Detection of BER Enzymes Based on Fluorescence Resonance Energy Transfer

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Recently, many sensing technologies using fluorescence resonance energy transfer (FRET) for detecting biomolecules have been developed.[1-4] Pertinent examples are probes that can detect specific DNA sequence using a FRET between nanoparticles and/or organic fluorophores.[5-8] In particular, molecular beacons,[9] which are probes that fluoresce upon hybridization, are a well-known technique in biotechnology that utilize the hybridization ability of DNA duplexes. There are numerous examples for the detection of DNA lesions such as abasic sites,[10-12] but few examples have been introduced regarding the detection of repair enzymes.[13]

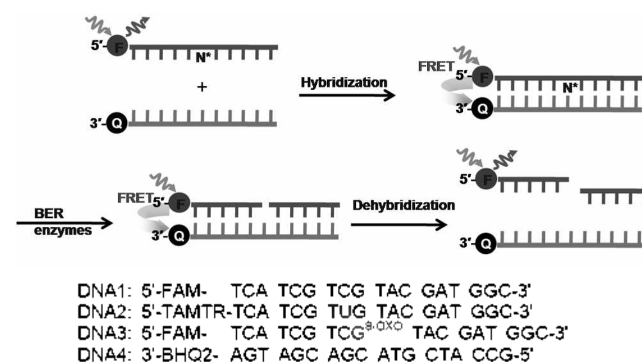
In the normal cellular condition, the expression level of base excision repair (BER) enzymes is maintained to a specific extent to repair damaged bases. However, in severe conditions such as a high concentration of reactive oxygen species or the presence of drugs that damage DNA, the level must be increased immediately to repair the DNA damages.[14] Therefore if the expression level of specific BER enzymes can be monitored, it would provide a means to understand drug responses in cancer chemotherapy. A general method for detecting the expression levels of specific enzymes involves the use of an immunoassay using a specific antibody. Although the specificity and sensitivity of this immunoassay are very high, a specific monoclonal antibody able to recognize a target antigen is required.[15]

This study introduces a novel method of detecting BER enzymes based on the fluorescence resonance energy transfer between an organic fluorophore and a quencher molecule. The basic idea for the detection of BER enzymes centers on a combination of the specific recognition and reaction of BER enzymes, the DNA hybridization ability, and the FRET between a fluorescence donor and an acceptor molecule. In this study, the target BER enzymes were uracil DNA glycosylase (UDG) and formamidopyrimidine DNA glycosylase (Fpg). UDG can recognize and excise a uracil base in a single- or double-strand DNA.[16] UDG has no endonuclease activity; hence it cannot excise the resulting abasic site. However, if coupled with Endo IV, which can recognize and cleave an abasic site,[17] a DNA strand containing a dU can be cleaved. This leads to the generation of two short DNA fragments. In contrast to UDG, Fpg has both excision and endonuclease activity for a damaged purine base in double-strand DNA.[18]

The hybridization ability of a DNA duplex is mainly dependent on the length of the DNA sequences in the presence of an adequate concentration of salts. In this work, the DNA sequence of probes was selected to hybridize fully with a complementary DNA at 37 °C. Accordingly, under normal reaction conditions, all DNAs remain as duplex forms. However, short DNA fragments resulting from the reaction of BER enzymes, in which the T_m values are lower than room temperature, were dehybridized spontaneously under normal reaction conditions. Finally, a FRET technique was applied to detect the signal indicating the presence of BER enzymes. Within the Förster distance, acceptor molecules can effectively absorb the light of donor fluorescence molecules if the emission of donor molecules and the absorption spectrum of acceptor are sufficiently overlapped.

Comprehensibly, an organic fluorophore-tagged 18-mer oligonucleotide, which has one damaged base at the central site, was hybridized with a complementary quencher-tagged oligonucleotide. In the hybridized condition, the fluorescence was suppressed by FRET between the fluorophore and the quencher. However, in the presence of BER enzymes, the damaged base was excised and an abasic site was produced. This abasic site was further cleaved by additional Endo IV and by Fpg itself. The T_m values of the short DNA duplexes were much lower than room temperature due to the cleavage of the fluorophore-tagged DNA strand. The resulting short DNA duplexes were dehybridized spontaneously, which resulted in the generation of fluorescence signals (Scheme 1).

