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ARTICLE *in* CHEMBIOCHEM · NOVEMBER 2006

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G Quadruplex-Based FRET Probes with the Thrombin-Binding Aptamer (TBA) Sequence Designed for the Efficient Fluorometric Detection of the Potassium Ion

Satoru Nagatoishi,[a] Takahiko Nojima,[a] Elzbieta Galezowska,[b] Bernard Juskowiak,[b] and Shigeori Takenaka*[c]

*The dual-labeled oligonucleotide derivative, FAT-0, carrying 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA) labels at the 5′ and 3′ termini of the thrombin-binding aptamer (TBA) sequence 5′-GGTTGGTGTGGTTGG-3′, and its derivatives, FAT-*n (n=*3, 5, and 7) with a spacer at the 5′-end of a TBA sequence of T_mA (m = 2, 4, and 6) have been designed and synthesized. These fluorescent probes were developed for monitoring K^+ concentrations in living organisms. Circular dichroism, UV-visible absorption, and fluorescence studies revealed that all FAT-*n probes could form intramolecular tetraplex structures after binding K^+. Fluorescence resonance energy transfer and quenching results are discussed taking into account dye–dye contact interactions. The relationship between the fluorescence behavior of the probes and the spacer length in FAT-*n was studied in detail and is discussed.*

Introduction

The monitoring of K^+ in biological samples by using homogeneous fluorescent systems is an important challenge.[1,2] Recently, selective and sensitive K^+ assays based on quadruplex-forming oligonucleotides have been reported.[3,4] Guanine-rich oligonucleotides are known to form G quadruplex structures.[5] It is also known that a G quadruplex has a channel at its center with a diameter that correlates well with the ionic radius of K^+ (1.3 Å).[6] The ionic radius and charge are the parameters that determine how well various cations stabilize guanine tetrads. Our first K^+ sensor exploited fluorescence resonance energy transfer (FRET) as a transduction process. The human telomere sequence, $d(G_3(TTAG_3)_3)$, was labeled with fluorophores 6-carboxyfluorescein (6-FAM) and 6-carboxytetramethylrhodamine (6-TAMRA). The FRET probe obtained was called a potassium-sensing oligonucleotide (PSO)[3,7,8] and has been recommended for the monitoring of K^+ in an excess of Na^+.[3] Application of the PSO for real-time K^+ monitoring at physiological conditions appeared to be difficult because of the high stability constant of the potassium complex, which caused saturation of the probe signal at submillimolar concentrations of K^+. One of the major points of the system was that changes in observed fluorescence with changing K^+ concentration were much more complex than could be explained by FRET. For example, a higher FRET signal was observed for the PSO/Na^+ complex; this suggested stacking interactions of fluorophores in the chair- or propeller-type K^+-quadruplex (static quenching of TAMRA).[8,9]

In a subsequent fluorescent probe (PSO-py), we exploited pyrene excimer emission for the transduction of the cation binding by the 15-mer thrombin-binding aptamer (TBA; d(GGTTGGTGTGGTTGG)).[10] Unlike in the FRET approach, stacking interactions between fluorophores (pyrene) were advantageous and produced efficient excimer emission. Due to the orientation of pyrene rings the PSO-py/K^+ complex emitted excimer fluorescence, while the random-coil structure of PSO-py in the absence of K^+ gave only monomer emission. This sensor possesses many advantages; however, the short excitation wavelength of pyrene is a drawback for the application of PSO-py in biological systems.

In this paper, we have extended the studies with the TBA oligonucleotide on a FRET approach with the use of FAM and TAMRA dyes as fluorescent labels (Figure 1). Table 1 shows the designed probes, FAT-*n* (*n* = 0, 3, 5, and 7, where *n* is the length of an additional sequence attached to the 5′-end of TBA oligonucleotide). Oligonucleotides singly labeled with FAM or TAMRA (F-TBA and TBA-T, respectively) were used as refer-

[a] *S. Nagatoishi, Dr. T. Nojima*
Department of Applied Chemistry, Kyushu University
Motooka, 819-0395 (Japan)

[b] *E. Galezowska, Prof. Dr. B. Juskowiak*
Department of Analytical Chemistry, A. Mickiewicz University
Grunwaldzka 6 Poznań, 60-780 (Poland)

[c] *Prof. Dr. S. Takenaka*
Department of Materials Science, Kyushu Institute of Technology
Kitakyushu-shi, 804-8550 (Japan)
Fax: (+81) 93-884-3322
E-mail: shige@che.kyutech.ac.jp

