Synthesis of a Peptide-Human Telomere DNA Conjugate as a Fluorometric Imaging Reagent for Biological Sodium Ion

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A peptide-oligonucleotide conjugate (I) was synthesized by the attachment of FAM, TAMRA, and biotin moieties to a telomere DNA sequence of 5′-TAG GGT TAG GGT TAG GG-3′. This conjugate was induced to be an antiparallel structure in the presence of sodium ion (Na⁺), whereas a hybrid one was formed under potassium ion (K⁺) as a monitoring by circular dichroic spectra. The conformation change of this conjugate gave an effective FRET signal change upon the addition of NaCl, compared with the case of KCl. Under 5 mM KCl as an extracellular condition, a FRET change was observed upon addition of NaCl and quantitative FRET change was observed in 0 - 250 mM NaCl. This conjugate was immobilized on the cell surface through a sugar chain on the cell, biotinyl concanavalin A and streptavidin. This conjugate was utilized for Na⁺ sensing based on anti-parallel tetraplex formation with Na⁺.

Keywords Peptide-oligonucleotide conjugate, FAM, TAMRA, sodium ion, potassium ion, antiparallel tetraplex, hybrid tetraplex, fluorometric imaging

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GG-3'. This ligand was designed as made up of TA-core having FRET dye pairs, where one of the dye is attached through a neutral peptide spacer as shown in Fig. 1. This spacer is useful to separate the distance between FRET dye pairs and to retain the stability of the TA-core with Na⁺. This paper evaluates the performance of this conjugate. Also we developed a method to localize this ligand on the cell surface in the following order: sugar chain on the cell surface, biotin-modified concanavalin A, streptavidin (StAv), and biotin-modified conjugate (I).

**Experimental**

**Reagents and chemicals**

The peptide-oligonucleotide conjugate (I) used in the present work was synthesized according to a procedure similar to PSO. Synthesis of the peptide-oligonucleotide conjugate

An oligonucleotide carrying fluorescein (FAM) and amino moieties, NH₂-5'-TAG GGT TAG GGT TAG GGT TAG GG-3' FAM (2), was custom-synthesized by Genosys (Sigma-Aldrich, Louis, MO). Figure S1 (Supporting Information) shows the synthetic route; 2 (45 nmol) was dissolved in 90 μL of 1x PBS (Ambion) and 10 μL of 0.36 mg/μL sulfo-SMCC (sulfo-succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate) (Thermo) was added to this solution and mixed with a Vortex for 1 h. The reaction mixture was diluted to 500 μL total, and the obtained oligonucleotide (3) was purified with a NAP-5 column (GE Healthcare Life Science, Japan) by elution with 750 μL of water. This eluent was added to 75 μL of a 1.0 M phosphate buffer (pH 7.0) and 2 mg of biotin-aminocaproic acid (6)-GGGGKGGGGGC, which was synthesized by Fmoc chemistry using a Model 433A peptide synthesizer (Applied Biosystems, Foster City, CA) and mixed with a Vortex for 3 h. The target conjugate was chromatographed by reversed-phase HPLC with a Mightysil RP-18 column using gradient elution from 10 to 70% acetonitrile in a aqueous 0.1 M TEAA buffer (pH 7.0) for 30 min, monitored at 260 nm. The fraction containing the conjugate (4) was lyophilized. The DMSO solution (22 μL) containing 0.4 mg of 5(6)-carboxytetramethyl-rhodamine succinimidyl ester (Biotium) was mixed with a 500 mM phosphate buffer (pH 8.8) (75 μL) containing the conjugate (4) and the mixture was vortexed for 45 h at room temperature. Unreacted 5(6)-carboxytetramethyl-rhodamine succinimidyl ester was removed by NAP-10 (eluent: Milli-Q water) and the target conjugate was chromatographed by the reversed-phase HPLC with a Mightysil RP-18 column using gradient elution from 10 to 70% acetonitrile in 0.1 M TEAA buffer (pH 7.0) for 30 min, monitored at 260 nm. The fraction containing the target conjugate (I) was lyophilized and obtained with 4.4 nmol in 10% yield. The MALDI-TOF mass spectrum was measured by the negative mode with 3-hydroxypropionic acid as a matrix and the obtained peak at m/z = 9930.889 was in agreement with the theoretical value of [C₁₅₀H₂₃₀O₁₇₇N₁₁₇P₂₄S₂-H]⁻ = 9929.539. The concentration of the obtained I was estimated using ε₅₆₀nm (103000 cm –1 mol–1 L) based on TAMRA and kept with freezing preservation.

