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Reviews

Fluorescence Detection of Potassium Ion Using the G-Quadruplex Structure

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Oligonucleotides with sequences of human telomere DNA or thrombin binding aptamer (TBA) are known to form tetraplex structures upon binding the K^+ ion. Structural changes associated with the formation of tetraplex assemblies led to the development of potassium-sensing oligonucleotide (PSO) probes, in which two fluorescent dyes were attached to both termini of particular oligonucleotide. The combination of dyes included fluorescence resonance energy transfer (FRET) and excimer emission approaches, and the structural changes upon binding K^+ ion could be monitored by a fluorescence technique. These systems showed a very high preference for K^+ over Na^+ ion, which was suitable for fluorescence imaging of the potassium concentration gradient in a living cell. In the case of human telomere DNA, it was also possible to follow the polymorphism of its tetraplex structures.

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1 Introduction

Several different metal ions are present in a living cell, and potassium ion (K^+) together with Na^+ and Ca^{2+} play a very important role in cells. K^+ exists in cells with the highest concentration, and together with the Na^+ ion, they control the osmotic pressure in each cell and maintain homeostasis of the cell volume; also, their concentrations are balanced with the concentrations of other ions, such as Ca^{2+} or Cl^- in cells.[1] Thus, any abnormality in the K^+ concentration in a cell or blood causes several kinds of disorders.[2] Any abnormality or defects in the K^+ channels, which cause decreasing K^+ concentration, lead to several diseases. For example, irregular heartbeat is known to result from a deficiency of the channel binding protein.[3,4] The concentration of K^+ in blood serves as an indicator in the diagnosis of heart disorders, and tests of electrolyte concentrations are conducted routinely in health checks.[5] For example, diseases connected with rising K^+ concentration (hyperkalemia) include cardiac arrest, congenital hemiplegia,

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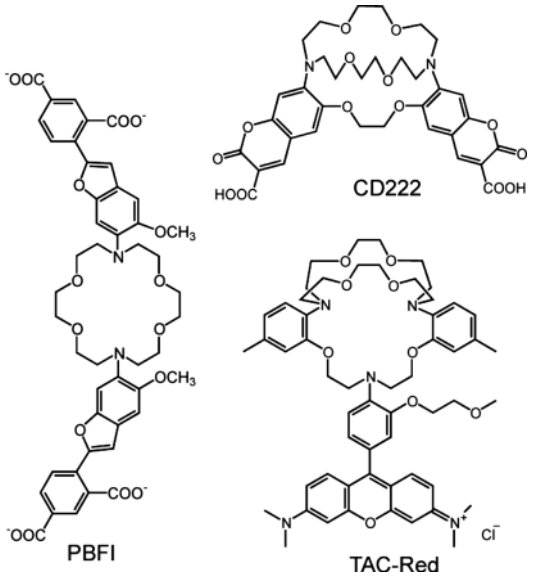

Fig. 1 Fluorescence dyes having binding preferences for potassium ion.

diabetic acidosis and others.[6]

The detection of K^+ in blood is carried out using a potassium-selective electrode.[7,8] The required feature of such an ion-selective electrode is high preference for K^+ over Na^+. Very selective K^+ electrodes were developed by applying crown ether derivatives as a potassium ionophore.[8]

On the other hand, the detection of K^+ in a cell is also important, and some fluorescence K^+ probes have been developed; their chemical structures are shown in Fig. 1. PBFI is well known as a fluorescence K^+ probe.[9] However, its preference for K^+ over Na^+ was *ca*. 1.5, and there are some problems with the detection of the K^+ concentration in cells.[10] CD222 has also been reported as another type of fluorescence K^+ probe, but its K^+/Na^+ preference was *ca*. 3.4, which is not enough for an efficient fluorescence K^+ probe to be suitable for cell-related measurements.[10] In 2003, He *et al*. reported a highly sensitive fluorescence K^+ probe, which was constructed with triazacryptand as a K^+ binding unit and 4-amino-naphthaleneimide as a chromophore part.[11] The probe showed a dynamic range for K^+ detection of 2 – 10 mM in the presence of 160 mM Na^+, which is a clinically important concentration region of K^+ under an extracellular condition. This probe has been used in an immobilized form on a polymer support for the automatic determination of potassium in Roche OPTI CCA, a commercially available whole-blood analyzer. In 2005, Padmawar *et al*. developed a fluorescence K^+ probe, known as TAC-Red, that carried the triazacryptand K^+ recognition unit and rhodamine fluorophore.[12] TAC-Red probe and other TAC derivatives exhibited *ca*. 30-times higher preference for K^+ over Na^+, and have been successfully applied for the fluorescence visualization of K^+ waves in brain cortex.[12-14]