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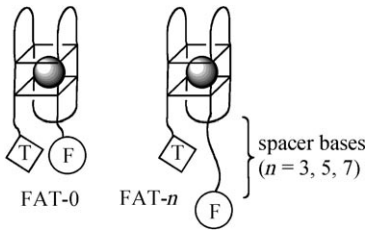
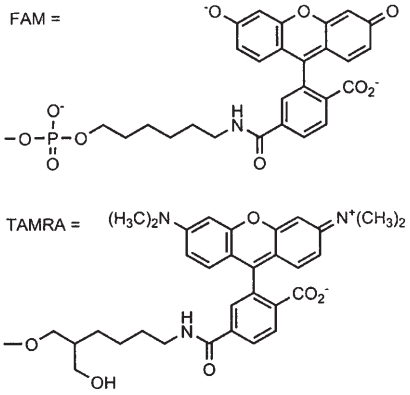


Figure 1. Differences in the locations of fluorophores for FAT-0 and FAT-n quadruplex structures.

Table 1. Oligonucleotides used in this experiment.

Abbreviation	Sequence
TBA	GGT TGG TGT GGT TGG
F-TBA	FAM-GGT TGG TGT GGT TGG
TBA-T	GGT TGG TGT GGT TGG-TAMRA
FAT-0	FAM-GGT TGG TGT GGT TGG-TAMRA
FAT-3	FAM-TTA GGT TGG TGT GGT TGG-TAMRA
FAT-5	FAM-TT TTA GGT TGG TGT GGT TGG-TAMRA
FAT-7	FAM-T TTT TTA GGT TGG TGT GGT TGG-TAMRA



ence probes. The extent of quenching of donor fluorescence and the enhancement of acceptor emission in this family of FRET probes should allow the estimation of FRET efficiencies and identification of other quenching phenomena in the system. Probes with a longer spacer were expected to show improved performance due to the elimination of unwanted contact quenching of TAMRA emission.

Results

A thrombin-binding aptamer (TBA) used in this study (Table 1) is known to form a chair-type intramolecular tetraplex structure in the presence of K^+,[12–14] as confirmed by circular dichroism (CD) spectroscopy.[12,15,16] Characteristic negative and positive bands are typically observed at 265 and 295 nm, respectively, upon addition of K^+.

Singly labeled probes, F-TBA and TBA-T

TBA oligonucleotides carrying FAM dye at the 5′-end or TAMRA at the 3′-end were synthesized to examine the effect of an attached oligonucleotide on the spectral behavior of FAM or TAMRA labels. CD spectra measured in 20 mM Tris-HCl (pH 7.4) in the presence of varied concentrations of K^+ showed the appearance of the characteristic G quadruplex signature: negative and positive bands at 265 and 295 nm respectively (Figure S1), similar to the case of the unmodified TBA/K^+ complex. Small negative bands observed in the CD spectra of F-TBA or TBA-T in the 450–600 nm range can be assigned to FAM or TAMRA absorption.

Formation of the quadruplex structure for F-TBA and TBA-T was confirmed by hyperchromicity of the UV absorption band at 295 nm.[17] The different extent of hypochromicity for F-TBA and TBA-T in the presence of KCl is probably linked to the effect of the nature of fluorescent label on the binding affinity for potassium.

A modest hypochromic effect ($H = -15\%$) and a small red shift ($\Delta\lambda = 4$ nm) for TBA-T absorption band at 560 nm were observed upon addition of potassium (0–5 mM), whereas the fluorescein band at 495 nm in the F-TBA spectrum exhibited a hyperchromic effect ($H = +15\%$) without any noticeable shift of the band maximum (Table 2).

Fluorescence enhancement was observed for both probes upon addition of K^+ (Figure S2). The origin of fluorescence enhancement seems to be different for each probe. Fluorescence changes for F-TBA were expected due to the effect of ionic strength on the protolytic equilibria of the FAM dye,[18,19] whereas the enhancement for TBA-T can be explained by a dequenching effect (quenching operates with unfolded oligonucleotides).[8] In conclusion, fluorescence emission of FAM or TAMRA dyes attached to TBA as single labels appeared to be sensitive to the formation of tetraplex structures in the presence of K^+, and these effects should be taken into account when FRET is considered for FAT-*n* probes.

FAT-0 probe

The spectral characteristics of FAT-0 carrying FAM and TAMRA dyes at both its termini (Table 1) were studied in the presence of different concentration of K^+ by using several techniques.

Table 2. Spectral characteristics of the fluorescent oligonucleotide probes

Absorption band	F-TBA	TBA-T	FAT-0	FAT-3	FAT-5	FAT-7
$\lambda = 495$ nm						
ε [10^4 mol^{-1} L cm^{-1}]	4.2	–	3.0	4.2	4.2	4.3
H [%][a]	+15	–	+30	+42	+41	+39
$\Delta\lambda$ [nm][a]	0	–	6	0	0	0
$\lambda = 560$ nm						
ε [10^4 mol^{-1} L cm^{-1}]	–	7.2	6.9	7.4	7.5	7.3
H [%][a]	–	−15	−32	−30	−24	−21
$\Delta\lambda$ [nm][a]	–	4	4	4	4	4

[a] In the presence of 50 mM KCl.

