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An Electrochemical Assay for Restriction Endonuclease Activity Using Graphene Monolayer

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Herein, we report a new electrochemical assay for the endonuclease activity using a graphene monolayer. The approach involves a methylene blue (MB) tagged double-stranded (ds) DNA as an enzyme substrate with a single-stranded (ss) segment at the end for anchoring on graphene through π-π stacking. The nuclease enzyme mediates DNA hydrolysis following the release of dsDNA-MB from the graphene surface, which reduces the electrochemical current signal. The protocol was validated for the HaeIII and EcoRV restriction endonuclease enzyme. We also assayed the activity of methyltransferase enzyme and inhibition of restriction by HaeIII. The method allows real-time measurement and quantitative assay for endonuclease activities in a simple manner.
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Restriction endonucleases (ENases) are one of the most important groups of enzymes that hydrolyze the phosphodiester bonds in DNA or RNA at a specific nucleotide sequence. ENases play crucial roles during cellular events and physiological processes, such as gene expression, replication, DNA repair, recombination of nucleic acid, gene cloning and DNA mapping; the assays of ENases are essential in molecular biology, and drug discovery.[1,2] Conventional methods for assaying activity involve gel electrophoresis, high-performance liquid chromatography (HPLC), filter binding, radioactive labeling, and immunoaffinity.[3–6] Regardless of great success for enzyme analysis, most of these traditional assay platforms are however, time-consuming, laborious, discontinuous and costly.

Several alternative approaches using fluorescent or colorimetric methods have been developed for simple, continuous real-time assay for ENase activity, but these require rather bulky, fragile, and costly instruments.[7–9] Recently, Lee et al. reported a similar assay method utilizing the preferential interaction of graphene oxide surface with ssDNA.[10] Their approach suggests several advantages over conventional methods, but still has typical limitations relied on fluorescent detection.

Electrochemical based biosensors have shown much advancement in the detection and analysis of biomolecules. These biosensors offer highly sensitive and selective detection schemes with more robust, less expensive, and easy to miniaturize non-optical approach.[11] Additionally, graphene has also emerged as a material of interest for use in biosensor because it possesses conductive yet transparent property with cost effectiveness and biocompatibility.[12]

Graphene is a two-dimensional monolayer of sp^2-hybridized carbon atoms into a dense honeycomb crystal structure.[13] The electrons in the un-hybridized $2p_z$ orbitals, which are responsible for the optical and electronic properties of graphene, delocalize along the plane of the graphene surface to form π (bonding) and π* (anti-bonding) bonds.[14] Because of its remarkable properties such as electrical and biocompatibility based on carbon composite has been regarded as a promising material in the chemical and biological sensors. After single-layer graphene was separated from graphite, much progress has been made in developing graphene devices in various types of sensors, in particular chemical and biological sensors because it is chemically robust and bio-inert. Above all, graphene is extremely sensitive to electric fields and charges around it, which makes it a suitable material for sensing application.[15]

Herein, we demonstrate a method of electrochemical assay for the ENases activity with graphene monolayer as an electrode and substrate for immobilization. The redox tagged DNA complex composed of ssDNA and dsDNA was deposited on the graphene. Recently, Lee et al. demonstrated an electrochemical assay for methyltransferase activity.[16] Their method includes modification of glassy carbon electrode with gold nanoparticle and deposition of graphene dioxide/ thionine functionalized DNA using gold-thiol chemistry. Though they succeeded in selectively detecting the enzyme activity, multiple labeling of electrode and DNA makes the assay laborious and expensive. However, our strategy is based on the fact that ssDNA adsorbs preferentially over dsDNA by the π-π staking interaction between the hexagonal structure of graphene and aromatic ring structure of exposed nucleobases. In contrast to that, dsDNA is unable to adsorb onto the graphene surface as the nucleobases are occupied inward the double helix.[17] The methylene blue tagged to the DNA gave the higher electrochemical signal in its native form. The signal reduced drastically after the reporter was cut-off from the DNA, due to the restriction enzyme's activity. A difference in the signal was used to analyse the ENases activity. Later the work was continued to detect the activity of methyltransferase enzyme of protecting the DNA from HaeIII enzyme. Finally, we also studied the inhibition of HaeIII using ethylenediamine tetraacetic acid. The inhibition assay can be useful in designing new pharmaceutical drug against the enzyme.

