Elucidation of a “Signal-ON” Mechanism of Aggregation-Induced Emission Dye-Labelled DNA/DNA Duplexes and Application of a Repeat DNA Detection Method

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Abstract
We reveal a fluorescence enhancement mechanism of an aggregation-induced emission (AIE)-DNA probe for target DNA detection. The enhancement is caused by the restriction of intramolecular rotation (RIR) effect that arises from steric hindrance between the AIE dye moiety in a hybridized probe and the dangling end (free-ssDNA) or peripheral dsDNA. Thus, these AIE-DNA probes can selectively detect two or more DNA repeat sequences, such as telomeric repeats.

Keywords: Aggregation induced emission; Telomeric repeats; Restriction of intramolecular rotation

Introduction
Aggregation-induced emission (AIE) dyes have attracted significant attention in the fields of bio-imaging [1,2] and bio-sensing [3-5]. These dyes exhibit drastically enhanced fluorescence due to aggregation and concomitant RIR [6,7]. The AIE phenomenon triggered by RIR is also observed when AIE dyes bind to target molecules [8,9]. Thus, when AIE dyes recognize and bind to target molecules the fluorescent intensity is enhanced. Therefore, AIE dye-based sensing is intrinsically a “Signal-On” mode, and is suitable for sensitive detection of biomolecules. AIE dye-modified biomolecular probes can be prepared easily to be selective for a target molecule owing to the high recognition ability of biomolecules, such as DNAs, peptide nucleic acids (PNAs), peptides, and proteins. Furthermore, the AIE dye has a signal switchover function. Thus, the AIE-labeled probe does not require the dual modification of the probe, such as a DNA molecular beacon, which has two different fluorophores at both ends of the DNA: the 5’- and 3’-termini [10,11].

The first report describing an AIE-labeled DNA probe was revealed by Li et al. [12]. More recently, we have reported an AIE-dye labeled PNA probe [13]. The behaviors of these two probes were completely different in aqueous media. In the case of the AIE-DNA, non-hybridized probes were dispersed and did not emit. However, once these probes hybridized with a DNA target, the fluorescent intensity was enhanced. In contrast, in the case of the AIE-PNA, non-hybridized probes aggregated to form nanoparticles because of the high hydrophobicity of PNA compared with DNA. These nanoparticles were found to emit brightly, but once hybridized, fluorescence quenching was observed. The explanation to this phenomenon was that the fluorescence quenching observed in duplex formation was attributed to the collapse of the PNA probe nanoparticles, which exhibited strong emission. From a sensing viewpoint, the signal-ON mode system is desirable and the fluorescence of an AIE-PNA/DNA duplex should also increase as well as an AIE-DNA/DNA duplex, according to the above-mentioned intrinsic property of AIE dyes. Thus, we have studied the mechanism of “Fluorescence-ON” in AIE-labeled DNA/DNA and AIE-labeled PNA/DNA duplexes, and the possible applications of AIE-labeled probes.

Materials and Methods

Materials and instruments

All reagents for the synthesis of AIE dye were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and used without further purification. DNA phosphoramidite monomer and coupling agents were purchased from Glen Research (VA, USA). Telomeric DNA and negative control DNA and other sequences were custom-synthesized by Hokkaido System Science Co., Ltd. (Hokkaido, Japan). DNA strands conjugated to AIE-probe was synthesized with 3400 DNA synthesizer (Applied Biosystem, USA). Synthesized AIE dye and AIE-probe were identified by ECA-500 NMR spectrometer (JEOL, Japan), ZQ 2000 LCMS (Waters, USA) or MALDI-TOF-MS (Bruker Daltonics, USA). Concentrations of DNAs and AIE-probes were determined using V630 spectrophotometer (JASCO, Japan). Fluorescence spectra were measured at 4°C using RF-5300PC spectrofluorometer (Shimazu, Japan) or FP-6200 spectrofluorometer (JASCO, Japan).