As described above, some important K^+ probes have been developed, but they have not been designed to be allocated in a living cell, as for example many fluorescent Ca^{2+} probes. Therefore, when easy-to-use fluorescent probes for living cell applications with a high preference for K^+ over Na^+ are developed, the research connected with K^+ functions in living cell will make progress.

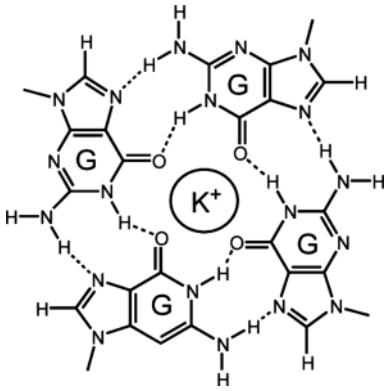

Fig. 2 Complex of potassium ion with the G-quartet structure.

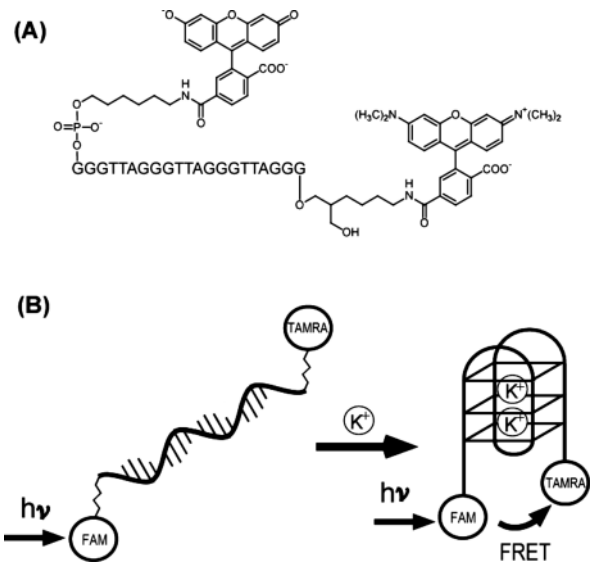

Fig. 3 Chemical structure of potassium-sensing oligonucleotide, PSO-1 (A) and mechanism of fluorescence detection of potassium ion using PSO-1 (B).

2 Potassium Sensing Oligonucleotide Probe Based on Human Telomere Sequence

Telomere DNA, which possesses a sequence of many TTA GGG repeats is known to be located in the termini of human chromosomes.[15] This DNA sequence is known to fold intramolecularly to form guanine quadruplexes with a guanine quartet (G-quartet) as a building element. Figure 2 shows the G-quartet structure formed by four guanines in a plane having a hole, which has a suitable size for K^+ ion accommodation, which is expected to stabilize the quadruplex structure.[15] Taking advantages of preferential binding of potassium ion by G-quadruplexes, we have synthesized oligonucleotide derivative, PSO-1, carrying part of the human telomere DNA sequence of $G_3TTAG_3TTAG_3TTAG_3$ and FAM and TAMRA dyes attached at its 5′- and 3′-termini, respectively (Fig. 3A).[16] It was expected that FRET (fluorescence resonance energy transfer) from a FAM energy donor to a TAMRA energy acceptor would be efficient upon K^+ complexation by the PSO-1 probe, since the Forster radius of this dye combination is known to be R_o = 54 – 60 Å.[17] Since PSO-1 was constructed with the 21-meric oligonucleotide, the FAM and TAMRA separation