5′-Tagged TAMRA and FAM were used as FRET donors for detecting BER enzymes. Black hole quencher 2 (BHQ2) as a FRET acceptor was selected and attached to the 3′-complementary strand due to the broad absorption of visible light in the range of 500 ~ 650 nm (Fig. 1). The length (18-mer, calc. T_m = 62 °C) and sequences (GC contents = 56%) of the DNA[19] were selected for perfect hybridization at 37 °C. The substrate



Scheme 1. Detection strategy for BER enzymes. Modified base N*; 2′-deoxyuridine or 8-oxo-2′-deoxyguanosine, organic fluorophores; TAMRA, FAM. A quencher molecule; BHQ2

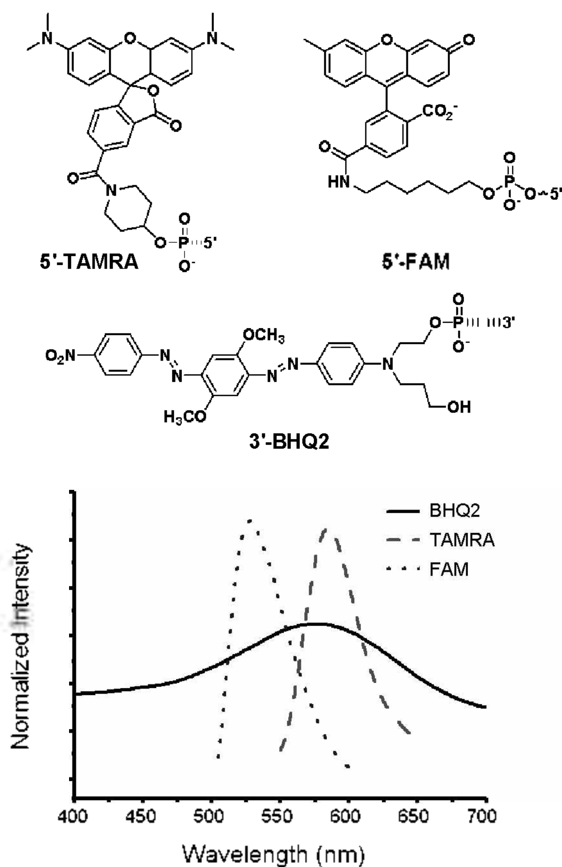


Figure 1. Emission spectra of TAMRA and FAM fluorophores (λ_{ex} = 510, 460 nm respectively), absorption spectrum of the Black Hole Quencher 2 (BHQ2).

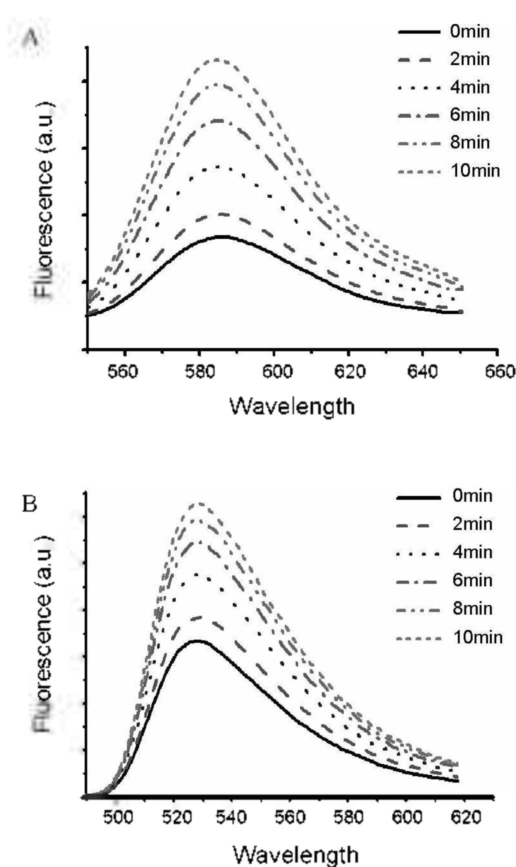


Figure 2. Time-dependent-fluorescence spectra after the addition of BER enzymes: A) DNA2/DNA4 duplex, 2 units of UDG, 20 units of Endo IV, B) DNA3/DNA4 duplex, 5 units of Fpg.

base of the BER enzymes was inserted into the 7th or 8th base site from the 5' site of the DNA. The dehybridization of the fragmented DNA duplex occurs after an enzymatic reaction due to the low melting temperature.

Initially, a control experiment was carried out to confirm that there was no reaction between the hybridized normal DNA1/DNA4 and UDG/Endo IV enzymes. As expected, an increase in the fluorescence signal was not observed, but a slight decrease in the time course due to the photo bleaching of the fluorophore was noted.

Figure 2 shows the time-dependent-fluorescence spectra in the presence of BER enzymes. Before the addition of BER enzymes, the fluorescence signal was suppressed by FRET between a fluorophore and a quencher in the fully hybridized DNA duplex (0 min bottom lines in Fig. 2). An addition of Fpg to the hybridized DNA3/DNA4 duplex resulted in an increase of the fluorescence signal, indicating the DNA cleavage of the fluorophore-tagged DNA strand, as Fpg has both excision and endonuclease properties (Fig. 2B).

