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Detection of average methylation level of specific genes by binary-probe hybridization

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ABSTRACT

We developed a simple and highly-selective method for 5-methylcytosine detection of specific gene sequence based on binary-probe DNA hybridization. The sequence complementary to the target was designed into two probes, and each fragment of binary probes bound to a relatively short sequence of the target, which made it sensitive to the base mismatches introduced by bisulfite treatment. The advantages of a low detection limit of methylation abundance of 0.1% for the fully methylated target and high sensitivity of 10 pM have been proved by the successful design of binary-probe hybridization. The successful design of the binary probes makes it possible to quantify the average methylation levels of five CpG sites. Thirty-two DNA strands containing 5, 4, 3, 2, 1 and 0 CpG sites were successfully analyzed with the same pair of binary probes. The higher the average methylation level of the target was, the higher the degree of the hybridization reaction. Based on the simple construction of the binary-probe hybridization, the developed biosensor exhibited signals proportional to the average methylation level of the *vimentin* gene and could evaluate the average methylation level of artificial mixtures. Furthermore, the method has been used to detect *vimentin* methylation in a genomic context with good specificity, which indicated its potential in the pre-diagnosis of methylation related disease.

1. Introduction

DNA methylation is an essential epigenetic modification, of which 5-methylcytosine is the most common and well-studied methylation modification [1–3]. Alterations of 5-methylcytosine methylation have been considered as a key role in epigenetic regulation [4], which can influence the gene activities, embryonic development and chromosome stability [5–8]. DNA methylation of some specific genes has been reported as cancer related [9]. To date, a variety of DNA methylation detection methods have been proposed, however, most of them can only distinguish fully methylated DNA and unmethylated DNA at methylation sites. Since DNA methylation is a dynamic and reversible process, and the state of a CpG site can be methylated or demethylated [10], the methylation status of a specific gene which always contains several CpG sites is very complicated. Compared to distinguish fully methylated DNA and unmethylated DNA at methylation sites, it is more important to determine the average methylation level of several CpG sites.

Some methods have been reported to determine the average methylation level in a specific gene sequence, such as methylation-sensitive restriction endonuclease (MSRE)-assisted methods [11], pyrosequencing [12], mass spectrometry [13], and hybridization-based methods [14–16]. MSRE cleaves unmethylated sequence, leaving methylated DNA intact [17]. Although MSRE-assisted methods can provide the average methylation level of one CpG site which can be recognized by the restriction endonuclease, some CpG sites cannot be analyzed due to the lack of sequence-corresponding enzyme [11,18,19]. Moreover, simultaneous analysis of multiple CpG sites in a specific sequence makes the average methylation level lower than expected [20]. Bisulfite pyrosequencing offers average methylation level at each CpG site [21,22]. Although the assay is a sensitive and quantitative method for methylation information, it is limited due to high cost. Besides, the amplification difference of different methylation sites makes the measurement of average methylation level of several sites less accurate [20]. Mass spectrometry provides the average methylation level

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of several CpG sites within the PCR products [13]. However, different CpG sites with the same molecular weight make the analysis difficult [23]. DNA hybridization probes provide a convenient means of detecting nucleic acids without the need of complex enzymatic conditions. Some hybridization-based methods have been proposed for average methylation level detection making use of single capture probes which hybridize to the target sequence [14–16]. However, most methods are limited by the number of CpG sites that can be determined. So far, the average methylation level was monitored up to three CpG sites by hybridization-based methods. In theory, the more CpG sites result in a more complicated epiallele samples. Besides, there is a concentration of CpG-rich regions in CpG islands, which is related to tumorigenesis [24, 25]. Therefore, there is a need for simple, low-cost and high-selective methods that can detect average methylation levels with more CpG sites.

In terms of DNA methylation, the aberrant methylation occurs with high frequency in the CpG islands of genes interested in, which can introduce multiple base mismatches after bisulfite treatment, resulting in a big difference on the thermodynamics [26]. Based on this, our method includes a novel strategy that introduces binary probes designed for various CpG sites of the target gene. Only when both probe sequences are hybridized to the gene targets, the detection signal is generated. By splitting the sequence complementary to the target into a binary probe, the selectivity of the probe can be improved, which is sensitive to the base mismatches introduced by bisulfite treatment. The use of binary probes can not only maintain the high specificity of hybridization [27, 28], but also offer the possibility for the detection of average methylation levels. Taking the advantage of a self-assemble DNA sandwich structure on the DNA-BIND 96-well plate, we developed a simple, high-throughput, low-cost and high-selective method for the detection of the gene-specific methylation based on binary-probe DNA hybridization. Taking the *vimentin* sequence containing five CpG sites as the target, it achieved a limit of detection of 10 pM and the fully methylation abundance was as low as 0.1%. Moreover, the difference in hybridization thermodynamics and reduced stability between the targets and probes were utilized to determine the average methylation levels. This simple construction of binary-probe hybridization was applied to the sequence with five CpG sites and exhibited signals proportional to the average methylation level of thirty-two synthesized *vimentin* genes with the same pair of binary probes, indicating that this study had the potential in the pre-diagnosis of DNA methylation related disease.