**Circular dichroism (CD) spectra measurement**

Two micromolar of I in 20 mM Tris-HCl buffer (pH 7.4) was placed in a quartz cell with a 1-cm light path length and the CD spectra were measured by a Jasco J-820 Spectropolarimeter (JASCO Corp., Tokyo, Japan) after the addition of a KCl or NaCl solution, and subsequently mixing by moving the cell up and down ten times and keeping for 3 min using the following conditions: sensitivity, 100 mdeg; scan rate, 50 nm/min; response, 4 s; data collecting interval, 0.2 nm; band width, 2 nm; accumulated time, 4 times.

**Fluorescence measurement**

A 2-mL volume of 0.2 μM I in a 20 mM Tris-HCl buffer (pH 7.4) in the presence or absence of 0.3 μM StAv was placed in a quartz cell (1 cm light path) and the fluorescence spectra.
were measured in the 500–700 nm spectral range (excitation wavelength at 495 nm, PMV = 700 V, and 10 nm slits) by an F-7000 fluorescence spectrometer (HITACHI) upon the addition of a KCl or NaCl solution (the volume change was neglected) and subsequently mixing by moving the cell up and down ten times and keeping 1 min at 25°C.

Calculation of dissociation constant of $K_d$ for metal cation

The oligonucleotide sequence of $I$ formed a tetraplex structure with a three G-quartet plate with a 1:2 complex with K⁺ or Na⁺. Spectra changes of CD or fluorescence titration upon the addition of K⁺ or Na⁺ were fitted with the following equation, (1) or (2), to detect the dissociation constant, $K_d/M$, where $\Phi_0$ and $\Phi_i$ refer to the quantum yield of the chromophore part in the absence and presence of metal cation (M⁺), while $\varepsilon_0$ or $\varepsilon_i$ also refers to molar absorptivity of chromophore part in the absence or presence of M⁺:

$$\frac{(\theta_1 - \theta_0)}{\theta_0} = \frac{(\Delta \varepsilon_1 - \Delta \varepsilon_0)}{\Delta \varepsilon_0} \times \frac{[M^+]^2}{K_d + [M^+]}.$$

$$\frac{(R_0 - R_1)}{R_0} = \frac{\varepsilon_0 \Phi_0 - \varepsilon_i \Phi_i}{\varepsilon_i \Phi_0} \times \frac{[M^+]^2}{K_d' + [M^+]}.$$

Fluorescence imaging of $I$ immobilized on a cell

A HeLa cell was cultivated in a collagen-cored glass bottom dish in a DMEM medium. This medium was removed and washed with 1 mL of PBS two times. Two hundred microliters of 2 µM concanavalin A (concanavalin A biotin conjugate, Type IV, lyophilized powder, SIGMA-AALDRICH, 4 - 8 mol biotin per mol protein) in DMEM (+ 10% FBS) was added into this dish and incubated for 5 min at 37°C under 5% CO₂. After removing this solution and washing with 1 mL PBS two times, 50 µL of 5.0 µM StAv in DMEM (+ 10% FBS) was added to this dish and incubated for 5 min at 37°C under 5% CO₂. Finally 50 µL of 2.0 µM $I$ in DMEM (+ 10% FBS) was added and incubated for 5 min at 37°C under 5% CO₂ after washing with 1 mL PBS two times. Two mL of DMEM (−) was added in this dish and confocal laser fluorescence inverted microscopy was carried out using ECLIPSE TE2000-U (Nikon Instruments Inc.). An oil immersion lens at 60-fold magnification was used as an object glass and CCD camera imaging (ORCA-AG, Hamamatsu Photonics K. K.) was carried out on an incubation stage for a living cell at 37°C under a 10% CO₂ atmosphere.

