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

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Using the different adsorption properties of ssDNA and dsDNA to GO, this study used a real time and efficient fluorescence assay to detect the enzymatic activity of the Klenow fragment with the adsorbed DNA to GO. Results showed that adsorption of fluorescein-tagged ssDNA to GO resulted in fluorescence quenching and DNA was released from GO by adding complementary DNA. In addition, fluorescence restoration was increased through a polymerization reaction by the Klenow fragment in the presence of a fluorescein-attached template, GO, and primer. Gel electrophoresis was conducted to confirm the hybridization and DNA polymerization reactions on GO.

Keywords: Graphene oxide, platform, biosensor, Klenow fragment

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 $MgCl_2$, 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.

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

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

Flc means Fluorescein

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

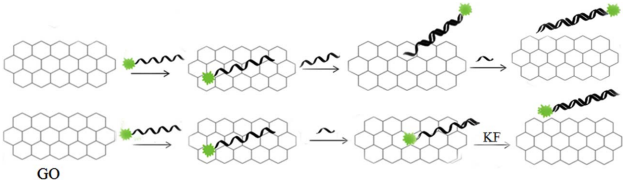

Fig. 1. Schematic diagrams of hybridization of ssDNA 1 with complementary DNA (ssDNA 2) (top) and DNA polymerization reaction of ssDNA 1 by Klenow fragment in the presence of ssDNA 3 (bottom).

ssDNA and ssDNA 1 but also DNA polymerization reaction by Klenow fragment in the presence of ssDNA 1and ssDNA 3 on GO. As seen in Fig. 2, ssDNA 1 itself showed strong fluorescence emission due to the fluorescein-attached DNA (curve a). By adding GO to ssDNA 1 solution, fluorescence intensity was quenched up to 98.9% (curve b) in an hour. Li *et al*. reported that fluorescence was quenched with 98% efficiency with MB probe in the presence of GO [24]. In case of ssDNA, fluorescence emission was quenched up to 97% with FAM-labeled 23 bases ssDNA in the presence of GO [9]. It indicated that ssDNA 1 molecules were stably and strongly adsorbed to GO surface due to the hydrophobic and π-stacking interaction between the bases of ssDNA and the hexagonal rings of GO.

Detection sensitivity for duplex DNA formation

According to the previous studies [5, 9], ssDNA was adsorbed to GO with high efficiency, but dsDNA was not adsorbed due to lack of the exposed bases of DNA. The specific binding of complementary DNA to probe results in the release of duplex DNA from GO. It means that the fluorescence intensity is increased in proportion to the extent of duplex DNA formation. For the gradient addition of ssDNA 2 to ssDNA 1-GO mixture (curves b, c, d, and e in Fig. 2), the fluorescence emissions were increased depending on the concentration of ssDNA 2 (1~5 times). As it was expected, duplex DNA was formed gradually by the increased addition of ssDNA 2. Fluorescence was restored to 53.8% in fivefold excess of ssDNA 2 (curve e) in comparison with ssDNA 1 alone (curve f).

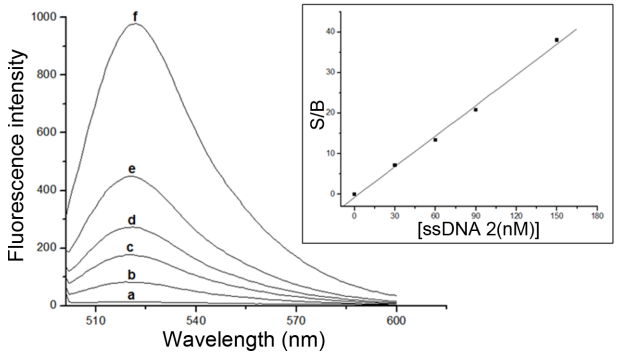

Fig. 2. Fluorescence emission spectra of ssDNA 1 in the presence of different ssDNA 2 concentrations.
Curve a shows the fluorescence emission of ssDNA 1 in the presence of GO. Curves b, c, d, and e represent the fluorescence emissions in the presence of one, two, three, and fivefold excess of ssDNA 2, respectively. Curve f is fluorescence intensity of ssDNA 1 only. Inset: S/B ratio plotted against the different concentrations of ssDNA 2.

