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Letter

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Design of Split G-quadruplex-based DNA–Bridged Nucleic Acid Chimera Nanotweezers That Recognize Short Nucleic Acids with a Single-base Mismatch

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The design and feasibility of split G-quadruplex-based DNA nanotweezers (split Gq-based DNA-NTs) that can recognize short nucleic acids with a single-base mismatch are discussed. The split Gq-based DNA-NTs consist of three single-stranded DNA sequences forming a tweezers shape with split Gq sequences at the edge of each arm. In response to target recognition such as specific nucleic acids, the split Gq sequences come close to each other and then regain their ability to form a Gq/hemin complex, which exhibits peroxidase activity. Therefore, specific nucleic acids can be detected by measuring the peroxidase activity of split Gq-based DNA-NTs in a homogeneous assay format. Although split Gq-based DNA-NTs have been utilized as a biosensing molecule that recognizes a specific target and generates a sensing signal, the condition of target recognition was not well elucidated. Therefore, it was yet unclear whether the split Gq-based DNA-NTs can recognize short nucleic acids with a single-base mismatch. In this study, by selecting microRNA (miRNA) sequences of let-7a and let-7c as a single-base mismatch model, we successfully developed split Gq-based DNA–bridged nucleic acid chimera NTs (split Gq-based DNA–BNA chimera NTs) that can distinguish these miRNAs on the basis of the difference in melting temperature. It is expected that the split Gq-based DNA–BNA chimera NTs will be applicable to the development of point-of-care testing devices as target recognition and signal generation elements.

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1. Introduction

The point-of-care testing (POCT) of nucleic acids has wide applications in fields such as diagnostics and food safety.(1–3) Nucleic acids consist of simple combinations of four bases, and thus, a difference of even one base can result in a huge difference in function. Therefore, the discrimination of nucleic acids with a single-base mismatch is an important topic. MicroRNAs (miRNAs), one of the representative nucleic acids that exhibit a significance difference in function based on single-base differences, are noncoding small RNA molecules composed of approximately 20–30 nucleotides. Correlations have been found between many diseases such as cancer(4–6) and miRNAs in bodily fluids including blood, urine, tears, and saliva.(7,8) Accordingly, miRNAs have attracted attention as promising biomarkers for POCT in many diseases because they can be collected with no or low invasiveness. However, conventional methods for detecting nucleic acids such as reverse transcription polymerase chain reaction (RT-PCR)-based methods are not ideal for POCT because they often require complex procedures and expensive instruments. Thus, it is necessary to develop a simple method for detecting miRNAs, even those with a single-base mismatch in order to use miRNAs as biomarkers in a POCT format.

We have been developing a DNA nanotweezer technique for detecting nucleic acids.(9–11) A tweezer shape made via the self-assembly of three single-stranded DNA oligonucleotides alters its structure from an open state to a closed state after recognizing a target nucleic acid with two arms, inducing the edges of each arm in a proximity relationship. The split G-quadruplex-based DNA nanotweezers (split Gq-based DNA-NTs) possess split Gq sequences(12–15) at the edges of each arm. In response to target recognition, the split Gq-based DNA-NTs drag the split Gq sequences in a proximity relationship, and then the split Gq sequences recover their ability to form a Gq/hemin complex, which exhibits peroxidase activity.(16,17) Thus, a simple homogeneous assay with a signal based on the peroxidase activity for the detection of nucleic acids can be realized by utilizing split Gq-based DNA-NTs.(10) Because of a simple structure and principle, the split Gq-based DNA-NTs and their derivatives have also been utilized by other research groups for the detection of a protein(18) and applied to the electrochemical detection of target nucleic acids.(19–21) However, the target recognition ability of split Gq-based DNA-NTs has not been precisely investigated yet. Specifically, it is unclear whether they can recognize short nucleic acids with a single-base mismatch. Therefore, to apply this DNA-NT technique to the detection of short miRNAs with a single-base mismatch, it is necessary to discuss the design of target recognition sites in its structure.