2. Experimental sections

2.1. Materials and reagents

Streptavidin-horseradish peroxidase (SA-HRP) and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Sigma-Aldrich (St. Louis, MO USA). Penicillin-Streptomycin was purchased from TransGen Biotech Co., Ltd. (Beijing, China). Fetal bovine serum was purchased from ExCell Biotech Co., Ltd. (Shanghai, China). Bovine serum albumin (BSA) and 3, 3′, 5, 5′-Tetramethy benzidine dihydrochloride (TMB·2HCl) were purchased from Coolaber Technology Co., Ltd. (Beijing, China). CpG Methyltransferase (M.SssI), Lambda exonuclease and Lambda Exonuclease Reaction Buffer were purchased from New England Biolabs (MA, USA). DNA-BIND 96-well plates were purchased from Corning Incorporated (USA). Micro Bio-Spin columns were purchased from Bio-Rad Laboratories Co., Ltd. (Shanghai, China). Methylated DNA, dNTP Mixture (2.5 mM for each), *TaKaRa Ex Taq* HS and TaKaRa MiniBEST Universal Genomic DNA Extraction Kit Ver.5.0 were acquired from Takara Biomedical Technology Co., Ltd. (Beijing, China). EZ DNA Methylation-Gold Kits and Genomic DNA Clean & Concentrator-10 were purchased from Zymo Research. NucleoSpin Gel and PCR Clean-up was acquired from Macherey-Nagel. AidQuick Oligo Purification Kit was purchased from Aidlab Biotechnologies Co., Ltd (Beijing, China). DNA probes and oligonucleotides were acquired from Sangon Biotech Co., Ltd. (Shanghai, China). The sequences of all the oligonucleotides were listed in Table S1.

2.2. Genomic DNA extraction and methylation

MDA-MB-231 cell line was cultured in DMEM, containing 10% fetal bovine serum and 5% penicillin-streptomycin. Genomic DNA (gDNA) was extracted by TaKaRa MiniBEST Universal Genomic DNA Extraction Kit Ver.5.0. The extracted gDNA was methylated by M.SssI according to the following steps: the reaction mixture contained 1 μg gDNA, 250 μM S-adenosyl-methionine (SAM), 5 μL 10 × NEB buffer 2, 10 U M.SssI, and the volume was brought up to 50 μL by UltraPure water. The methylation reaction was incubated at 37 °C for 3 h by a C1000 Touch Thermal Cycler (USA). Then, 0.375 μL 32 mM SAM and 5 U M.SssI were added to the reaction mixture and incubated at 37 °C for an additional 1 h to ensure complete methylation. M.SssI was inactivated at 65 °C for 20 min. After M.SssI treatment, the products were purified by Genomic DNA Clean & Concentrator-10, and the DNA concentration was quantified by NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific).

2.3. Procedure of DNA bisulfite treatment

Bisulfite treatment of synthetic ssDNA. DNA bisulfite treatment was performed according to the reported method [29]. 3.2 M $NaHSO_3$ and 0.5 mM hydroquinone were freshly prepared, and reacted with 1 μg ssDNA at 50 °C for 16 h. Then the bisulfite treated DNA was desalted by Micro Bio-Spin columns. The recovered DNA was treated with 0.3 M NaOH at 37 °C for 15 min and neutralized with ammonium acetate, and then purified by ethanol precipitation. The treated DNA was verified by electrospray ionization mass spectrometry according to the molecular weight (Fig. S1) and kept at −20 °C for long-term storage.

Bisulfite treatment of gDNA. The M.SssI treated and untreated gDNA were bisulfite treated by the EZ DNA Methylation-Gold Kits. In each individual treatment, the total amount of gDNA is 500 ng. After the treatment, the DNA concentration was quantified by NanoDrop 2000.

2.4. Detection of the methylated and unmethylated DNA

Coupling of the amine-modified capture probe with the DNA-BIND 96-well plate was performed in the Na_2HPO_4–NaH_2PO_4 buffer (pH 8.5, obtained from Coolaber) at 37 °C for 1 h. After being washed with washing buffer (1 × PBS, 0.05% Tween, pH 7.4), 5% BSA dissolved in PBS (pH 8.5) was used to block the unmodified sites. The methylated or unmethylated target with 50 nM biotin-modified probe was prepared in 10 mM PBS (pH 7.4). Hybridization was performed at 25 °C for 2 h, and then unhybridized targets were removed by nonspecific washing. 0.4 μg/mL SA-HRP dissolved in 10 mM PBS was added and incubated at 25 °C for 0.5 h. Each well was carefully washed three times to reduce the false positive signal. Then, TMB-H_2O_2 solution (0.12 mg/mL TMB·2HCl, 18 mM H_2O_2 in 100 mM Na_2HPO_4–NaH_2PO_4 buffer, pH 6.0) was added to trigger colorimetric reaction. TMB was oxidized by H_2O_2 with the catalysis of HRP and output a colorimetric signal. After 40 min, 2 M H_2SO_4 was used to terminate the reaction. The absorbance values at 450 nm and 620 nm were recorded by the microplate reader (BioTek, Winooski, USA).

