Analytica Chimica Acta 869 (2015) 74–80

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Fluorescence quenching of graphene oxide combined with the site-specific cleavage of restriction endonuclease for deoxyribonucleic acid demethylase activity assay

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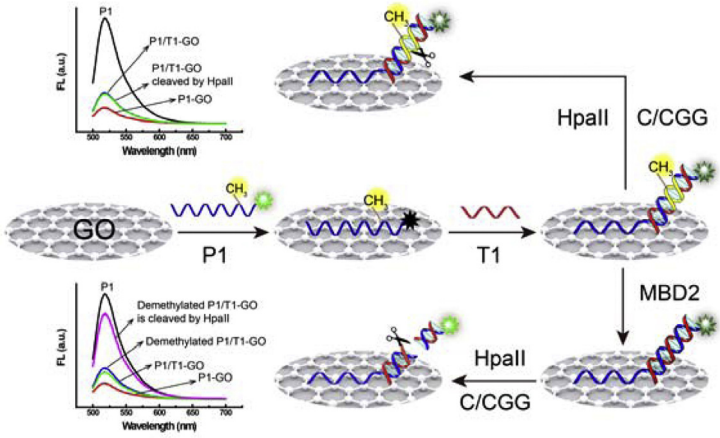
HIGHLIGHTS

- An approach for sensitive and selective DNA demethylase activity assay is reported.
- This assay is based on the fluorescence quenching of GO and site-specific cleavage of endonuclease.
- It can determine as low as 0.05 ng mL^{-1} of MBD2 with a linear range of 0.2–300 ng mL^{-1}.
- It has an ability to recognize MBD2 from other possibly coexisting proteins and cancer cell extracts.
- It can avoid false signals, requiring no bisulfite conversion, PCR amplification, radioisotope-labeling.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 31 December 2014
Received in revised form 4 February 2015
Accepted 10 February 2015
Available online 12 February 2015

Keywords:
Deoxyribonucleic acid methylation
Deoxyribonucleic acid demethylase
Graphene oxide
Restriction endonuclease
Fluorescence quenching

ABSTRACT

We report on the development of a sensitive and selective deoxyribonucleic acid (DNA) demethylase (using MBD2 as an example) activity assay by coupling the fluorescence quenching of graphene oxide (GO) with the site-specific cleavage of HpaII endonuclease to improve the selectivity. This approach was developed by designing a single-stranded probe (P1) that carries a binding region to facilitate the interaction with GO, which induces fluorescence quenching of the labeled fluorophore (FAM, 6-carboxyfluorescein), and a sensing region, which contains a hemi-methylated site of 5′-CmCGG-3′, to specifically recognize the target (T1, a 32-mer DNA from the promoter region of *p*53 gene) and hybridize with it to form a P1/T1 duplex. After demethylation with MBD2, the duplex can be specifically cleaved using HpaII, which releases the labeled FAM from the GO surface and results in the recovery of fluorescence. However, this cleavage is blocked by the hemi-methylation of this site. Thus, the magnitude of the recovered fluorescence signal is related to the MBD2 activity, which establishes the basis of the DNA demethylase activity assay. This assay can determine as low as ~(0.05 ± 0.01) ng mL^{-1} (at a signal/noise of 3) of MBD2 with a linear range of 0.2–300 ng mL^{-1} and recognize MBD2 from other possibly

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http://dx.doi.org/10.1016/j.aca.2015.02.021

coexisting proteins and cancer cell extracts. The advantage of this assay is its ability to avoid false signals and no requirement of bisulfite conversion, PCR amplification, radioisotope labeling, or separation.

1. Introduction

DNA methylation at the carbon 5 (C5) position of the pyrimidine ring in cytosine residues in the 5′-CG-3′ sequence (CpG) is one of the most important epigenetic modifications and has broad and critical biological functions, particularly in transcriptional regulation, gene silencing, and genome reprogramming [1–3]. Abnormal methylation has been closely associated with human diseases such as cancer [4–7]. The DNA methylation pattern is maintained by DNA methyltransferase (MTase) [8–13]. However, studies have proposed that DNA demethylase, which catalyzes the removal of the methyl group from the C5 position of 5-methylcytosine, 5mC, may also participate in the process, and the methylation pattern is a balance of reversible methylation–demethylation reactions [14–27]. Dysfunction of DNA demethylase has been implicated in many important diseases such as cancer, imprinting-related diseases, and psychiatric disorders [28,29]. Thus, sensitive activity assays for DNA demethylase represent a valuable strategy for understanding the regulation of DNA demethylation and developing drugs targeting demethylation, which are particularly useful for clinical diagnostics and therapeutics and for studying the response of various organisms to environmental conditions.

