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A New Assay for Endonuclease/Methyltransferase Activities Based on Graphene Oxide

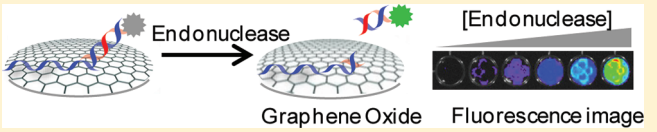
Jieon Lee,[†] Young-Kwan Kim,[†] and Dal-Hee Min*[,‡]

[†]Department of Chemistry, KAIST, Daejeon, 305-701, Republic of Korea

[‡]Department of Chemistry, Seoul National University, Seoul, 151-747, Republic of Korea

Supporting Information

ABSTRACT: A new endonuclease/methyltransferase activity assay method based on graphene oxide (GO) is developed. Substrate DNA is designed to possess a double-stranded part to serve as a nuclease substrate and a single-stranded part for anchoring the DNA to the GO surface via strong noncovalent binding. Nuclease-mediated DNA hydrolysis induces the recovery of fluorescence intensity of the dye attached to the end of the double-stranded DNA region. This GO-based method allows real-time measurement and quantitative assay for endonuclease/methyltransferase activities in short time.



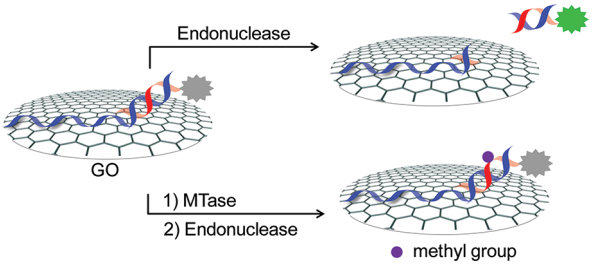
Endonucleases (ENases) are a family of enzymes that hydrolyze the internal phosphodiester linkages in deoxyribonucleic acid (DNA) or ribonucleic acid (RNA).[1] These nucleases are involved in many important biological processes related to genetic information transfer and gene expression, such as replication, DNA repair, and recombination of nucleic acids.[2−4] Virus replication processes require an enzyme called integrase,[5] which has nuclease activity, for viral genome insertion into host chromosomal DNA. Thus, nucleases have been a target for antiviral drug development. Furthermore, some nucleases, called restriction enzymes,[6] which cleave DNA at specific recognition sites, are essential in the field of molecular biology, especially in gene cloning and DNA mapping.[7,8]

DNA methyltransferase (MTase) is another important enzyme that modifies nucleic acids by transferring a methyl group from *S*-adenosyl methionine (SAM) to adenine or cytosine residues in DNA.[9] MTases play a crucial role in cellular differentiation and development, gene suppression, tumorigenesis, and genetic diseases.[10−14] Thus, evaluating ENase/MTase activities is important in the fields of drug discovery and clinical diagnostics.

Conventional assay methods include gel electrophoresis, high-performance liquid chromatography (HPLC), filter binding, radioactive labeling, and enzyme-linked immunosorbent assay (ELISA).[15−19] Although gel electrophoresis and isotope labeling are chosen routinely for ENase/MTase activity assays in most laboratories, these methods are time-consuming and/or not cost-effective. Recently, alternative approaches such as colorimetric and fluorescence methods, based on nanomaterials, have been developed for simple and real-time monitoring of ENase activity.[20,21] For example, an enzyme-responsive system using DNA−gold nanoparticle assembly provides a measure of ENase activity.[21]

Here, we report a new ENase/MTase activity assay method based on the preferential binding of single-stranded DNA (ssDNA) over double-stranded DNA (dsDNA) to graphene oxide (GO; Scheme 1). Graphene is a one-atom-thick 2D carbon nanomaterial with extraordinary electronic, thermal, and mechanical properties.[22] Graphene oxide (GO) is a water dispersible version of graphene presenting oxygen-containing functional groups such as hydroxyl, carboxyl, and epoxy groups. GO has been harnessed recently in biological applications, such as drug delivery, biosensors, and enzyme activity assays.[23−25] Strategies in these applications rely on the strong binding of hydrophobic small molecules and ssDNA with GO and/or the fluorescence-quenching capability of GO.[24,26,27]

Scheme 1. Strategy for ENase/MTase Activity Assays, Based on Fluorescence Quenching by GO



In this study, we prepared DNA substrates of ENases that carry both a single-stranded region for binding GO and a double-stranded region for the sequence-specific recognition of ENases. Here, the single-stranded region provides an anchoring function to facilitate the interaction between GO and DNA, inducing fluorescence quenching of a dye that is conjugated at the end of the double-stranded part. Recently, GO was reported to interact

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with ssDNA through π-stacking interaction between nucleobases and GO surface, whereas dsDNA cannot be strongly adsorbed on the GO due to shielding of nucelobases within its double-helix structure.[28] In the presence of an ENase, the dye-conjugated dsDNA part is subject to release from GO, resulting in the recovery of fluorescence of the dye (Scheme 1). The strategy can be further employed in a DNA MTase activity assay in which the DNA strands methylated by MTases cannot be hydrolyzed by ENases.