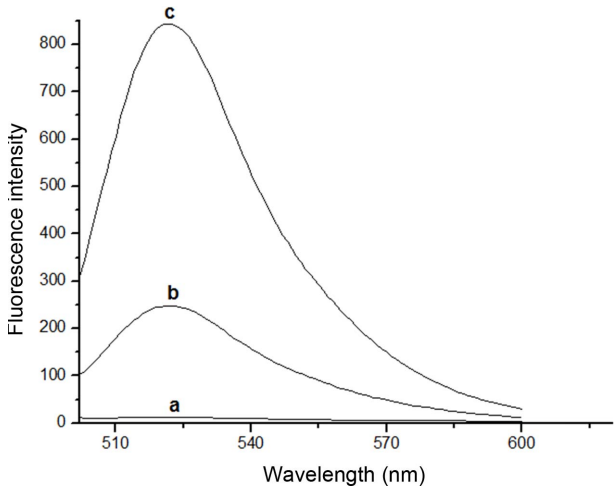

Fig. 3. Change of fluorescence emission of ssDNA 1 by DNA polymerization with Klenow fragment.
Curve a contains ssDNA 1 in the presence of GO. Curve b retains ssDNA 1 and GO in the presence of ssDNA 3 and Klenow fragment (0.34 units/μl). Curve c has ssDNA 1 only.

In addition to hybridization, DNA polymerization was conducted with Klenow fragment in the presence of ssDNA 1, ssDNA 3, and GO (Fig. 3). The quenched fluorescence was restored about 30% in comparison with that of ssDNA 1 alone. As shown in publications [9, 13, 24], fluorescence restoration was monitored by duplex DNA formation via second strand synthesis of ssDNA 1 by Klenow fragment. Thus, duplex DNA was released from GO due to lack of the interaction between nucleobases of DNA and hexagonal rings of GO.

To confirm GO-based detection sensitivity of molecules, it is important to enhance the significant restoration of fluorescence in the presence of molecules [13, 24]. As shown above, fluorescence intensity of ssDNA 1 was strongly restored by adding fivefold excess of ssDNA 2. It was further investigated with S/B (signal to background) ratio to estimate the restoration efficiency of fluorescence at different concentration of ssDNA. S/B ratio was defined as $(F_{ssDNA\text{-}ssDNA\ 1\text{-}GO} - F_{buffer}) / (F_{ssDNA\ 1\text{-}GO} - F_{buffer})$, where $F_{ssDNA\text{-}ssDNA\ 1\text{-}GO}$, $F_{ssDNA\ 1\text{-}GO}$, and F_{buffer} represent the fluorescence intensities for the hybridization of ssDNA to ssDNA 1-GO complex, adsorption of ssDNA 1 to GO, and reaction buffer, respectively.

S/B ratio was plotted against the concentrations of ssDNA 2 in the presence of ssDNA 1-GO complex (Inset of Fig. 2). S/B ratio was linearly proportional to the concentra-

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tion of ssDNA 2. S/B ratio values were 17.8 and 37.7 in twofold and fivefold excess of ssDNA 2, respectively. Based on Fig. 3, S/B ratio was 22.1 for the polymerization reaction with Klenow fragment (0.34 units/μl). In addition, S/B ratio was increased by the raise of enzyme concentration (Fig. 5B). It was because there was no change of $F_{ssDNA\ 1\text{-}GO}$ and F_{buffer} except the increase of fluorescence restoration as a result of the polymerization reaction at the increased concentration of enzyme.

This study showed that Klenow fragment synthesized the second strand DNA of adsorbed ssDNA in the presence of primer. However, it was reported that GO and carbon nanotube protected the adsorbed DNA from digesting with DNase I, because resistance to enzymatic cleavage could be a steric hindrance that prevent DNase I from binding to the MB and ssDNA [14, 18, 24]. In contrast, human thrombin proteins recognized specifically human thrombin aptamer DNA which was adsorbed onto GO[9]. In addition, DNase I digested FAM-labeled double stranded DNA (ds DNA) which was adsorbed to GO, resulting in the restoration of fluorescence emission from the quenched fluorescence [24]. It suggests that adsorbed DNA is not completely protected from enzymatic digestion and DNA polymerization.