CD spectra of FAT-0

The changes in the CD spectra of FAT-0 induced by the addition of K^+ were similar to those observed for singly labeled probes and suggested that FAT-0 also adopts only two possible structures: the extended and the tetraplex forms (Figure S3 A).[10,15] Interestingly, upon addition of K^+, negative and positive peaks appeared (490 and 560 nm respectively) that corresponded to the FAM and TAMRA dyes, respectively. Human telomere tetraplexes carrying FAM and TAMRA dyes described in previous papers exhibited a similar peculiarity,[3,8,20] which was attributed to the interactions between FAM and TAMRA dyes after forming the tetraplex structure.

Absorption spectra of FAT-0

Tetraplex formation by the FAT-0 probe was supported by the hyperchromic effect at 295 nm (Figure S4) and, similar to the case of singly labeled reference probes, the absorption bands of the labels were significantly affected by folding of the oligonucleotide in the presence of K^+ (Table 2, Figure S4 A). The FAM absorption band exhibited a 6 nm blue shift upon addition of K^+ (0–10 mM), and the molar absorptivity increased by 30%, while the hypochromicity of the TAMRA band reached −32% with a 4 nm red shift. These results are consistent with other reports,[21,22] in which observed spectral changes were ascribed to dye–dye interactions.

Fluorescence spectra of FAT-0

Fluorescence spectra of FAT-0 changed upon addition of K^+ (Figure 2 A). Upon excitation at the FAM absorption band (495 nm), fluorescence bands at both 518 and 585 nm (FAM and TAMRA emission, respectively) were observed. This result evidences the occurrence of FRET from FAM to TAMRA even in the absence of K^+. This is reasonable if one compares the distance between fluorophores in a 15-mer oligonucleotide (ca. 62 Å)[4] with the expected Förster radius, (R_0 = 45–60 Å).[11,20,22,23] Surprisingly, addition of K^+ resulted in fluorescence quenching of both bands (Figure 2 A). Excitation spectra monitored at the wavelength of TAMRA emission (576 nm) exhibited two bands that corresponded to TAMRA and FAM absorption maxima, thus supporting the presence of FRET both in the absence and presence of K^+ (Figure 2 B). Although fluorescence intensities of FAM and TAMRA labels decreased with an increase in K^+ concentration, the intensity ratio (F_{495}/F_{560}) increased from 0.190 to 0.35 in the 0–10 mM concentration range of K^+. The apparent quenching of TAMRA emission can be accounted for by the interplay of two factors: a decrease in the molar absorptivity of TAMRA (Table 2) and a possible static quenching due to interactions with the FAM moiety. The importance of these two factors was confirmed by recording the emission spectra of FAT-0 excited at the TAMRA absorption band, λ_{ex} = 560 nm (Figure 2 C). The decrease in fluorescence intensity approaches 50% at 10 mM K^+, which is too high to be accounted for exclusively by the absorbance changes (H = −32%). Furthermore, the opposite effect of the K^+ ion on

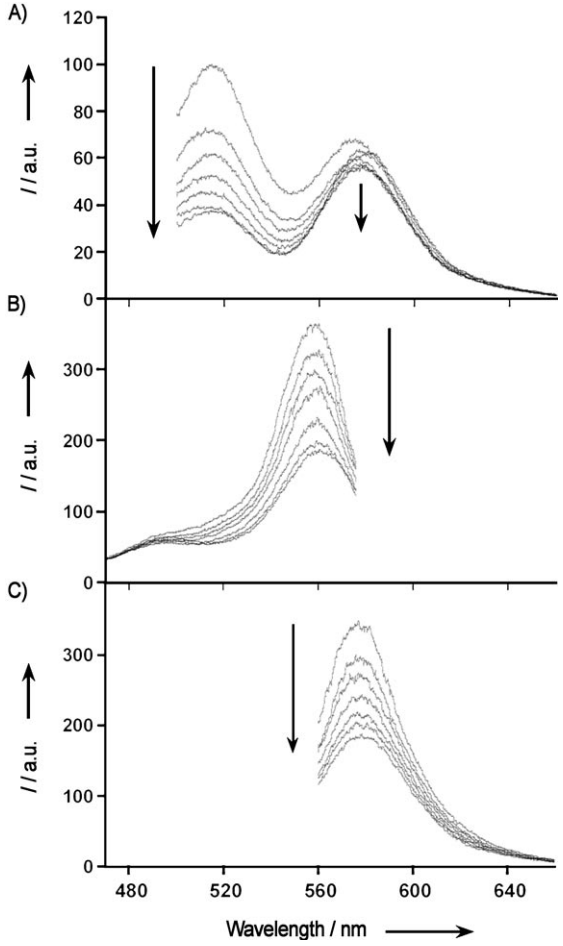


Figure 2. KCl effect on the fluorescence spectra of 0.2 μM FAT-0. A) Emission spectra with λ_{ex} = 495 nm; B) Excitation spectra with λ_{em} = 576 nm; C) Emission spectra with λ_{ex} = 560 nm. Conditions: Tris-HCl buffer (20 mM, pH 7.4), KCl varied from 0 to 100 mM.