In contrast to Fpg, UDG causes only excision activity for the uracil base. Consequently, there was no significant spectral change after the addition of UDG enzyme into the hybridized DNA2/DNA4 duplex. The activity of UDG was confirmed by polyacrylamide gel electrophoresis (PAGE) after the hydrolysis of the abasic site with 0.1 *N* NaOH. For the detection of the UDG enzyme, a second enzyme, able to recognize and cleave the abasic site was required. Endo IV is a specific enzyme for cleaving abasic sites. Indeed, after the reaction of UDG with the hybridized DNA2/DNA4 duplex, the addition of Endo IV into the solution resulted in an increase in the fluorescence signals (Fig. 2A). Therefore, this hybridized DNA2/DNA4 duplex containing Endo IV can specifically detect the UDG enzyme.

Kinetically, the reaction rate of Endo IV is much slower compared to the rate of the UDG reaction; therefore, it is impossible to determine the concentration-dependent-kinetic data for the UDG detection. However, the concentration of the Fpg enzyme can be determined quantitatively from both the excision and endonuclease activities of the Fpg enzyme. Figure 3A shows the fluorescence spectra in the time courses with various enzyme units for the detection of Fpg. The addition of a high concentration of Fpg led to a much faster increase of the fluorescence signal. Under the substrate excess conditions, the reaction was well fixed to the exponential growth kinetics.

The initial velocity was calculated from the slopes of each zero time and was then plotted based on the added units of Fpg (Fig. 3B). The result was well fitted to the linear correlation. Hence, if the initial velocity of Fpg detection is determined, the present units of Fpg in a solution can be calculated easily from the kinetic data. For Fpg detection, the detection limit of Fpg is calculated as a lesser than 2.5 units/mL. The most characteristic information from these biosensors is signal amplification by the enzyme itself. At a very low level of enzyme concentration,

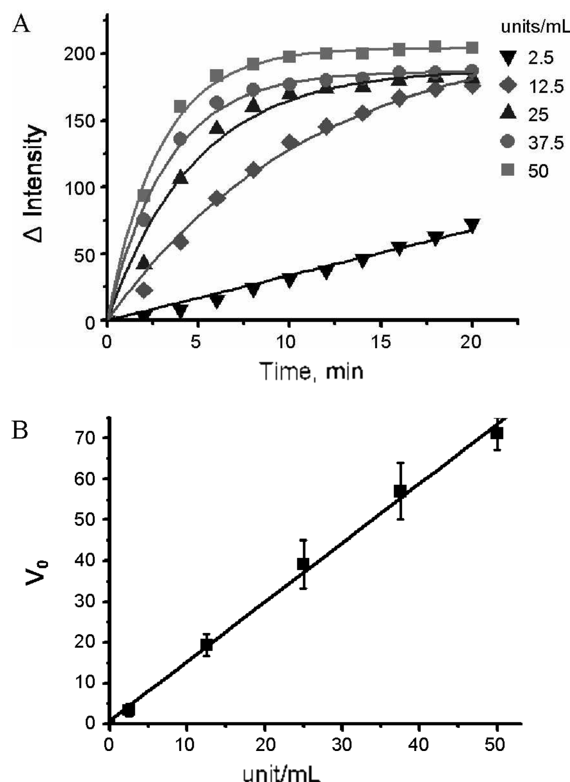


Figure 3. Kinetic analysis of Fpg detection, A) unit-dependent-fluorescence spectra after the addition of the Fpg enzyme into the hybridized DNA3/DNA4 duplex, λ_{ex} = 460 nm, λ_{em} = 527 nm, B) plotting over the initial velocity versus the enzyme units in the solution.

the fluorescence signal gradually increases as the enzyme reacts with the substrate upon an extended reaction time.

In conclusion, biosensors for the specific detection of BER enzymes were originally constructed based on FRET technology. The enzymatic cleavages of a target DNA containing a damaged base resulted in the dehybridization of a DNA duplex and an increase in the fluorescence signals. Fluorescence signals can be amplified by BER enzymes as catalysts. This technology is faster and simpler because it avoids PAGE and the specific antibodies used in typical protein detection techniques. If organic fluorophores are replaced by quantum dots, which are adequate for multi-color detections of several enzymes simultaneously, a very useful biosensor able to monitor the expression level of BER enzymes simultaneously in cancer chemotherapy would be achievable.