Current methods for DNA demethylase assay are mainly based on the detection of the methylation state of a DNA sequence, such as the bisulfite mapping method, which involves the conventional sodium bisulfite treatment of DNA followed by a polymerase chain reaction (PCR) assay or sequencing [14,15], and the affinity-based method, which uses antibodies or methyl-CpG-binding domains to capture a methylated DNA substrate followed by hybridization detection or immunoassays [21,24]. Although these methods are well established, they are generally laborious, technically demanding, and involve expensive equipment and time-consuming multi-step treatments, which restrict their utility for routine assays. The electrochemical technique offers the attractive advantages of low-cost, simple operation and fast response; however, it usually involves complicated and tedious labeling or conjugation procedures, which may limit its assay throughput, and its sensitivity and selectivity heavily depend on the redox features of electrochemical reporters [29]. Thus, the development of highly sensitive, specific, and low-cost methods for DNA demethylase activity assays remains a great challenge.

This work reports on the development of a rapid, sensitive, and selective DNA demethylase (using methyl-CpG-binding domain protein 2, MBD2, as an example) activity assay by coupling the fluorescence quenching of graphene oxide (GO) with the site-specific cleavage of a restriction endonuclease (HpaII) to improve selectivity (Fig. 1). We designed a single-stranded probe DNA (P1) that carries both a binding region (64 bases) and a sensing region (32 bases). The binding region provides an anchoring function to facilitate the interaction between GO and the probe, which induces fluorescence quenching of the labeled fluorophore (FAM, 6-carboxyfluorescein). The sensing region, which contains a hemi-methylated site of 5′-CmCGG-3′, specifically recognizes the target DNA (T1, a 32-mer DNA sequence from the promoter region of *p*53 gene) and hybridizes with it to form a duplex (P1/T1). This duplex serves as the substrate of MBD2 and contains the symmetrical sequence 5′-CCGG-3′, which is specifically recognized by HpaII endonuclease and catalytically cleaved between the cytosines [30]. However, the cleavage is blocked by the hemi-methylation of this site [31,32]. After this barrier is eliminated using the demethylation under treatment with MBD2, the duplex can be cleaved using HpaII. As a result, the labeled FAM is released from the GO surface, which results in the recovery of the fluorescence intensity of the fluorophore and produces a readily detectable signal. Thus, the fluorescence signal recovered after the HpaII cleavage is related to the MBD2 activity, which establishes the basis of the DNA demethylase activity assay. The extraordinarily high quenching efficiency of GO, which results in a high signal-to-background ratio, and high site-specific cleavage of HpaII, which results in high selectivity, make the method a promising assay for DNA demethylase activity with high sensitivity and selectivity.

2. Materials and methods

2.1. Materials

1,4-Dithiothreitol (DTT), human serum albumin (HSA), tris (hydroxymethyl)-aminomethane (Tris), and disodium ethylenediaminetetraacetic acid (EDTA), phenylmethylsulfonyl fluoride (PMSF), and 4-(2-hydroxyerhyl)piperazine-1-erhanesulfonic acid (HEPES) were purchased from Sigma–Aldrich and used as received. DNA demethylase (methyl-CpG-binding domain protein 2, MBD2), MBD1, MBD4, and MeCP2 were purchased from Epigentek Group Inc. (Farmingdale, NY) and stored at −80 °C. HpaII endonuclease was purchased from New England BioLabs (Ipswich, MA) and stored at −20 °C in 10 mM Tris–HCl (pH 7.4), which contained 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.2 mg mL^{-1} BSA, and 50% (v/v) glycerol.

The oligonucleotides were purchased from BioSune Biological Engineering Technology Co. (Shanghai, China). They were FAM-labeled single-stranded 96-mer probe DNA (P1, 5′-FAM-TTC TCT TCC TCT GTG CG***C mCGG*** TCT CTC CCA GGA CTA TGT GCC GAA TAT CAA GGA CAG TTG TAG CTA TGT GCC GAA TCG TAC CGT GAG TAA TGC GAC-3′. The underlined bases (32-mer) are responsible for hybridizing with the target; the italicized and bolded sequence (CCGG) is the

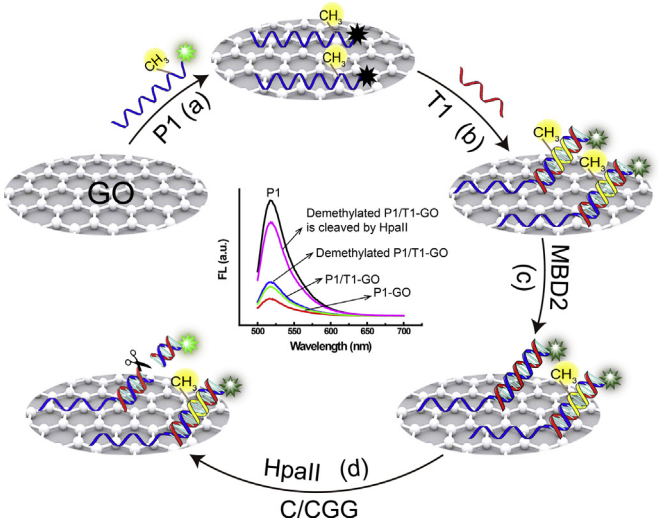

Fig. 1. Schematic illustration of the DNA demethylase activity assay based on coupling the fluorescence quenching of GO with the site-specific cleavage of a restriction endonuclease.