Fluorescence energy transfer probes based on the guanine quadruplex formation for the fluorometric detection of potassium ion

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Abstract

Dual-labeled oligonucleotide derivative, FAT-0, carrying 6-carboxyfluorescein (FAM) and 6-carboxy-tetramethylrhodamine (TAMRA) labels at 5′- and 3′-termini of thrombin-binding aptamer (TBA) sequence 5′-GGTTGGTGTGGTTGG-3′ and its derivatives, FAT-*n* (*n* = 3, 5, and 7) were designed and synthesized. FAT-*n* derivatives contained a T_mA spacer (*m* = 2, 4, and 6, respectively) at 5′-end of TBA sequence. The probes were developed to estimate the spacer effect on FRET efficiency and to identify the best probe for sensing of K^+. Circular dichroism (CD), UV–vis absorption, and fluorescence studies revealed that all FAT-*n* probes could form the intramolecular tetraplex structures after binding K^+. Association constants of particular K^+/FAT-*n* complexes were determined using different experimental approaches. Suitability of particular probes for sensitive monitoring of K^+ in intra- and extracellular conditions was examined and discussed. Calibration graphs of fluorescence ratio were linear in the K^+ concentration range of 2–10 mM for extracellular conditions showing sensitivity of 1.2% mM^{-1} K^+ and for intracellular conditions in the range of 100–200 mM with sensitivity of 0.49% mM^{-1} K^+.
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1. Introduction

Potassium and other alkali metal ions play an important role in biological systems. For example, K^+ participates in the regulation of membrane potential of a cell [1], potassium affects the physiological function of a nerve [2], or influences the apoptosis of a cell [3]. Since the abnormal K^+ concentration in blood was correlated with several diseases, the monitoring of K^+ in blood has became a very important challenge. Numerous studies were reported concerning K^+ sensors based on the ionophore-containing membrane but homogeneous fluorescent K^+-sensing systems are limited to few examples [4,5]. Their selectivity for K^+ against Na^+ is rather low, which limit applications to the conditions under the high concentration of K^+ (intracellular). The

difficulty in the monitoring of K^+ in extracellular conditions comes from the coexistence of an excess of sodium ion (Na^+), where small variations in K^+ concentration (around 5 mM K^+) should be monitored in the presence of 100 mM Na^+ concentration. Recently, we and other group reported very selective and sensitive K^+ assays based on the quadruplex-forming oligonucleotides [6,7].

The idea behind these sensors was to exploit peculiar metal cation binding abilities of quadruplex-forming oligonucleotides [8], which labeled with fluorescent tags were suitable for the transduction of a metal cation binding event (Fig. 1). In our first strategy, we developed a sensor that exploited fluorescence resonance energy transfer (FRET) as a transduction process. The G-rich oligonucleotide possessing the telomere sequence from human $G_3(TTAG_3)_3$, was labeled with fluorophores suitable to cooperate as FRET partners [6,9,10]. The application of this sensor for real time K^+ monitoring at physiological conditions appeared to be not facile task because of saturation of the probe response at submillimolar concentration of K^+ and lower FRET

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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
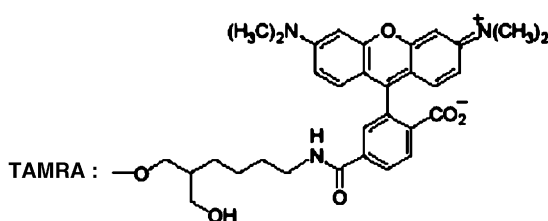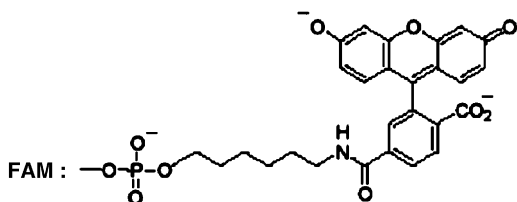
efficiency observed in the presence of K^+ comparing with that for Na^+ [10,11].