Methods and Materials

Reagents and samples.— Upper ssDNA substrate for EcoRV, HaeIII and HaeIII Methyltrasferase; and methylene blue-labeled bottom ssDNA substrate for EcoRV, HaeIII and HaeIII Methyltrasferase were purchased from Bioneer (Korea). HaeIII and EcoRV were purchased from Enzynomics (Daejon, Korea). HaeIII methyltransferase was purchased from New England BioLabs (MA, UK). Ethylenediamine tetraacetic acid (EDTA) was purchased from Bio-Rad (UK). Other chemicals and reagents were of analytical grade and purchased from Sigma-Aldrich, Korea.

Preparation of graphene electrode.— Monolayer graphene was synthesized by chemical vapor deposition (CVD) on a rectangular piece of Cu foil (25 μm thick) using the procedure described elsewhere.[18] A "wet transfer" process was adopted to transfer the graphene on Au coated glass substrate (Fig. S1).[19] Firstly, a PMMA layer was spin-coated onto the graphene/copper foils followed by chemical etching of Cu layer using 0.1 M ammonium per sulfate solution for 3 hours until Cu is no longer visible. The PMMA/graphene film was then dipped in the ultrapure water to get rid of etchant residue. The floating PMMA/graphene layer was then slowly drawn onto the Au coated glass substrate with a needle on the top and putting the glass substrate underneath the water tilted in 30° angle. Note that an edge of the rectangular graphene should touch the Au electrode. PMMA/graphene on the glass substrate was then blown dry with N_2 and baked in the air on a hot plate at 220°C for 5 min. Once the sample cooled down, PMMA was stripped using acetone at 25°C. Lastly, this working electrode was deposited with 100 nM dsDNA-MB complex,

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and after washing several times, it was completely soaked in 2 mL enzyme reaction mixture (pH 7.9, 10 mM Tris-HCl) which contained various amounts of ENases. After the hydrolysis, the electrode surface was rinsed with ultrapure water to remove the leftover reaction components.

Electrochemical method.— Differential pulse voltammetry (DPV) measurements were performed with a CHI 830b electrochemical analyzer (CH Instruments, Inc. USA). The parameters applied were: scanning range: 0.0 — −0.5 V, amplitude: 0.05 V, pulse width: 0.2s, and pulse time: 0.5s. All measurements were conducted at room temperature (25°C) with graphene modified electrode as working, Ag/AgCl as a reference electrode and Pt as counter electrode in 0.1 M PBS buffer solution (pH 7.4) unless otherwise stated.

Methyltransferase activity.— First, the reaction mixtures were prepared in 1X MTase reaction buffer (pH 8.5, 50 mM Tris-HCl) containing the 100 nM dsDNA-MB complex, 1.6 mM S-adenosyl-l-methionine (SAM) and various amounts of HaeIII MTase. After incubating at 37°C for 30 min, reaction mixtures were mixed with HaeIII buffer containing 0.3 units of HaeIII and again incubated at room temperature for 20 min. Finally, the electrodes were soaked in the reaction mixtures, and after several washing steps, DPV was carried out in 0.1 M phosphate buffer (pH 7.4).

Results and Discussion

As shown in Fig. 1a, we prepared a graphene monolayer as a working electrode and substrate for redox-tagged DNA. The synthesized graphene was characterized using Raman spectroscopy and is discussed in supporting information (Fig. S3). The DNA substrate consists of a single-stranded region for binding to the graphene surface and a double-stranded region for the sequence specific recognition for the ENase catalysis. When reduction potential is applied to the graphene surface, the redox tag conjugated at the end of dsDNA part efficiently accepts an electron from graphene surface, which results in high reduction current in the voltammetry. In the presence of ENase, however, the tag is detached from the graphene because of enzymatic hydrolysis at the dsDNA region, exhibiting attenuated current signal. Our electrochemical approach is illustrated in Fig. 1b. The assay was performed with the differential pulse voltammetry with the manner of time-dependent (or concentration-dependent) approach, and it successfully exhibited the change in the current signal originated from the enzyme activity.

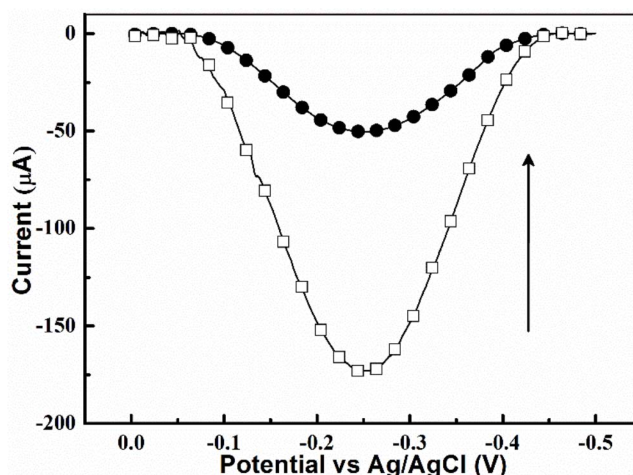

Figure 2. Differential pulse voltammogram from the MB tagged DNA substrate using graphene electrode when HaeIII is absent (□) or present (●).