TAMRA emission for the TBA-T probe (TAMRA emission was enhanced by ca. 15%) indicates clearly that dye–dye interactions play a crucial role in the quenching process. The quenching of TAMRA fluorescence has already been suggested for similar dual-labeled systems: duplex DNA,[21] i-motif structures,[22] and tetraplex species.[8]

FAT-*n* probes (*n* = 3, 5 and 7)

To reduce the contact quenching by dye–dye interaction, FAT-*n* (*n* = 3, 5, and 7) derivatives were designed by introducing oligonucleotide spacers at the 5′-end of the TBA sequence (Table 1). Investigated probes were expected to show improved performance due to the elimination of unwanted contact quenching of the TAMRA emission.

CD spectra of FAT-*n*

The negative and positive bands around 265 nm and 295 nm in the CD spectra of FAT-*n* probes evidenced quadruplex formation upon addition of K^+ (Figure S3 B). However, unlike the

FAT-0 probe, only negative bands were present at 490 and 560 nm (FAM and TAMRA absorption, respectively). Thus, the CD spectra of FAT-*n* can be regarded as linear combinations of the CD spectra of F-TBA and TBA-T. This is evidence that the insertion of a spacer in FAT-*n* diminished dye–dye contact interactions.

Absorption spectra of FAT-*n*

Spectral characteristics of FAM and TAMRA absorption bands for all FAT-*n* probes are summarized in Table 2. The molar absorptivities of FAM and TAMRA in the absence of potassium are larger than those for FAT-0. Different sequences at the 5′ termini of FAT-*n* and FAT-0 (T_mA vs. GGG, see Table 1) may be responsible for changes in FAM absorbance. The molar absorptivity of TAMRA is probably affected by the different extent of interactions with the oligonucleotide bases, especially adjacent guanines.[24] Alternatively, FAT-0 might form a hairpin structure in the absence of K^+ as previously suggested.[8] Such a hairpin might be stabilized by dye–dye interactions in the case of the blunt-end FAT-0 structure. Fluorescence anisotropy measurements for the TAMRA label in TBA-T and FAT-0 in the absence of K^+ showed that the *r* value for FAT-0 was slightly higher than that for TBA-T (0.1560 vs. 0.1542). The restricted rotational depolarization of TAMRA in FAT-0 is in agreement with the above discussion.

Hypochromicity of the TAMRA band observed upon addition of K^+ probably reflects dye–dye interactions, since its value decreases with an increase in the spacer length (FAT-3 > FAT-5 > FAT-7, Table 2).

Fluorescence spectra of FAT-*n*

FRET is also expected to operate in the extended FAT-*n* probes (in the absence of K^+) since the addition of spacer groups (3 to 7 bases) does not change the donor–acceptor separation distance dramatically. Figure 3A supports this: in the absence of K^+, the fluorescence intensity of the FAM emission band increases with the length of the spacer, and the relative fluorescence intensity of the TAMRA band decreases. These spectral changes reflect a drop in FRET efficiency caused by the increase in the distance between FRET partners. One cannot, however, exclude other factors: for example, an increase in molar absorptivity of FAM (Table 2), or in the fluorescence quantum yield of this fluorophore, as well as the avoidance from quenching of FAM by adjacent G-bases.[24–26] Upon addition of K^+, all probes exhibited significant quenching of a donor band (FAM), which was accompanied by the enhanced fluorescence of the acceptor (TAMRA; Figure 3B).