To circumvent above limitations, a new fluorescent probe was developed, which exploited the pyrene excimer emission for the transduction of the cation-binding event. Contrary to the FRET approach, stacking interactions between fluorophores (pyrene) were advantageous and produced efficient excimer emission [12]. To achieve optimal cation-binding properties, the 15-meric thrombin-binding aptamer (TBA) with a d(GGTTGGTGTGGTTGG) sequence was chosen as tetraplex-forming oligonucleotide. However, the short excitation wavelength of pyrene is a drawback for the applications of this probe in biological systems.

Recently, we have extended the studies with the TBA oligonucleotide on a FRET approach with the use of FAM and TAMRA dyes as fluorescent labels [13]. A family of FAT-*n* probes, where

n means the length of a spacer sequence attached to 5′-end of TBA oligonucleotide ($n = 0$, 3, 5, and 7 bases), was designed and evaluated as K^+ FRET probes (Table 1). Fluorescence measurements allowed an estimation of the spacer effect on FRET efficiencies and identified the best probes for sensing of K^+ ion. The most promising probes, FAT-5 and FAT-7, showed improved performance due to the elimination of unwanted contact quenching of TAMRA emission sensitized by FRET.

In this paper, we have carried out detail studies on the performance of the FAT-*n* probes in intra- and extracellular conditions. Binding affinities of FAT-*n*/K^+ complexes have also been evaluated.

2. Experimental

2.1. Materials

Oligonucleotides modified with fluorescence dyes were custom-synthesized by Sigma-Genosys (Tokyo, Japan) and characterized as described previously [13]. Other chemicals were of analytical grade and water for all experiments was purified using MilliQ apparatus (Millipore Inc., Billerica, MA).

2.2. Measurements and methods

Fluorescence measurements were carried out with a Hitachi F-4500 spectrofluorimeter. Experimental conditions were as follows: 0.2 μM oligonucleotide probe, 20 mM Tris–HCl buffer (pH 7.4) at 25 °C, slit widths of 10 nm. Excitation wavelengths in the emission spectra measurements were 495 nm for FAM and 560 nm for TAMRA. For the excitation spectra, the emission wavelength was set at 576 nm (TAMRA emission). The cell compartment equipped with a magnetic stirrer was thermostated at 25 °C. All measurements were carried out using a 10 mm quartz cell.

The efficiency of FRET was expressed as a (ratio) calculated from excitation spectra, which is a normalized measure of the enhancement of the fluorescence from the acceptor due to FRET
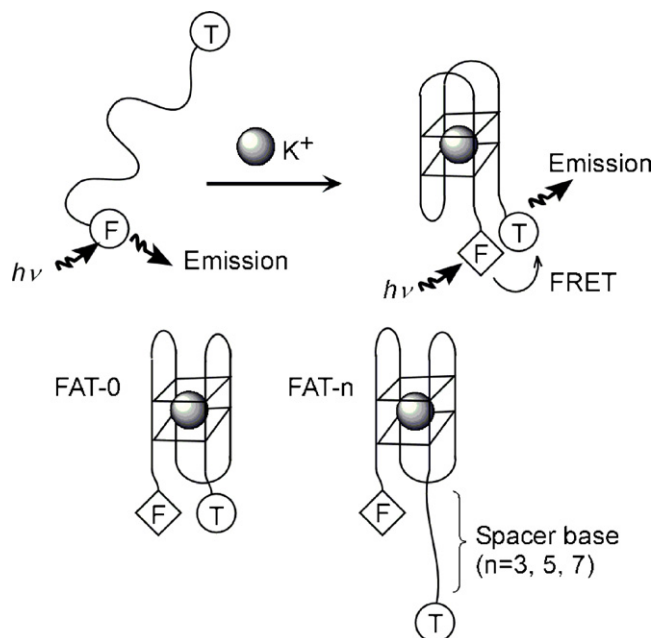

Fig. 1. Expected structural changes of FAT-*n* exerted by the complex formation with K^+ and differences in FAT-*n* structures.

