Fluorescent Probes

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A Pyrene-Labeled G-Quadruplex Oligonucleotide as a Fluorescent Probe for Potassium Ion Detection in Biological Applications**

*Satoru Nagatoishi, Takahiko Nojima, Bernard Juskowiak, and Shigeori Takenaka**

Alkali metal ions contribute to several physiological functions in living organisms.[1] Fluorescent indicators, particularly for Na^+ and K^+ ions, should permit the continuous or intermittent optical determination of these ions and visualization of their dynamic changes in living cells, extracellular spaces, vesicles, vascular tissue of plants and animals, biological fluids, and environmental samples.[2]

We have focused on fluorescent probes for the potassium ion, which plays a significant role as a transmitter in cells and in the regulation of membrane potential, nerve stimulation, and hormone secretion.[3] Potassium-binding benzofuran isophthalate (PBFI)[4] and coumarin diacid cryptand [2.2.2] (CD222)[5] have been recommended for the fluorimetric detection of K^+ ions in biological fluids, although these indicators show rather poor selectivity against sodium (a 1.5- and 3.4-fold excess of Na^+ ions is tolerated in each case, respectively).[4,5] Therefore, they can only be used for intracellular applications at sufficiently high K^+ concentrations. Other fluorescent probes for K^+ ions have recently been reported,[6] but only some of them are suitable for real-time monitoring applications at high Na^+ concentrations.[6c] Recently, we reported the potassium-sensing oligonucleotide (PSO)[7] as a FRET (fluorescence resonance energy transfer) probe for K^+ ions. It consists of two fluorophores (*N*,*N*,*N*′,*N*′-tetramethyl-6-carboxyrhodamine (TAMRA) and 6-carboxyfluorescein (FAM)) that are attached to the termini of a 21-mer oligonucleotide (human telomeric DNA sequence), which can form stable G-quadruplex complexes with metal cations.[8] Although PSO has been suggested to be useful for the monitoring of K^+ in an excess of Na^+ ions, its application

[*] Prof. S. Takenaka
Department of Materials Science, Faculty of Engineering
Kyushu Institute of Technology
Kitakyushu-shi 804-8550 (Japan)
Fax: (+81) 93-884-3333
E-mail: shige@che.kyutech.ac.jp
S. Nagatoishi, Dr. T. Nojima
Department of Applied Chemistry, Faculty of Engineering
Kyushu University, Fukuoka 812-858 (Japan)
Prof. B. Juskowiak
Faculty of Chemistry, A. Mickiewicz University
Poznan 60-780 (Poland)

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for real-time K^+ ion monitoring under physiological conditions was rather difficult. Two factors hampered such an application: 1) the binding constant of the potassium complex was too high, resulting in saturation of the probe at a submillimolar concentration of K^+ and 2) FRET for the PSO complex was more efficient with Na^+ than that with PSO/K^+ (apparent quenching).

Herein, we report a novel PSO probe for the facile detection of potassium in the presence of an excess of Na^+ ions, in which these disadvantages are circumvented. The idea behind this new fluorescent probe (termed PSO-py) is to exploit the pyrene excimer emission for the transduction of a cation-binding event. Unlike the FRET system, in which stacking interactions between FRET partners resulted in unwanted quenching phenomena,[7] the pyrene moieties are expected to produce efficient excimer emission when stacked by π interactions.[9] However, potassium and sodium binding preferences were carefully tuned by selection of a quadruplex-forming oligonucleotide. The 15-mer oligonucleotide with the sequence d(GGTTGGTGTGGTTGG), known as a thrombin-binding aptamer (TBA),[10] was chosen for this purpose. TBA forms a chair-type quadruplex structure upon binding to thrombin protein, and this quadruplex incorporates K^+ ions with a 1:1 stoichiometry.[11] Therefore, binding of potassium ions should organize the 5′ and 3′ termini of TBA so that the two attached pyrene moieties are arranged face-to-face (Figure 1). As a result of this spatial orientation