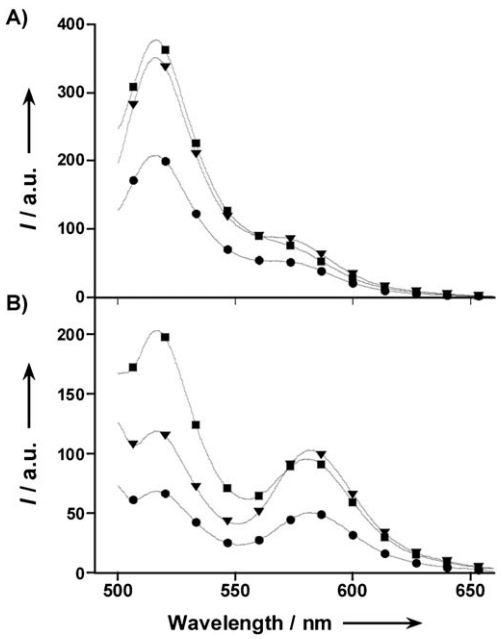


Figure 3. Effect of the spacer length on the fluorescence emission spectra of FAT-*n* probes ($n=3$ (●), $n=5$ (▼), $n=7$ (■) in the A) absence or B) presence of 100 mM KCl. Conditions: 20 mM Tris-HCl buffer (pH 7.4), $\lambda_{ex}=495$ nm.

Discussion

FRET and dye–dye interactions in FAT-0

Fluorescence enhancement for the FAT-0 probe was not observed after formation of the tetraplex structure because of the suspected dye–dye interactions. Although similar dye–dye interactions were observed in previously reported studies with human telomere tetraplex systems carrying FRET dyes, the enhancement of acceptor fluorescence was observed at that time.[3,20] The difference between the spectral behaviors of these two systems (TBA vs. human telomeric DNA) can be explained in terms of the different structures of the quadruplexes. The human telomere oligonucleotide can form an intramolecular "basket"-type quadruplex, where two fluorophores are separated by a loop that diagonally crosses the quadruplex and inhibits the dye–dye interactions. On the other hand, the TBA oligonucleotide forms only the compact intramolecular "chair"-type tetraplex structure. In this quadruplex, fluorophores are positioned side by side on neighboring strands, and such a location facilitates direct dye–dye interactions (static quenching). In the similar i-motif tetraplex system reported by Mergny et al.,[22] two dyes were located on adjacent DNA strands; this led to quenching of acceptor fluorescence. Such dye–dye interactions are regarded as the main causes of static quenching or ground-state quenching,[27,28] as well as with the formation of H-type heterodimer species.[29,30] The proximity of labels in the TBA tetraplex has been proved in a sensing probe based on pyrene excimer emission, where a TBA oligonucleotide carrying pyrenes at its termini showed very strong excimer emission after the formation of a tetraplex structure with K^+.[10]

FRET process in FAT-*n* probes

Förster showed that the efficiency of FRET depends on the inverse sixth power of the distance between the two fluoro-

phores [Eq. (1)], and this is the basis of the use of this technique to provide structural information:[11,31]

$$E = (1 + R^6/R_0^6)^{-1} \quad (1)$$

where R is the distance between the fluorophores. R_0 is the characteristic Förster radius for a donor–acceptor pair, which is given by:

$$R_0^6 = 8.8 \times 10^{-28}\, \Phi_D \kappa^2 n^{-4} J(\nu) \quad (2)$$

where Φ_D is the fluorescence quantum yield of the donor in the absence of the acceptor, κ^2 is a parameter that depends on the relative orientation of the donor and acceptor transition moments, n is the refractive index of the medium, and $J(\nu)$ is the spectral overlap between donor emission and acceptor absorption. From Equation (1), it is easily seen that the efficiency of FRET is 50% when $R = R_0$. Quantitative measurements of fluorescence energy transfer give information on spatial orientation of the donor and acceptor; thus, FRET should provide structural parameters for the particular topological forms of tetraplexes.

FRET efficiency (E) can be measured from a decrease in fluorescence of the donor ($E = 1 - F_D/F_{0,D}$) or from an increase in sensitized fluorescence of the acceptor.[11] To assess FRET efficiency from the sensitized fluorescence of the acceptor (TAMRA), one needs to calculate (ratio) values from excitation spectra.[11] FRET efficiency can then be determined by using the following expression:

$$\text{ratio} = \frac{F_{495}^A}{F_{560}^A} = \frac{\varepsilon_{495}^D E + \varepsilon_{495}^A}{\varepsilon_{560}^A} \quad (3)$$

where F_{495}^A is the fluorescence of the acceptor (TAMRA) measured when the FAT-n probe is excited by FRET (at 495 nm), F_{560}^A is the acceptor emission directly excited at 560 nm, ε^D and ε^A are the molar absorption coefficients of the donor and the acceptor at the indicated wavelengths.