[14], using the following expression:

$$(\text{ratio}) = \frac{F_{\text{ex},495}}{F_{\text{ex},560}} \tag{1}$$

where $F_{\text{ex},495}$ is the fluorescence of the acceptor (TAMRA) measured when the probe is excited by FRET (at 495 nm) and $F_{\text{ex},560}$ is the acceptor emission directly excited at 560 nm. For analytical purposes, the fluorescence ratio values were also calculated from emission spectra using the expression:

$$R = \frac{F_{\text{em},585}}{F_{\text{em},518}}. \tag{2}$$

Hitachi U-3310 spectrophotometer was used for absorbance measurements. Sample solutions contained 1.4–2.0 μM of fluorescent oligonucleotide in 20 mM Tris–HCl buffer (pH 7.4) at 25 °C. All measurements were carried out using a 10 mm quartz cell.

Jasco J-820 spectrophotometer was used for recording of CD spectra using the following conditions: 2.0 μM probe; 20 mM Tris–HCl buffer (pH 7.4) at 25 °C; a 10 mm quartz cell.

2.3. Calculation of the binding constant (K_{ass})

Association constants (K_{ass}) of oligonucleotide complexes with K^+ were calculated using several experimental data: molar ellipticities (CD spectra), absorbances and fluorescence intensities. Assuming a two-component model of complexation: an unfolded probe (no KCl) and a folded quadruplex/K^+ complex (excess of K^+) and 1:1 stoichiometry [15–17], the association constant (K_{ass}) can be defined by the following general equation:

$$K_{\text{ass}} = \frac{1 - \alpha}{\alpha \times c_{\text{KCl}}} \tag{3}$$

where α denotes a molar fraction of the unfolded probe. Equilibrium concentration of K^+ could be replaced by total KCl concentration (c_{KCl}) since an excess of KCl was present comparing with the probe concentration in our experimental conditions. Substitution of the molar fraction, α, with an analytical signal from CD, absorption or fluorescence spectra gave equations, which were exploited for determination of K_{ass}:

$$\frac{\theta - \theta_0}{\theta_S - \theta} = K_{\text{ass}} \times c_{\text{KCl}}, \tag{4a}$$

$$\frac{A - A_0}{A_S - A} = K_{\text{ass}} \times c_{\text{KCl}}, \tag{4b}$$

$$\frac{F_0 - F}{F - F_S} = K_{\text{ass}} \times c_{\text{KCl}} \tag{4c}$$

where θ, A and F correspond to the molar ellipticity, absorbance, and fluorescence intensity, respectively. Subscripts '0' and 'S' denote the value in the absence of K^+ and in an excess of KCl (saturation), respectively. Plotting of the left side of above equations against KCl concentration allowed K_{ass} determination as a slope of the obtained linear dependence. Calculations of K_{ass} from quenching data were carried out using both original fluorescence intensities and those after correction for absorbance changes ($F_{\text{corr}} = F/(1 - 10^{-A})$).

3. Results and discussion

3.1. Spectral properties of FAT-n probes

A thrombin-binding aptamer used in this study (Table 1) is known to form a chair-type intramolecular tetraplex structure in the presence of K^+ [15–17]. Characteristic negative and positive bands at 265 and 295 nm, respectively, were observed in circular dichroism (CD) spectra upon addition of K^+ for all probes listed in Table 1 [13]. The same typical CD bands are present in an unmodified TBA/K^+ complex [12,18,19], suggesting the tetraplex formation in all investigated systems. Thus, after labeling with fluorescent reporter groups, the oligonucleotides preserved their abilities to fold into a quadruplex structure and to interact with metal cations.

Absorption spectra of FAT-*n* probes exhibited spectral changes upon addition of KCl. Tetraplex formation was supported by hyperchromic effect at 295 nm [20]; the absorption bands of FAM and TAMRA (at 495 and 560 nm, respectively) were also affected by folding of the oligonucleotide (data not shown). FAM absorption band exhibited small blue shift upon addition of K^+ (0–10 mM) and the molar absorptivity increased by 30–40%, while TAMRA band exhibited hypochromicity (15–30%) with a red shift. These results are consistent with other reports [21,22], in which similar spectral changes were ascribed to the dye–dye interactions. Since hypochromicity of TAMRA band decreased with an increase in the spacer length (FAT-0 > FAT-3 > FAT-5 > FAT-7), one can conclude that this order represents diminished dye–dye interactions between FAM and TAMRA.