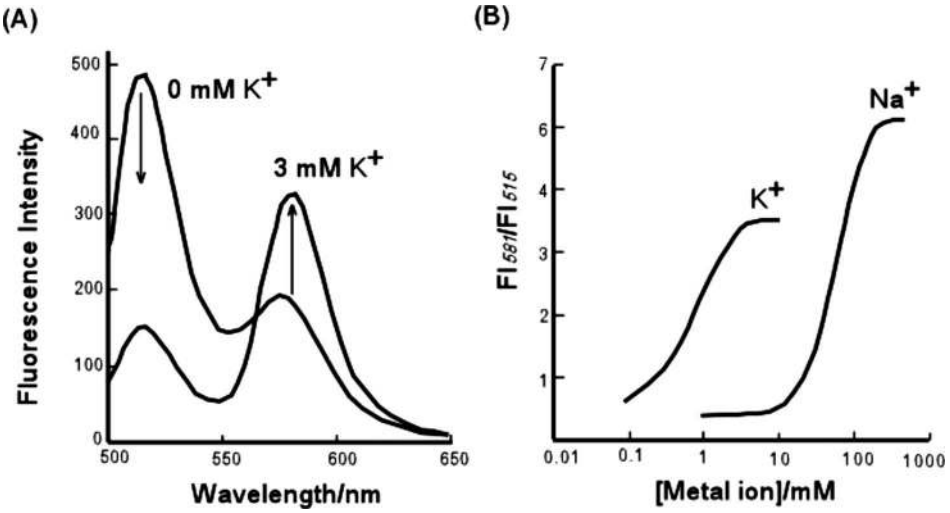

Fig. 4 (A) Fluorescence spectra of 0.2 μM PSO-1 in the absence and presence of 3 mM potassium ion in 5 mM Tris–HCl (pH 7.0) at 25°C. λ_{ex} = 492 nm. (B) Fluorescence intensity ratio (FI_{581}/FI_{515}) of 0.2 μM PSO-1 plotted against K^+ or Na^+ concentration.

distance was limited to 87 Å (an extended form). Upon the addition of K^+, the tetraplex structure should be adopted by PSO-1, and FRET from FAM to TAMRA was expected to occur (Fig. 3B). Figure 4A shows the fluorescence spectra of PSO-1 in both the absence and presence of K^+ ion under FAM donor excitation (λ_{ex} = 492 nm). The first fluorescence band observed at λ_{em} = 515 nm is characteristic of the FAM emission, and a second emission band at λ_{em} = 581 nm is typical for TAMRA fluorophore. The relative high intensity of TAMRA emission in the extended form (no K^+ ion) of the PSO-1 probe suggested that PSO-1 might have a shrunken conformation (less than 60 Å rather than its extended one), although the direct excitation of TAMRA acceptor could not be neglected. Nevertheless, the addition of potassium ion caused an increase in the fluorescence band based on TAMRA at λ_{em} = 581 nm with a decrease in that based on FAM at λ_{em} = 515 nm, as expected (Fig. 4A). The dissociation constant of the PSO-1 complex with K^+ was calculated from florescence changes observed upon PSO-1 titration with potassium ion. Good fitting was achieved with a binding model based on the PSO-1 interaction with two metal ions; the dissociation constants for PSO-1 complexes with potassium and sodium ions were respectively, $K_{d,K+}$ = 0.28 mM and $K_{d,Na+}$ = 57.7 mM. Thus, the PSO-1 probe showed a 208-times higher binding preference for K^+ over Na^+, which was the highest preference for K^+ compared with those reported previously for other probes.[9–14] Figure 4B shows the variation of the FRET efficiency expressed as a fluorescence intensity ratio (FI_{581}/FI_{515}) for PSO-1 plotted against the concentration of K^+ and Na^+. An increase in the FRET signal was observed for K^+ and Na^+ ions in the range of 0.1 – 1 mM and 10 – 100 mM, respectively, which is consistent with the binding affinities of these cations. Interestingly, a plateau region was observed for both plots at higher cation concentrations, but the value of FI_{581}/FI_{515} for the sodium plateau was 1.5-times higher than that for K^+. This result is consistent with our observation that an addition of K^+ to a solution containing an excess of Na^+ (145 mM) caused a drop in the FRET signal. These effects were difficult to be explained when we published our first paper (Ref. 16), since at that time it was accepted that human telomere DNA formed the basket- type tetraplex structure in the presence of K^+ and Na^+ (Fig. 5A).[18] Around the 2006s, it was proposed