In the unfolded probe form some FRET is expected since the average distance between the donor and acceptor (about 60–90 Å) corresponds to the critical radius of the FAM/TAMRA pair (ca. 60 Å).[23] Folding of the oligonucleotide into a quadruplex under increasing concentration of cations brings these two fluorophores closer, and energy transfer becomes more efficient (Figure 4A). All (ratio) plots exhibit a saturation tendency at high KCl concentrations. FAT-3, −5, and −7 show similar plots, but the dependence for FAT-0 is shifted significantly to lower ratio values. However, for a quantitative analysis of the structural parameters of the folding process, it is necessary to calculate FRET efficiency [Eq. (3)] since the investigated systems exhibited significant variation in the absorption spectra with KCl concentration (Table 2). Figure 4B shows the K^+ effect on the FRET efficiency for particular probes calculated by using Equation (3). There is no simple reason that can explain the obtained dependences. Apparently, FAT-0 exhibits lower FRET than other probes that disagree with the expected smaller separation distance between fluorophores. Moreover, the observed FRET efficiency order (FAT-7 > FAT-5 > FAT-3 > FAT-0) is opposite to that representing a decrease in separation distance. As can be easily calculated by using Equation (1), FRET efficiency values attained by the probes (0.30–0.44 in Figure 4B) correspond to separation distances (R) of 69–62 Å (assuming $R_0 = 60$ Å),[23] which are much too large for folded quadruplexes. Thus, the FRET plots shown in Figure 4A do not represent pure folding processes of the probes. It should be noted that these observations are consistent with results reported by Mergny et al. for the 21-mer oligonucleotide of human telomeric DNA sequence, where the R value of 40–50 Å was calculated for the folded quadruplex.[20] Undoubtedly, these systems cannot be discussed in terms of the FRET process exclusively; one should take into account a significant contribution from quenching processes that are not related to the FRET mechanism.

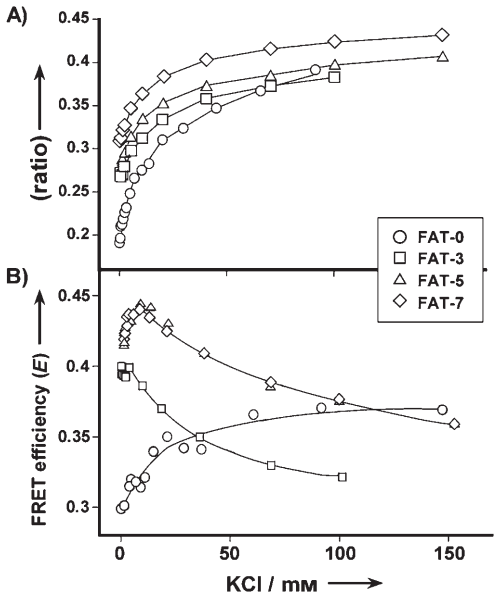


Figure 4. A) KCl effect on the ratio value for FAT-n probes. The (ratio) = $F_{ex,495}/F_{ex,560}$ was calculated from excitation spectra with $\lambda_{em} = 576$ nm. B) Effect of KCl on the FRET efficiency (E) calculated from excitation spectra by using Equation 3.

Figure 5 shows the effect of KCl on quenching efficiency ($E = 1 - F_D/F_{D,0}$) calculated from donor fluorescence changes (F_{518}). The dependences were plotted on the assumption that the singly labeled oligonucleotide (F-TBA) can serve as the reference probe to obtain $F_{0,D}$ for particular concentrations of KCl. Plots in Figure 5 perfectly reflect the expected effect of distance between donor–acceptor pair on FRET efficiency, giving the following order: FAT-0 > FAT-3 > FAT-5 > FAT-7. Assuming a two-component system composed of an unfolded probe (no KCl) and a folded quadruplex structure (complexed with K^+), one can derive information on the distances between the donor and acceptor in both conformations of probes.

From a model of an extended probe, the separation distance (R) can be expressed by:

$$R = l + n \times d \quad (4)$$

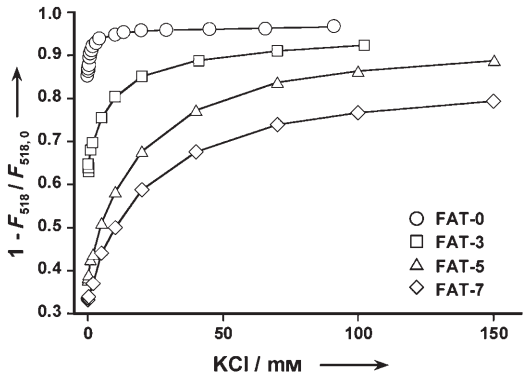


Figure 5. Effect of KCl on the FRET efficiency (E) of FAT-n probes. FRET efficiency was calculated from emission spectra, $E = 1 - F_{518}/F_{518,0}$.

where l is the contribution from the length of the TBA oligonucleotide (15-mer) and $n \times d$ stands for the spacer contribution with n being a number of nucleotides in a spacer and d a unit distance for one nucleotide. Combining Equations (1) and (4) gives the following relationship:

$$\sqrt[6]{1/E - 1} = l/R_0 + n(d/R_0) \quad (5)$$

The left side of this equation was plotted against the number of nucleotides in the spacer (n) of FAT-n probes (Figure 6). Two