2.5. Evaluation of the detection sensitivity and discrimination of the fully methylated target

Sensitivity evaluation. Fully methylated DNA with concentrations of 0, 10, 25, 50 and 1000 pM were prepared respectively. The hybridization reaction was performed under optimized conditions.

Discrimination evaluation. Fully methylated and unmethylated DNA were mixed to prepare the mixed DNA strands composed of different methylation abundance. The total DNA concentration was 10 nM. The proportions of fully methylated DNA were 0%, 0.1%, 0.25%, 0.5%, 1%, 10% and 100%, respectively. Hybridization reaction was performed

with 10 nM mixed DNA strands and 50 nM biotin-modified probes for the experimental group, while biotin-modified probes only for the control experiment under optimized conditions. The following steps were described above.

2.6. Determination of the methylation level of specific gene sequence

We synthesized DNA strands containing 5, 4, 3, 2, 1 and 0 cytosine to thymine base change (Table S1), which were used to mimic the gene sequences with 0, 1, 2, 3, 4 and 5 methylated CpG sites treated with bisulfite, and the corresponding numbers of DNA strands were C_5^0, C_5^1, C_5^2, C_5^3, C_5^4 and C_5^5. Hybridization reaction was performed with 10 nM targets and 50 nM biotin-modified probes under optimized conditions.

2.7. Evaluation of the average methylation level of artificial mixtures

According to Ref. [15], DNA strands with the same methylation level were mixed to obtain standard samples, and the concentration of a standard sample was 10 nM. The total number of strands with a methylation level of 40% or 60% is ten, while the total number of target strands with a methylation level of 20% or 80% is five. Therefore, to obtain a standard sample representing an average methylation level of 20% or 80%, the concentration of each strand is 2 nM. To obtain a standard sample representing an average methylation level of 40% or 60%, the concentration of each strand is 1 nM. To demonstrate the feasibility of the assay in the evaluation of the average methylation level of artificial mixtures, twenty-four mixed samples with different average methylation levels were prepared using the standard samples to obtain the calibration curve of the absorption intensity vs the average methylation level of *vimentin* (Table S4). Each sample was prepared in parallel three times and analyzed once (n = 3). Hybridization reaction was performed with 1 nM mixed targets and 50 nM biotin-modified probes under optimized conditions.

2.8. Detection of vimentin methylation of cell derived samples

The PCR amplification of bisulfite treated gDNA with or without M. SssI pretreatment was performed by a C1000 Touch Thermal Cycler. The total volume of PCR amplification was 20 μL, containing 2 μL 10 × *Ex Taq* Buffer (Mg^{2+} plus), 0.5 U *TaKaRa Ex Taq* HS, 1.6 μL dNTP Mixture (2.5 mM each), 30 ng gDNA, 0.2 μL 50 μM forward primers, 0.2 μL 50 μM reverse primers, and the rest volume was made up by UltraPure water. The PCR procedure was 95 °C for 5 min, 55 °C for 25 s, 72 °C for 30 s, 40 cycles, and 72 °C for 5 min. The PCR products were purified using the NucleoSpin Gel and PCR Clean-up kit. Then, the purified products were digested by Lambda exonuclease according to the protocol. The digestion products were purified by an AidQuick Oligo Purification Kit and the concentration was determined by NanoDrop 2000. After purification, 5 nM of the digested PCR products with a length of 137 nt and 50 nM P2 were prepared in 10 mM PBS (pH 7.4), and then the mixture was denatured at 95 °C for 5 min and cooled on ice for 15 min to ensure better hybridization. The following detection steps were described above.