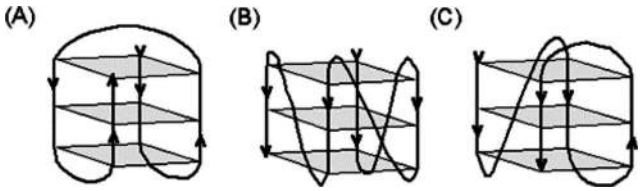

Fig. 5 Structural polymorphism of human telomere DNA quadruplexes; a basket type (A), a propeller type (B), and a hybrid type (C) structures.

that human telomere DNA exhibits polymorphism of tetraplex structures.[19] Figure 5 shows examples of such structures that include propeller and hybrid-type quadruplexes in addition to the basket-type structure. The separation of FAM and TAMRA moieties that are linked to the termini of PSO-1, estimated for particular quadruplex structures of PSO-1, revealed closer proximity of both moieties for the basket-type structure, which is consistent with higher FRET efficiency observed for sodium tetraplex. Therefore, the FRET results for PSO-1 are sensitive to the polymorphism of the human telomere DNA structures, because the FRET signal is coupled with the distance between the 3′- and 5′-termini of oligonucleotide. One should remember, however, that quenching/dequenching processes of FAM and TAMRA fluorophores may operate upon the folding of oligonucleotide, and such phenomena also affect the FRET signal. Nevertheless, one can assume that the increased fluorescence ratio signal reflects the higher fraction of the basket-type structure in equilibrium with other quadruplexes. The above assumption was supported by EPR results of Singh *et al*., who proposed a 1:1 complex between basket and propeller-type structures in an aqueous solution for an oligonucleotide, A G_3 TTA G_3 TTA G_3 TTA G_3, carrying TEMPO as a spin probe at the underlined thymine bases.[20] Although the addition of nucleic bases at the oligonucleotide terminal and different experimental conditions are known to induce the formation of different tetraplex structures, our result suggested that the FRET results are sensitive to the polymorphism of tetraplex structures of human telomere DNA.

3 Potassium Sensing Oligonucleotide Probe Based on a Thrombin Binding Aptamer Sequence

To avoid problems with tetraplex polymorphism, we focused on a thrombin binding aptamer (TBA) sequence of GGTTGGTGTGGTTGG, which formed only a chair-type structure.[21] The dissociation constants of TBA with K^+ or Na^+ were estimated from changes in the intensity of the peak at 295 nm (positive Cotton effect) in the circular dichroism spectra, caused by the addition of K^+ or Na^+, respectively. The calculated values, $K_{d,K+}$ = 0.29 mM and $K_{d,Na+}$ = 104 mM, indicated a binding preference for K^+ over Na^+ of 350.[22] Thus, we synthesized a FAT-0 probe, which possessed a TBA sequence carrying FAM and TAMRA groups at the 5′- and 3′-termini, respectively (Table 1).[23,24] As shown in Fig. 6, the FAT-0 probe formed a chair-type structure upon the addition of K^+ with two dyes located in a close proximity. Figure 7A shows the fluorescence spectra of FAT-0 (λ_{ex} = 495 nm) with well-separated fluorescence bands based on FAM and TAMRA emission. Interestingly, the fluorescence ratio (FI_{581}/FI_{515}) was larger than that for PSO-1. This is reasonable when considering that two dyes should be closer than in the case of PSO-1, since the length of a 15-meric TBA is only 63 Å. Upon the addition of K^+, fluorescence based on FAM decreased, but that based on TAMRA also slightly decreased. An analysis of the UV-Vis absorption spectra suggested that the fluorescence decrease of TAMRA was caused by quenching, resulting from direct contact interactions of these two dyes (Fig. 8A). To increase the separation distance between dyes, a spacer of five nucleotides (21 Å) was introduced between FAM and 5′-terminal of the TBA sequence to obtain a FAT-5 probe (Table 1). Figure 7B shows the fluorescence spectra of FAT-5 in the absence and the presence of potassium ion. The emission band of TAMRA (λ_{em} = 581 nm) decreased in FAT-5 alone, as compared to that for free FAT-0, in agreement with a lower FRET efficiency expected for an increase in the separation distance between the donor and the acceptor. A much better FRET response was observed upon the addition of potassium due to a significant decrease in FAM emission and a distinct increase in the TAMRA band intensity (Figs. 7B and 8B). However, the binding affinity of FAT-5 with K^+ decreased dramatically. This was expected as result of more effective electrostatic repulsion interactions upon folding of the DNA polyanion after attaching an additional