Excitation of FAT-*n* probes at the FAM absorption band (495 nm) gave two fluorescence bands at 518 and 585 nm (FAM and TAMRA emission, respectively) both in the absence and presence of K^+ (Fig. 2A). Similarly, the excitation spectra monitored at 576 nm (TAMRA emission band) exhibited one band for direct excitation of TAMRA (560 nm) and another band at 495 nm representing FRET process (Fig. 2B). This evidences the occurrence of FRET process from FAM to TAMRA even in the absence of K^+, which is reasonable if one compares the distance between fluorophores in a 15-meric oligonucleotide (ca. 62 Å) [7] and the expected Förster radius, ($R_0 = 45–60$ Å) [14,22,23].

3.2. Association constants of quadruplex complexes with K^+

The presence of fluorophores attached to the oligonucleotide termini may affect cation-binding affinity of the probe and change its selectivity towards other cations. The association constants (K_{ass}) of the complexes between K^+ and quadruplex-forming fluorescent probes were calculated from several experimental data: molar ellipticities (CD spectra), absorbances (UV and vis absorption bands) and fluorescence intensities, using Eqs. (4a)–(4c). There are controversial reports in literature concerning stoichiometry of TBA/K^+ complexes. Generally, an 1:1 stoichiometry is accepted [15,16,18], although the 2:1 or 3:1 potassium/DNA complexes were observed at higher KCl concentration using NMR and calorimetric titration [19,24,25]. Our
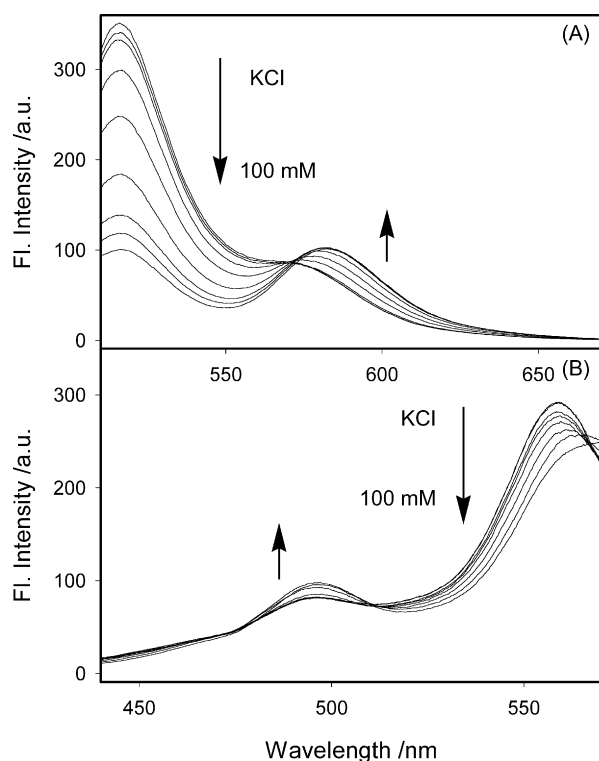
Fig. 2. KCl effect on the fluorescence spectra of 0.2 μM FAT-5. (A) Emission spectra with $\lambda_{ex} = 495$ nm and (B) excitation spectra with $\lambda_{em} = 576$ nm. *Conditions*: 20 mM Tris–HCl buffer (pH 7.4).

experimental techniques (CD, spectrophotometry and fluorescence) are sensitive only to the folding process of quadruplex DNA, which is completed after accommodation of a single potassium ion, therefore, we assumed an 1:1 stoichiometry in binding affinity calculations. Obtained results are presented in Table 2. Association constants calculated from CD spectra (positive band at 295 nm) and those calculated from absorbance changes at 295 nm are in good agreement (within experimental error) and should be regarded as reference values. The K_{ass} values based on the spectral changes of the fluorescent labels (absorbance—A^{495} or fluorescence—F^{518}) are generally much lower when compared with the reference values. One can conclude that spectral changes associated with fluorescence labels are not caused exclusively by the folding of oligonucleotide but

Table 2
Association constants (K_{ass}) of the quadruplex complexes of fluorescent probes with K$^+$ determined using different methods