3. Results and discussion

3.1. Principle of the methylated DNA detection based on binary-probe hybridization

The CpG islands are rich in CpG dinucleotides, and are associated with gene regulations. For this reason, we chose the target region containing 5 CpG sites within a length of 23 nucleotides, which could be considered as a CpG-rich region. The detection targets are the *vimentin* genes, which are related to various tumors, including colon and gastric cancers [30,31]. This region has been reported to be unmethylated in normal colonic epithelial cells, but highly methylated in colon cancer tissues [32]. The principle of this binary-probe hybridization-based method for the discrimination of the fully methylated target (M) and fully unmethylated target (UM) is illustrated in Scheme 1A. When the *vimentin* gene was treated with bisulfite, unmethylated cytosines were converted into uracils while 5-methylcytosines were not [33,34]. In order to improve the recognition specificity, the sequence complementary to the target was split into two short probes P1 and P2, which were designed to be complementary to M. Therefore P1 had two mismatches with UM and P2 had three mismatches. Amine-labelled P1 was immobilized on the 96-well plate, performing as a capturing probe. Upon the subsequent addition of the targets and P2, the specific hybridization between M and the binary probes formed the sandwich structure. Meanwhile, UM could not form the stable sandwich structure due to the thermodynamic difference. Since P2 was labelled with biotin, HRP could be easily introduced through the interaction between biotin and SA, and acted as a catalyst for the oxidation of TMB. The colorimetric signal was visible and could be accurately determined by a microplate reader, which was related to the methylation levels. As previously described, the methylation state of each individual CpG site is reported to be dynamically regulated, therefore the methylation levels are almost complicated for each methylated gene in real samples. The principle of gene-specific average methylation level detection based on binary-probe hybridization is illustrated in Scheme 1B. Theoretically, the degree of hybridization is related to the number of mismatches. On this basis, we further used the binary probe hybridization-based method to determine the average methylation level of different methylation sites.

3.2. The feasibility of methylated DNA detection

The performance of the probes toward M and UM was investigated. Upon the addition of M or UM, the specific hybridization among target, P1 and P2 happened spontaneously. The designated probes were complementary to M, but had five mismatches with UM resulting in poor hybridization. As shown in Fig. 1, the signal response (A_{450}–A_{620}) generated by M was much higher than that of UM, indicating a higher degree of the hybridization reaction with the methylated target, which proved that the method was feasible for methylation detection.

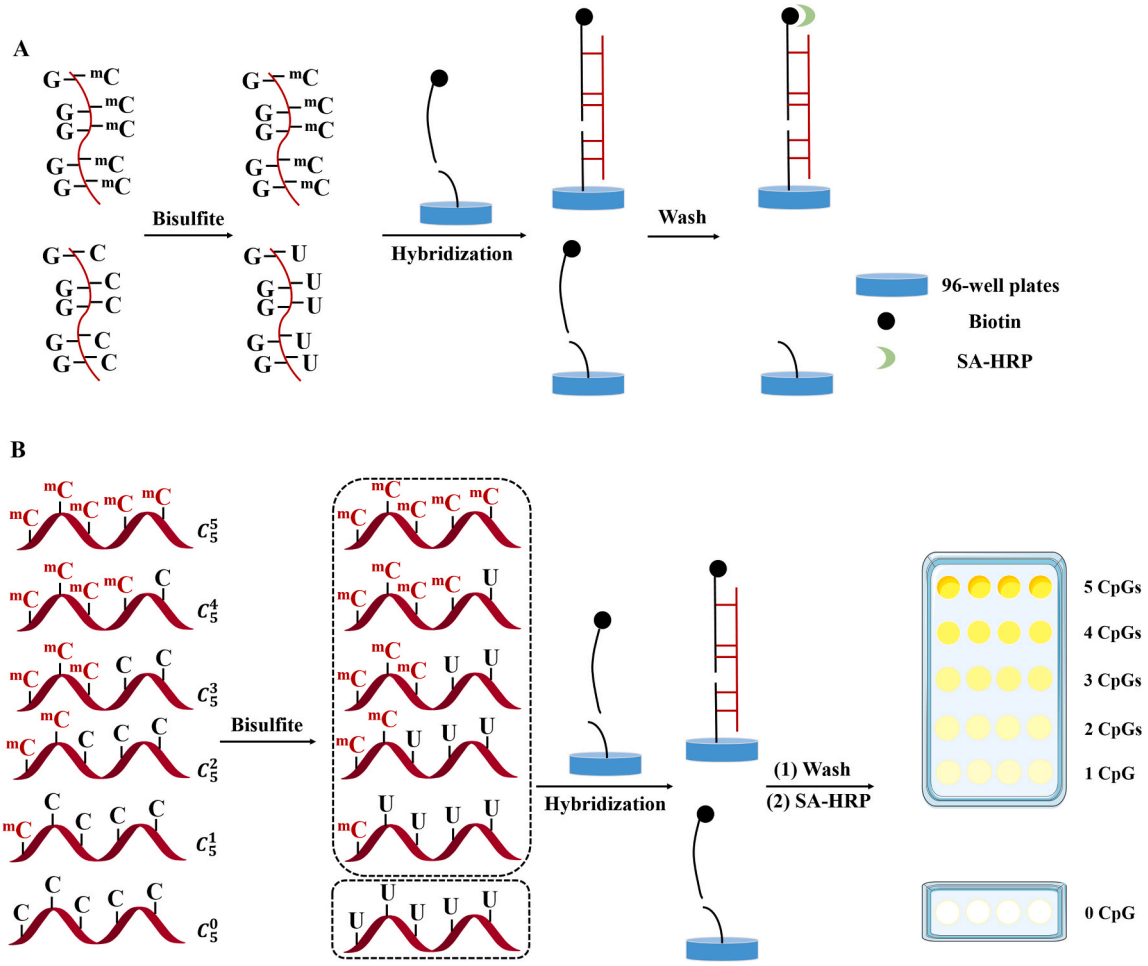
3.3. Optimization of the hybridization conditions

