Analytical Methods

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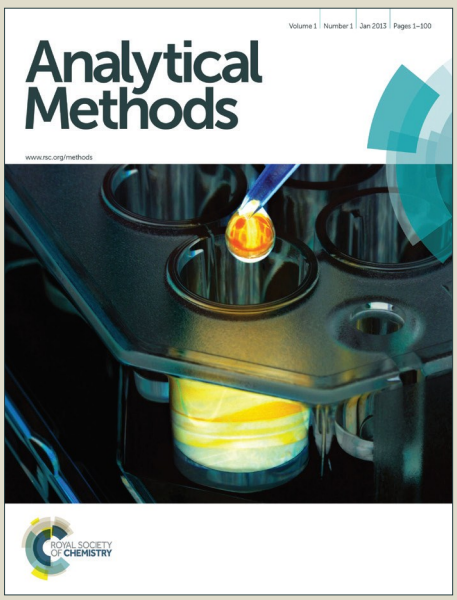

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PAPER

Label-free simple fluorescence assay of DNA polymerase using G-quadruplex structure

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In this study, the G-quadruplex structure was used to detect the enzymatic activity and inhibition of Klenow fragment exo$^-$ (KF-) in the presence of N-methyl mesoporphyrin IX (NMM) which binds specifically to the G-quadruplex. We found KF- synthesized the complementary strand of the template DNA, leading to a decrease in fluorescence intensity in the presence of NMM due to the difficulty of G-quadruplex formation. Thus, KF- activity was sensitively detected using NMM. In addition, daidzein, the major phytoestrogens in soy, inhibited DNA synthesis mediated by KF-, and the IC_{50} was determined to be 15.6 μM by nonlinear regression analysis.

Introduction

DNA polymerase plays an important role in various biological processes including DNA replication, DNA repair, genetic recombination, and reverse transcription.[1] The Klenow fragment exo$^-$ (KF-) has been utilized extensively in a variety of biological and biotechnological DNA manipulations such as DNA end blunting,[2] cDNA second-strand synthesis for RT-PCR,[3] DNA sequencing[4], and enzyme-assisted amplification.[5,6] In traditional methods, KF- activity is detected by incorporating radiolabeled nucleotides and gel electrophoresis.[7,8] However, recently KF activity was measured using the different adsorption properties of graphene oxide (GO) between single-stranded and double-stranded DNA.[9] In addition, KF- has been frequently exploited as an assisting enzyme for signal amplification assays to enhance the limit of detection of various enzymes and biomolecules.[5,6]

G-quadruplex structures are stacked arrays of G-quartets (four-stranded structure) formed at G-rich sequences through Hoogsteen hydrogen-bonding.[10,11] G-quadruplex structures are stabilized by specific binding to hemin molecules, forming a complex, called DNAzyme, which possesses peroxidase-like activity.[12,13] Fluorescent dyes specific for G-quadruplex enable utilization of this complex in sensitive detection assays of

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biomolecules and enzymes. As an enzyme with multiple uses, KF- requires simple and sensitive detection methods without laborious and time-consuming work. This study was designed to detect the enzymatic activity and inhibition of KF- by measuring fluorescence emission from a G-quadruplex structure bound to its specific dye, N-methyl mesoporphyrin IX (NMM).[14,15]

Our results showed that DNA synthesis was sensitively detected by NMM. In addition, daidzein, the major phytoestrogens in soy, inhibited DNA synthesis of KF- and the estimated IC_{50} was determined to be 15.6 μM through nonlinear regression analysis.

Experimental

Materials

KF- (5 U/μL) was obtained from TaKaRa Biotech (Shiga, Japan). Template DNA (5'-GGGTAGGGCGGGTTGGGAAGATACGCATAGAACT-3') and primer (5'-AGTTCTATGCGTATC-3') were synthesized and purified by Bioneer (Chungwon, Korea) using PAGE. Daidzein, KCl, and dNTP were purchased from Sigma-Aldrich (MO, USA). Triton X-100 (extra pure grade) was purchased from Yakuri (Kyoto, Japan). Hemin was obtained from Tokyo Chemical Industry (Tokyo, Japan). N-methyl mesoporphyrin IX (NMM) was purchased from Frontier Scientific (Logan, USA). All chemicals were of analytical grade.