HaeIII, one of the representative type-II ENases was employed to evaluate our method. The DNA substrate was composed of a long upper strand (5′-CTA GCT ATG TGC CGA ATT TCA AGG ACA GTT GTA TGG CCC TCG T-3′) with methylene blue (MB)-tagged short bottom strand (5′-MB-ACG AGG GCC ATA-3′). The enzyme recognizes the palindromic 4-base DNA sequence 5’-GGCC-3’ of the bottom strand and makes a cut between G and C (Fig. S2). In Fig. 2 of preliminary experiments, strong reduction peak current appeared at −0.25 V (vs Ag/AgCl) owing to the electrochemical reduction of MB at the end of dsDNA. However, drastically decreased signal, reaching to 30% of its original value, was obtained when the excess amount of HaeIII was present. The DNA substrates were successfully hydrolysed by the enzyme catalysis following to desorption of MB-tagged DNA cut from the graphene surface. In Fig. 3a, enzyme-concentration-dependent assay was performed by collecting the peak current level in various concentrations of HaeIII after 20 min incubation. As expected, the higher amount of the DNA substrates was cleaved with the higher concentration of HaeIII. The current signal gradually decreased, which suggested that the hydrolysis became saturated around 0.6 units/μL

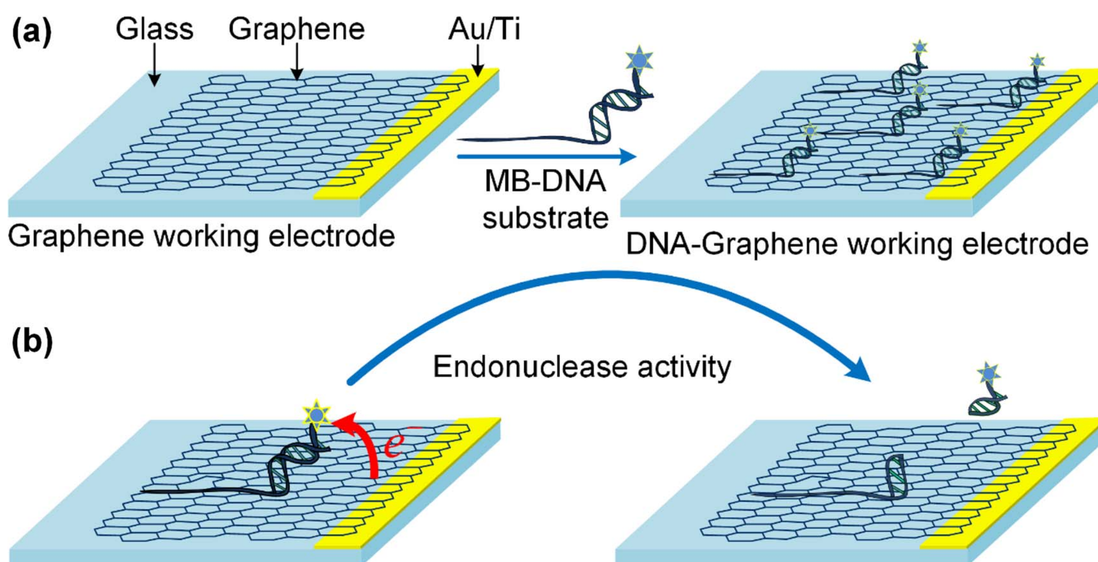

Figure 1. (a) Fabrication of MB tagged DNA- graphene working electrode and (b) Schematic of the electrochemical assay for ENase activity.