EXPERIMENTAL SECTION

Materials. Natural graphite (FP 99.95%) was purchased from Graphit Kropfmühl AG (Hauzenberg, Germany). Sulfuric acid (H_2SO_4) was purchased from Samchun chemical (Seoul, Korea). Sodium nitrate ($NaNO_3$) and hydrogen peroxide (30% in water) (H_2O_2) were purchased from Junsei. Potassium permanganate ($KMnO_4$), aurintricarboxylic acid (ATA), and *S*-(5′-adenosyl)-L-homocysteine (SAH) were purchased from Sigma-Aldrich (St. Louis, MO). Upper strand DNAs for *Eco*RV substrate (5′-CTA GCT ATG TGC CGA ATT TCA AGG ACA GTT GTA TGG ATA TCA TAC T-3′) and fluorescent-dye-labeled bottom strand (5′-FAM-AGT ATG ATA TCC A-3′) were purchased from Genotech (Daejon, Korea). Upper strand DNAs for *Hae*III/*Hae*III Methyltrasferase substrate (5′-CTA GCT ATG TGC CGA ATT TCA AGG ACA GTT GTA TGG CCC TCG T-3′) and fluorescent-dye-labeled bottom strand (5′-FAM-ACG AGG GCC ATA-3′) were also purchased from Genotech (Daejon, Korea). *Hae*III and *Eco*RV were purchased from Enzynomics (Daejon, Korea). *Hae*III methyltransferase was purchased from New England BioLabs (MA, UK). Ethylenediamine tetraacetic acid (EDTA) was purchased from Bio-Rad. Fluorescence intensity was measured by a SynergyMx fluorometer (Biotek)

Preparation of Graphene Oxide. Graphene oxide (GO) nanosheets were prepared according to previously reported methods. A mixture of 0.5 g of natural graphite, 0.5 g of $NaNO_3$, and 23 mL of H_2SO_4 was vigorously stirred in an ice bath. Then, 3 g of $KMnO_4$ was slowly added. After the addition, the mixed solution was transferred to a 35 °C water bath for 1 h with stirring. Next, 40 mL of distilled water was added and the bath temperature was increased up to 90 °C for 30 min. Another 100 mL of distilled water was then added. Next, dropwise addition of 3 mL of 30% H_2O_2 changed the color of the solution from dark brown to yellow. Synthesized GO solution was filtered by Buchner funnel and washed with copious amounts of distilled water. The filter cake was dried in a desiccator and redispersed in distilled water.

Endonuclease Activity Assay by GO-Based Platform. To prepare duplex DNA substrate of *Eco*RV (DNA1), 2.5 μL of 100 μM fluorescent-dye-labeled short bottom strand DNA was mixed with a 1.2-fold excess of long upper strand DNA in pH 8.0 buffer containing 50 mM Tris-HCl and 50 mM NaCl. Then, the mixture was annealed by heating to 90 °C for 5 min and followed by slow cooling at room temperature for 1 h. For *Eco*RV activity assay, reaction mixture was prepared by mixing *Eco*RV enzyme stock with 200 nM annealed DNA1 in 1× *Eco*RV reaction buffer (pH 7.9 Tris-HCl buffer containing 50 mM Tris, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol). After incubation at room temperature, 30 μL of reaction mixture and 30 μL of 60 μg/mL GO solution were mixed by equal amount in a 96-well plate. In case of *Hae*III, duplex DNA substrate of *Hae*III (DNA2) was annealed by mixing 2.5 μL of 100 μM fluorescent-dye-labeled short bottom strand DNA and a 1.2-fold excess of long upper strand DNA in pH 8.0 buffer containing 50 mM Tris-HCl and 50 mM NaCl. Then, the mixture was annealed by heating to 90 °C for 5 min, followed by slow cooling at room temperature for 1 h. For *Hae*III activity assay, reaction mixture was prepared by mixing *Hae*III enzyme stock with 200 nM annealed DNA2 in 1× *Hae*III reaction buffer (pH 7.9 Tris-HCl buffer containing 10 mM Tris, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol). After incubation at room temperature, 30 μL of reaction mixture and 30 μL of 20 μg/mL GO solution were mixed by equal amount in a 96-well plate. Fluorescence intensity was measured at 520 nm.