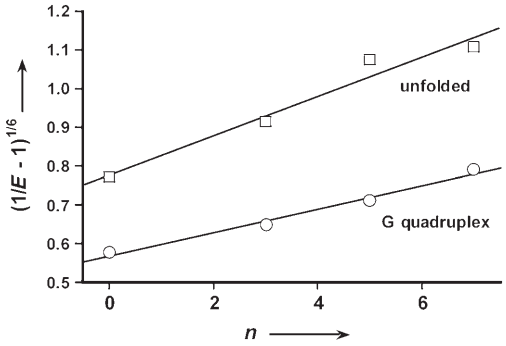
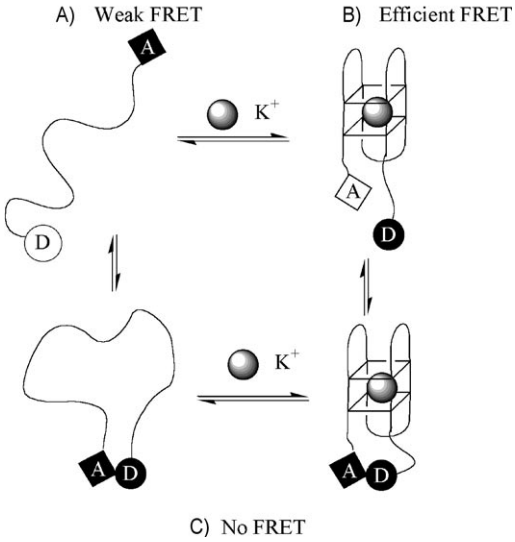


Figure 6. Correlation of the FRET efficiency and the length of spacer in FAT-n probes ($n = 0, 3, 5, 7$) according to Equation (5).

limiting cases were considered according to the two-state model: an unfolded probe (no KCl) and a folded quadruplex structure (excess of K^+). From slope and intercept values of linear plots in Figure 6 and with $R_0 = 60$ Å, the parameters l and d were calculated for the unfolded probes as 46 Å and 3.2 Å, respectively. Good agreement for the obtained structural parameters with those predicted from molecular modeling ($l \sim 50$ Å and $d = 3.4$ Å for the TBA 15-mer with stacked bases; Nucleic Acids builder implemented in HyperChem 3.0, Hypercube Inc.) indicates that FRET is mainly responsible for quenching of donor fluorescence in the unfolded structures of the probes. On the other hand, parameters obtained for the folded quadruplex ($l \sim 35$ Å and $d = 1.8$ Å) clearly show that in the presence of KCl, FRET plays a minor role and that other quenching mechanisms (e.g., static quenching) dominate. Interestingly, even in the FAT-7 probe, which contains a relatively long spacer that should protect fluorophores from direct interactions, non-FRET quenching operates (the calculated $R = 48$ Å cannot reflect the actual separation distance). An alternative explanation for the peculiar FRET results for FAT-n probes in the presence of KCl may involve other factors connected with the proper parameterization of Equation (2). For example κ^2 may take a value between 0 and 4 for DNA-attached fluorophores,[9,32] variations in fluorophore spectra influence the spectral overlap $J(\nu)$[11] and finally, the critical radius R_o may be different in the case of folded and unfolded probes.[20,22] Nevertheless, other evidence (CD, UV/Vis absorption) strongly suggests that static quenching is a major process that disturbs energy transfer.

A four-component model of equilibrium, schematically shown in Scheme 1, may explain the observed peculiarities. In



Scheme 1. Schematic representation of a four-component model of the equilibrium of FAT-n probe in the presence of K^+ ion. Fluorescent structures (A) and (B) are equilibrated with the non-fluorescent conformations (C).

the absence of K^+, an unfolded probe (A) exhibits an intrinsic FRET that depends of the length of the oligonucleotide and the flexibility of the probe. Addition of KCl promotes formation of a quadruplex (B) that shows efficient FRET, the extent of which depends of the spacer length in the probe. Both structures (A) and (B) are equilibrated with the nonfluorescent conformations (C). The nonfluorescent "dark" structures (C), with very closely positioned fluorophores, can be achieved due to both thermal motions and attractive interactions between fluorophores. This model is consistent with fluorescence and absorption spectra as well as with calculated binding parameters.[33] Surprisingly low FRET efficiency for FAT-0 in the absence of KCl can be explained by the high fraction of a "dark" form. A hairpin structure is suspected to promote this process as already suggested.[8]