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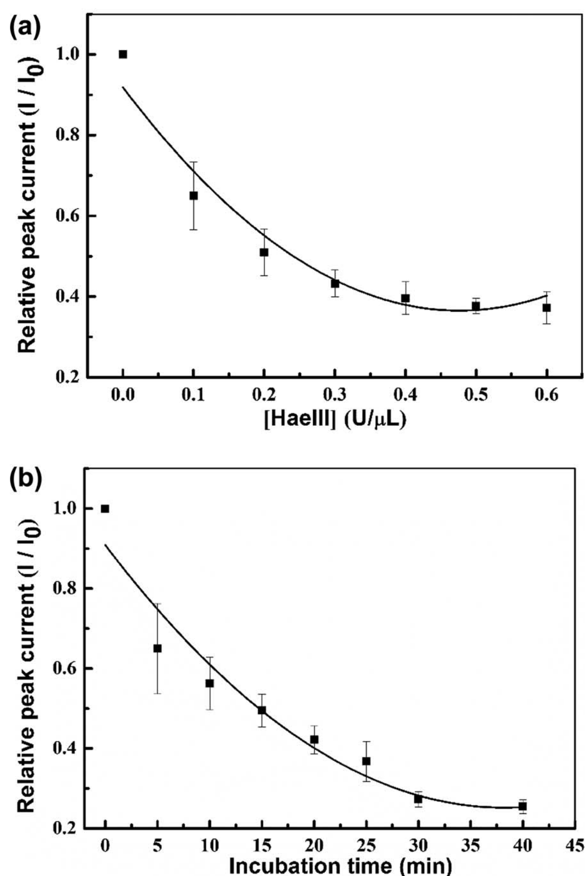

Figure 3. Differential pulse voltammogram from the MB tagged DNA substrate using graphene electrode in presence of HaeIII; (a) enzyme-concentration-dependent and (b) time-dependent peak current (–0.25 V vs Ag/AgCl).

of HaeIII. We found that 0.3 units/μL was appropriate to investigate the time-dependent assay of HaeIII, ranging from 0 to 40 minutes (Fig. 3b).

EcoRV, another representative of a type-II ENases, was additionally testified in a similar condition (Fig. 4). The DNA substrate containing a hydrolytic sequence specific to EcoRV (Fig. S2) was employed, and the enzyme cuts off the short strand from the DNA substrate following the attenuation of the reductive current. The signal decreased with the excessive amount of EcoRV reaching down to about 15% value. The concentration-dependent and time- dependent assays showed almost the similar characteristics to those from HaeIII (Fig. 5a and 5b).

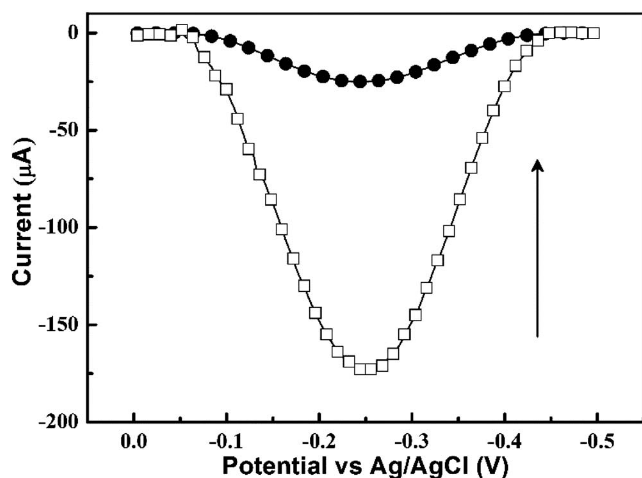

Figure 4. Differential pulse voltammogram from the MB tagged DNA substrate using graphene electrode when EcoRV is absent (□) or present (●).