***Hae*III Methyltrasferase Activity Assay by GO-Based Platform.** To measure the *Hae*III MTase activity, reaction mixture was prepared by mixing *Hae*III MTase enzyme stock with 600 nM annealed DNA2 in 1× MTase reaction buffer (pH 8.5 Tris-HCl buffer containing 50 mM Tris, 50 mM NaCl, 10 mM dithiothreitol, 1.6 mM SAM) and incubated at 37 °C. Then, 10 μL of reaction mixture was mixed with 30 μL of 1× *Hae*III reaction buffer containing 20 units of *Hae*III and incubated at room temperature for 10 min. Finally, 40 μL of reaction mixture and 20 μL of 30 μg/mL GO solution were mixed in a 96-well plate. Fluorescence intensity was measured at 520 nm.

ENase/MTase Inhibition Assay by GO-Based Plarform. For EDTA−*Hae*III inhibition assay, EDTA solutions with various concentrations were mixed with 200 nM DNA2 in 1× *Hae*III reaction buffer followed by addition of 10 units of *Hae*III. After incubation at room temperature for 1 h, 30 μL of each reaction mixture was mixed with 30 μL of 20 μg/mL GO solutions in a 96-well plate. In the case of ATA−*Hae*III, ATA solutions with various concentrations were mixed with 200 nM DNA2 in 1× *Hae*III reaction buffer followed by the addition of 10 units of *Hae*III. After incubation at room temperature for 1 h, 30 μL of each reaction mixture was mixed with 30 μL of 20 μg/mL GO solutions in a 96-well plate.

For ATA−*Eco*RV inhibition assay, various concentrations of ATA solution were mixed in 1X× *Eco*RV reaction buffer containing 200 nM DNA1 followed by addition of 2.5 unit of *Eco*RV. After incubation at room temperature for 1 h, 30 μL of each reaction mixture was mixed with 30 μL of 60 μg/mL GO solutions in a 96-well plate.

For SAH−*Hae*III MTase inhibition assay, SAH solutions with various concentrations were mixed with 600 nM DNA2 in 1× *Hae*III MTase reaction buffer followed by addition of 5 units of *Hae*III MTase. After incubation at 37 °C for 1 h, 10 μL of reaction mixture was mixed with 30 μL of 1× *Hae*III reaction buffer containing 20 units of *Hae*III and incubated at room temperature for 10 min. Finally, 40 μL of reaction mixture and 20 μL of 30 μg/mL GO solution were mixed in a 96-well plate. Fluorescence intensity was measured at 520 nm.

RESULTS AND DISCUSSION

First, we prepared an aqueous suspension of GO according to a modified Hummers method.[29] The formation of the single-layer GO sheet was confirmed by atomic force microscopy (AFM) images (Figure 1a), and the height profile (Figure 1b) showed GO sheets with about 1 nm thickness. Next, we further characterized the GO using Raman and FT-IR spectroscopy. The Raman spectrum of GO showed characteristic peaks at 1361 and 1598 cm^{-1}, assigned as a D peak, attributed to structural disorder of the sp^2 carbon domain, induced by oxidation and exfoliation, and a G peak from the ordered sp^2 carbon domain, respectively (Figure 1c). Several characteristic peaks of functional groups containing oxygen were observed in the FT-IR