Several reaction conditions, which might influence the hybridization efficiency, were investigated to obtain optimal conditions for methylation detection. The hybridization time was optimized to achieve the maximum relative absorption difference between M and UM. As shown in Fig. S2A, with the increase of reaction time, the signal of M increased while that of the UM sequence was almost unchanged. By taking both signal intensity and time efficiency into account, the reaction time was set at 2 h.

Considering that the reaction temperature could have an effect on the stability of the complex formed by binary probes and target, the absorption signals were tested at different temperature. As shown in Fig. S2B, the signal difference between M and UM decreased with temperature increasing, which was presumably due to unstable hybridization under higher temperatures. Reaction exhibited optimal at 25 °C.

3.4. The detection sensitivity and discrimination of fully methylated DNA

Under the optimal condition, the sensitivity of the method for M was investigated, which was directly related to the discrimination ability of DNA methylation abundance. We studied the variance of signal response with the concentration of M. As shown in Fig. 2A, the signal response increased with the increase of the concentration of M. When the amount of M was as low as 10 pM, it could still be sensitively detected. The high sensitivity might come from the enzyme amplification endowed by HRP



Scheme 1. Principle of the methylated DNA detection based on binary-probe hybridization. (A) Schematic principle of DNA methylation detection based on binary-probe hybridization. (B) Schematic principle of average methylation level detection based on binary-probe hybridization.

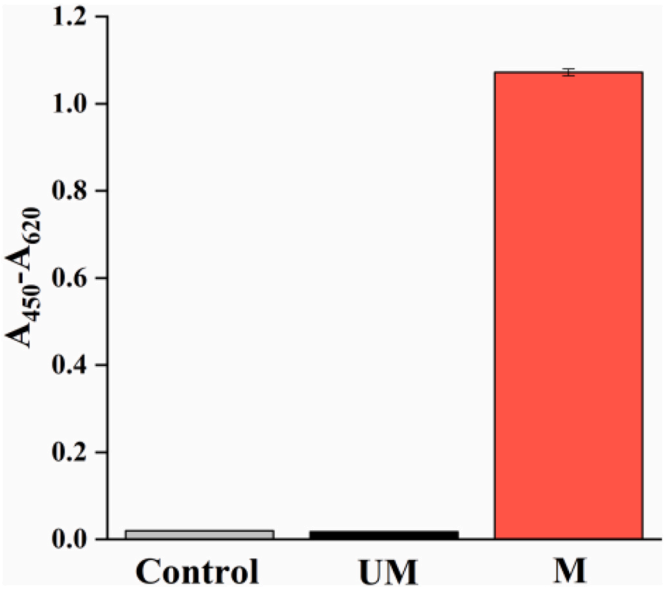


Fig. 1. The colorimetric responses for the feasibility of the detection of methylated target based on hybridization. Experimental conditions: 250 nM P1, 50 nM P2 with 10 nM UM or M incubated at 25 °C for 2 h.

[35]. By PCR amplification, 30 ng gDNA can be amplified to 100 nM PCR products approximately (the elution volume is 20 μL). Although the trace amount target can be amplified by PCR, DNA is prone to loss during purification and digestion. Therefore, a higher sensitivity is more desirable in trace sample analysis.

To evaluate the ability of this method to detect the low abundance of M, a series of samples with different methylation abundance of M were prepared. As shown in Fig. 2B, the colorimetric signal increased with the decrease of the abundance of UM. For the methylated *vimentin* sequence, the detectable methylation abundance was as low as 0.1%, which meant the method could detect 0.1% fully methylated DNA in the presence of large amount of unmethylated DNA with a total concentration of 10 nM. The performances of some current approaches for the detection of gene-specific methylation were summarized in Table S2. The approach provided a lower detection limit of methylation abundance compared to most of the assays for methylation detection reported in recent years [15,36–40]. The low LOD of methylation abundance might derive from the specificity of the hybridization between binary-probes and targets. Moreover, the binary-probe hybridization method was verified to have good reproducibility for methylation detection (Table S3).