In this study, we demonstrated the feasibility of DNA-NTs to distinguish miRNAs with a single-base mismatch by controlling the melting temperature (Tm) at the target recognition sites of DNA-NTs utilizing a bridged nucleic acid (BNA).(22) A BNA is an artificial nucleic acid possessing a rigid structure attributed to a bridge between the 2′- and 4′-positions of the ribose, and it exhibits a higher affinity for its complementary base than native nucleic acids such as DNA and RNA. Therefore, by inserting a BNA at the target recognition site of mismatched sequences, it is expected that the affinity of split Gq-based DNA-NTs for a specific miRNA will increase, permitting the discrimination of single-base mismatched miRNAs (Fig. 1). In this

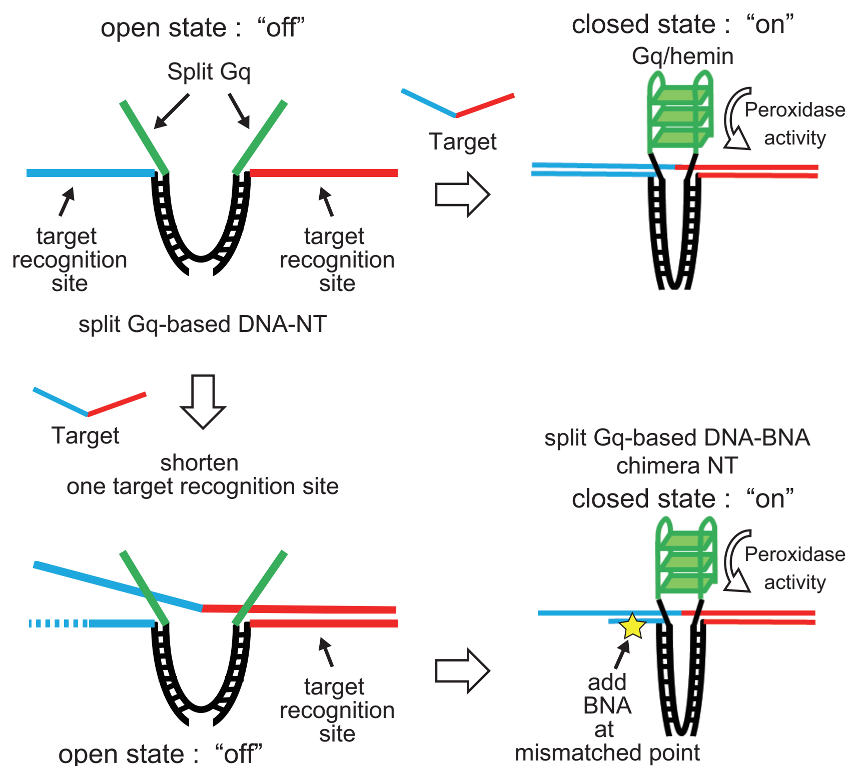

Fig. 1. (Color) A strategy for designing split G-quadruplex-based DNA–bridged nucleic acid nanotweezers (split Gq-based DNA–BNA NTs) for the specific detection of short nucleic acids such as microRNAs with a single-base mismatch. Typical split Gq-based DNA-NTs alter their shape from an open state to a closed state in response to a target recognition, exhibiting a peroxidase activity (upper side). The split Gq-based DNA-NTs do not respond to a shortened target recognition site because of a decreased melting temperature at the site (left side). After adding a BNA at the mismatched point, the split Gq-based DNA–BNA chimera NTs can recognize the short target with a single-base mismatch (bottom side).

study, we designed a strategy to distinguish let-7a and let-7c[23,24] as a single-base mismatched model to conceptually prove the feasibility of the split Gq-based DNA-NT technique for miRNA detection.

2. Materials and Methods

2.1 Materials

All nucleotide sequences used in this study are listed in Table 1. Synthetic DNA and RNA were purchased from Integrated DNA Technologies (MBL, Nagoya, Japan). DNA–BNA[22] chimera oligonucleotides were synthesized by GeneDesign, Inc. (Osaka, Japan). Among several different types of BNAs, we selected BNA^{NC} because it was expected to exhibit the highest Tm according to the manufacturer. 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and hemin were purchased from Wako (Osaka, Japan).