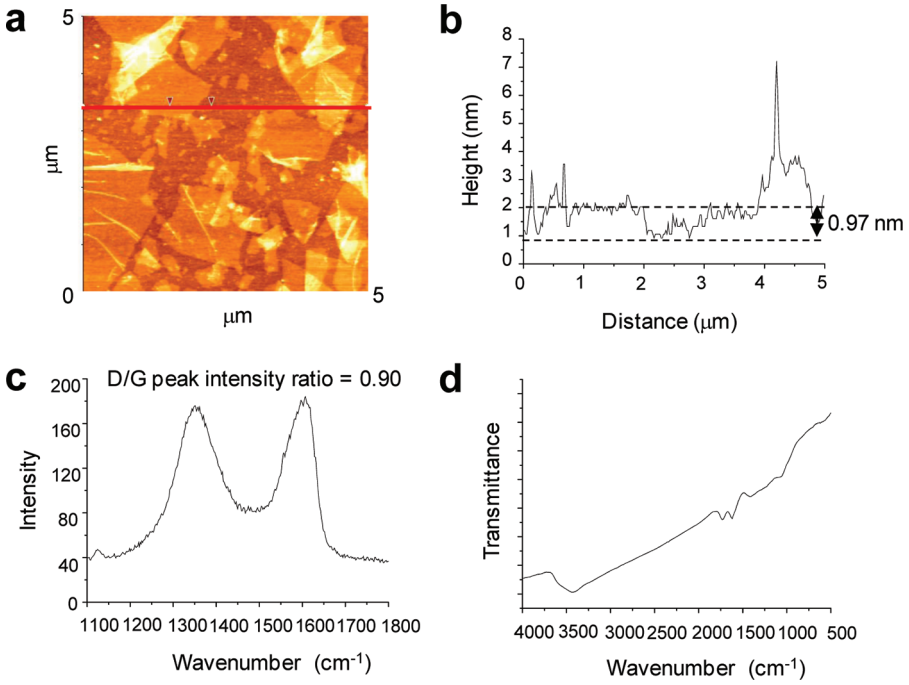

Figure 1. Characterization of GO. (a) AFM image and (b) height profile of GO showing the dimensions of prepared GO as 0.1−3 μm in width and ca. 0.97 nm in height. (c) Raman and (d) FT-IR spectra of GO, indicating the structural disorder of the sp^2 carbon domain and characteristic peaks of oxygen functional groups in GO, respectively.

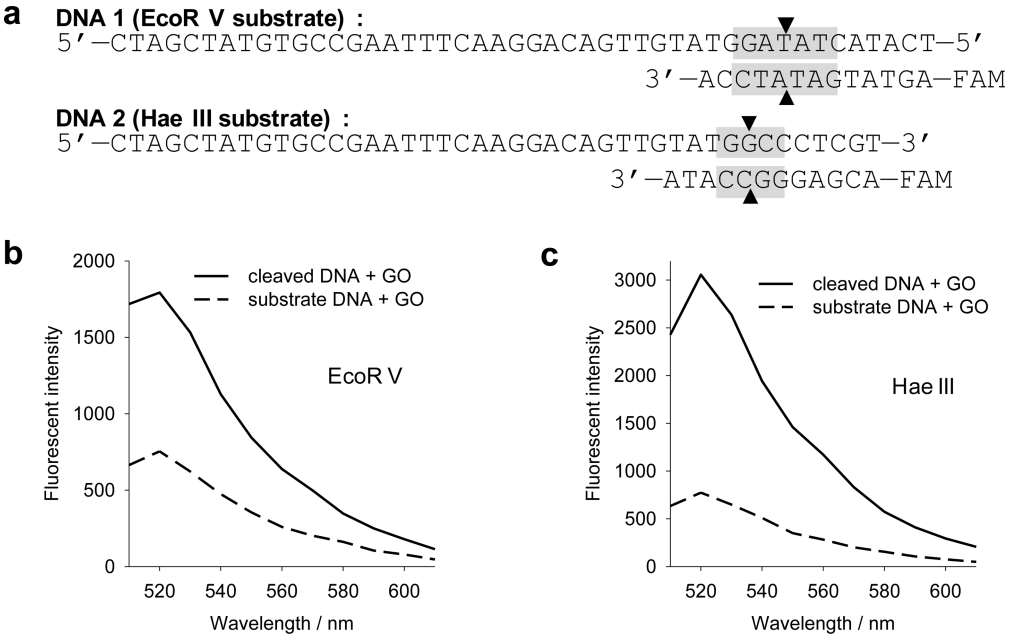

Figure 2. FAM fluorescence quenching by GO. (a) Sequences of substrate DNAs for *Eco*RV and *Hae*III ENases. DNA1 and DNA2 possess the recognition sites of *Eco*RV and *Hae*III, respectively, indicated as shaded regions. The *Hae*III substrate is also subject to methylation by *Hae*III MTase. Fluorescence spectra of substrate DNA and the cleaved product DNA by (b) *Eco*RV and (c) *Hae*III at each optimal GO concentration (30 and 10 μg/mL, respectively).

spectrum of GO, including peaks at 1716 and 1079 cm^{-1} from C═O and C−O stretching, respectively (Figure 1d).