Syntheses of TPA-COOH

p-(Diphenylamino)benzaldehyde (277 mg, 1.01 mmol) and malonic acid (350 mg, 5.10 mmol) were dissolved in pyridine (5 mL), and piperidine (0.25 mL) was added to the solution. The mixture was heated at 80°C for overnight. After the reaction solution was cooled to room temperature, 1M-HCl aq. was added to the mixture. The resulting precipitate was collected by suction filtration and washed with distilled water. The obtained light yellow powder was recrystallized from MeOH and the resulting orange crystal was dried under vacuo. Yield: 78% (248 mg, 0.787 mmol). 'H-NMR (CDCl3, 500 MHz): δ (ppm) 7.72 (d, J = 16 Hz, 1H), 7.39 (d, J = 8.5 Hz, 2H), 7.30 (t, J = 8.0 Hz, 4H), 7.13 (d, J = 7.0 Hz, 4H), 7.12 (t, J = 7.3 Hz, 2H), 7.01 (d, J = 8.5 Hz), 6.29 (d, J = 16 Hz, 1H), 13C-NMR (CDCl3, 500 MHz): δ (ppm) 172.57, 150.27, 146.78, 146.65, 129.58, 129.51, 126.89, 125.51, 124.15, 121.41, 114.11. ESI-MS: m/z calc for C19H14N2O3: 315.13, found 316.20 (M+H+).

Synthesis of Fmoc-Acp-Phosphoramidite

Fmoc-Acp-ol (102 mg, 0.300 mmol) was suspended in DCM/

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ACN (4 mL/1 mL), and then DIPEA (137 µL, 0.800 mmol) was added dropwise to the solution. After the mixture was stirred for 5 min at room temperature, 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (134 µL, 0.600 mmol) was dropped and stirred for 10 min. The solvent was evaporated off, and the residue was purified by column chromatography (DCM/ethyl acetate/TEA=5/1/0.01). The resulting phosphoramidite was immediately used for DNA solid phase synthesis.

Syntyes of TPA-DNA probe using solid-phase peptide synthesis

2-Cyanoethyl groups of oligonucleotides prepared were deprotected by flushing with 2% diisopropylamine in ACN (3 mL × 2) without cleavage from the solid support. Subsequently, Fmoc group was deprotected by using 20% piperidine in DMF (2 mL) for 15 min. The prepared resin was washed with ACN, and dried by blowing N₂ gas.

TPA-COOH (15 mg) was dissolved in NMP containing 0.15 M HATU, 0.15 M HOBT and 0.3 M DIPEA. This solution was passed and filled on column with terumo syringes containing the prepared resin at either end of column. Coupling reaction was performed for 1 hr (× 2). After the coupling reaction was finished, the resin was washed with ACN and dried by blowing N₂ gas. Synthesized AIE-dye-labeled oligonucleotide was cleaved from the resin with 25% NH₄OH, and deprotected for overnight at 55°C. Crude TPA-DNA was purified by Sep-Pak C18 cartridge column (Waters) and HPLC using 0.01 M TEAA in MilliQ water and 0.01 M TEAA in MeOH. Total reaction scheme was shown in Figure 1. The TICT (Twisted-Intramolecular Charge Transfer) property of obtained TPA-DNA probe was confirmed by the observation of fluorescence quenching in polar solvents. Consequently, TPA-DNA probe emitted weak fluorescence in aqueous media.

Results and Discussion

In this paper, we report the behavior of AIE-labeled telomeric DNA (CCCTAA)ₙ₂₂ probes in the presence of various types of DNA, including fully-matched DNA with the same length to probe sequence, telomeric repeat DNA of various lengths, mismatched DNA, and fully-matched/mismatched fused sequences. To investigate the relationships between fluorescence enhancement and probes/target interactions, the target sequence chosen was telomeric DNA [5’-(TTA GGG)-₃’] [14]. Telomeric DNA is a repeat sequence found at the end of chromosomes, and the telomere is elongated by telomerase in tumor cells [16,17]. Therefore, telomere sequences are suitable for considering the assembly effect of an AIE dye and in the understanding of the “Fluorescence-ON” mechanism in AIE-labeled probe/DNA duplexes. Moreover, by using this target, we could investigate the applicability of the AIE-based assay for cancer diagnosis as an alternative to the gel electrophoresis based TRAP assay [18]. The AIE dye chosen was a triphenylamine (TPA)-derivative, and it was linked to the complementary strands of a 12-mer human telomeric repeat DNA (5’-CCC TAA CCC TAA-3’).

