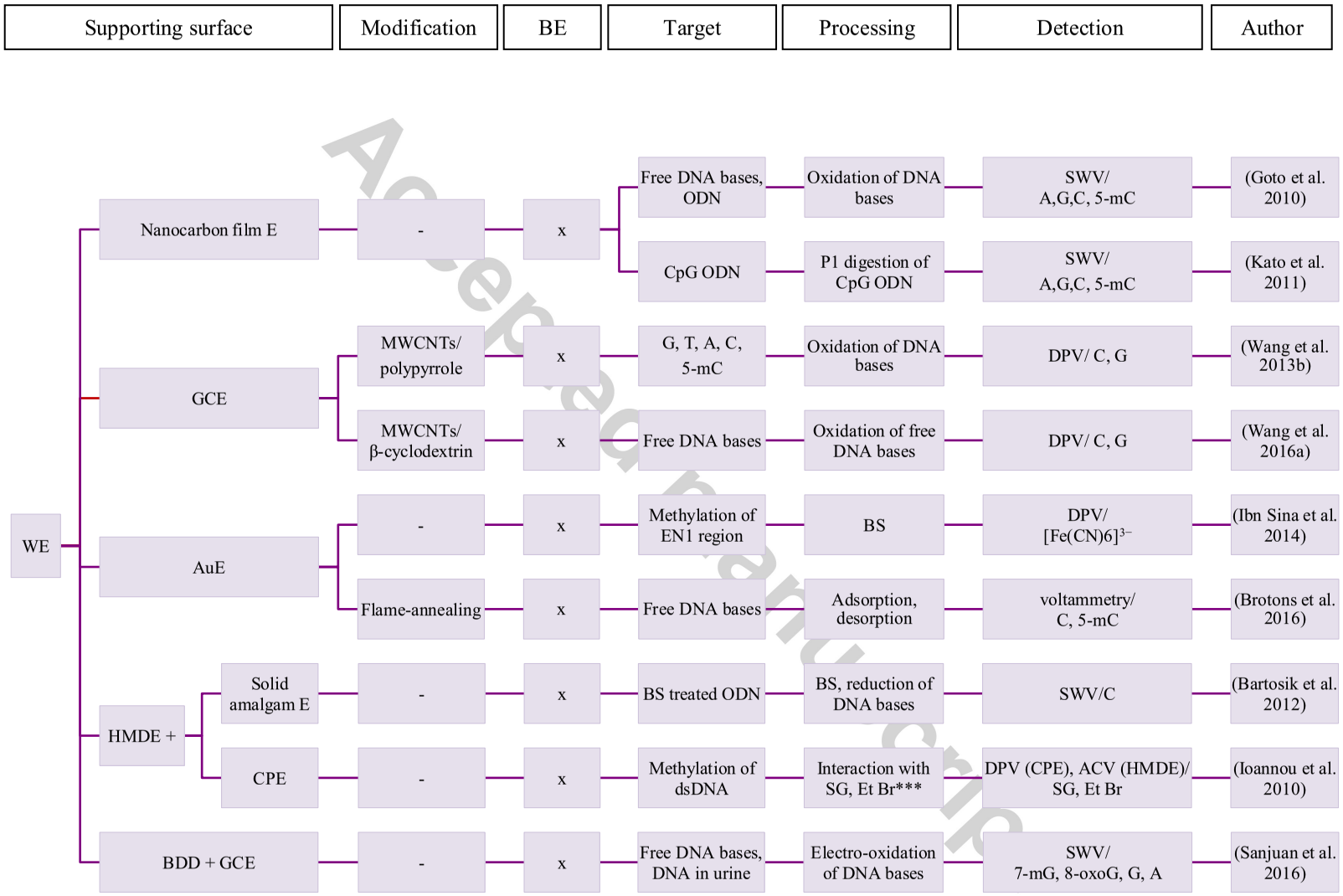
*MUA + APBA = 4-aminophenylboronic acid + 11-mercaptoundecanoic acid

Scheme 4



**AET + GA = 2-aminoethanethiol and glutaraldehyde

Scheme 5



*** SG, EtBr = SYBR green I, ethidium bromide

Scheme 6