In the present report, we demonstrate our strategy using two different types of restriction ENases (*Eco*RV and *Hae*III) and a DNA MTase (*Hae*III MTase). We first performed enzymatic hydrolysis reactions of substrate DNAs catalyzed by *Eco*RV and *Hae*III. For the hydrolysis by *Eco*RV, the substrate DNA (DNA1) was prepared by annealing the long upper strand DNA (5′-CTA GCT ATG TGC CGA ATT TCA AGG ACA GTT GTA TGG ATA TCA TAC T-3′) with a FAM-labeled

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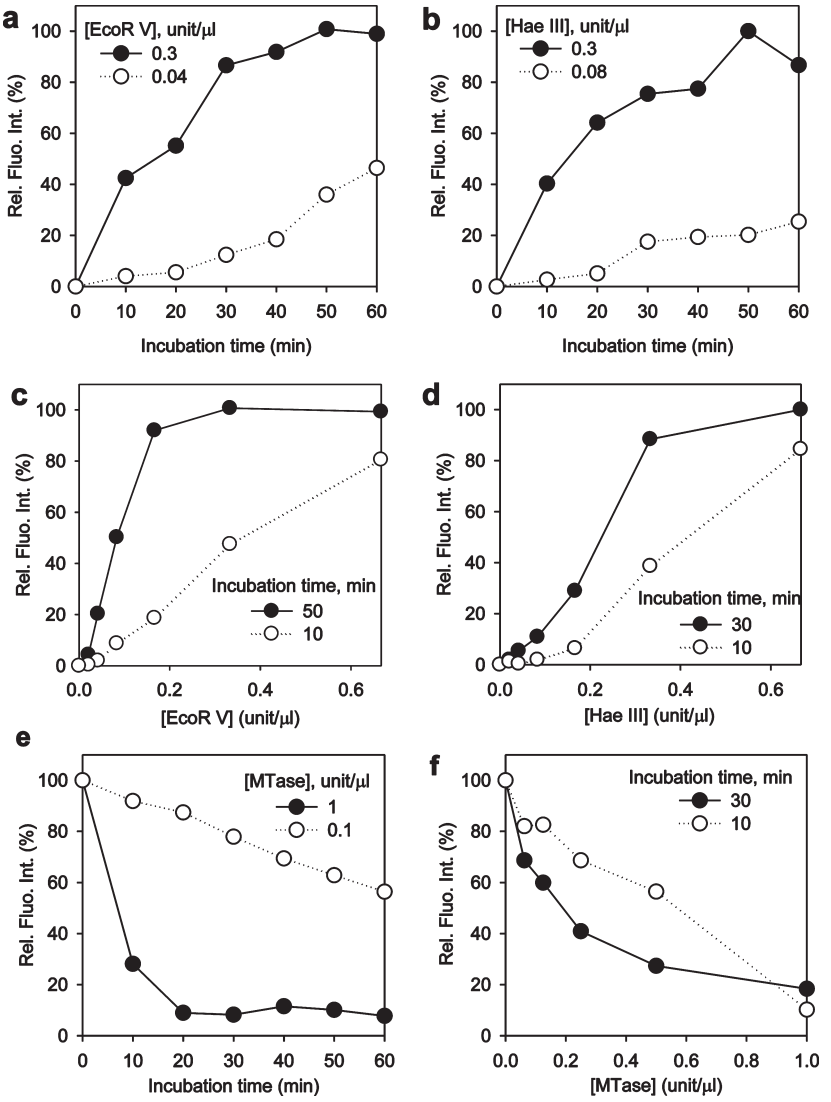

Figure 3. Time-dependent and enzyme-concentration-dependent fluorescence intensities of reaction mixtures of DNA1 with *Eco*RV (a, c), DNA2 with *Hae*III (b, d), and DNA2 with *Hae*III MTase (e, f). MTase activity was evaluated after the addition of *Hae*III to the mixture of DNA2 and *Hae*III MTase to allow cleavage of unmethylated DNA2. The DNA concentration was 600 nM for all experiments.

