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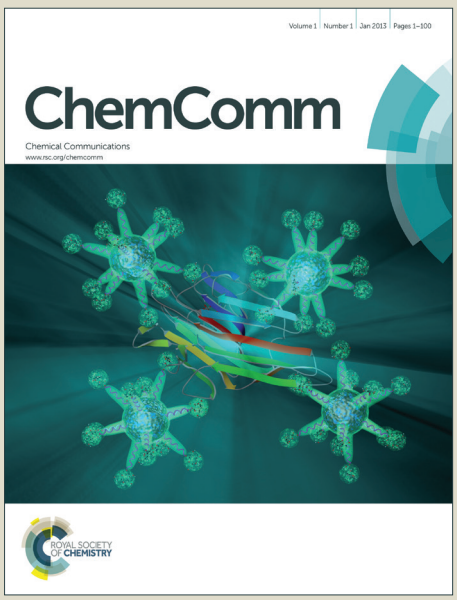

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Blinking triggered by the change in the solvent accessibility of the fluorescent molecule

Cite this: DOI: 10.1039/xoxxoooooox

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Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/xoxxoooooox

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The more a fluorescent molecule is exposed to a solvent, the faster its triplet excited state is quenched by molecular oxygen. The changes in the solvent accessibility of the fluorescent molecule were probed by measuring the duration of the off time during the blinking of the fluorescence, which enabled analysis of the function of a molecular beacon-type probe.

Environmentally sensitive fluorescent molecules are widely used as universal tools in molecular biology and diagnostic imaging. These fluorescent molecules report information regarding their surrounding local microenvironment such as pH, viscosity, and polarity by changing their fluorescence properties, *i.e.*, fluorescence wavelength, intensity, and lifetime.[1-7] In order to maximize the spatial resolution and/or to minimize the number of fluorescent molecules needed for detection, one strategy would be to focus on a detection method that relies on the properties of fluorescent molecules that become highlighted when we look at molecules at the single-molecule level. On the single-molecule level, fluorescent molecules often exhibit fluctuating emissions between bright ''on'' and dark "off" states, which is referred to as "blinking." During the repetitive cycles of excitation of the S_0 grand state to the singlet excited state (S_1) and subsequent emissions that allow a return to S_0, fluorescent molecules occasionally enter non-fluorescent off states, such as a radical ion state, an isomerized state, and a triplet state (T_1), resulting in a blinking of the fluorescence. By understanding the factors that affect the blinking, single fluorescent molecules can serve as reporters of their local environment.[8-12] We have recently reported that information about single-nucleotide variations in DNA sequences can be extracted by monitoring the blinking of fluorescence originated by the photo-induced formation of the radical ions and subsequent charge-recombination in DNA.[13,14]

Triplet state blinking results from intersystem crossing from the S_1 to the T_1 state. Since the triplet to singlet transition is spin-forbidden, the lifetime of T_1 is much longer than that of S_1. Therefore, the lifetime of the triplet state (τ_T) can be more sensitive to the concentration of a quencher such as molecular oxygen (O_2) in its microenvironment. Taking into account that the diffusion-controlled rate constant between O_2 and T_1 of fluorescent molecules is approximately $k_{O2} \approx 10^{10}$ M^{-1} s^{-1},[15] and that the concentration of O_2 in aqueous solution is approximately $[O_2] = 0.3$ mM,[16] the triplet quenching reaction occurs with a time constant slower than 0.3 μs. Conventionally, triplet state kinetics of fluorescent molecules have been investigated by transient absorption measurements, which typically require a significant amount of sample (>1 nmol). Pioneering work led by Widengren and Rigler has demonstrated that τ_T can be accessed by measuring the off time (τ_{OFF}) during blinking using fluorescence correlation spectroscopy (FCS).[8,9] They suggested that FCS can be utilized for the analysis of $[O_2]$. Based on this concept, Schwille and co-workers have reported that $[O_2]$ can be measured by FCS on the level of single fluorescent molecules.[17] Recently, Pack and co-workers have reported that a fluorescent molecule attached to DNA is also able to report $[O_2]$.

The triplet quenching reaction can also be affected by the accessibility of a fluorescent molecule to O_2, *i.e.*, the solvent accessibility of the fluorescent molecule.[18] Austin and co-workers have reported that k_{O2} of methylene blue (MB) significantly decreases when it is intercalated in the DNA duplex.[19] In this study, we investigated whether DNA conformational change can be monitored by the blinking triggered by the change in the solvent accessibility of a fluorescent molecule. We designed a DNA site specifically modified with a fluorescent molecule, methylene blue (MB) and Rhodamine 6G (R6G), and investigated how the change in the solvent accessibility of fluorescent molecules affects τ_T and τ_{OFF}.

