A fluorescence method for detection of DNA and DNA methylation based on graphene oxide and restriction endonuclease HpaII

Wei Wei a, Chunjian Gao a, Yanxiang Xiong a, Yuanjian Zhang a, Songqin Liu a,⁎, Yuepu Pu b,1

a Key Laboratory of Environmental Medicine Engineering, Ministry of Education, School of Chemistry and Chemical Engineering, Southeast University, Jiangning District, Nanjing 211189, PR China
b Key Laboratory of Environmental Medicine Engineering, Ministry of Education, School of Public Health, Southeast University, Jiangning District, Nanjing 211189, PR China

Abstract

DNA methylation plays an important role in many biological events and is associated with various diseases. Most traditional methods for detection of DNA methylation are based on the complex and expensive bisulfite method. In this paper, we report a novel fluorescence method to detect DNA and DNA methylation based on graphene oxide (GO) and restriction endonuclease HpaII. The skillfully designed probe DNA labeled with 5-carboxyfluorescein (FAM) and optimized GO concentration keep the probe/target DNA still adsorbed on the GO. After the cleavage action of HpaII the labeled FAM is released from the GO surface and its fluorescence recovers, which could be used to detect DNA in the linear range of 50 pM–50 nM with a detection limit of 43 pM. DNA methylation induced by transmethylation (Mtase) or other chemical reagents prevents HpaII from recognizing and cleaving the specific site; as a result, fluorescence cannot recover. The fluorescence recovery efficiency is closely related to the DNA methylation level, which can be used to detect DNA methylation by comparing it with the fluorescence in the presence of intact target DNA. The method for detection of DNA and DNA methylation is simple, reliable and accurate.

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1. Introduction

Genomic DNA methylation is one of the most important epigenetic modifications in eukaryotes, which serves a wide variety of biological functions. DNA transmethylase (Mtase) can catalyze the transfer of a methyl group to DNA and all the known DNA methyltransferases use S-adenosylmethionine (SAM) as the methyl donor. In animals, most of the methylations occur at the 5′ position of the pyrimidine ring of the cytosine. The methylcytosine (mC) is mainly found in cytosine–guanine (CpG) dinucleotides. The presence of 5-mC in the promoter of specific genes alters the binding of transcriptional factors and other proteins to DNA and recruits methyl-DNA-binding proteins and histone deacetylases that compact the chromatin around the gene-transcription start site. Both mechanisms block transcription and cause gene silencing. Thus methylation of cytosine residues in genomic DNA plays a key role in the regulation of gene expression in many biological events and is closely associated with various diseases, especially cancer [1–3]. So, it is significant to develop a simple and sensitive method for the detection of DNA methylation.

The traditional method for detection of DNA methylation is based on bisulfite. Bisulfite modification converts unmethylated cytosine to uracil, while methylated cytosine cannot react. After denaturation and bisulfite modification, double-strand DNA is obtained by primer extension and the fragment of interest is amplified by PCR. There is an extensive range of methods based on the sodium bisulfite treatment for quantifying the methylation status of cytosines located in specific DNA regions [4,5]. The bisulfite-based methods are currently considered gold standard assay techniques because they are reliable, accurate, and can help understand the methylation status of each CpG in target DNA, while complex and expensive clone and sequencing procedures are needed in this method. A lot of new methods, including gel electrophoresis [6], real-time quantitative polymerase chain reaction [7], high-performance liquid chromatography (HPLC) [8,9] and gas chromatography/mass spectrometry (GC/MS) [10], are also developed. They are also complicated to operate, time consuming and expensive. Recently, alternative approaches such as electrochemical [11–13], electrogenerated chemiluminescence strategy [14,15], colorimetric [16–19] and fluorescence methods [20–25]
have been developed to detect DNA methylation. Endonucleases can recognize and cleave specific short DNA sequences. Combined with methyltransferase they are usually used to detect DNA methylation [26–29]. GO has been reported as an excellent quenching material for the fluorescence dye due to fluorescence resonance energy transfer. The other superior property is that GO has a strong binding force with ssDNA strands due to π-stacking interaction between nucleobases and GO surface [30–33].