The target sequences chosen for our study were 12L, 24, 36, 48, 60, 72, and 84-mer human telomeric sequences [HT12, HT24, HT36, HT48, HT60, HT72, and HT84: 5’-(TTA GGG)-₃’, n=2, 4, 6, 8, 10, 12, 14], negative control sequences [NC48: 5’-(TGA GTG)-₃’, n=2, 8], complementary strand of the negative control sequences [NCC12, NCC48: 5’-(CAC TCA)-₃’, n=2, 8], and a fully-matched/mismatched fused control sequence (HT-NC36: 5’-TGA GTG TGA GTG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TGA GTG TGA GTG-3’). The corresponding TPA-labeled probe sequences used were TPA-DNA6 (5’-TPA-linker-(CCC TAA)-₃’) and TPA-DNA12 (5’-TPA-linker-(CCC TAA)ₙ₂₂-3’). Details of the AIE dye and AIE probe syntheses are presented in the supplementary information (ESI).

The preparation of DNA samples for measuring was carried out as follows: i) HT, NC, NCC, and HT-NC DNA were diluted with ultra-pure water and prepared to the required concentrations; ii) 5x Tris-HCl buffer (250 mM LiCl and 100 mM Tris-HCl, pH 7.5) was added (40 µL); iii) the probe solution was added to each sample solution (total volume was 200 µL). Then, the mixtures were heated to 90°C for 2 min and then cooled to 4°C for 2 h in a thermal cycler.

First of all, we have investigated the fluorescent enhancement of an AIE probe in the presence of different lengths of telomeric DNA. Figure 2 shows the fluorescence spectra and increase ratio in the presence of each length of telomeric DNA (HT12-HT84). Each telomeric DNA sample concentration is as follows: HT12: 1 µM, HT24: 0.5 µM, HT36: 0.33 µM, HT48: 0.25 µM, HT60: 0.20 µM, HT72: 0.167 µM, and HT84: 0.143 µM. When each concentration was converted to the telomeric
unit (12-mer: 5’-TTA GGG TTA GGG-3’) concentration, all samples had the same concentration [e.g., HT48: 4 (Unit number) × 0.25 µM (Concentration)=1 µM (Unit concentration); HT72: 6 (Unit number) × 0.167 µM (Concentration)=1 µM (Unit concentration)]. F1 and F0 represent the fluorescence intensity at 505 nm of the sample with telomeric DNAs (HT12-HT84) and without telomeric DNA (AIE probe only), respectively.

According to our previous studies, [19] the fluorescent intensities were proportional to the concentrations of repeated DNA unit (12-mer: 5’-TTA GGG TTA GGG-3’). Thus, our prediction in advance was that all samples would exhibit approximately the same increase of fluorescence. In the case of HT24-HT84, the fluorescence were certainly comparable enhancement, while no fluorescence enhancement was observed with HT12. This is because when TPA-DNA12 (12-mer) and HT12 (12-mer) formed a duplex structure, the intramolecular rotation of the AIE dye moiety in the hybridized probe was not restricted owing to no steric hindrance (Figure 3). While in the case of HT24-HT84, the AIE moieties of the hybridized probes, except for termini, were influenced by the adjacent duplex and intramolecular rotations were restricted and fluorescence enhancement was observed.

When TPA-DNA6 (6-mer) was used as a probe towards HT12, an enhancement in the fluorescence was observed because two probe molecules can bind to HT12 (ESI). The essential point for fluorescence enhancement was the existence of peripheral dsDNA beside the AIE dye moiety, and consequent interference of intramolecular rotations. Fluorescence enhancement was found to not be concentration dependent for each TPA-DNA12/HT48 duplex concentration examined (ESI). Hence, intermolecular hydrophobic interactions among hybridized probes has not occurred, even multiple hydrophobic AIE probes bound to single telomeric DNA strands.

To investigate the effects of free-ssDNA and/or dsDNA at both ends of the target (telomeric) DNA, we have mixed a strand of telomeric DNA with the negative control sequence, which has a 12-mer telomeric of the target (telomeric) DNA, we have mixed a strand of telomeric DNA strands.

AIE probes bound to single telomeric DNAs.

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To investigate the effects of free-ssDNA and/or dsDNA at both ends of the target (telomeric) DNA, we have mixed a strand of telomeric DNA with the negative control sequence, which has a 12-mer telomeric unit and two 12-mer negative control sequence regions at both ends of the telomeric sequence (HT-NC36). The chosen negative control sequence showed no fluorescence enhancement when using NC48 and TPA-DNA12 as probes (ESI). Figure 4 shows the fluorescence increase ratio (F1/F0) of HT12/TPA-DNA12, HT-NC36/TPA-DNA12 (where ssDNA was present at the 3’ and 5’ dangling ends) and HT-NC36/TPA-DNA12+2eq. NCC12 (where dsDNA existed at both ends). When the dangling end or negative control dsDNA existed at the 3’ end of the target strand, the fluorescence intensities were found to increase, and this was dependent on the HT-NC36 concentration.