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† Electronic Supplementary Information (ESI) available: Experimental procedures, absorption and fluorescence spectra of MB- and R6G-modified DNA, and melting temperature (T_m) measurements for MB-modified DNA. See DOI: 10.1039/c000000x/

It was demonstrated that the conformational change of DNA from the hairpin form to the duplex form, which causes a decrease in the solvent accessibility of the fluorescent molecule, can be monitored by measuring τ_{OFF} during blinking.

In order to bury a fluorescent molecule in the π-stacks in the context of a double helical structure, a fluorescent molecule was attached to DNA by an aminolinker (X) (Fig. 1). In contrast, we assumed the fluorescent molecule to be exposed to the solvent when it was placed at the loop region of a hairpin. First we selected MB as a fluorescent molecule due to its high intersystem crossing yield (Φ_T) and high triplet molar extinct coefficient suitable for transient absorption measurements to obtain τ_T.[19-22] The UV Melting-temperature (T_m) was measured at 260 nm and 676 nm corresponding to the absorption of nucleobases and MB, respectively. Similar T_m melting values were obtained at two wavelengths in the duplex form, while the melting transition at 676 nm was not obvious when MB was placed at the hairpin loop region, suggesting that MB is buried in DNA in the duplex form while it is exposed to the solvent in the hairpin form as designed (see Supporting Information). The flash excitation of MB-modified DNA with a 532-nm laser pulse led to the formation of absorption bands at 430 nm and 830 nm, which were assigned to the T_1-T_n absorption of MB ($^3MB^*$, Fig. 2A).[19-22] While τ_T showed a long lifetime under the Ar-saturated condition (τ_T > 50 μs), its decay rate was accelerated under aerobic conditions due to the bimolecular quenching reaction between $^3MB^*$ and O_2. τ_T and the bimolecular reaction rate constant between ^3MB and O_2 (k_{O2}) obtained according to eq (1) are summarized in Table 1.

$$k_{O_2}[O_2] = 1/\tau_{T(\text{under air})} - 1/\tau_{T(\text{under argon (Ar)})} \quad (1)$$

Interestingly, τ_T decreased along the order of duplex > hairpin loop ~ single strand > monomer MB (Figure 2. Table 1), which correlates well with the increase in the solvent accessibility of MB. These results showed that τ_T reports the changes in the microenvironment around the fluorescent molecule. The k_{O2} value obtained for MB placed at the loop region was still about five fold slower than that obtained for monomer MB. These results suggest that MB π-stacks with the adjacent bases to some extent even when it is positioned at the loop region.

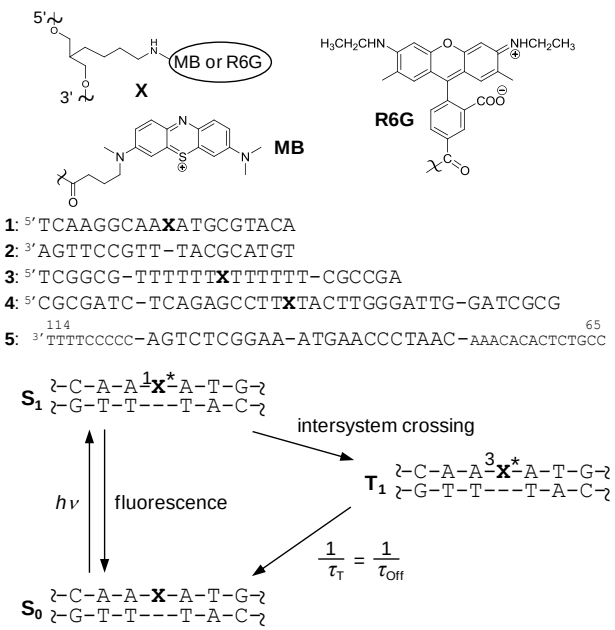

Fig. 1. Chemical structures of an amino-linker (X), methylene blue (MB), Rhodamine 6G (R6G), DNA sequences used in this study, and a schematic representation for emission and formation and decay of fluorescence molecule in the triplet excited state attached to DNA.