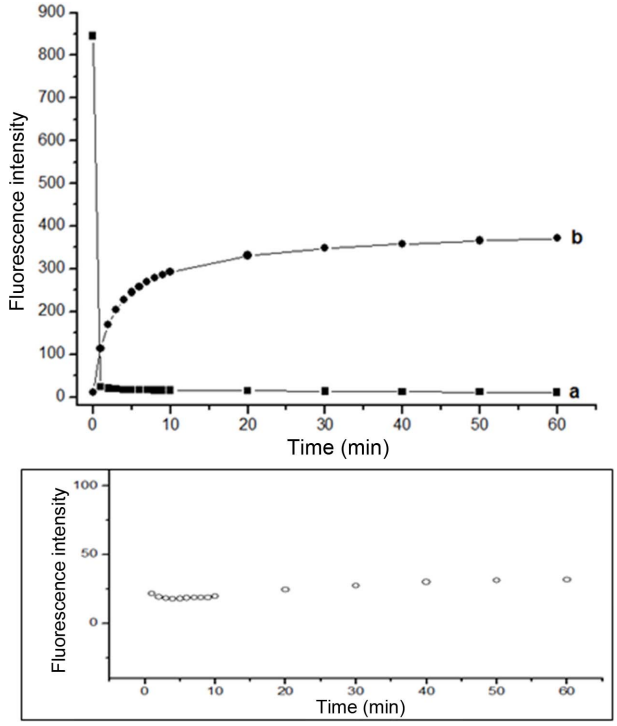

Fig. 4. Fluorescence quenching of ssDNA 1 (30 nM) by GO as a function of time (curve a) and fluorescence restoration of ssDNA 1-GO with fivefold excess of ssDNA 2 (curve b). The lower graph shows change in fluorescence emission by the addition of ssDNA 3 to ssDNA 1-ssDNA 2 complex.

Kinetics of duplex DNA formation

To study the kinetic behaviors of adsorption and desorption between DNA and GO, fluorescence emission was measured as a function of incubation time. As seen in curve a (Fig. 4), the fluorescence quenching of ssDNA 1 was 98.3% at 5 min in the presence of GO. It indicated that adsorption of ssDNA 1 reached to equilibrium almost within 5min at room temperature (~20°C). The duplex DNA of ssDNA 1-ssDNA 2 was formed to 43.9% (curve b in Fig. 4) in the presence of fivefold excess of ssDNA 2. It showed that duplex DNA was not formed fast enough to compare

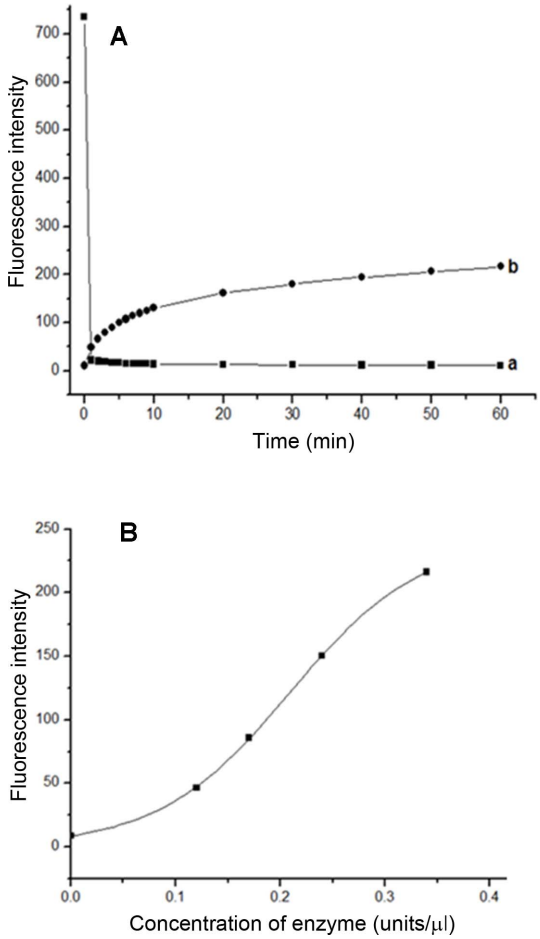

Fig. 5. (A) Fluorescence quenching of ssDNA 1 (30 nM) by GO as a function of time (curve a) and fluorescence restoration of ssDNA 1-GO by DNA polymerization with Klenow fragment (0.34 units/μl) as a function of time (curve b). (B) Fluorescence enhancement of DNA polymerization reaction at different concentration of Klenow fragment.

with the adsorption of ssDNA 1 to GO. At the increase of ssDNA 2 from twofold to fivefold excess, duplex DNA formation was increased by 26.2% (data not shown).

In addition, the low plot of Fig. 4 represented change of fluorescence emission by adding ssDNA 3 to ssDNA 1-ssDNA 2 –GO complex. Addition of ssDNA 3 leaded to the complete duplex DNA formation by hybridization of ssDNA 3 to ssDNA 1-ssDNA 2, resulting in the release of duplex DNA from GO. It indicated that ssDNA 1-ssDNA 2-ssDNA 3 resulted in small change of fluorescence restoration in comparison with that of duplex DNA with single stranded region. It was demonstrated that fluorescence emission was fully restored by duplex DNA whether its end was adsorbed or released from GO by single stranded region.