Results and Discussion

Structural change of $I$ upon addition of Na⁺ or K⁺ ion

The CD spectra of part of telomere sequences of TA-core, 5′-TA GGG TTA GGG TTA GGG-3′ was measured upon the addition of NaCl and KCl. The TA-core showed a positive Cotton effect at 295 nm and a negative one at 265 nm upon addition of NaCl and showed a positive Cotton effect at 290 nm with a shoulder at 270 nm and negative one at 240 nm. This result suggested that the TA-core forms an anti-parallel or hybrid structure upon the addition of NaCl or KCl, respectively (Fig. S2, Supporting Information). Although the above result suggested that one base difference for human telomere DNA leads to different tetraplex conformation, the CD spectra of $I$ modified by FAM, TAMRA, and biotin was measured in the presence of varied amounts of KCl or NaCl. Figure 2 shows the CD spectra of 2.0 µM $I$ in a Tris–HCl buffer (pH 7.4) upon the addition of NaCl or KCl. This result suggested that the TA-core forms an anti-parallel or hybrid structure upon the addition of NaCl or KCl, respectively (Fig. S2, Supporting Information). The CD spectra of $I$ modified by FAM, TAMRA, and biotin was measured in the presence of varied amounts of KCl or NaCl. Figure 2 shows the CD spectra of 2.0 µM $I$ in a Tris–HCl buffer (pH 7.4) upon the addition of NaCl or KCl. This result suggested that the TA-core forms an anti-parallel or hybrid structure upon the addition of NaCl or KCl, respectively (Fig. S2, Supporting Information). The CD spectra of $I$ modified by FAM, TAMRA, and biotin was measured in the presence of varied amounts of KCl or NaCl. Figure 2 shows the CD spectra of 2.0 µM $I$ in a Tris–HCl buffer (pH 7.4) upon the addition of NaCl or KCl. This result suggested that the TA-core forms an anti-parallel or hybrid structure upon the addition of NaCl or KCl, respectively (Fig. S2, Supporting Information). The CD spectra of $I$ modified by FAM, TAMRA, and biotin was measured in the presence of varied amounts of KCl or NaCl. Figure 2 shows the CD spectra of 2.0 µM $I$ in a Tris–HCl buffer (pH 7.4) upon the addition of NaCl or KCl. This result suggested that the TA-core forms an anti-parallel or hybrid structure upon the addition of NaCl or KCl, respectively (Fig. S2, Supporting Information).
suggesting the formation of a hybrid tetraplex structure. These results show that 1 forms an anti-parallel or hybrid tetraplex structure under NaCl or KCl, respectively.

The dissociation constant, $K_d$, of 1 for Na$^+$ or K$^+$ was estimated by Eq. (1), a fitting of the curve of Fig. 2C or Fig. 2D obtained from an ellipticity change upon addition of NaCl or KCl, respectively. Figures 2C and 2D showed the sigmodal change of their ellipticity as a suggesting positive cooperativity. This is in agreement with the fact that tetraplex DNA was formed in a step-by-step manner in Na$^+$ or K$^+$.19 These changes have a good fitting with 1:2 bindings of 1 for both cations. Here, we arranged as an apparent stability constant of $K_d$ without any distinction of the first and second coordination of Na$^+$ or K$^+$ with 1. The obtained $K_d$ of 1 for Na$^+$ or K$^+$ was 19.7 ± 0.6 mM or 1.04 ± 0.04 mM, respectively, upon the addition of either salt. Although 1 has a 20-times higher affinity for K$^+$ than for Na$^+$, a structural change of 1 does not occur at over 4 mM KCl, as shown in Fig. 2D, suggesting no effect for K$^+$ under the intracellular condition.

Fluorescence change of 1 upon addition of Na$^+$ or K$^+$

A fluorescence change of 2.0 μM 1 in Tris-HCl buffer (pH 7.4) was measured upon the addition of NaCl or KCl with 495 nm of the excitation wavelength as shown in Fig. 3. The fluorescence increase and decrease of 1 was observed at 518 and 585 nm, respectively, upon the addition of NaCl and KCl. Although the fluorescence decreasing rate at 518 nm, based on FAM, was similar in the case of NaCl (Fig. 3A) and KCl (Fig. 3B), the fluorescence increasing rate at 585 nm based on TAMRA was two times larger for NaCl than for KCl (Fig. 3).