short bottom strand DNA (5′-FAM-AGT ATG ATA TCC A-3′; Figure 2a). The substrate DNA (DNA1, 200 nM) was prepared in Tris-HCl buffer solution (pH 7.9) containing 50 mM Tris, 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol (*Eco*RV reaction buffer). To determine the optimal GO concentration, DNA product completely cleaved by *Eco*RV was prepared, as well as control DNA substrate without cleavage. Each DNA sample was mixed with varying amounts of GO solution to make final GO concentrations from 0 to 40 μg/mL. The FAM-labeled DNA showed strong fluorescence at around 520 nm. We found that 30 μg/mL GO showed ca. 60% quenching efficiency, giving a maximum difference in fluorescence intensities between intact uncleaved substrate DNA and DNA completely cleaved by *Eco*RV (Figure 2b and Supporting Information, Figure S1a). In the case of *Hae*III, the substrate DNA (DNA2) was prepared by annealing the long upper strand DNA (5′-CTA GCT ATG TGC CGA ATT TCA AGG ACA GTT GTA TGG CCC TCG T-3′) with FAM-labeled short bottom strand DNA (5′-FAM-ACG AGG GCC ATA-3′; Figure 2a). Then, the substrate DNA was prepared in a buffer solution (pH 7.9) containing 200 nM DNA2, 10 mM Tris, 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol (*Hae*III reaction buffer). *Hae*III enzyme stock (10 U/mL) was added to DNA solution and the reaction mixture was incubated at room temperature. From the quenching experiment in the presence of various GO concentrations, 10 μg/mL GO, which showed 75% quenching of fluorescence, was the optimal GO concentration for assaying *Hae*III activity (Figure 2c and Supporting Information, Figure S1b).

Next, we measured the time-dependent hydrolysis of DNA1 catalyzed by *Eco*RV. Reaction mixtures were prepared by adding *Eco*RV stock solution to DNA1 solution to give 600 nM DNA1 and 0.3 or 0.04 U/μL *Eco*RV as final concentrations in *Eco*RV reaction buffer. Then, 30 μL of the *Eco*RV reaction mixture was removed at 10 min intervals and mixed immediately with 30 μL of 60 μg/mL GO solution in a 96-well plate. The fluorescence spectrum was collected for each well using a fluorometer. Fluorescence intensity was normalized using the equation, $I'FX = IFX - IB$, where IB represents the background fluorescence intensity. The relative amount of product was represented by calculating the

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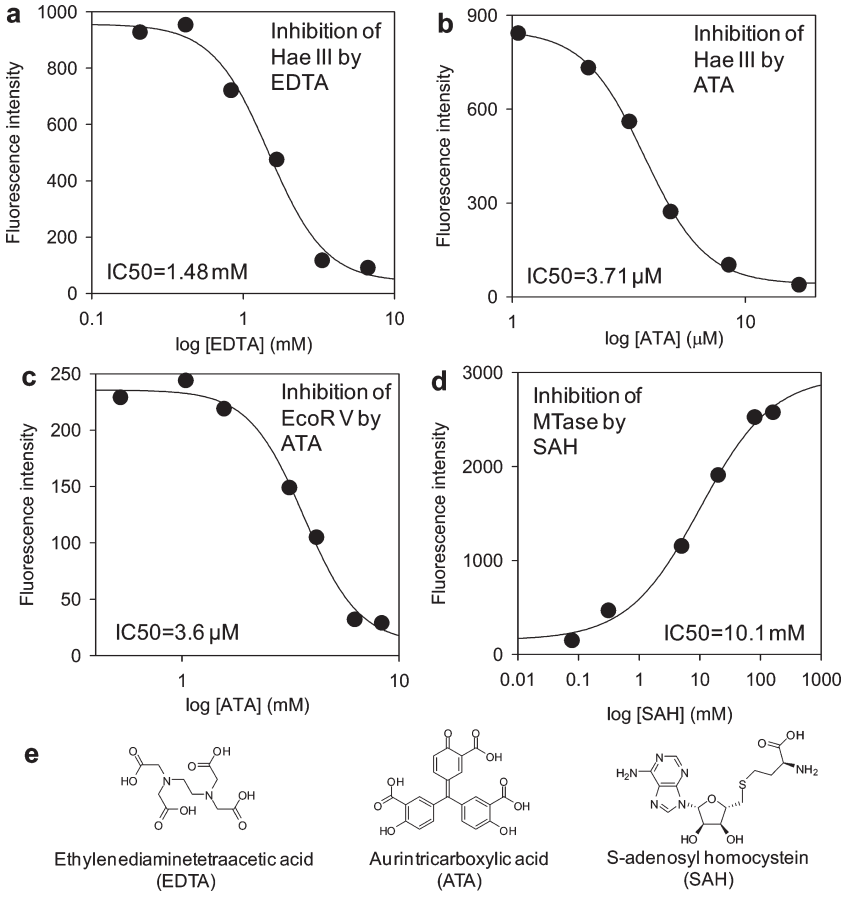