3.5. The determination of the methylation level

In the *vimentin* gene, the methylation level was reported to be 30% and 50% approximately in the diffuse and intestinal tumor tissues in gastric cancers, respectively [31]. The design of binary hybridization probes was used to determine the average methylation level. Each

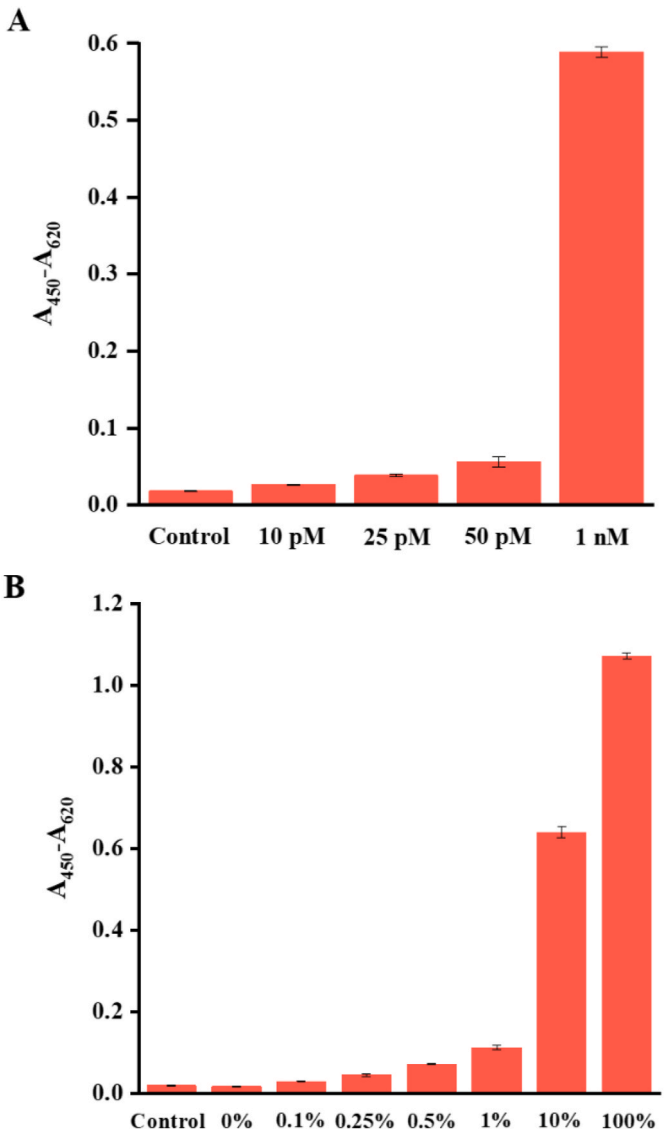


Fig. 2. (A) Variance of the signal responses (A_{450}–A_{620}) as a function of fully methylated DNA concentration. (B) Variance of the signal responses (A_{450}–A_{620}) as a function of the abundance of fully methylated DNA. Each experiment repeated three times.

fragment of binary probes binds to a relatively short sequence of the target, which makes it sensitive to the base mismatches and provides the possibility to detect average methylation level of specific gene sequence with more CpG sites. Genes with more CpG sites result in a more complicated composition of methylation epialleles. For the tested *vimentin* gene, there are three types of methylation, the fully methylation, partially methylation and unmethylation. The *vimentin* gene with five CpG sites has 2^5 permutations. Binary probes hybridize to the partially methylated targets to produce 1, 2, 3 and 4 mismatches, corresponding to the different methylation state of each CpG site on the target. The higher the average methylation level of the target, the higher the degree of the hybridization reaction. The methylation sites of *vimentin* gene are located at X_1, X_2, X_3, X_4 and X_5, where X represented C (synthetic *vimentin* gene mimicking bisulfite treated DNA with methylated CpG site) and T (mimicking unmethylated CpG site) (Fig. 3A). According to this, we synthesized thirty-two DNA strands containing 5, 4, 3, 2, 1 and 0 cytosine to thymine base changes, which were used to mimic the gene sequences with 0, 1, 2, 3, 4 and 5 methylated CpG sites treated with bisulfite, and the corresponding numbers of DNA strands

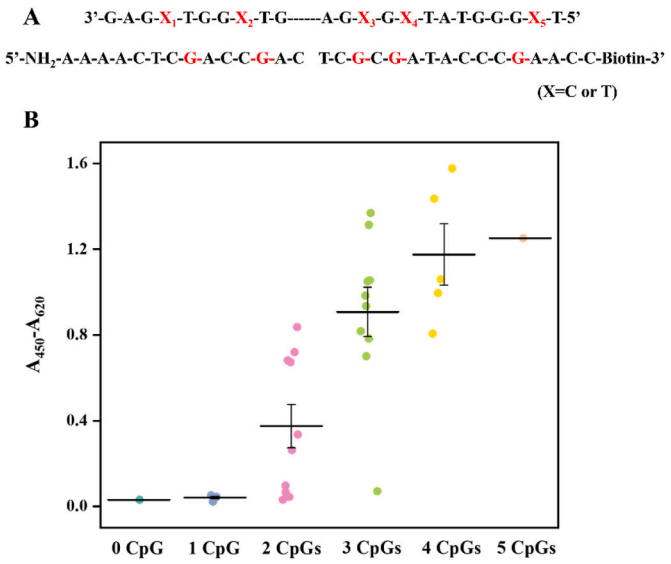


Fig. 3. (A) The binary probe and targets with five CpG sites. (B) The absorption intensity of the binary probes to the synthesized targets containing 0, 1, 2, 3, 4 and 5 methylated CpG sites, respectively.

were C_5^0, C_5^1, C_5^2, C_5^3, C_5^4 and C_5^5, respectively, where C_5^y represented that there were y matched CpG sites which were methylated in five mismatches. The average methylation level is 0, 20, 40, 60, 80 and 100% of five CpG sites with 0, 1, 2, 3, 4 and 5 sites methylated, respectively.