Fig. 3  Fluorescence spectra change of 2.0 μM 1 in Tris-HCl buffer (pH 7.4) upon the addition of NaCl (A) or KCl (B). FRET efficiency change ($F_{585\text{nm}}/F_{518\text{nm}}$) was plotted for NaCl (C) or KCl (D) as obtained from (A) or (B).

Fig. 4  (A) Fluorescence spectra change of 2.0 μM 1 upon the addition of NaCl in Tris-HCl buffer (pH 7.4) under 5 mM KCl as an extracellular condition and (B) FRET efficiency change ($F_{585\text{nm}}/F_{518\text{nm}}$) was plotted for NaCl (C) as obtained from (A).
This shows that the FRET of 1 was more effective for NaCl than for KCl, which shows that 1 formed an anti-parallel tetraplex complex with Na+ and a hybrid one with K+ and that FAM and TAMRA existed at a closer position for the former than the latter showing more effective FRET signal. The fluorescence intensity ratio of 585 nm over 518 nm was plotted against the Na+ or K+ concentration as shown in Figs. 3C and 3D. Sigmoidal changes were observed in Fig. 3 as a similar manner in the CD spectra titration of Fig. 2 and we discussed this as an apparent $K_d$ value. Fitting of these curve with Eq. (2) gave $K_d$ value of $47.8 \pm 0.4$ or $4.03 \pm 0.08$ mM for Na+ or K+, respectively. Since the binding affinity of 1 was higher for K+ than for Na+, the FRET signal for K+ was gently increasing up to 8 mM KCl, 1 might be utilized for Na+ monitoring under an extracellular condition. Figure 4A shows the fluorescence change upon the addition of NaCl under an extracellular condition and shows a fluorescence increase at 585 nm under 10 – 250 mM of Na+. The fluorescence intensity ratio of 585 nm for 518 nm increased upon the addition of NaCl under 5 mM KCl and $K_d$ was obtained as 90.2 ± 7.5 mM using this curve fitting as shown in Fig. 4B. Under the extracellular condition, the Na+ concentration was changed over the range of 120 – 150 mM, which is the range to monitor with 1.

Localization of 1 on the cell surface and its fluorescence imaging

To perform the localization of 1 on the surface of HeLa cell, we focused on the interaction of the sugar chain on the cell. The immobilization concept is shown in Fig. 5A and this procedure was conducted in the following way. Biotin-modified concanavalin A21 was added to the cell to localize biotin on the cell surface and was subsequently treated with avidin and biotin-modified 1 in this order. Since avidin has four binding sites of biotin, avidin on the cell surface has enough binding sites of biotin-modified 1 (Fig. 5A). Finally, 1 was immobilized on the surface of a cell cultivated in collagen-coated dish, where a DMEM (−) medium containing 110 mM NaCl and 5 mM KCl is in agreement with the extracellular condition. Figure 5B shows the fluorescence imaging of cells using confocal laser fluorescence microscopy. The cell surface showed fluorescence and florescence derived from FAM at 518 nm and from TAMRA at 585 nm observed at the same position on the cell. This result gave evidence that 1 is localized on the cell surface and responded for Na+. From this photographic image, the fluorescence intensity ratio of 585 nm for 518 nm was estimated to be 1.22, which is in agreement with an expected value of 1.20 (5 mM KCl and 110 mM NaCl).

Conclusions

The conjugate 1, having human telomere DNA sequence with FAM and TAMRA at its termini and biotin, was designed and synthesized to monitor Na+ on the cell surface. Although the binding affinity of 1 is higher for K+ than for Na+, the FRET signal is larger for Na+ than for K+ because of the difference in their tetraplex structure of anti-parallel or hybrid. 1 is utilized for Na+ imaging in a living cell, even under the presence of a 5 mM KCl condition, based on the difference of the FRET efficiency depending on the tetraplex structure. In this paper, we developed a localization method of 1 on the cell surface by a treatment with the following procedure: sugar chain on the cell surface, biotin-modified Concanavalin A, StAv, and biotin-modified 1 in this order.

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Supporting Information

This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.

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