Figure 4. Dose-dependent inhibition of *Hae*III, *Eco*RV, and *Hae*III MTase reactions by small molecules. Inhibition of *Hae*III by (a) EDTA and (b) ATA, inhibition of *Eco*RV by (c) ATA, and inhibition of *Hae*III MTase by (d) SAH were examined and the IC_{50} values were obtained. (e) Molecular structures of the inhibitors.

relative intensity (relative amount of MX = I′FX/I′FM × 100, where I′FM indicates the normalized maximum or saturated fluorescence intensity after the cleavage of all substrate DNA). In the experiments on the time-dependent DNA hydrolysis catalyzed by *Hae*III, the reaction mixtures were prepared to contain 600 nM DNA2 and 0.3 or 0.08 U/μL *Hae*III in *Hae*III reaction buffer. Then, 30 μL of the *Hae*III reaction mixture was removed and mixed with 30 μL of 20 μg/mL GO solution in a 96-well plate at intervals of 10 min. With both the *Eco*RV and *Hae*III reactions, time-dependent substrate hydrolysis was observed, and the relative amounts of products could be calculated conveniently from the fluorescence intensities at 520 nm (Figure 3a,b). Next, enzyme-concentration-dependent DNA hydrolysis assays were performed using various concentrations of *Eco*RV or *Hae*III (Figure 3c,d). As expected, the DNA hydrolysis rate was faster with higher concentrations of ENase.

For the MTase activity assay, we used both a restriction enzyme (*Hae*III) and DNA MTase (*Hae*III MTase). *Hae*III MTase catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionine (SAM) to a cytosine residue at the recognition site of the substrate DNA. Methylated DNA substrate is expected to be resistant to *Hae*III-mediated hydrolysis. We carried out a time-dependent enzymatic methylation reaction of substrate DNA, catalyzed by *Hae*III MTase. To measure MTase activity, substrate DNA was prepared in a reaction buffer solution (pH 8.5) containing 600 nM DNA2 and Tris-HCl buffer containing 50 mM Tris, 50 mM NaCl, 10 mM dithiothreitol, and 1.6 mM SAM. *Hae*III MTase enzyme stock solution (10 U/mL) was added to DNA solution and the reaction mixture was incubated at 37 °C. Then, 10 μL of reaction mixture was mixed with 30 μL of *Hae*III buffer containing 20 U of *Hae*III at 10 min intervals and incubated at room temperature for 10 min. Finally, fluorescence spectra were obtained after mixing reaction mixtures and GO solutions to make the final GO concentration 10 μg/mL (Figure 3e). In the enzyme-concentration-dependent experiment, substrate DNA was incubated with various concentrations of *Hae*III MTase, followed by treatment with *Hae*III and GO (Figure 3f). In both the time- and enzyme-concentration-dependent assays, the relative amounts of product DNA were calculated successfully by measuring the fluorescence intensity at 520 nm.

We next performed inhibition assays of *Eco*RV, *Hae*III, and *Hae*III MTase using known inhibitors to confirm that the present method was quantitative and applicable to the evaluation of inhibitors. Ethylenediaminetetraacetic acid (EDTA) and aurintricarboxylic acid (ATA) were used as *Hae*III inhibitors.[30,31] *Hae*III (10 U) was added to substrate DNA solution in the presence of various concentrations of the inhibitors, and incubated at room temperature for 50 min. Fluorescence spectra were obtained after mixing reaction mixtures with GO solution. Dose-dependent inhibition curves were plotted on the basis of fluorescence intensities. The IC_{50} values of EDTA and ATA were estimated to be 1.48 mM and 3.71 μM, respectively (Figure 4a,b). Similarly, ATA