Table 1 Potassium sensing oligonucleotides based on thrombin binding aptamer (TBA)

Abbreviation	Sequence
TBA	GGT TGG TGT GGT TGG
FAT-0	FAM-GGT TGG TGT GGT TGG-TAMRA
FAT-5	FAM-**TT TTA** GGT TGG TGT GGT TGG-TAMRA

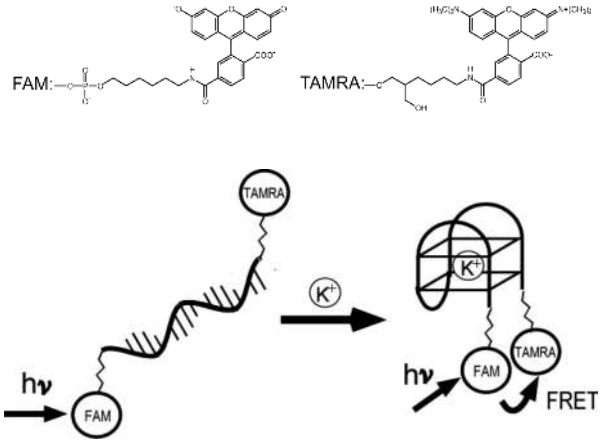

Fig. 6 Mechanism of fluorescence detection of potassium ion using FAT-0.

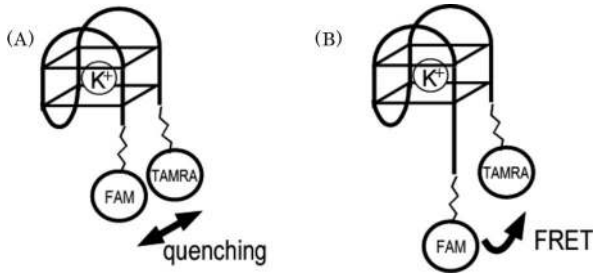

Fig. 8 Expected structural arrangement of fluorescent labels for FAT-0 (A) and FAT-5 (B) caused by the complex formation with K^+ ion.

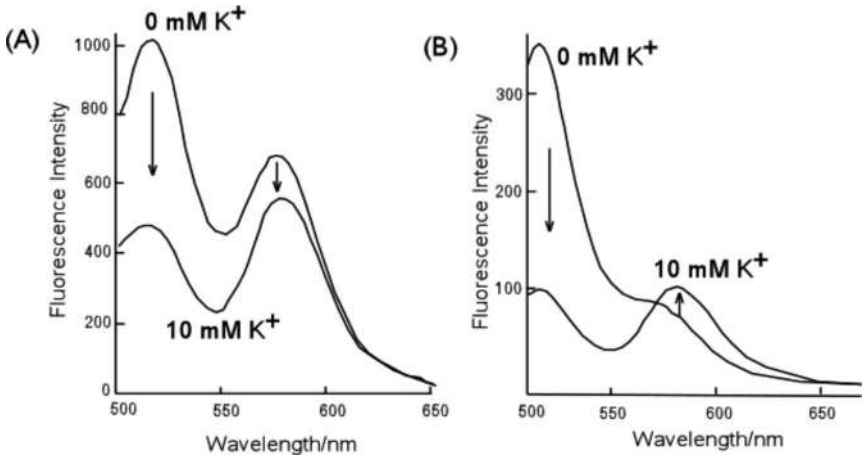

Fig. 7 Fluorescence spectra of 0.2 μM FAT-0 (A) or FAT-5 (B) in the absence and presence of 10 mM potassium ion in 5 mM Tris–HCl (pH 7.0) at 25°C. λ_{ex} = 495 nm.