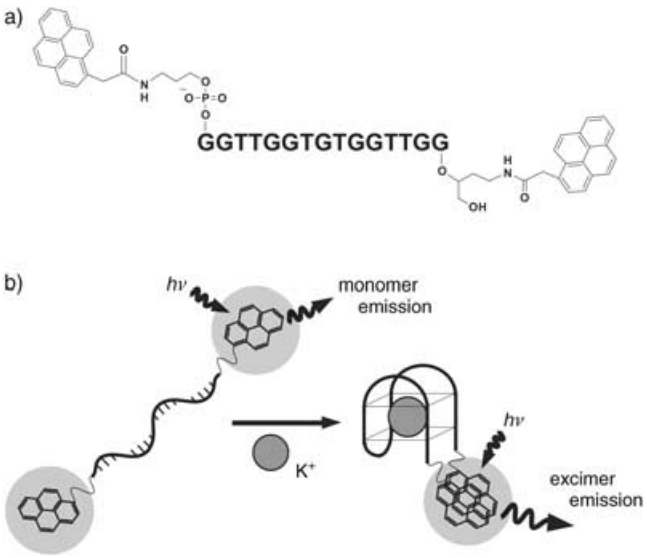

Figure 1. a) Chemical structure of PSO-py and b) the expected quadruplex formation induced by K^+ ions.

of the pyrene rings, the PSO-py/K^+ complex emits only excimer fluorescence, whereas the random-coil structure of PSO-py in the absence of K^+ ions gives only monomer emission. Consequently, PSO-py is expected to exhibit an excimer-emission intensity that depends on the K^+ concentration.[12] The presence of other metal cations is expected to cause minor interferences, as TBA forms weak complexes with Na^+, Mg^{2+}, and Ca^{2+} ions.[11c,13] PSO-py was obtained by modification of the synthetic TBA oligonucleotide termini with the appropriate derivatives of 1-pyrenylacetic acid: the phosphoramidite form for the 5′ end and the succinimidyl ester for the 3′ end. Modification and purification procedures were similar to those described by Fujimoto et al.[9d]

The suitability of PSO-py for the detection of K^+ ions was tested by fluorimetric titration with KCl (Figure 2). The

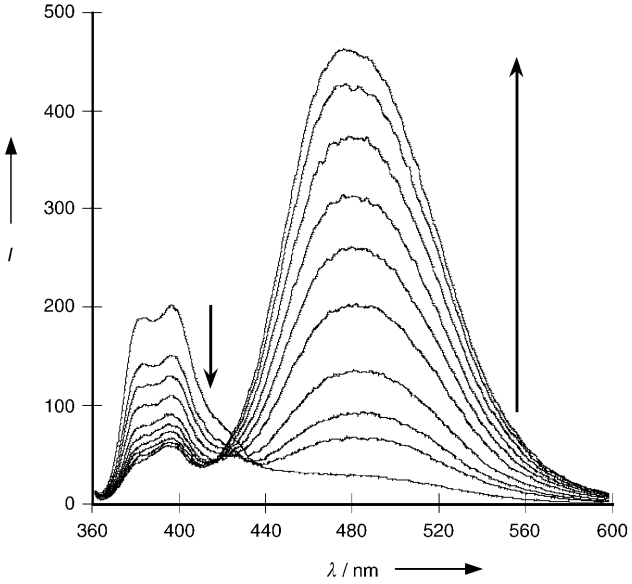

Figure 2. Fluorescence titration spectra of PSO-py (0.2 μM) with KCl (0–200 mM) in Tris·HCl solution (5 mM, pH 7.2) at 25 °C; λ_{ex} = 347 nm. Tris = tris(hydroxymethyl)aminomethane.

fluorescence spectrum of PSO-py in the absence of K^+ exhibits only a weak monomer band at 390 nm. The addition of K^+ ions results in the appearance of a new broad excimer band around 480 nm,[9] accompanied by quenching of the monomer emission. Interestingly, the weak monomer band at 390 nm lacks the vibronic structure that is probably connected with stacking interactions of the pyrene fluorophore with nucleobases.[9e,f] Notably, there is no clear isoemissive point in the system, which suggests a more complex situation than the simple two-component excimer–monomer equilibrium. Besides excimer emission, an additional fluorescence is most likely present from the excited dimer, for example. Contrary to the classic "dynamic excimer" observed in nonrestricted systems, some pyrene-bridged structures were reported to exhibit a "static excimer" or "fluorescent dimer" emission.[14] Circular dichroism (CD) recorded for PSO-py upon the addition of K^+ ions showed that the excimer fluorescence resulted from a structural change from a random coil to a chair-type quadruplex structure (the negative band near 265 nm and the positive band at 290 nm with an isoelliptic point at 280 nm).[8,13c]