Probe	K_{ass} (10^2 M^{-1})				
	CD spectra	A^{295}	A^{495}	F^{518}	F^{518a}_{corr}
TBA	49	–	–	–	–
F-TBA	2.6	2.5	0.41	0.74	–
TBA-T	108	111	33[b]	91[b]	–
FAT-0	15.5	8.4	3.4	3.3	1.9
FAT-3	0.93	0.82	0.39	0.98	1.1
FAT-5	0.80	0.59	0.37	0.47	0.70
FAT-7	0.45	0.50	0.28	0.20	0.45

[a] Corrected for absorbance variation $F^{518}_{corr} = F^{518}/(1 - 10^{-A})$.
[b] A^{560} or F^{585}.

also by other processes. One of such processes already identified, was the protolytic effect of ionic strength on FAM absorbance and emission [10,26,27]. Correction of the fluorescence intensities (F^{518}) for the absorbance changes exerted by an addition of K$^+$ gave much better agreement of calculated K_{ass} values with the reference constants (F^{518}_{corr} in Table 2). Only in the case of FAT-0, this correction was not effective that indicated the importance of other factors, most probably connected with dye–dye interactions. The following conclusions can be drown from the results presented in Table 2: (i) spectral changes in the UV region of CD and absorption spectra can be successfully exploited for the characterization of quadruplex folding process, (ii) the proper correction of fluorescence data is needed to obtain convinced association constants, (iii) attachments of a negatively charged label (FAM) or a nucleotide spacer inhibit formation of quadruplexes, and (iv) the positively charged TAMRA label stabilizes quadruplexes. Association constants decrease according to the following order: TBA-T > TBA > FAT-0 > F-TBA > FAT-3 > FAT-5 > FAT-7, which agrees perfectly with the presence and number of stabilizing or destabilizing groups embedded into the 15-meric thrombin aptamer (TBA). Mergny et al. studied the effect of spacer bases on the stability of tetraplex structures of human telomere oligonucleotide [28] and C-rich oligonucleotide (i-motif) [22] and they showed that an increase of number of nucleotides in a spacer lead to the decrease in thermal stability of the tetraplex structure, which agrees with our observations.

One should remember that in living organisms, four elements dominate as free cations whose approximate concentrations are as follows: 145 mM NaCl, 5 mM KCl, 2.5 mM CaCl$_2$, and 1.5 mM MgCl$_2$. Because of high content of Na$^+$, the efficient formation of potassium-quadruplex complex requires significant binding preferences for K$^+$ over Na$^+$. Thus, significantly lower binding affinity of probes with the spacers (FAT-3, FAT-5, and FAT-7) may deteriorate the sensitive detection of K$^+$ in biological systems. Supposedly, this drawback is compensated by an advantageous FRET behavior of FAT-n probes.

3.3. Fluorescence resonance energy transfer in FAT-n probes

Fig. 2 shows spectral changes in emission and excitation spectra of FAT-5 probe in the presence of added KCl. Other probes showed similar behavior. As expected for folding process in this FRET system (Fig. 1), the FAM fluorescence ($\lambda = 518$ nm) is quenched, while TAMRA emission at 585 nm is enhanced due to FRET sensitization. FRET process is supposed to operate also in the extended FAT-n probes (in the absence of K$^+$) and an addition of spacer groups (3–7 bases) that changes the donor–acceptor separation distance should affect the FRET efficiency. Fig. 3A supports this expectation: in the absence of K$^+$, fluorescence intensity of the FAM emission band increases with the length of spacer (solid bars). Accordingly, the relative fluorescence intensity of TAMRA band decreases only slightly (patterned bars). The main reason for such spectral changes is undoubtedly associated with a drop in FRET efficiency caused by the increase in the separation distance of two dyes. Upon addition of K$^+$, all probes exhibited substantial quenching of
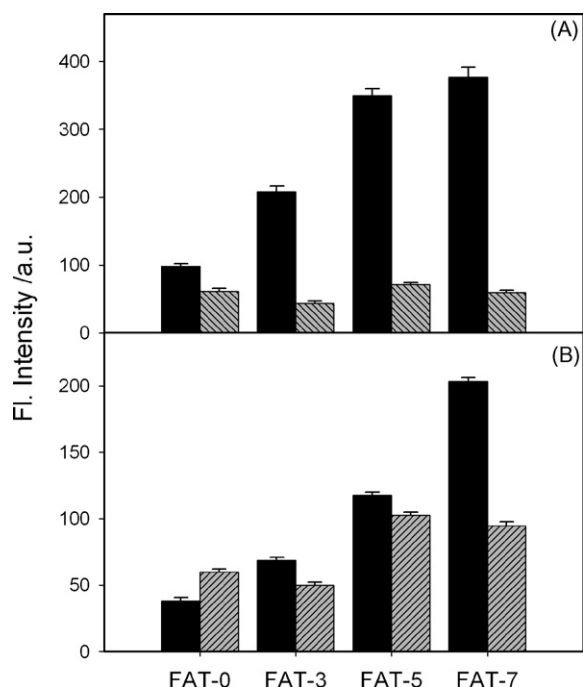
Fig. 3. Effect of the spacer length on the fluorescence intensity (donor emission ($\lambda = 518$ nm)—solid bars; acceptor emission ($\lambda = 583$ nm)—patterned bars) of FAT-*n* probes in the absence of KCl (A) and in the presence of 100 mM KCl (B). *Conditions*: 20 mM Tris–HCl buffer (pH 7.4), $\lambda_{ex} = 495$ nm. Error bars show standard deviation of triplicate measurements.