DNA polymerization reaction

Reactions were performed in KF- buffer (10 mM Tris, pH 7.5, 5 mM $MgCl_2$, 7.5 mM DTT) at room temperature (~20 ^{o}C) for 1 h. For the appropriate concentration of primer, reactions were conducted with 0.5 μM template DNA at different primer concentrations (0 - 1.0 μM) and 25 U/mL KF-. To determine the optimal concentration of dNTP, reactions were conducted with 0.5 μM template DNA and cDNA at different concentrations of dNTP (0 - 200 μM).

Fluorescence measurement

DNA polymerization was carried out by adding KF- (0 - 50 U/mL) to a reaction mixture containing 0.5 μM oligomer and cDNA. After the addition of salt buffer (10 mM KCl, 25 mM NaCl, 0.003% triton X-100) and hemin (1 μM) to reaction mixture, reaction was incubated for 1 h to allow for the formation of the G-quadruplex structure. Then, fluorescence intensity was measured 30 min after the addition of NMM to the reaction. Fluorescence intensity was measured with a Molecular Devices XPS fluorescence microplate reader (Sunnyvale, USA) with excitation and

emission wavelengths of 399 nm and 614 nm, respectively. Fluorescence spectra were scanned from 590 nm to 650 nm at room temperature.

KF- inhibition

The inhibition reaction was carried out with 20 U/mL KF- at different concentrations of daidzein (0 - 200 μM) for 1 h at room temperature. Salt buffer and hemin were added to each reaction, and the reactions were further incubated for the formation of the G-quadruplex structure. The reaction was measured 30 min after NMM addition to obtain data on the inhibition reaction and its kinetics.

Results and Discussion

Figure 1 shows a schematic representation of a G-quadruplex-based fluorescence assay of KF-, assisted by NMM. The template strand contains the G quadruplex sequence at the 5' region and cDNA is annealed at the 3' region of the template strand. The G-quadruplex structure can be formed in the absence of KF- and thus fluorescence emission is strongly enhanced by the specific binding of NMM to G-quadruplex (top). In addition, G-quadruplex is not formed in the presence of KF- because of the synthesis of complementary DNA caused by KF-. As a result, fluorescence emission is weakened (middle). In the presence of a KF- inhibitor, daidzein, G-quadruplex is formed while DNA synthesis is inhibited (bottom).

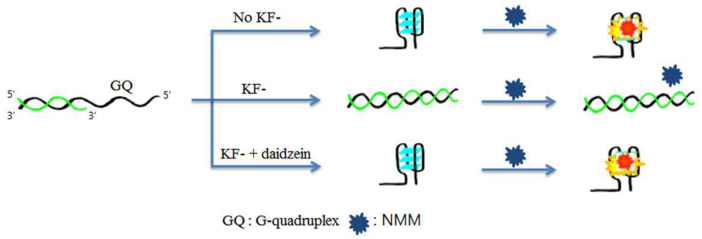

Figure 1. Schematic diagrams of fluorescence assay of KF-. Without KF- (top) in the reaction, G-quadruplex was formed in the absence of complementary strand synthesis, leading to the strong fluorescence intensity assisted by NMM. In the presence of KF- (middle), G-quadruplex was not formed as duplex formation occurred in the G-quadruplex region. In the presence of daidzein (bottom), KF- was inhibited and G-quadruplex was formed.

In Figure 2, template DNA was incubated in the absence of KF-, and high fluorescence intensity was shown as a result of the formation of G-quadruplex (a). By contrast, the addition of 25 U/mL KF- resulted in a decrease in fluorescence intensity by 199.0, which was 28.5% of that in the absence of KF- (b). This result indicated that G-quadruplex was not formed as a result of the synthesis of complementary stranded DNA mediated by KF-. As shown in Figure 3A, NMM-assisted fluorescence intensity was linearly proportional to the concentrations of template DNA without KF-. It is indicated that G-quadruplex was formed stably, and the

fluorescence emission correlated linearly with the specific binding of NMM to G-quadruplex, demonstrating that G-quadruplex-forming DNAs significantly enhanced fluorescence emission in the presence of NMM, while duplex calf thymus DNA did not.[16]