Without regard to the concentrations, F1/F0 of HT12 samples were approximately 1. This means no fluorescence enhancement was observed owing to the lack of RIR effect, as mentioned above. In both ssDNA and dsDNA, the F1/F0 values between 8 µM and 4µM were almost identical. These results indicate the intramolecular interaction did not affect the rotation of AIE moieties.

While, the clear difference of F1/F0 was observed between ssDNA and dsDNA. As already reported, [20,21] dsDNA is much more rigid than ssDNA, and the short length dsDNA. Thus, the reason for these differences in F1/F0 may be attributed to the difference in steric hindrance between flexible ssDNA and relatively rigid dsDNA.

We have reported previously the detection of telomeric DNA using an AIE-PNA probe [13]. In this report, we have shown that the fluorescent intensity diminishes as a function of the target DNA concentration. However, the fluorescence was not completely quenched, even in the saturated region. This indicates that the intramolecular rotation of AIE dyes is still restricted by steric hindrance of neighboring DNA/PNA hybrid duplexes in a similar manner to the present case.

Judging from these results, the AIE probe cannot detect the same length-fully-matched target DNA without a dangling end or peripheral dsDNA that interferes with the intramolecular rotation; therefore, two or more repeated DNAs are selectively detected. Otherwise, if the AIE probe can replace the corresponding strand, including a genomic duplex, the duplex may be detectable. Therefore, this system
may be applied to determine single-nucleotide polymorphisms [22]. When these probes are applied to biological samples, it is necessary to consider the effect of chromosomal DNA. In other words, a DNA detection probe requires high selectivity towards the target DNA, even in the presence of other DNAs. For example, in our previous report describing a telomeric DNA detection method with an induced FRET system, ethidium bromide [23] and the intercalator were found to interact with chromosomal DNA, thereby influencing the signal response [19]. Thus, we investigated the efficiency of the AIE probe for quantification of telomeric DNA under the coexistence of decoy dsDNA. Figure 5 shows the FI/Fo without (blue) or with (red) 10 µM dsDNA (NC48/NCC48). The result shows that the AIE probe can quantify telomeric DNA using either condition. In other words, the AIE dye moiety did not intercalate between DNA base pairs, and the restriction of intramolecular rotation of the AIE probe was not influenced by the coexistence of dsDNA.

**Conclusions**

We have revealed a fluorescence enhancement mechanism of an AIE probe during target detection. The enhancement is caused by the RIR effect, which arises from steric hindrance of a dangling end (free-ssDNA) or peripheral dsDNA. In the case of an AIE probe/fully-

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**Figure 3:** Schematic of the fluorescence enhancement mechanism by restriction of intramolecular rotations. (a) TPA-DNA12 and HT12. (b) TPA-DNA12 and HT24-HT84.

**Figure 4:** Fluorescence increase ratio of 3 µM of TPA-DNA12 and 0.12–8 µM of HT12, HT-NC36, and HT-NC36/NCC12 in 50 mM LiCl and 20 mM Tris-HCl buffer (pH 7.5), r.t., λex=360 nm (left). Strength of the RIR effect of the AIE dye in HT12, HT-NC36 (ssDNA), and HT-NC36/NCC12 (dsDNA) (right). Error bars indicate the standard error of the mean for n=3.
matched DNA duplex, the degrees of free intramolecular rotation and the surrounding environment were approximately equal to the AIE probe. Therefore, at least two or more repeat DNA units are required for selective detection with the AIE probe, and non-repeat DNA was also detected when a dangling end or peripheral dsDNA existed in the probe-target duplex, e.g., TPA-DNA12/HT-NC36 and TPA-DNA12/HT-NC36/NCC48.

Because the AIE-labeled probe does not require dual modification with two different fluorophores or a fluorophore and quencher such as a DNA molecular beacon, probe preparation is rather convenient. Furthermore, the AIE-labeled probe is not influenced by other dsDNA molecules, which is unlike the induced FRET method with an intercalator. Many AIE dyes with various emission wavelengths have been reported [24-28]; therefore, we can choose the most suitable dye for a particular purpose. In addition, this detection concept can be applied to a DNA assay using an AIE PNA probe that has higher dye for a particular purpose. In addition, this detection concept can be applied to a DNA assay using an AIE PNA probe that has higher

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