a donor band (FAM) comparing with unfolded probe, but the quenching effect decreases with an increase in spacer length in accordance with expected less efficient FRET (Fig. 3B). Accordingly, one could expect relative drop in TAMRA emission as a consequence of reduced FRET efficiency. Instead, an enhanced fluorescence of acceptor (TAMRA) can be observed for FAT-5 and FAT-7 probes (Fig. 3B). Several factors can be considered to explain this effect: an increase in molar absorptivity of FAM, an enhanced quantum yield of the fluorophore, or quenching interactions between the donor and acceptor [29–31].

3.4. Effect of a spacer length in FAT-n probes on the detection of K+

In order to evaluate suitability of the probes for the sensitive detection of K^+, several dependences were examined using both fluorescence intensities and ratio of intensities. Fig. 4A shows effect of potassium on the normalized intensity ratio of acceptor (Eq. (1)) $\Delta(\text{ratio})/(\text{ratio})_0 = ((\text{ratio}) - (\text{ratio})_0)/(\text{ratio})_0$, where subscript '0' denotes the (ratio) value in the absence of K^+. These dependences were plotted using data from excitation spectra, thus they are supposed to reflect FRET efficiency for particular FAT-*n* probes. Interestingly, plots for FAT-3, FAT-5, and FAT-7 exhibit very similar runs, while FAT-0 shows quite different behavior. Much higher ratio values for FAT-0 and lower but the same values for other FAT-*n* probes may indicate stacking interactions between the donor and acceptor molecules. Additionally, FRET efficiency is probably seriously affected by the absorbance changes of the donor and acceptor. The shapes of
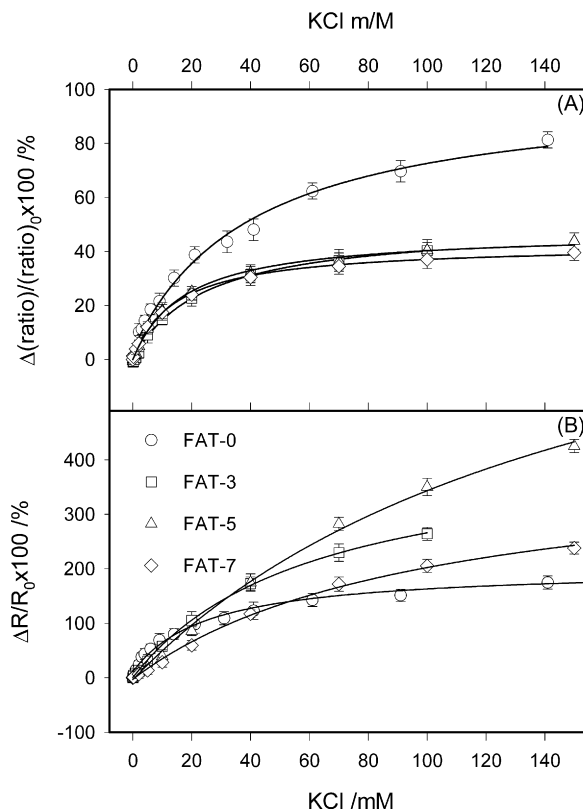