spacer nucleotides with negative charges. Fortunately, no fluorescence changes were observed upon the addition of Na^+ excess, and the FAT-5 probe exhibited quite good binding selectivity for K^+ over the Na^+ ion.[23,24]

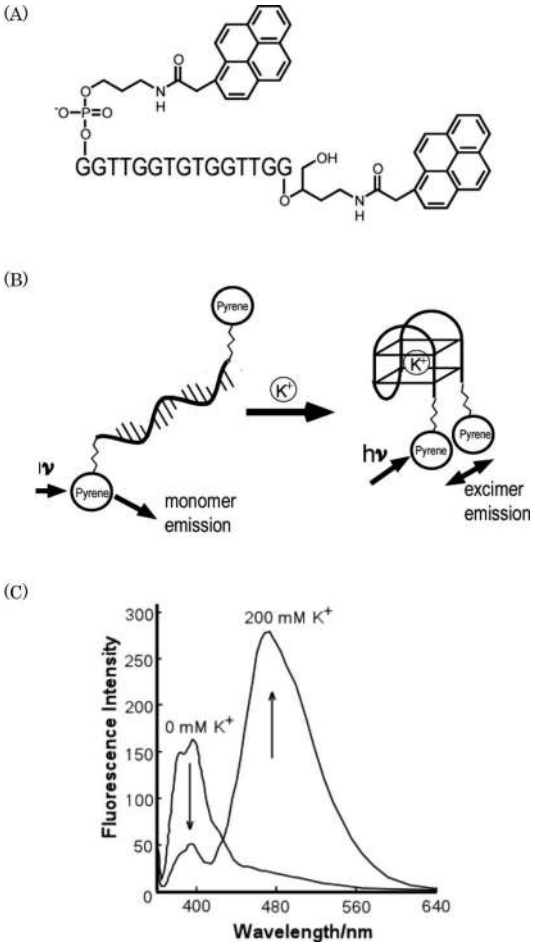

Fig. 9 (A) Chemical structure of PSO-py, (B) expected mechanism of fluorescence detection of potassium ion using PSO-py, and (C) fluorescence spectra of 0.2 μM PSO-py in the absence and the presence of 200 mM K^+ in 5 mM Tris–HCl (pH 7.2) at 25°C.

4 Potassium Sensing Using Pyrene Excimer Approach

The binding preference for K^+ over Na^+ for TBA alone was of 350, which is higher than that for PSO-1. Therefore, one can expect to obtain a highly selective K^+ fluorescence probe with TBA as a K^+ recognition element. However, the chair-type tetraplex structure arranges the two dyes very close to each other, which is disadvantageous for FRET approach, as described in the case of FAT-0. Therefore, we introduced pyrene groups at both termini, expecting excimer emission after chair-type quadruplex formation. Figure 9A shows the chemical structure of PSO-py, and the K^+ induced structural rearrangement of the PSO-py probe into the quadruplex structure with the expected excimer formation is shown in Fig. 9B.[25] Figure 9C shows a fluorescence spectra of PSO-py with the pyrene monomer emission at 390 nm for a free probe, and a large pyrene excimer emission observed at 480 nm upon the addition of K^+. The dissociation constants determined from the fluorescence changes upon the addition of K^+ and Na^+, were $K_{d,K+}$ = 7.3 mM and $K_{d,Na+}$ = 272 mM with a binding preference for K^+ over Na^+ of 37. The excimer fluorescence changes were reversible, and the PSO-py showed a very fast response in fluorescence to the stepwise variations in the potassium concentration. The response of the relative fluorescence change was 8.3%/mM under the range of 2 – 10 mM K^+ in the presence of 145 mM Na^+, which is superior compared to 1.5%/mM for PBFI. Furthermore, we synthesized a probe based on the human telomere oligonucleotide carrying pyrenes at both termini. The fluorescence spectra of this probe showed only monomer emission in both the absence and the presence of potassium ion. The lack of excimer emission after quadruplex formation indicated that pyrene groups were too far from each other to adopt the excimer arrangement, which is consistent with the discussion for PSO-1.[26]