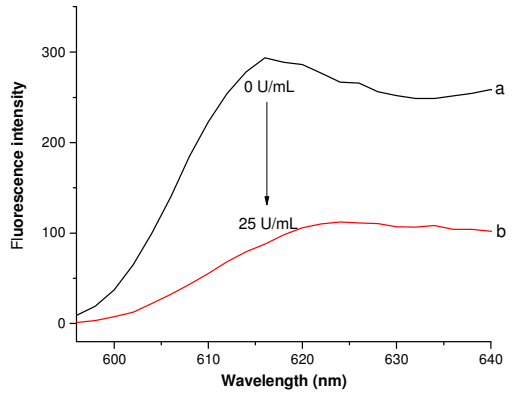

Figure 2. Fluorescence emission spectra of NMM-assisted KF- assay. Fluorescence assays were conducted either in the absence (a) or presence (b) of KF- at 0.5 μM template DNA. Spectra were scanned from wavelengths of 590nm to 640 nm at an excitation wavelength of 399 nm.

Recently, the G-quadruplex structure has been utilized as a sensitive detection platform. Terminal deoxynucleotidyl transferase (TdT) activity was detected by thioflavin T-assisted fluorescence assay of G-quadruplex formed from random G-rich DNA sequences synthesized by TdT under conditions of a G-rich dNTP pool.[17] In addition, telomerase and helicase activities were sensitively and selectively monitored by the formation of G-quadruplex in the presence of SYBR Green I or fluorescent quenchers (Dabcyl).[18,19]

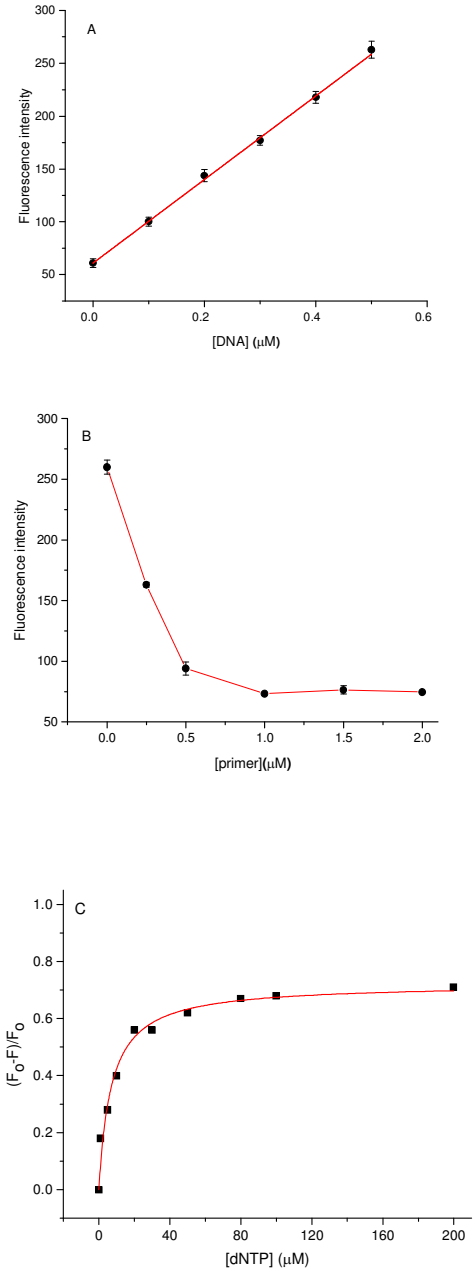

Figure 3. (A) Direct correlation between the amount of template DNA and fluorescence intensity assisted by NMM. (B) Dependence of complementary DNA synthesis on the amount of cDNA at 25 U/mL KF-. (C) Dependence of

complementary DNA synthesis on dNTP concentrations at 25 U/mL KF-.

In addition, to determine the optimal concentration of cDNA for DNA synthesis mediated by KF-, DNA polymerization was carried out at different cDNA concentrations. Figure 3B shows that in the presence of KF- (25 U/mL), fluorescence intensity decreased rapidly by 64% from that of template DNA without KF- at 0.5 µM cDNA, which was 1x the concentration of the template DNA. Furthermore, KF- synthesized complementary DNA to an extent of 72 % of template DNA at 1.0 mM cDNA. According to GO-based DNA polymerase assay,[20] the fluorescence intensity was increased to ~78 % that of optimal cDNA in the case of 2× cDNA concentration compared with template DNA.