According to recent publication [22], GO-based DNA polymerization was conducted with Klenow fragment to detect DNA polymerization activity. Polymerization was measured by using the different adsorption affinity between single stranded DNA and duplex DNA to GO. The reported method was discontinuous because each reaction was monitored at the specific time by the addition of GO. In addition, the reported method did not monitor the whole processes of DNA adsorption and enzymatic reaction in a tube.

Fig. 5 represented change of fluorescence emission for the adsorption of ssDNA 1 to GO and duplex DNA formation by Klenow fragment in the presence of ssDNA 3. Fluorescence emission of ssDNA 1 was quenched (curve a) due to the rapid adsorption of ssDNA 1 to GO but fluorescence emission was restored as a result of complementary DNA synthesis by Klenow fragment (curve b). Restoration of fluorescence emission was 31.3% by polymerization in an hour.

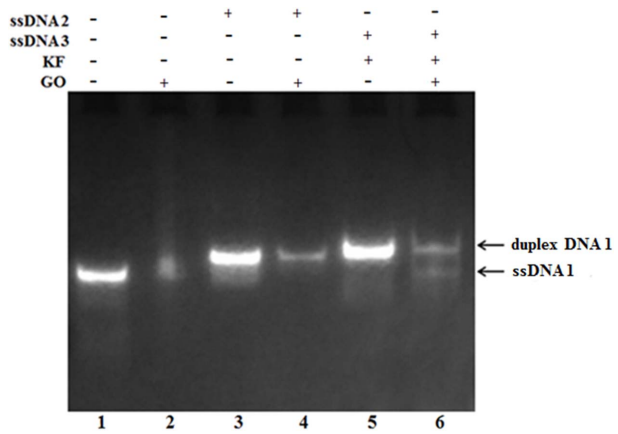

Fig. 6. Gel electrophoresis of hybridization and DNA polymerization reactions.
All lanes contain ssDNA 1 (300 nM) in reaction buffer. Fivefold excess of ssDNA 2 was added in lanes 3 and 4. Lane 5 and 6 retain KF (Klenow fragment) of 0.1 and 1.0 units/μl, respectively. + and – represent reactions with or without the indicated molecule, respectively. The ds ssDNA 1 and ss ssDNA 1 represent double stranded ssDNA 1 and single-stranded ssDNA 1, respectively.

Gel electrophoresis was conducted to confirm GO-based DNA polymerization reaction by Klenow fragment (Fig. 6). It showed that ssDNA 1 was hybridized with ssDNA 2 either in the absence (lane 3) or presence (lane 4) of GO. Second stranded DNA was synthesized in the presence of ssDNA 3 by Klenow fragment either in the absence (lane 5) or presence (lane 6) of GO. It showed that Klenow fragment functioned to synthesize complementary strand of DNA to form duplex DNA 1 adsorbed to GO.

Conclusions

In GO, fluorescein-tagged probe DNA was strongly adsorbed and fluorescence emission was quenched rapidly. However, adsorbed ssDNA 1 was released from GO in the presence of complementary ssDNA, leading to the fluorescence emission restoration. It showed that DNA hybridization was sensitive and sequence specific in the presence of GO. For the measurement of polymerization reaction, fluorescence restoration was increased as polymerization reaction was proceeded by Klenow fragment in the presence of fluorescein-attached probe, GO, and primer. Gel electrophoresis was conducted on 12% native polyacrylamide gel to confirm hybridization and DNA polymerization reactions in the presence of GO.

Acknowledgments

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국문초록

산화 그래핀 플랫폼을 이용한 DNA 중합효소의 실시간 형광에세이. *강종백*. 가천대학교 나노 화학과*

단일가닥 DNA와 이중가닥 DNA의 흡착 율의 차이를 이용하여, 본 연구는 산화 그래핀에 흡착된 단일 가닥 DNA을 사용하여 Klenow fragment의 효소 활성을 검출하기 위하여 실시간 형광에세이 방법을 사용했다. 실험 결과에 의하면, 산화그래핀에 흡착된 형광표지 ssDNA는 형광이 퀜칭(quenching) 되지만, cDNA 첨가에 의해서 흡착된 단일가닥 DNA가 유리되었다. Klenow fragment의 활성을 측정하기 위해서 형광표지 틀(template) DNA, 산화그래핀과 시발체(primer)가 존재할 때, 고분자 반응이 진행됨에 따라 퀜칭된 형광세기가 증가하였다. 그리고 겔 전기영동 실험은 산화 그래핀에서 DNA 합성과 hybridization 반응을 확인하였다.