5 Fluorescence Imaging of Potassium Ion in a Living Cell

Fluorescence imaging of the K^+ ion concentration in a living cell was first attempted with a FAT-5 probe. FAT-5 was introduced to the HeLa cell using a bead-load method. Recorded fluorescence images showed an exporting process of FAT-5 to the nucleus and cell death for 70% of the introduced cell population (N = 57).[27] Figure 10 shows the DIC and

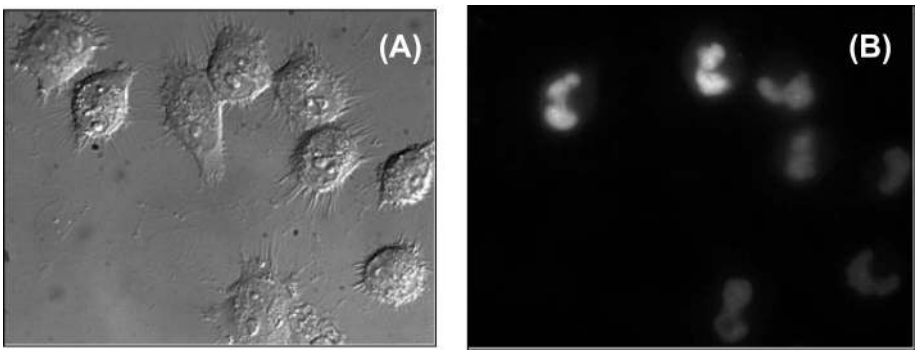

Fig. 10 (A) DIC and (B) fluoresce images of HeLa cell loaded with FAT-5. FAT-5 was localized in the nuclei of the cells and caused cytotoxicity.

Table 2 Dissociation constants and selectivity for K^+ sensing reagents

Compd.	Dissociation constant, K_d/mM		Selectivity of K^+ over Na^+ [$K_{d,Na+}/K_{d,K+}$]	Ref.
	K^+	Na^+		
PBFI	8	21	2.6	10
CD222	0.54	1.74	3.2	10
PSO-1	0.28	57.7	208	16
PSO-py	7.3	272	37	25
FAT-0	9.0			23, 24
FAT-5	14			23, 24
TAC-Red	60[a]		30[b]	12, 13

a. Estimated with dissociation and association rate constants from Ref. 13.
b. The ratio of fluorescence intensities for potassium and sodium complexes.

fluorescence images of the HeLa cell after loading with the FAT-5 probe, where FAT-5 is located in the nucleus. Goodchild *et al*. reported that G-rich oligonucleotides introduced into the cell were exported to the nucleus after binding to nucleolin, and exhibited high cytotoxicity.[28] The result obtained by us should be explained by similar phenomena. In a next approach, we conjugated the FAT probe with biotin to prevent nucleolin binding, and to reduce any cytotoxicity. As a result, cell death was diminished markedly, but the probe was still exported to the nucleus. Finally, when biotin-modified nuclear export peptide was conjugated to the FAT probe, we succeeded to observe fluorescence imaging of K^+ in the cytoplasm, since this probe remained in the cytoplasm.[27]

6 Conclusions

Table 2 summarizes the binding preferences of the potassium fluorescence probes described here. One can conclude that G-quadruplex based probes exhibit higher potassium ion binding affinity and advantageous K^+/Na^+ selectivity. Fluorescence imaging of K^+ was achieved using the G-quartet structure formed by four guanine bases. Especially, the fluorescence probes based on the thrombin binding aptamer (TBA) sequence were superior, since they allowed us to achieve a high binding preference for K^+ over Na^+ with a wide dynamic range of K^+ response that was consistent with the potassium level in a cell. After we reported our research results, many other papers connected with K^+ sensing based on TBA oligonucleotide have been reported.[28–31] Some of them showed a very high preference for K^+, but they presented multi-reagent approaches, which are not suitable for the fluorescence imaging of K^+ in a living cell.[28–30] Recently, a cell-based fluorescence screening assay for the K^+ channel inhibitors was developed with a fluorescence probe based on triazacryptand immobilized on a polymer support.[14] In the near future, the research field related to the fluorescent K^+ probes will expand not only to studies concerning the functions of K^+ in a cell, but also to applications in diagnosis connected with K^+ channels and drug discovery and screening the series of drugs.

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