As shown in Figure 3C, $(F_o-F)/F_o$ was plotted at different concentrations of dNTP at 1× cDNA and 0.5 µM template DNA. F and F_o represent the fluorescence intensity in the presence and absence of dNTP, respectively. F_o-F indicates the change in fluorescence intensity at a specific concentration of dNTP following DNA synthesis. Thus, $(F_o-F)/F_o$ is the ratio of fluorescence intensity in the presence of KF- to that in the absence of KF- at any dNTP concentration. It was demonstrated that KF- was fully maximal at dNTP concentrations of greater than 80 µM. This result was consistent with those obtained from GO-based DNA polymerase assay.[20]

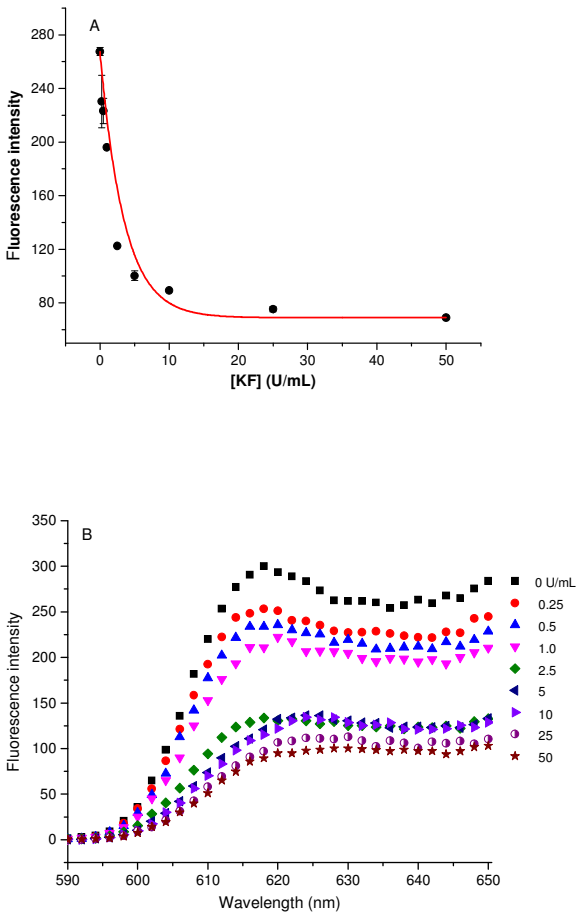

Figure 4. (A) Complementary DNA synthesis at different concentrations of KF- in the presence of 100 µM dNTP and 0.5 µM template and cDNA. (B) Fluorescence spectra at different concentrations of KF- from 0 U/mL to 50 U/mL.

Under the same reaction conditions, DNA synthesis was performed at different concentrations of KF-. As shown in Figure 4A, reactions were conducted with 0.5 µM template and cDNA in the presence of 100 µM dNTP at

room temperature for 1 h. The fluorescence intensity was found to have dramatically decreased until the KF- concentration reached 5.0 U/mL. To further analyze the degree of DNA synthesis, $(F_o\text{-}F)/F_o$ was utilized at different concentrations of KF-. $(F_o\text{-}F)/F_o$ was increased to 14%, 27%, 66.7%, and 74% at 0.25 U/mL, 1.0 U/mL, 10 U/mL, and 50 U/mL KF-, respectively. This shows that DNA synthesis increased steeply up to a KF- concentration of 10 U/mL according to the change in fluorescence. Figure 4B shows the fluorescence spectra at different concentrations of KF- from 0 U/mL to 50 U/mL. At low KF- concentrations, changes in fluorescence intensity were large compared with those at high KF concentrations because of the saturation of KF- with substrates. It was indicated that DNA synthesis reached its maximum at a KF- concentration of 25 U/mL.