Changes in the excimer fluorescence of PSO-py were also monitored in the presence of Na^+, Mg^{2+}, and Ca^{2+} ions. Excimer emission intensities were plotted against concentration of the tested ions (Figure 3). Only the potassium ion gave a positive response, but excimer emission was detectable for concentrations of Na^+ ions exceeding 100 mM. The small

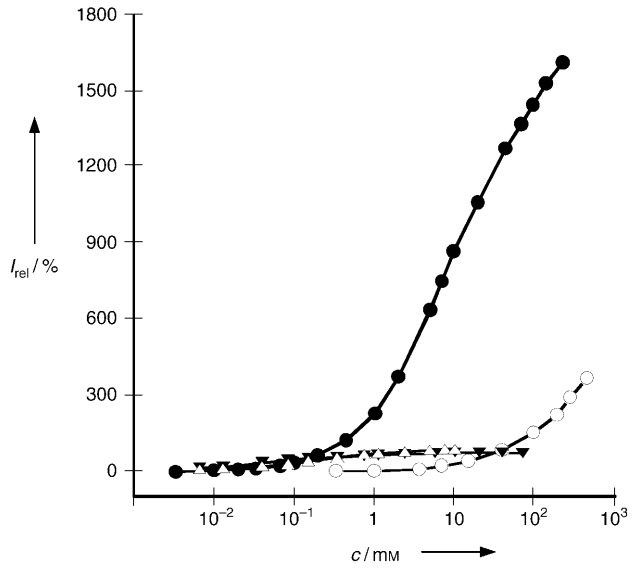

Figure 3. Plots of the relative fluorescence intensity of PSO-py (0.2 μM) against the concentration of KCl (●), NaCl (○), $CaCl_2$ (△), and $MgCl_2$ (▼).

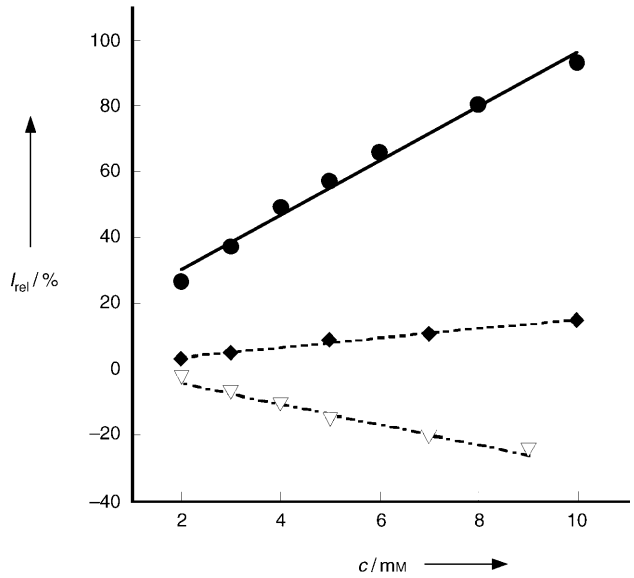

Figure 4. Comparison of the sensitivity of potassium detection for PSO-py (0.2 μM, ●), PBFI (0.2 μM, ◆), and PSO (0.2 μM, ▽) in Tris·HCl (5 mM, pH 7.2) containing 145-mM Na^+, 1.5-mM Mg^{2+}, and 2.5-mM Ca^{2+}.

changes observed in the CD spectra of PSO-py in the presence of Na^+ and Ca^{2+} ions confirmed the low binding affinity of PSO-py toward these cations. The dissociation constants (K_d) of PSO-py complexes were calculated from the plots shown in Figure 3 by fitting experimental data to an equation derived as before,[7] but for a complex with 1:1 stoichiometry. Values of 7.33 and 272 mM were determined for the K^+ and Na^+ complexes, respectively. The selectivity coefficient of 37, obtained from the ratio of sodium and potassium dissociation constants, is much higher than those for PBFI and CD222 (1.5 and 3.4, respectively).[4,5]