The hybridization was performed with 10 nM targets and 50 nM P2 under optimized conditions to investigate the discrimination ability of the probes towards targets with different average methylation levels. As shown in Fig. 3B, the hybridization-based method verified that the average methylation level of gene-specific target can be quantitatively determined. The following facts were in agreement with the conclusions:

(1) The target with five CpG sites methylated (5 CpGs) was detectable, because the probes were designed to be complementary to the fully methylated target;
(2) Although the targets with four CpG sites methylated (4 CpGs) formed a mismatch with the probe, there was little influence on the base pairings. Among the targets with four methylated CpG sites, the signals of two of them were slightly higher than that of the target with five methylated CpG sites, which might arise from the comparable thermodynamic stability of GT mismatches and perfectly matched base pairs [41]. In general, the signal of the targets with an average methylation level at 80% was lower than that of 100%;
(3) When three CpG sites were methylated in the sequence, the signal response of only one target was extremely low because the two mismatched bases fell on P1, which made poor pairing between the target and P1.

It was found that the signal intensity of the duplex having a fixed mismatch at X_1 with a random mismatch at $X_{3,\ 4\ or\ 5}$ was slightly lower than that of X_2 (Fig. S3). It can be explained by the perspective that the sequence selectivity is maximized with a single mismatch locating at the center position of short probes, where P1 can be regarded as the short probe with a 10 nt length [42,43]. Compared with X_2, X_1 was closer to the center, resulting in higher specificity between P1 and targets;

(4) Four targets containing two CpG sites methylated (2 CpGs) displayed insignificant signals. Three of them had two mismatches with P1, and one mismatch with P2. Another target formed three mismatches with P2, resulting in low hybridization efficiency with P2;

(5) It was found negligible signal intensities of targets with one or zero methylated CpG site (1 CpG or 0 CpG) due to weak hybridization.

Conclusively, the thirty-two target strands were divided into six groups, and the sequences with the same methylated CpG site number were listed in the same group, indicating the same methylation level. The more the methylated site number, the higher the methylation levels. In most cancer-related genes, promoter hypermethylation is a common event in tumorigenesis, with dynamic regulation of methylation at the CpG sites [44]. So, the signal intensity of one strand was not sufficient to represent the methylation level. For this reason, we took the average of the signal intensity of all strands in each group with the same methylated site number to show the average methylation level. As shown in Fig. 3B, with the increase of the methylated CpG sites from 0, 1, 2, 3, 4 to 5, that was, the average methylation levels rising from 0, 20, 40, 60, 80 to 100%, the absorption intensity increased.

3.6. The applicability of the method to evaluate average methylation levels in artificial mixtures

In the determination of the methylation level, only one single target was used in each experiment. As mentioned above, DNA methylation is a dynamic process, and the samples to be detected were composed of different strands with different methylation levels. To demonstrate the applicability of the assay in the average methylation level evaluation, twenty-four specific compositions of targets were mixed by the standard samples shown in Table S4. As shown in Fig. 4, the absorption intensities of the mixed targets were measured by the same pair of binary-probes, and the correlation between the average intensity and the average methylation levels was fairly linear. Three artificial mixtures which contained low (20%, Sample 1), medium (40%, Sample 2) and high (80%, Sample 3) average methylation level were prepared (Table S5), and the evaluation results of these artificial mixtures were close to the theoretical level (Table S6). The result indicated that our method owned the potential to detect the average methylation levels of regional DNA in real samples.