Herein, a graphene oxide (GO) based platform coupled with Hpall and Mtase M.SssI is studied to detect DNA methylation. First, GO was used to quench the probe DNA that was modified with 6-carboxyfluorescein (FAM) on its 5′ end. The probe DNA is skillfully designed to have 10 bases longer than that of target DNA in order to make their hybridism still adsorbed on the GO surface (Scheme 1, a). After the cleavage of specific site 5′-CCGG-3′ through Hpall, the labeled FAM is released to the solution and the fluorescence recovers (Scheme 1, b). The DNA detection limit of this method is 43 pM without signal amplification strategy. When the target DNA is methylated, the fluorescence cannot recover because Hpall digestion function is blocked by methylated cytosine base (Scheme 1, c). Therefore, the fluorescence intensity of FAM is closely related to the methylation level. Based on this, a sensitive fluorescence method for detection of DNA and DNA methylation is proposed. The method avoids bisulfite treatment of DNA and is simple, reliable and selective.

2. Experimental

2.1. Reagents and apparatus

The GO was synthesized by our group following the Hummers method [34]. Tris(hydroxymethyl)aminomethane (Tris) was purchased from Sigma-Aldrich. Hydrogen peroxide (30% in water) (H2O2) was purchased from Sinoreagent. S-adenosylmethionine (SAM), Escherichia coli CpG methyltransferase (M.SssI), and E. coli restriction endonuclease (Hpall) were obtained from New England BioLabs (NEB, UK). All these standard solutions were prepared and stored at under 4 °C. Milli-Q water (18 MΩ cm) was used throughout.

The DNA oligonucleotides were synthesized by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd., China. 1 μM DNA stock solution was prepared by using 33 mM Tris–acetate buffer solution (pH 7.4) containing 66 mM NaAc and 10 mM Mg(AC)2. The sequences of oligonucleotides used in this work are shown in Table 1.

The buffers employed in this work were as follows: DNA hybridization buffer was 33 mM Tris–acetate (pH 7.4) containing 66 mM NaAc and 10 mM Mg(AC)2. The buffer for MTase work was 10 mM Tris–HCl (pH 7.9) containing 50 mM NaCl, 10 mM MgCl2, and 1 mM DTT. The buffer for endonuclease Hpall digestion experiment was 33 mM Tris–acetate (pH 7.9) containing 10 mM Mg(AC)2, 66 mM KAc and 0.1 mg/mL BSA.

Safety note: dimethyl sulfoxide (DMSO) and acetaldehyde (CH3 CHO) are suspected human carcinogens and somewhat volatile. Gloves were worn; manipulations were done under a closed hood. All reactions were done in closed vessels. A fluoromax 4 spectrophotometer (Horiba, Japan) was used in the experiment.

2.2. Endonuclease digestion of probe/target DNA on GO surface

FAM-labeled probe DNA was mixed with target DNA (10 bases longer than probe DNA) in 33 mM pH 7.4 Tris–acetate buffer solution containing 66 mM NaAc and 10 mM Mg(AC)2. Then, the solution was heated to 90 °C for 5 min followed by slow cooling to room temperature to ensure complete hybridization. Then, Hpall was incubated with probe/target DNA in 33 mM pH 7.9 Tris–acetate buffer solution containing 10 mM Mg(AC)2, 66 mM NaAc and 0.1 mg/mL BSA at 37 °C. Finally, 25 μg/mL GO was added to the mixture and the fluorescence intensity of the FAM was monitored.

2.3. DNA cytosine methylation induced by M.SssI Mtase

The methylation was prepared by incubating M.SssI Mtase with prepared double strands DNA in 1× MTase reaction buffer (10 mM pH 7.9 Tris–HCl containing 50 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol and 160 μM SAM) at 37 °C.

2.4. DNA methylation induced by chemical reagents

25 μL of 1 μM double strands DNA was mixed with 5.0 μM FeSO4, 20.0 μM H2O2, 10.0 μM L-ascorbic acid, 3.0 μM ethylene diamine tetraacetic acid (EDTA)–2Na and 100 μM DMSO or CH3 CHO. The solution reacted under N2 atmosphere. After reaction, the mixture solution was centrifuged to separate chemical reagents from DNA. The followed detection procedure was the same as that for M.SssI methyltransferase.