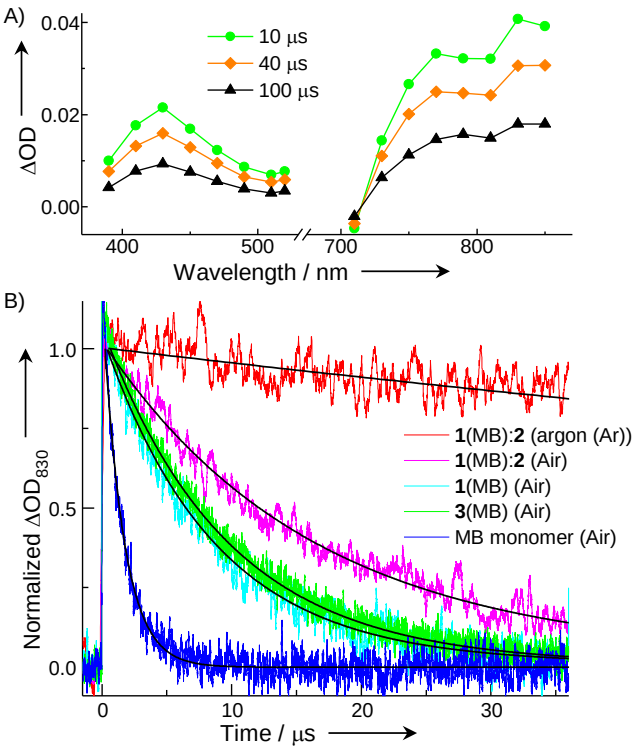

Fig. 2. Formation and decay of methylene blue (MB) in the triplet excited state. (A) Transient absorption spectra for **3** observed after the laser flash excitation. (B) Time profiles of formation and decay of MB in the triplet excited state. The sample aqueous solution contained 30 μM DNA, 100 mM NaCl in 10 mM Na phosphate buffer (pH 8.0). The smoothed black curves superimposed on the experimental data are the single exponential fit from which the lifetime of MB in the triplet excited state (τ_T) was determined.

Table 1. Lifetime of the Triplet Excited State (τ_T) and Bimolecular Reaction Rate Constant between $^3MB^*$ and O_2 (k_{O_2}) Measured by Transient Absorption

entry	τ_T (μs)	k_{O_2} (M^{-1} s^{-1})
MB monomer	1.6 ± 0.1	1.9×10^9
1(MB)	8.5 ± 0.4	3.4×10^8
1(MB):**2**	16 ± 0.4	1.9×10^8
3(MB)	9.5 ± 0.1	3.3×10^8

We next tested whether changes in the solvent accessibility of fluorescent molecule can be extracted by monitoring the blinking utilizing FCS. To enable observation of the blinking, a fluorescent molecule should have a high fluorescence quantum yield (Φ_F) and a moderated Φ_T.[8,9] For this reason MB is inadequate for the FCS measurement (MB: Φ_T = 0.52,[23] Φ_F = 0.02[24] in H_2O). We therefore selected R6G as a fluorescent molecule for observation of the triplet blinking (R6G: Φ_T = 0.002 in EtOH,[25] Φ_F = 0.89 in H_2O[26]). τ_{OFF} was obtained by fitting the data using the following equation (2),[27-33]

$$G(\tau) = 1 + \left(\frac{1}{N}\right)\left(\frac{1}{1+\tau/\tau_D}\right)\left(\frac{1}{1+(1/S^2)(\tau/\tau_D)}\right)^{\frac{1}{2}}\left(1+\left(\frac{\tau_{Off}}{\tau_{On}}\right)\exp\left(-\tau\left(\frac{1}{\tau_{Off}}+\frac{1}{\tau_{On}}\right)\right)\right) \quad (2)$$

where N is the number of molecules within the sample volume element, τ_D is the translational diffusion time, $S = \omega_1/\omega_o$ is the radius of the measurement area ω_0 and half of its axial length ω_1, τ_{ON} is the length of the duration of the "on" time, and τ_{OFF} corresponds to τ_T. Consistent with the results of transient absorption measurements

obtained for MB-modified DNA, τ_{OFF} decreased with increases in the solvent accessibility of R6G (Fig. 3), showing that the solvent accessibility of a fluorescent molecule can be evaluated by monitoring the blinking of the fluorescence.