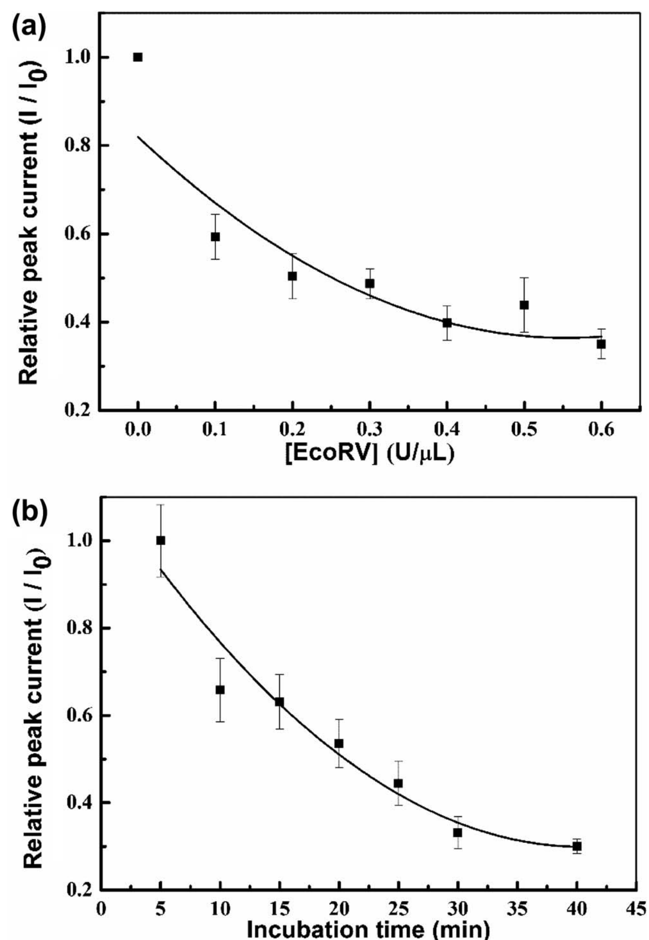

Figure 5. Differential pulse voltammogram from the MB tagged DNA substrate using graphene electrode in presence of EcoRV; (a) enzyme-concentration-dependent and (b) time-dependent peak current (–0.25 V vs Ag/AgCl).

The approach was further employed in a DNA methyltransferase (MTase) activity assay in which the DNA strands methylated by MTases cannot be hydrolysed by ENases. HaeIII and its DNA MTase (HaeIII MTase) were chosen for the assay. Since HaeIII MTase catalyzes the transfer of a methyl group from S-adenosyl-L-methionine to a cytosine residue at the recognition site of the substrate DNA, the methylated DNA substrate was expected to be resistant to HaeIII -mediated hydrolysis. In Fig. 6a, the peak current increased with the amount of MTase (inset), which is in accordance with the fact that the higher the amount of MTase, the more DNA substrates methylated. The signal increased linearly with the amount of MTase from 0.1 to 0.4 units/μL, and then reached its plateau. This means most of the DNA substrates are methylated at a high concentration of MTase and subsequently cannot be cleaved by HaeIII.

Next, we performed inhibition assay of HaeIII using a known inhibitor to confirm that the method was quantitative and applicable to the evaluation of inhibitors. Ethylenediamine tetraacetic acid (EDTA) was employed as a HaeIII inhibitor.[20] The graphene electrode deposited with the DNA substrates was soaked in the mixed solution of HaeIII and EDTA following the incubation for 50 min. A dose-dependent inhibition curve from the DPV measurements was plotted on the basis of the peak current level at around –0.25 V. As per the

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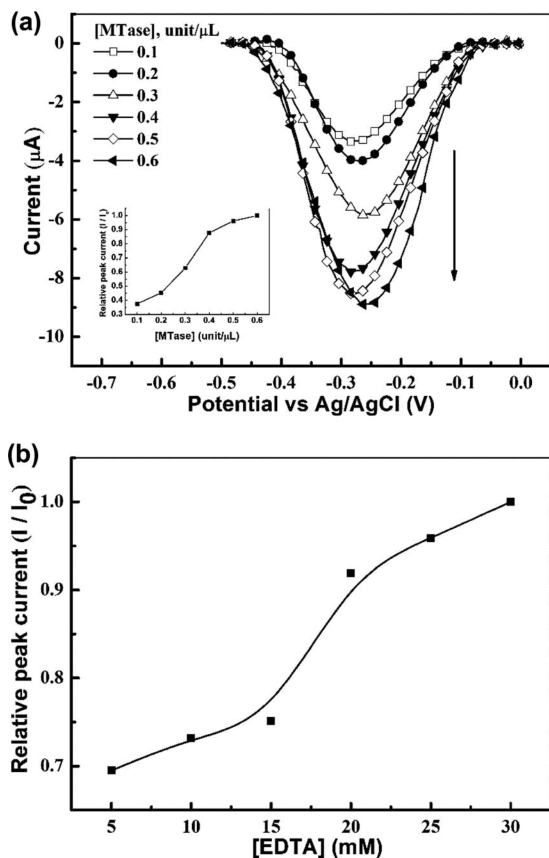

Figure 6. (a) Differential pulse voltammogram of the DNA substrate using graphene electrode with 0.3 U of HaeIII upon the addition of MTase in the reaction mixture (inset: the changes in the peak current at –0.25 V), and (b) dose-dependent inhibition of HaeIII by EDTA.

graph obtained, about 20 mM of EDTA inhibited more than 50% of the enzyme activity (Fig. 6b).

Conclusions

A new graphene-based electrochemical method for ENase and MTase activity assays has been developed. The approach allows cost and time-efficient assay for restriction enzyme activity. It is also technically straightforward and compatible with parallel assay formats, and versatile for other ENase or MTase assays by changing the recognition/methylation site in the double-stranded region of the substrate. We expect that our approach will suggest an alternative assay tool in ENase-related basic research or drug development.

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