3.7. Vimentin methylation detection of cell derived samples

We further applied the method to detect the methylation level of *vimentin* of human breast cancer cell line, MDA-MB-231. The gDNA was extracted and then treated with bisulfite. The bisulfite treated sample was amplified by PCR and then digested by Lambda exonuclease to convert dsDNA into ssDNA. The signal of the target with a length of 137 nt was close to the signal of 0 CpG, which indicated that *vimentin* derived from MDA-MB-231 was hypomethylated (Fig. 5). The result was also confirmed by bisulfite genomic sequencing (Fig. S4). Bisulfite genomic sequencing can give the methylation status of a single DNA molecule at each CpG site and has been used in the methylation level analysis of the interested gene sequence [37,45]. The sequencing results showed that the five cytosines in the CpG sites of the target region were unmethylated and all converted into uracils during bisulfite treatment. In order to verify that the developed method has the ability to detect methylation in a genomic context, the extracted gDNA was treated with M.SssI. After gDNA methylation, the average methylation level of the target increased significantly (Fig. 5). Bisulfite genomic sequencing was also used to confirm a higher methylation level of *vimentin* after methylase treatment (Fig. S5). Among the five potential CpG sites in the region, the *vimentin* was read out to be five cytosines in average, which showed a complete methylation. These sequencing data was in consistent with the binary-probe hybridization results, indicating that the method could be applied to detect *vimentin* methylation in a genomic context with good specificity.

4. Conclusions

Methylation detection of gene-specific sequence is of great significance for cancer diagnosis since it has been identified as early biomarkers. To sum up, we developed a method for DNA methylation detection based on specific binary-probe hybridization, and the biosensor is specific for the target containing 5 CpG sites, which is superior to most methods based on hybridization for average methylation detection. Firstly, specific hybridization of the binary probes with methylated targets endows this method with highly specific recognition. The method can achieve a high sensitivity with a detection limit of 10 pM, and is able to distinguish as low as 0.1% methylation abundance for the fully methylated target. Secondly, taking advantage of the high selectivity of binary probes, the hybridization-based method is beneficial for average methylation levels evaluation especially when the targets are hypermethylated, which is an important molecular marker of cancers. Lastly, ninety-six individual experiments can be performed on

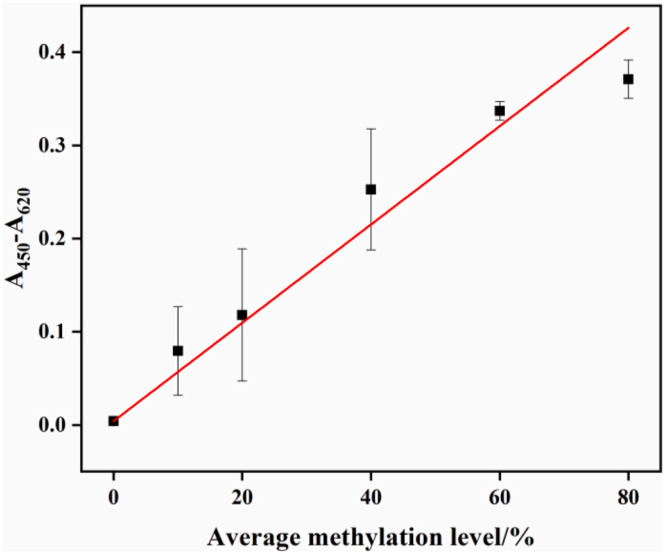


Fig. 4. The linearity of the absorption intensity vs the average methylation level of *vimentin* from 0% to 80%.

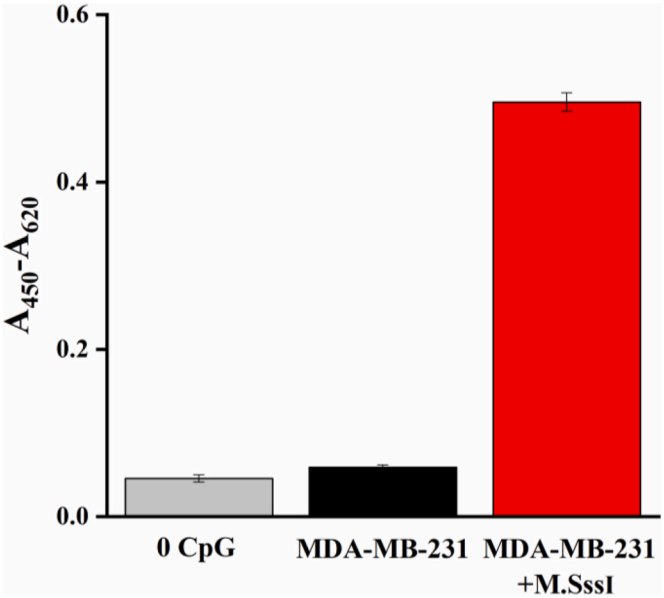


Fig. 5. The absorption intensity of the target derived from MDA-MB-231 cell line.

the DNA binding plate simultaneously, which is likely to realize multiplex detection by probe designing. Although the method is simple in design, the validity of DNA hybridization guarantees the method to be highly effective. We expect that this study has the potential in the disease pre-diagnosis of DNA methylation related.

Credit author statement

Xin-Ying Zhong, all the experiment and original draft preparation. Qian-Yu Zhou, conceived research and paper revision. Jia-Hui Dong, data repetition. Yue Yu, mass spectrometry test. Ying-Lin Zhou, conceived research, supervision and paper revision. Xin-Xiang Zhang, supervision and paper revision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2021.122630.

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