Real-time monitoring of the extracellular concentration of K^+ ions (2–10 mM) requires the PSO-py probe to exhibit a sufficiently high response in the presence of a complex matrix containing several ions (Na^+, Mg^{2+}, Ca^{2+}, and Cl^-) at millimolar concentrations. Therefore, the performance of PSO-py was studied by examining the K^+ calibration graph in the presence of a synthetic mixture containing 145-mM Na^+, 1.5-mM Mg^{2+}, and 2.5-mM Ca^{2+}. The increase in excimer emission of PSO-py was observed upon the addition of K^+ ions in the concentration range of 0–200 mM with linear dependence up to 10 mM K^+. The slope of the linear plot was +8.3% per 1 mM K^+, which is an excellent result given the typical physiological level of extracellular potassium. Similar calibration graphs with PBFI and PSO probes were determined for comparison (Figure 4), and these plots exhibit much lower sensitivities of +1.5 and −3.1% per 1 mM K^+, respectively. Undoubtedly, the PSO-py probe exhibits the highest sensitivity for K^+ ions relative to other probes reported so far. The dynamics of the fluorescence response and the reproducibility of excimer emission were also tested for the PSO-py/K^+ system by monitoring fluorescence changes upon subsequent alteration of the K^+ concentration between 5 and 6 mM (sequential dilution of solution and addition of concentrated KCl). The results of this experiment (Figure 5) indicate that the response time to the millimolar

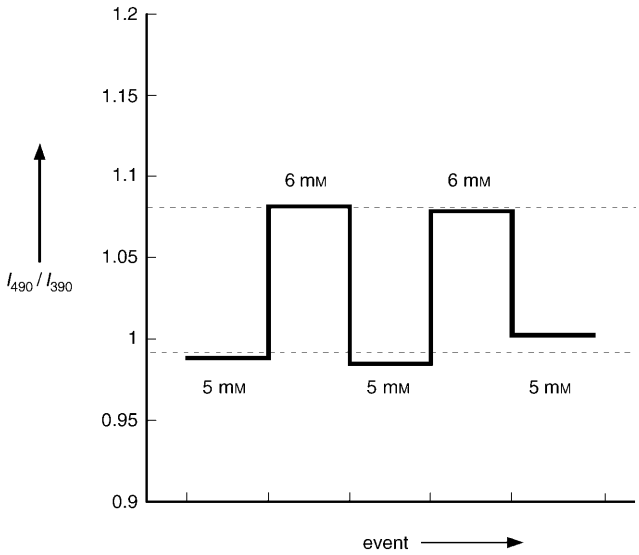

Figure 5. Fluorescence changes of PSO-py (0.2 μM) registered upon the subsequent variation of K^+ concentration between 5 and 6 mM in Tris·HCl (5 mM, pH 7.2).

variation in K^+ concentration is very short (within a few seconds), the excimer fluorescence of PSO-py/K^+ is reversible, and the probe shows reproducibility of the fluorescence signal within 5%.

In conclusion, we have designed the fluorescent probe PSO-py, which can be potentially exploited for the real-time monitoring of K^+ ions under extracellular conditions. The PSO-py ligand possesses the following advantages: 1) it binds potassium with a dissociation constant (K_d) of 7.33 mM, which perfectly matches the expected concentration range for K^+ ions and maximizes sensitivity; 2) it has sufficient discrimination against other ions, especially Na^+ (37-fold, K_d =

272 mM), so that variations in the physiological concentration of sodium and other ions have little effect; 3) it shows reasonably good sensitivity (ΔF of +8.3% per 1 mM K^+); 4) its excitation wavelength (>350 nm) avoids the need for expensive quartz optics (microscope) and minimizes light absorption by nucleic acids and aromatic amino acids; and 5) the long wavelength of excimer emission (>450 nm) decreases overlap with tissue autofluorescence. Further studies, including application of the PSO-py probe for K^+ monitoring in biological systems, are in progress.

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[12] First, we studied the FRET system containing the TBA sequence with FAM and TAMRA at both termini. However, we did not observe any FRET signal after quadruplex formation with K^+ ions. This effect was probably caused by quenching between dyes, which were prone to direct π-stacking interactions (data not shown). Furthermore, the TBA oligonucleotide carrying FAM and TAMRA showed residual FRET in the absence of K^+ ions.
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