Table 1

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Sequences (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probe DNA</td>
<td>FAM-ACCCCGATAACATCCTACTTCAC</td>
</tr>
<tr>
<td>Target DNA</td>
<td>AGCATCTATCCGCTG</td>
</tr>
<tr>
<td>One base mismatched target DNA (T1)</td>
<td>AGCATCTATCCAGGT</td>
</tr>
<tr>
<td>Non-complementary target DNA (T2)</td>
<td>GAACATCATACCGGAC</td>
</tr>
</tbody>
</table>

Mismatched base is underlined.
3. Results and discussion

3.1. Strategy for detection of DNA and DNA methylation based on GO and endonuclease HpaII

As demonstrated in Scheme 1, first, probe DNA was hybridized with target DNA to form double strands and labeled FAM that emitted strong fluorescence (Fig. 1a). As previously reported the signal of FAM labeled on the single strand DNA was quenched efficiently in the presence of certain amount of GO, while the fluorescence was recovered when it was hybridized with the perfect matched DNA due to the separation of double strands DNA from GO surface [28,31]. In our assay, the probe DNA are designed to have 10 bases longer than that of target DNA, which can keep their hybrids still adsorbed on GO and the fluorescence is still quenched (Fig. 1b). After incubation with HpaII that can recognize the double strands sequence of “CCGG” and cleave it (5’-CCmGG-3’), FAM is released from GO surface and the fluorescence is recovered (Fig. 1c). Thus, the target DNA can be detected based on the recovered fluorescence intensity. The cleavage action of HpaII is prevented by the presence of a 5-methyl group at the second C base of its recognition sequence of “CCGG” (i.e. “CcGG”). As a result, the fluorescence cannot recover (Fig. 1d). So, DNA methylation can be discriminated from the intact DNA by comparing their fluorescence recovery efficiencies.

3.2. Optimization of detection condition

GO concentration is one of the important factors for the DNA and DNA methylation detection. The fluorescence intensity of the probe DNA/target DNA varies considerably with the GO concentration. The quench efficiency increases with increasing amount of GO and begins to level off at 25 μg/mL (Fig. 2A). On the other hand, GO concentration has large impact on the fluorescence recovery efficiency induced by HpaII. Fig. 2B demonstrates that the most efficient fluorescence recovery is obtained at 25 μg/mL of GO, while further increased amount of GO decreases the fluorescence recovery efficiency. High fluorescence quench efficiency induced by GO decreases the background signal, while high recovery efficiency induced by HpaII increases the detection sensitivity. Thus, 25 μg/mL GO is chosen as the optimal condition. The cleavage time for HpaII is optimized.

The cleavage time for HpaII was optimized. Fig. 3 shows that the fluorescence cannot recover in the absence of HpaII, while it recovers in the presence of 0.02 U/μL of HpaII and the recovery efficiency is dependent on the incubation time of HpaII and probe/target DNA. The fluorescence intensity increases with increasing incubation time and levels off at 1 h. In order to obtain the best recognition and cleavage function of HpaII, 2 h incubation is chosen in the experiments.

3.3. Detection of target DNA

Fig. 4A shows the fluorescence spectra of 50 nM probe DNA in the presence of different concentrations of target DNA from 0 to 100 nM, 25 μg/mL of GO and 0.02 U/μL of HpaII. The fluorescence recovery efficiency increased with increment of target DNA. Fig. 4B reveals that the increased fluorescence intensity is linear with the target DNA concentration in the range of 50 pM–50 nM. The calibration equation is $y = 95.244x + 48.847$, with correlation coefficient $R^2 = 0.998$. The detection limit was 43 pM obtained in terms of 3 times deviation of blank sample, which was much lower than that of reported result based on GO induced fluorescence quench [35].

One-base mismatched (T1) and non-complementary (T3) target DNA were used to evaluate the selectivity of the method. Compared with the complementary DNA (Fig. 5a) the fluorescence...
intensity for T1 (b) and T3 (c) decreased 96%, only a little higher than that without target DNA (d). This demonstrated that Hpall and Exo III do not work on one-base mismatched or non-complementary target DNA, indicating that the proposed method has good selectivity. The method can make a distinction between one-base mismatched DNA and complementary target DNA, which possesses more advantages than those of previously reported methods.