DNA polymerase has been utilized as a target in therapeutic drug screening for diseases such as cancers and viral infections.[21] Thus, the sensitivity of the method described in this study was evaluated in the screening of DNA polymerase-target drug. Daidzein, the major phytoestrogens in soy, was chosen as an inhibitor of DNA polymerization. Figure 5 shows that the fluorescence intensity increased steeply up to daidzein concentration of 10 µM, indicating that DNA synthesis mediated by KF- was inhibited sensitively with the addition of daidzein.

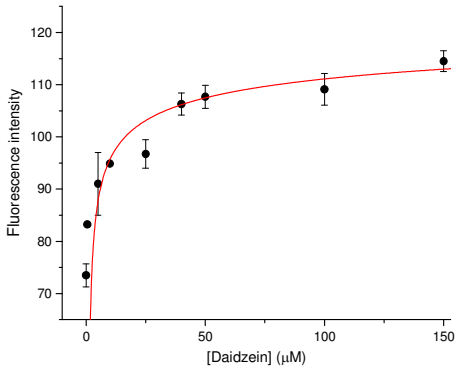

Figure 5. Effect of KF- activity on daidzein concentrations from 0 µM to 150 µM at 25 U/mL KF-.

The IC_{50} for daidzein was 15.6 µM, which was determined from the nonlinear regression of the data in Fig. 5. In addition, DNA synthesis mediated by KF- was not affected by phosphonoacetic acid (data not shown). It was reported that daidzein treatment of T47D: A18/PKCα tumor cells was initially stimulated, but growth inhibition was observed during a period of time.[22] Furthermore, the growth inhibition effects of daidzein appeared to be greater than those of genistein, a phytoestrogen in soy, like daidzein.[22] According to many publications,[23-26] genistein inhibited DNA synthesis and the proliferation of tumor cells at high concentrations (>10 µM).

Conclusions

Here, the G-quadruplex structure was used to detect the enzymatic activity and inhibition of KF- using NMM. DNA synthesis mediated by KF- led to the formation of double-stranded DNA from single-stranded DNA in the G-rich region. This region in single-stranded conformation was essential for G-quadruplex formation, and fluorescence was not emitted in the presence of NMM due to the difficulty of G-quadruplex formation. Thus, KF- activity was sensitively detected with the aid of NMM, a binding dye specific for G-quadruplex. In addition, daidzein, the major phytoestrogens in soy, inhibited DNA synthesis, and the IC_{50} was determined to be 15.6 μM by nonlinear regression analysis.

Acknowledgements

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Notes and references

1. J. yamtich and J. B. Sweasy, *Biochem. Biophys. Acta*, 2010, **1804**, 1136-1150.
2. S. Anderson, M. J. Gait, L. Mayol, and I. G. Young, *Nucleic. Acids Res.*, 1980, **8**, 1731-1743.
3. L. M. Houdebine, *Nucleic. Acids Res.*, 1976, **3**, 615-630.
4. F. Sanger, S. Nicklen, and A. R. Coulson, *Proc. Natl. Acad. Sci. USA*, 1977, **74**, 5463-5467.
5. W. Li, Z. Liu, H. Lin, Z. Nie, J. Chen, X. Xu, and S. Yao, *Anal. Chem.*, 2010, 82, 1935-1941.
6. H. Jia, Z. Li, C. Liu, and Y. Cheng, *Angew. Chem. Int. Ed.*, 2010, 49, 5498-5501.
7. D. L. Earnshaw and A. J. Pope, *J. Biomol. Screening*, 2001, **6**, 39-46.
8. S. Lutz, P. Burgstaller, and S. A. Benner, *Nucleic Acids Res.*, 1999, **27**, 2792-2798.
9. F. Xu, H. Shi, X. He, K. Wang, X. Ye, L. Yan, and S. Wei, *Analyst*, 2012, **137**, 3989-3994.
10. J. Xu, L.-L. Cai, D.-M. Kong, and H.-X. Shen, *Anal. Lett.*, 2011, **44**, 2582-2592.
11. A. T. Phan, V. Kuryavyi, and J. D. Patel, *Curr. Opin. Struct. Biol.*, 2006, **16**, 288-298.
12. P. Travascio, Y. Li, and D. Sen, *Chem. Biol.*, 1998, **5**, 505-517.
13. P. Travascio, A. J. Bennet, D. Y. Wang, and D. Sen, *Chem. Biol.*, 1999, **6**, 779-787.
14. Q. Chen, Q. Guo, Y. Chen, J. Pang, F. Fu, and L. Guo, *Talanta*, 2015, **138**, 15-19.
15. D. Monchaud and M. P. Teulade-Fichou, *Org. Biomol. Chem.*, 2008, **6**, 627-636.
16. H. Arthanari, S. Basu, T. L. Kawano, and P. H. Bolton, *Nucleic Acids Res.*, 1998, **26**, 3

724-3728.