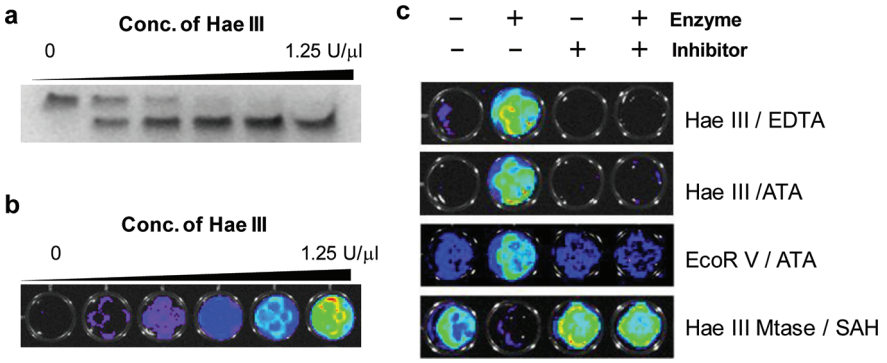

Figure 5. Comparison between the conventional gel-based assay method and the present GO-based method. (a) Gel electrophoresis was performed on a 15% polyacrylamide gel after loading samples containing substrate DNA and varying concentrations of *Hae*III. (b) Fluorometric assay based on GO was performed by obtaining a fluorescence image of the reaction mixtures in a 96-well plate. (c) Fluorescent images of reaction mixtures for the inhibition assay of ENases and MTase: row 1, inhibition of *Hae*III activity by EDTA; row 2, inhibition of *Hae*III activity by ATA; row 3, inhibition of *Eco*RV activity by ATA; row 4, inhibition of *Hae*III MTase activity by SAH.

and *S*-adenosylhomocysteine (SAH) were used as inhibitors for *Eco*RV and *Hae*III MTase, respectively.[31,32] Dose-dependent inhibition curves were plotted on the basis of fluorescence intensities, and the IC_{50} values for ATA inhibition of *Eco*RV and SAH inhibition of *Hae*III MTase were determined to be 3.6 μM and 10.1 mM, respectively (Figure 4c,d).

Next, we conducted an experiment to compare a conventional method (gel electrophoresis) with the present GO-based method. *Hae*III concentration-dependent activity was measured using both gel electrophoresis and the GO-based fluorometric method, visualized by fluorescence imaging. In a typical method, the cleaved DNA and intact DNA are resolved using urea−polyacrylamide gel electrophoresis (urea−PAGE). *Hae*III concentration-dependent DNA cleavage was observed in both experiments (Figure 5a, b). In our GO-based assay, addition of GO led to the quenching of nuclease activity and also induced differences in fluorescence intensities among mixed solutions containing different nuclease activities. In contrast, the conventional gel-based assay requires two steps involving the quenching of enzymatic activities by adding EDTA and, subsequently, running a gel to resolve the longer substrate DNA and short cleaved DNA.

Finally, we demonstrated the feasibility of conducting parallel assays using our GO-based platform by observing fluorescence images of multiple reaction mixtures. The samples were prepared by mixing the *Eco*RV or *Hae*III substrate DNA solutions and the corresponding enzymes with or without inhibitors in a 96-well plate. As expected, with both ENases, the enzyme-treated samples without the addition of any inhibitor showed the highest fluorescence, compared with the other samples. The *Hae*III MTase-treated samples without SAH, an inhibitor, displayed the lowest fluorescence, whereas other samples in which unmethylated DNAs were cleaved by *Hae*III showed high fluorescence in the same row (Figure 5c). It was clear that our GO-based ENase activity assay method was more compatible with parallel enzyme assay formats, such as inhibitor screening, than the conventional gel-based assay.

CONCLUSIONS

In conclusion, we have developed a new GO-based platform for ENase and MTase activity assays. In the present assay platform, GO plays two roles, stopping enzyme reactions and quenching the fluorescence of a dye that is attached to the substrate DNA. GO may inhibit enzyme activities due to its interaction with enzymes,[33] which can induce the loss of enzyme activities. However, it is unclear how GO inhibits enzyme activity at this time. Each substrate DNA was designed to possess a GO-binding part, consisting of a long single-stranded DNA region. That is, the long single-stranded DNA part was used to immobilize the double-stranded DNA part onto the GO. The present platform for restriction enzyme activity assays has important advantages over conventional methods. First, the GO makes the present method more cost- and time-efficient, because unlike conventional assays, expensive reagents, additional quenchers, and isotopes are not required. GO can be prepared from the oxidation of graphite at very low cost. Second, the present strategy showed technically new application of GO for enzyme assays by using the newly designed substrate DNA possessing both single and double stranded parts and by demonstrating sequential enzyme activity assay of two enzymes, ENase and MTase. Third, this method is also technically straightforward and compatible with parallel assay formats. Many samples can be prepared, and the quantitative analysis of their activity can be carried out by simply obtaining a fluorescence image of the samples. Finally, this platform, based on GO, is versatile and can be applied to other ENase and MTase assays by changing the recognition/methylation site in the double-stranded region of the substrate. We believe that this new GO-based ENase/MTase assay platform will become an important assay tool in ENase-related basic research and drug development.

ASSOCIATED CONTENT

Supporting Information. Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Tel: +82 2 880 4338. Fax: +82 2 889 1568. E-mail: dalheemin@snu.ac.kr.

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