Fig. 4. KCl effect on the relative fluorescence ratio values for FAT-*n* probes. (A) (ratio) $= F_{ex,495}/F_{ex,560}$ calculated from excitation spectra, $\lambda_{em} = 576$ nm. (B) $R = F_{em,585}/F_{em,518}$ calculated from emission spectra, $\lambda_{ex} = 495$ nm. Error bars as in Fig. 3.

dependences shown in Fig. 4A seem to be useless for analytical applications because of marked nonlinearity and saturation effect at the higher concentration range of K^+. More suitable for determination of K^+ appeared to be dependences plotted with data from the emission spectra ($\Delta R/R_0 \times 100\%$) with R defined by Eq. (2) as shown in Fig. 4B. Comparing with Fig. 4A, plots for FAT-3, FAT-5 and FAT-7 exhibit considerably wider range of signal change and better linearity, thus seem to be more suitable for analytical purposes. The highest slope and the best linearity are observed for FAT-5. The reason for such a sensitivity order may involve an interplay of many factors including binding affinity, distance dependence of FRET, variations in molar absorptivities and quantum yields of fluorophores as well as quenching processes.

3.5. Fluorescence detection of K+ under biological conditions

In the biological conditions it is important to detect discriminately between Na^+ and K^+. Fluorometric detection of K^+ by using FAT-*n* was studied under concentrations of cations that mimic biological environment both at intracellular conditions (high K^+ concentration) and in extracellular conditions (high Na^+ concentration). The experiments were carried out at cation compositions that corresponded to the common conditions of mammalian cells [1]. Under the intracellular conditions
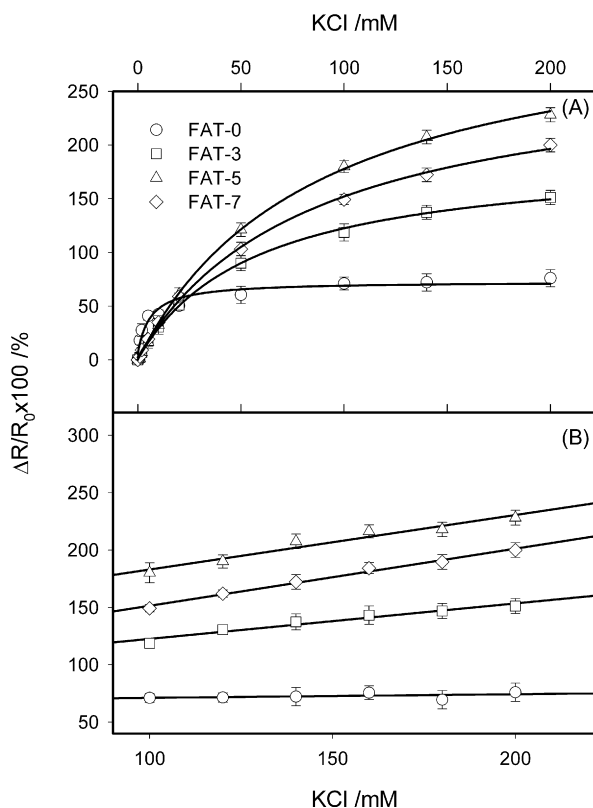
Fig. 5. (A) Plots of the relative R changes for FAT-n probes against KCl concentration under intracellular conditions. (B) Close-up view: axis X was extended from 100 to 200 mM KCl. Other conditions as in Fig. 4.
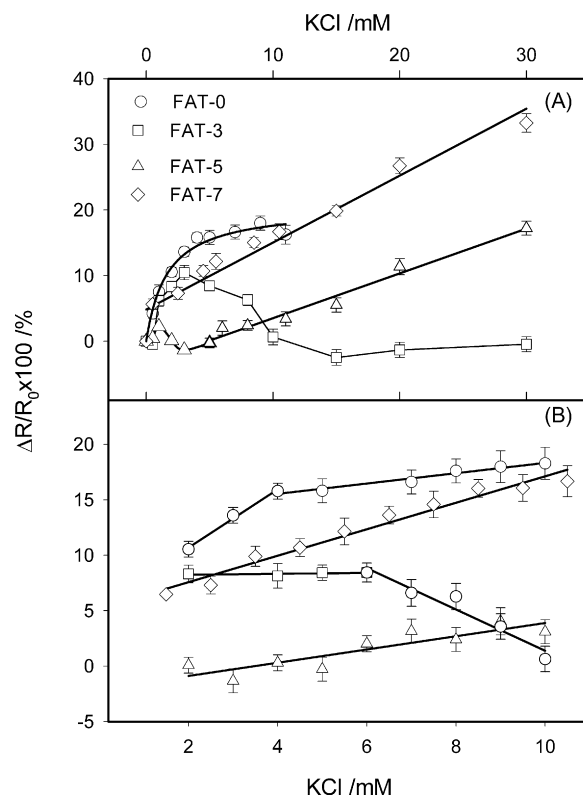