Experimental Section

All synthetic modified oligonucleotides were purchased from Bioneer Corp., Korea; 5′-fluorophore-tagged DNA sequences; 5′-FAM-TCA TCG TCG TAC GAT GGC-3′, 5′-TAMRA-TCA TCG TUG TAC GAT GGC-3′, 5′-FAM-TCA TCG TCG^{oxo} TAC GAT GGC-3′, complementary 3′-quencher-tagged DNA sequence; 5′-GCC ATC GTA CGA CGA TGA-BHQ2-3′. Uracil DNA glycosylase (5000 units/mL), formamidopyrimidine DNA glycosylase (8000 units/mL), and endonuclease IV (10,000 units/mL) were purchased from New England Biolabs Inc. All buffers for UDG (1x, 20 mM Tris-HCl, 1 mM dithiothreitol, and 1 mM EDTA, pH 8.0), Endo IV (1x, 50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol, pH 7.9), and Fpg (1x, 10 mM Bis-Tris-propane-HCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol, pH 7.0) were provided by NEB Inc. Deionized and doubly distilled water was used throughout. All Eppendorf tubes and tips, as well as water samples were sterilized before use. Hybridization experiments were conducted using a heat block. The spectroscopic measurements were carried out on a PerkinElmer LS55 device with a temperature controller.

5′-Fluorophore-tagged oligonucleotide (10 μL, 100 pmol) and 3′-quencher-tagged oligonucleotide (10 μL, 100 pmol) were mixed in 10x UDG buffer (10 μL) and 10 μL NaCl (1.0 M) was added. The final volume was adjusted to 100 μL with water. After thoroughly mixing the solution, it was incubated at 70 °C for 5 min using a heat block and was then slowly cooled to room temperature. The hybridized DNA duplexes (1 μM, pH 8.0) were kept in a dark freezer.

Water (221 μL), NaCl solution (28 μL, 1.0 M) and UDG buffer (10x, 28 μL) were mixed and the hybridized DNA duplex (20 μL, 1.0 μM) was added to the solution. The initial fluorescence spectrum was recorded at each excitation wavelength (460 nm for FAM, 510 nm for TAMRA). After the determination of an initial spectrum, the BER enzyme was immediately added (1 μL UDG (2 units), 2 μL Endo IV (20 units) or each units of Fpg). The fluorescence spectra were obtained in time courses at 37 °C.

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References

1. Zhang, C.; Johnson, L. W. *Anal. Chem.* **2009**, *81*, 3051.
2. Medintz, I. L.; Konnert, J. H.; Clapp, A. R.; Stanish, I.; Twigg, M. E.; Mattoussi, H.; Mauro, J. M.; Deschamps, J. R. *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 9612.
3. Patolsky, F.; Gill, R.; Weixmann, Y.; Mokari, T.; Banin, U.; Willner, I. *J. Am. Chem. Soc.* **2003**, *125*, 13918.
4. Voss, S.; Fischer, R.; Jung, G.; Wiesmuller, K.; Brock, R. *J. Am. Chem. Soc.* **2007**, *129*, 554.
5. Medintz, I. L.; Uyeda, H. T.; Goldman, E. R.; Mattoussi, H. *Nat. Mater.* **2005**, *4*, 435.
6. Maxwell, D. J.; Taylor, J. R.; Nie, S. *J. Am. Chem. Soc.* **2002**, *124*, 9606.
7. Chen, X.; Zehnbauer, B.; Gnirke, A.; Kwok, P. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 10756.
8. Dubertet, B.; Calame, M.; Libchaber, A. L. *Nat. Biotechnol.* **2001**, *19*, 365.
9. Tyagi, S.; Kramer, F. R. *Nat. Biotechnol.* **1996**, *14*, 303.
10. Kow, Y. W.; Dare, A. *Methods* **2000**, *22*, 164.
11. Kobu, K.; Ide, H.; Wallace, S. S.; Kow, Y. *Biochemistry* **1992**, *31*, 3703.
12. Atamna, H.; Cheung, I.; Ames, B. N. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 686.
13. Liu, B.; Yang, X.; Wang, K.; Tan, W.; Li, H.; Tang, H. *Anal. Biochem.* **2007**, *366*, 237.
14. Inoue, M.; Shen, G.; Chaudhry, M. A.; Galick, H.; Blaisdell, J. O.; Wallace, S. S. *Radiat. Res.* **2004**, *161*, 409.
15. Liu, X.; Dai, Q.; Austin, L.; Coutts, J.; Knowles, G.; Zou, J.; Chen, H.; Huo, Q. *J. Am. Chem. Soc.* **2008**, *130*, 2780.
16. Lindahl, T.; Ljungquist, S.; Siegert, W.; Sperens, B. *J. Biol. Chem.* **1977**, *252*, 3286.
17. Takeuchi, M.; Lillis, R.; Demple, B.; Takeshita, M. *J. Biol. Chem.* **1994**, *269*, 21907.
18. Rabow, L. E.; Kow, Y. W. *Biochemistry* **1997**, *36*, 5084.
19. http://www.appliedbiosystems.com/support/techtools/calc/.