All these spectral effects should be taken into account when designing an efficient FRET probe. However, one should re-

member that binding affinity and selectivity of the probe also play crucial roles. Binding affinity should be finely tuned to meet requirements associated with the sensitivity of the probe and the expected concentration measurement range. Binding affinity optimization can be achieved by proper design of the probe: oligonucleotide sequence, additional linkers, and characteristics of the fluorophores. It has been shown that incorporation of additional nucleotides into the G4 oligonucleotide reduces the binding affinity of the probe.[20,22,33] In addition, a complex biological matrix may hamper K^+ monitoring in real samples. Proteins can affect the probe folding equilibrium through binding a probe both in the extended form (e.g., single-stranded DNA binding proteins, SSB) and as a tetraplex structure (e.g., thrombin). Further difficulties one can encounter with the intracellular approach include transferring of the probe through cell membrane and the biological activity of guanine-rich oligonucleotides (GRO). For example, an antiproliferative activity of GRO to carcinoma cells has been reported recently,[34] and catalytic activity of thrombin is blocked by binding to the TBA quadruplex.[12,13] The latter phenomenon has been exploited by Willner et al.[35] to develop a fluorescent sensor for DNA.

Conclusion

Oligonucleotide derivatives carrying the TBA sequence and FAM or TAMRA fluorescent labels at each terminus, with a spacer DNA sequence introduced between FAM and the 5′-end, were synthesized as fluorescence probes for K^+ ions. FAT-0 did not show sensitized emission of acceptor (TAMRA) in the presence of K^+ because of the quenching of TAMRA fluorescence due to the dye–dye interactions with FAM. When the spacer was introduced, the fluorescence of TAMRA was increased due to prevention of the dye–dye interaction. The results presented here showed that both donor quenching and sensitized emission of the acceptor could be used as sensitive markers to monitor the folding processes of quadruplex DNA. However, fluorescence signals of labels should be treated with caution and corrected, when needed, for the effects of protolytic equilibria, environment, and static quenching. In agreement with previous reports,[20,22] our data unambiguously confirm that the addition of a spacer significantly reduces contact quenching and improves the performance of FRET probes.

Experimental Section

Materials: Water used in these experiments was purified by using MilliQ apparatus (Millipore Inc., Billerica, MA). Custom oligonucleotides with fluorescence dyes were synthesized by Sigma-Genosys (Tokyo, Japan). Fluorescent labels were attached to the termini of oligonucleotides by using six-carbon linkers. The purity of these oligonucleotides was checked by reversed phase high performance liquid chromatography (RP HPLC) and matrix-assisted laser desorption ionization time of flight mass spectroscopy (MALDI TOF MS, Perspective Voyager Elite, acceleration voltage 21 kV, negative mode). Concentrations of oligonucleotides were determined spectrophotometrically at 260 nm in Tris-HCl (20 mM, pH 7.4) at room temperature by using the following molar absorptivities: $1.43\times10^5\ cm^{-1}M^{-1}$ for FAT-0, F-TBA, and TBA-T; $1.7\times10^5\ cm^{-1}M^{-1}$ for FAT-3; $1.92\times10^5\ cm^{-1}M^{-1}$ for FAT-5; $2.09\times10^5\ cm^{-1}M^{-1}$ for FAT-7.

Measurements and methods: Fluorescence measurements were carried out with a Hitachi F-4500 spectrofluorimeter and a Perkin–Elmer LS-55 Luminescence Spectrometer. Measurement conditions were as follows: oligonucleotide probe (0.2 μM), Tris-HCl buffer (20 mM, pH 7.4) at 25 °C with 10 nm slit widths. Excitation wavelengths in the emission spectra measurements were 495 nm for FAM and 560 nm for TAMRA. For the excitation spectra measurements, the emission wavelength was set at 576 nm (TAMRA emission). The 10 mm quartz cell compartment was maintained at 25 °C and was equipped with a magnetic stirrer. Quenching or fluorescence enhancement effects were evaluated by using plots of the normalized fluorescence intensity (integrated emission spectrum) versus cation concentration. The efficiency of FRET was expressed as a (ratio), which is a normalized measure of the enhancement of the fluorescence from the acceptor due to FRET.[11] A Hitachi U-3310 spectrophotometer was used for absorbance measurements. Sample solutions contained fluorescent oligonucleotide (1.4–2.0 μM) in Tris-HCl buffer (20 mM, pH 7.4) at 25 °C in 10 mm quartz cells. Typical titration consisted of successive additions of small amounts (2–4 μl) of a concentrated solution of KCl, followed by stirring, thermal equilibration, and recording of the spectrum. A Jasco J-820 spectrophotometer was used for recording CD spectra under the following conditions: probe (2.0 μM), Tris-HCl buffer (20 mM, pH 7.4) at 25 °C in a 10 mm quartz cell.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

Keywords: dual-labeled oligonucleotide · dye–dye contact · FRET · potassium · thrombin-binding aptamer (TBA)

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Received: April 30, 2006
Published online on September 28, 2006