17. Z. Liu, W. Li, Z. Nie, F. Peng, Y. Huang, a nd S. Yao, *Chem. Commun.*, 2014, **50**, 687 5-6878.

18. Q. H. Quach, J. Jung, H. Kim, and B. H. C hung, *Chem. Commun.*, 2013, **49**, 6596-65 98.

19. O. Mendoza, N. M. Gueddouda, J.-B. Boul e, C. A. Bourdoncle, and J.-L. Mergny, *Nu cleic Acids Res.*, 2015, **43**, 0305-0315.

20. F. Xu, H. Shi, X. He, K. Wang, CX. Ye, L. Yan, and S. Wei, *Analyst*, 2012, **137**, 3989 -3994.

21. A. J. Berdis, *Biochemistry*, 2008, **47**, 8253-8260.

22. D. A. Tonetti, Y. Zhang, H. Zhao, S.-B. Li m, and A. L. Constantinou, *Nutr. Cancer*, 2 007, **58**, 222-229.

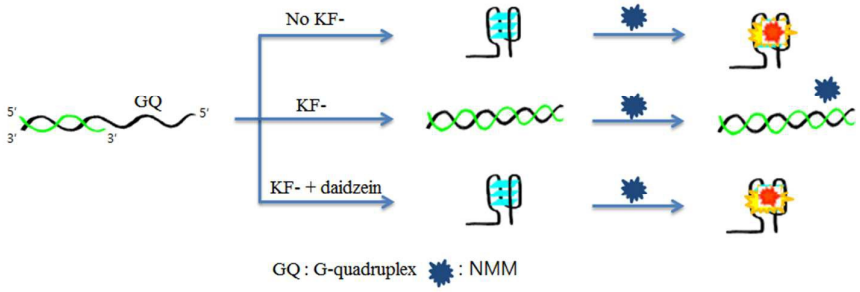
23. M. L. Lemos, *Ann. Pharmacother.*, 2001, **3 5**, 1118-1121.

24. S. Balabhadrapathruni, T. J. Thomas, E. J. Yurkow, P. S. Amenta, and T. Thomas, *On col. Rep.*, 2000, **7**, 3-12.

25. H. Nakagawa, D. Yamamoto, Y. Kiyozuka , K. Tsuta, Y. Uemura, K. Hioki, *J. Cancer Res. Clin. Oncol.,* 2000, **126**, 448-454.

26. R. C. Santell, N. Kieu, and W. G. Helferich , *J. Nutr.*, 2000, **130**, 1665-1669.

Figure 1.



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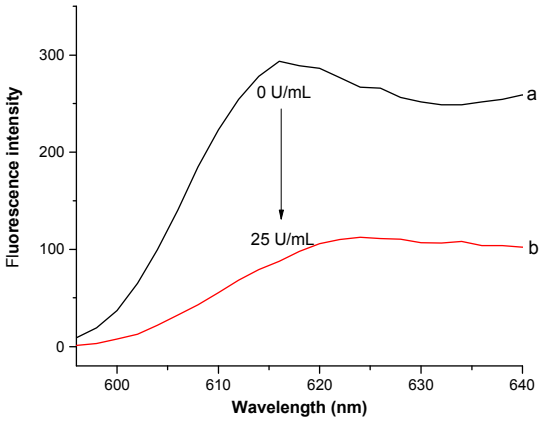

Figure 3A.