3.4. Detection of DNA methylation induced by M.SssI MTase

Based on the good selectivity of the method, it is used to detect the DNA methylation induced by M.SssI in the presence of SAM. The synthetic methylated DNA containing 5'–CCmGG–3' sequence is used as standard methylated DNA for comparison. Experiments showed that no fluorescence was recovered in the presence of Hpall because it cannot recognize and cleave the methylated DNA.

Fig. 3. Time-dependent fluorescence intensity of probe/target DNA in the presence of 0.02 U/μL Hpall and 25 μg/mL of GO.

Fig. 4. (A) Fluorescence spectra of the hybrid of 50 nM probe DNA and various concentrations of target DNA (0, 0.2, 0.5, 1, 2.5, 5, 10, 15, 20, 25, 30, 40, 50, 100 nM from a to n in order). (B) Dependence of fluorescence intensity on different target DNA concentrations; the inset shows the plot of fluorescence intensity versus target DNA concentration from 0 to 50 nM. Hpall concentration: 0.02 U/μL, incubation time: 2 h.

Fig. 5. Selectivity of the method. (A) Fluorescence spectra in the presence of (a) complementary target DNA, (b) one-base mismatched target DNA, (c) noncomplementary target DNA and (d) without DNA. (B) The histogram corresponds to the fluorescence spectra in (A). Hpall concentration: 0.02 U/μL, incubation time: 2 h.
Probe/target DNA are treated with various concentrations of M.SssI and 160 μM SAM for 2 h and 4 h followed by detection of their fluorescence in the presence of GO and HpaII. Fig. 6 shows that the fluorescence intensity of treated probe/target DNA decreases with the increase of M.SssI concentration from 0 to 1.0 U/μL. Both higher concentration and longer incubation time of HpaII lead to lower fluorescence recovery efficiency, which indicates that a higher level of DNA methylation occurs. When the probe/target DNA are treated for 4 h, their fluorescence intensity decreases linearly with the HpaII concentration in the range of 0–0.5 U/μL; the calibration equation is \( y = 7.83E^6 + 4.50E^6 \) with correlation coefficient \( R^2 = 0.996 \). So, the method can not only be used to detect DNA methylation but also has the potential to be used to detect the activity of M.SssI.

3.5. Detection of DNA methylation induced by chemical reagents

DNA methylation induced by chemical reagents such as DMSO and CH₃CHO in the presence of the Fenton reagents (Fe²⁺, H₂O₂) is detected by the proposed method. The fluorescence spectra of the double strands DNA treated by different reagents are shown in Fig. 7. Fluorescence in the absence of target DNA is very low, while it is very strong in the presence of intact target DNA. All the fluorescence recoveries corresponding to standard methylated DNA and the target DNA treated with chemical reagents or M.SssI are recovered slightly; however, their recovery efficiencies are still different from each other. This indicates that all of these reagents induce DNA methylation and the different methylation levels between them could be discerned by this method. CH₃CHO (curve a) or DMSO (curve b) in the presence of Fenton reagents gives rise to DNA methylation because of the methyl radicals that are generated by the reaction of OH radicals with CH₃CHO or DMSO [36–38], while their DNA methylation level is lower than that induced by M.SssI Mtase (curve c). Also, the DNA methylation level induced by M.SssI is lower than that of the synthetic standard methylated DNA (curve d). So, it is reasonable to judge DNA methylation level from their fluorescence intensity. Fig. 8 shows comparison of fluorescence intensity in the presence of different types of target DNA. It shows that the fluorescence intensity for intact complementary DNA is different obviously from those of various methylated DNA and the non-complementary target DNA. Therefore, the developed method has good selectivity to detect intact target DNA and methylated DNA.

4. Conclusions

In summary, a platform based on GO and endonuclease HpaII is constructed for detection of DNA and DNA methylation. The assay is based on the fluorescence recovery of probe/target DNA on the GO surface after the recognition and cleavage action of endonuclease HpaII. The method avoids complex bisulfitite treatment of DNA, PCR, and antibody, and is simple, reliable and of low-cost. On the other hand, the method has potential to be used to detect Mtase activity and is expected to screen appropriate inhibitor drugs in DNA Mtase and disease diagnosis related to DNA methylation.

Acknowledgments

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References
