Real-time Fluorescence Assay of DNA Polymerase Using a Graphene Oxide Platform

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Introduction

Graphene is an atom-thick and two-dimensional carbon material with high surface area, excellent electrical conductivity, biocompatibility and wide-ranged functionality [2, 4, 12, 16, 20]. Graphene has attracted the strong interest in the development of the biological platforms [5, 7, 9], biosensors [11, 21, 23], and biodevice [10]. Graphene has been utilized as the fluorescence resonance energy transfer (FRET) biosensors with the sensing application ranged from small molecules to DNA and proteins [17]. The first graphene-based FRET biosensor showed that a fluorescein-amidt (FAM)-labeled single strand DNA (ssDNA) was adsorbed onto GO and fluorescence was rapidly quenched [9]. In addition, fluorescence intensity was restored in the presence of complementary HIV1 DNA [9]. Fluorescence was quenched rapidly for FAM attached ssDNA in the presence of GO, but FAM attached dsDNA emitted strong fluorescence regardless of GO. Graphene-based FRET platform has been developed for the detection of duplex DNA unwinding by helicase [7]. As FAM attached dsDNA unwinding proceeded by helicase, the fluorescence was quenched and then helicase activity was monitored in real time. GO-based molecular beacon (MB) enhanced S/B (signal-to-background) ratio and sensitivity for the detection of complementary sequence of DNA in comparison with MB alone [8, 13]. It was also demonstrated that MB differentiated wild-type and single base mismatched duplex DNA molecules in the presence of GO.

In addition, FAM-labeled double stranded DNA (ds DNA) bound to GO and ds DNA-GO complex was accessible to DNase I, resulting in restoration of fluorescence emission from the fluorescence quenching [24]. Human thrombin protein bound specifically to human thrombin aptamer DNA which was adsorbed onto GO, resulting in the release of aptamer DNA from GO [9]. It suggests that ssDNA and dsDNA are adsorbed to GO depending on reaction condition and enzymes functions to synthesize second strand DNA of adsorbed DNA. Recently, GO-based Klenow fragment activity was measure by using the different adsorption property of GO between ssDNA and dsDNA [22]. The reported method was discontinuous because enzyme activity could be monitored at the specific time by the addition of GO to reaction. In addition, the method could not

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measure the continuous process of DNA adsorption and enzyme activity in a tube.

Using the different adsorption properties of ssDNA and dsDNA to GO, this study used real time and efficient fluorescence assay to detect enzymatic activity of Klenow fragment with the adsorbed probe DNA to GO in the presence of primer. It was known that Klenow fragment plays essential roles in DNA manipulation (making blunt end [1], DNA mutagenesis [19], cDNA second strand synthesis [6]), preparation of single stranded DNA probe for in situ hybridization [3], and DNA sequencing [15], etc. Klenow fragment is extensively used in a variety of biological and clinical studies. Thus, sensitive, selective, and efficient method is required to detect DNA polymerization activity of Klenow fragment.

Materials and Methods

Materials

GO (0.3~0.7 μm, 80% single layer) was purchased from graphene supermarket (USA). DNA polymerase (Klenow fragment, 5 units/μl) was obtained from Beams biotech (Korea) and oligomers were synthesized and purified by PAGE (Sigma-aldrich). All chemicals were of analytical grade.

Fluorescence emission measurement

All the oligomers used in this study (shown in Table 1) were fluorescein-labeled at 5’ end. Reactions were performed in Klenow fragment buffer (10 mM Tris, pH 7.5, 5 mM MgCl₂, 7.5 mM DTT, 0.2 mM dNTP) at room temperature (~20°C). Each reaction was conducted with 0.01 mg/ml of GO and 30 nM of fluorescein-labeled ssDNA 1. In the hybridization assay, fluorescence emission was measured at regular intervals for an hour after the addition of ssDNA 1 and GO to reaction. And ssDNA 1-GO complex was hybridized with the different concentration of complementary ssDNA 2 of 30, 60, 90, and 150 nM. Fluorescence intensity was measured with RF 5301PC (Shimadzu) with excitation at 485 nm and emission range from 502 to 600 nm. To release ssDNA 1-ssDNA 2 DNA from the surface of GO, fivefold excess of ssDNA 3 was added to reaction and checked in succession the fluorescence intensity for an hour. In DNA polymerization reaction, GO was incubated with ssDNA 1 in reaction buffer containing dNTP (200 μM). Fluorescence emission was monitored for 10 min and then fivefold excess of ssDNA 3 was added to ssDNA 1-GO complex. Reaction mixture was further incubated for 50 min. To initiate DNA polymerization reaction, Klenow fragment (0.12~0.34 unit/μl) was added to reaction mixture and fluorescence intensity was measured regularly for an hour.

Gel electrophoresis of GO-based duplex DNA formation

As mentioned in hybridization and polymerization reaction, reactions were conducted at room temperature (~20°C) in reaction buffer containing 300 nM ssDNA 1 and 0.1 mg/ml GO. After finishing reactions, gel loading buffer was added and incubated at 65°C for 5 min prior to loading on 12% native polyacrylamide gel. Gel was run at 80 volt for 40 min and dried for visualization with Kodak Image scanner at excitation of 470 nm and emission of 530 nm.

Results and Discussion

Adsorption efficiency of fluorescent probe to GO

Fig. 1 shows a schematic representation of sequence specific hybridization and polymerization reaction in the presence of GO. As reported in early studies [5, 9], dye-tagged ssDNA adsorbed strongly to the surface of GO through the noncovalent interaction between GO and ssDNA, resulting in the fluorescence quenching with high efficiency. Schematic representation in Fig. 1 is designed to show not only sequence specific hybridization between

Table 1. Sequences of the oligonucleotides and complementary DNA used.

<table>
<thead>
<tr>
<th>Type</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flc-labeled</td>
<td>Flc-CAT GCC TGC AGG TGG ACT CTC</td>
</tr>
<tr>
<td>ssDNA(1)</td>
<td>GAG CCC CGG GTA CCG AAA AAA AAA A-3'</td>
</tr>
<tr>
<td>ssDNA(2)</td>
<td>5'-CCG TAC CGG GGG ATC CTC GAG AGTC</td>
</tr>
<tr>
<td></td>
<td>CAC CTG CAG GCA TG-3'</td>
</tr>
<tr>
<td>ssDNA(3)</td>
<td>5'-TTT TTT TTT T-3'</td>
</tr>
</tbody>
</table>

Flc means Fluorescein