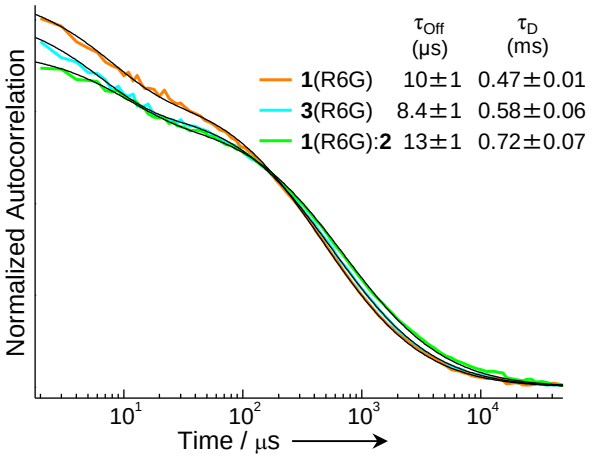

Fig. 3. Fluoresence correlation spectroscopy (FCS) time traces for **1**(R6G), **3**(R6G), and double–stranded **1**(R6G):**2**. The sample aqueous solution contained 5 nM DNA, 100 mM NaCl, 10 mM $MgCl_2$, 3% PEG-20,000 in 10 mM Na phosphate buffer (pH 7.0). The smoothed black curves superimposed on the experimental data correspond to the theoretical fitting curves.

Finally, we tested whether the function of a molecular beacon type probe can be detected by monitoring the blinking of the fluorescence. We selected Human T-cell lymphotropic virus type 2 DNA (AF412314)[34] as a target and synthesized the probe **4**. Probe **4** was designed to have an R6G group at its loop region to expose R6G in the absence of the target, while R6G is buried in the context of the duplex in the presence of the target gene **5**. Interestingly, in additon to the increase in τ_D due to the increase in the size of the complex upon hybridization, an increase in the τ_{OFF} was observed in the presence of the target gene **5** (Fig. 4), which could be measured using less than 2 fmol of probe (< 0.1 nM, 20 μL). These results clearly demonstrated that the function of a molecular beacon-type probe can be analyzed by monitoring the changes in the τ_{OFF} during the blinking of the fluorescence triggered by changes in the solvent accessibility of fluorescent molecules.

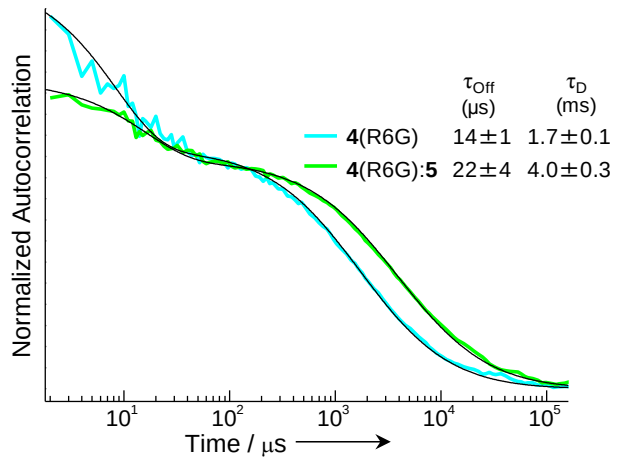

Fig. 4. FCS time traces for **4**(R6G) and **4**(R6G):**5**. The sample aqueous solution contained 0.1 nM DNA, 100 mM NaCl, 10 mM $MgCl_2$, 5% PEG-20,000 in 10 mM Na phosphate buffer (pH 7.0). The smoothed black curves superimposed on the experimental data correspond to the theoretical fitting curves.

Conclusions

In summary, we report that blinking triggered by the formation of the triplet state offers information regarding the solvent accessibility of the fluorescent molecule. It was demonstrated that the conformational changes of DNA structure can be monitored on the level of single fluorescent molecules traversing the detection volume element of FCS. While we tested two fluorescent molecules, MB and R6G, the effect of the conformational transition from the hairpin to the duplex on the τ_T or τ_{OFF} was more pronounced for MB-modified DNA. These results suggested that the properties of fluorescent molecules such as size and hydrophobicity, affect its solvent accessibility under a certain environment. The investigation of fluorescent molecules that sharply change their solvent accessibility, reflecting the local environment of interest, may lead to the development of an environmentally sensitive blinking probe that works on the single-molecule level.

We thank Dr. N. Kato of Olympus for assistance with the data analysis. This work has been partly supported by a Grant-in-Aid for Scientific Research (Innovative Areas ‘‘Nanomedicine Molecular Science’’ (No. 2306) and Projects 25220806, 23240074, 24350084 and 23655155) from the MEXT of Japanese Government, and the SENTAN project from Japan Science and Technology Agency. T.M. thanks to the WCU (World Class University) program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (R31-10035) for the support.

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