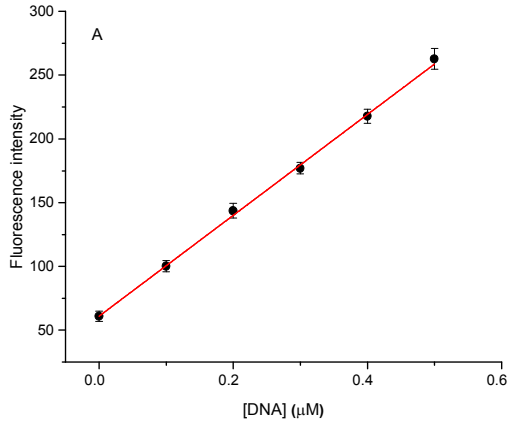

Figure 3B

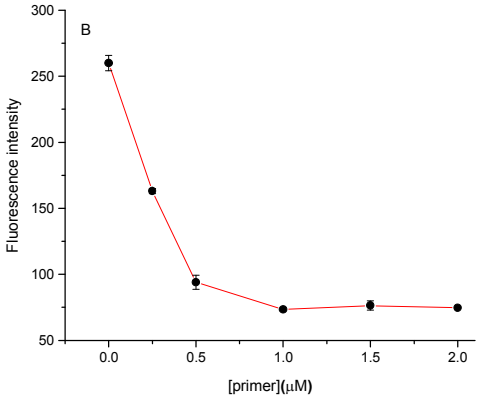

Figure 3C

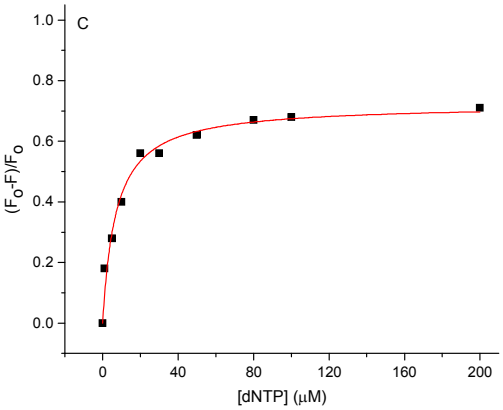

Figure 4A

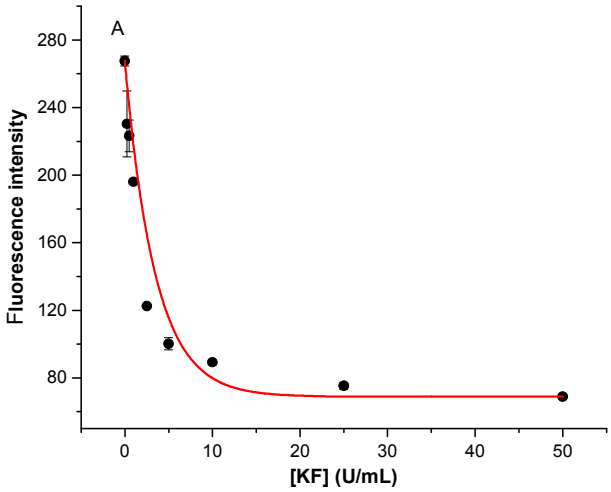

Figure 4B

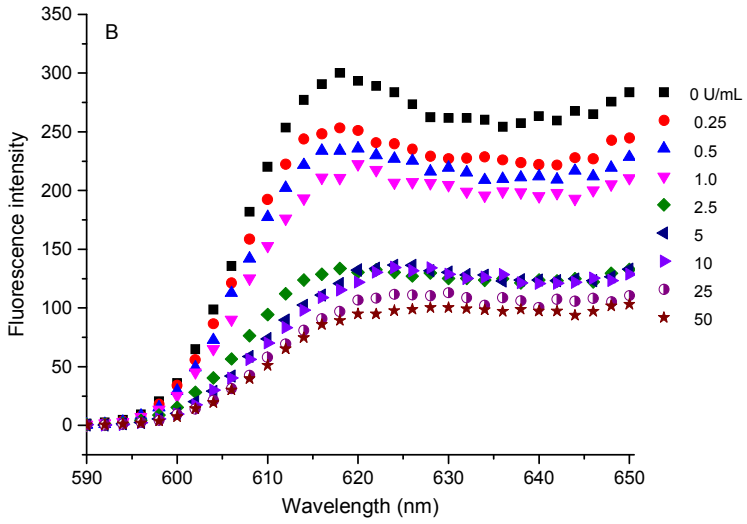

Figure 5