Fig. 6. (A) Plots of the relative R changes for FAT-n probes against KCl concentration under extracellular conditions. (B) Close-up view: axis X was extended from 2 to 10 mM KCl. Other conditions as in Fig. 4.

of 15 mM Na^+, 0.5 mM Mg^{2+}, 0.1 μM Ca^{2+} (without K^+), the fluorescence spectra of FAT-n showed spectral changes typical for the tetraplex formation (binding of Na^+, Mg^{2+} or Ca^{2+}) [17–20]. Significant changes in R values were observed upon addition of K^+ as illustrated in Fig. 5A. The plot for FAT-0 was saturated with 10 mM K^+, whereas R values for FAT-3, FAT-5, and FAT-7 increased gradually up to 200 mM K^+. Plots for FAT-5 and FAT-7 showed the highest slopes in the range of 100–200 mM K^+(Fig. 5B), suggesting their analytical suitability. Within the concentration range of K^+ that is useful for intracellular monitoring of K^+ (100–200 mM), the plots for FAT-5 and FAT-7 are linear and possess the highest slopes (+0.49% mM^{-1}), while FAT-0 probe does not respond to K^+.

Under the extracellular conditions of 145 mM Na^+, 1.5 mM Ca^{2+}, 1.5 mM Mg^{2+}, the large background fluorescence was observed because of the presence of high Na^+ concentration (data not shown). Thus, the relative R changes observed after addition of K^+, were modest (0–30%) and calibration graphs for potassium exhibited rather complex runs as shown in Fig. 6A. Within the K^+ concentration range typical for extracellular conditions (2–10 mM^{-1} K^+), only FAT-5 and FAT-7 exhibited linear correlation of relative R changes against K^+ concentration with slope approaching 1.2% mM K^+ for FAT-7 (Fig. 6B). FAT-0 and FAT-3 probes gave bimodal dependences that reflected rather complex processes including binding saturation of the probes. Since the increases in R values were largest for FAT-5 and FAT-7, these probes are suggested for K^+ determination under extracel-

lular conditions. In addition, these two probes exhibited linear calibration graphs in low K^+ concentration range of 2–10 mM (Fig. 6B).

4. Conclusions

Oligonucleotide derivatives FAT-n carrying TBA sequence and FAM or TAMRA fluorescent labels at either terminus were tested as FRET probes for K^+ Probes contained different spacers inserted between FAM and 5′-end in order to identify the best probe for sensing of K^+. FAT-0 (no spacer) showed the lowest 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. Probes with spacers showed in the presence of K^+ the enhancement of TAMRA fluorescence due to prevention of the dye–dye interaction. Presented here results unambiguously confirmed that the addition of a spacer significantly reduced contact quenching between FRET partners and improved the performance of the FAT-n probes. We found that FAT-5 and FAT-7 probes were suitable for detection of K^+ under intracellular and extracellular conditions. Practical applications of these probes needs further studies including evaluation of the effect of biological matrix, mainly proteins. The interferences from ssDNA-binding proteins (SSB) could be reduced by adding excess amount of oligonucleotides of random sequences. Another problem is a possible presence of G4 binding proteins, biological activity of the guanine-rich oligonucleotides (GROs), and delivery of the probe to the cell through membrane. All these

issues will be addressed in our further application studies of the probes.

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