Table 1
(Color) Sequences of nucleotides used in this study.

Name	Sequence (5′–3′)
O1 (Hes-1)[9]	CTCAACTTTTATAATACAAATACATTTTACGCCTGGTGCC
O2 (Hes-1)[9]	CCGACCGCAGGATCCTATAAGGCGCAATCCAATAT
O2-14 base	CCGACCGCAGGATCCTATAAGGCGCAATCCAATA
O2-13 base	CCGACCGCAGGATCCTATAAGGCGCAATCCAAT
O2-12 base	CCGACCGCAGGATCCTATAAGGCGCAATCCAA
O2-11 base	CCGACCGCAGGATCCTATAAGGCGCAATCCA
O2-10 base	CCGACCGCAGGATCCTATAAGGCGCAATCC
O2-9 base	CCGACCGCAGGATCCTATAAGGCGCAATC
O3 [2]	GGGTTGGGTTTTTATAGGATCCTGCGGTCGGAGGCACCAG GCGTAAAATGTATTTGGGTAGGG
O1 (let-7a)	AC **t** ATACAACCTACATTTTACGCCTGGTGCC
O1 (let-7c)	AC **c** ATACAACCTACATTTTACGCCTGGTGCC
O2 (let-7a, let-7c)	CCGACCGCAGGATCCTATAAT**a**CTACCTCA
Target (Hes-1)[9]	ATATTGGATTGCGCCTTTGTATTATAAAAGTTGAG
Target (let-7a)[23,24]	UGAGGUAGUAGGUUGUAU **A** GUU
Target (let-7c)[23,24]	UGAGGUAGUAGGUUGUAU **G** GUU

Nucleic acids colored in red in O1 sequences are the target recognition sites hybridized to the underlined red parts in the corresponding targets. Bold lowercase letters in O1 (let-7a) and O1 (let-7c) denote BNAs. The bold characters in Target (let-7a) and Target (let-7c) are mismatched points hybridized to BNAs. Nucleic acids colored in blue in O2 sequences are the target recognition sites hybridized to the underlined blue parts in the corresponding targets. Bold lowercase letters in O2 (let-7a, let-7c) denote BNAs. The green color in O3 indicates the split sequences of the G-quadruplex. The black parts in O1, O2, and O3 were hybridized to form the DNA nanotweezer shape.

2.2 Production of DNA-NTs[10]

Each single-stranded DNA oligonucleotide (O1, O2, and O3; 5 μM) mixed in phosphate-buffered saline (Sigma-Aldrich) was incubated at 95 °C for 5 min and then gradually cooled (−1 °C/3 min) to 10 °C to form the DNA-NT structure via self-assembly. The prepared DNA-NTs were stored at 4 °C until needed.

2.3 Evaluation of effect of Tm at target recognition site on target recognition ability of split Gq-based DNA-NTs

The effect of Tm at the target recognition site on the target recognition ability of split Gq-based DNA-NTs was evaluated by measuring its peroxidase activity. The length of the target recognition site in one arm of the FRET-based DNA-NTs for Hes-1,[9] which was previously identified as valid DNA-NTs, was gradually reduced, after which its peroxidase activity was measured as described elsewhere. The Tm for each target recognition site was estimated using the following equation:

$$\mathrm{Tm} = 4\ ^\circ\mathrm{C} \times (\text{number of G + C in target recognition site}) + 2\ ^\circ\mathrm{C} \times (\text{number of A + T in target recognition site}). \quad (1)$$

2.4 Measurement of peroxidase activity[10]

The peroxidase activity of each DNA-NT with target recognition sites of various lengths was measured via a typical colorimetric method utilizing 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). The DNA-NTs (final, 50 nM), target (final, 50 nM), and hemin (final, 1 μM) were mixed in a working buffer (50 mM Tris-HCl, 150 mM NH_4Cl, 20 mM KCl, and 0.03% Triton X-100, pH 7.5) to a total volume of 89 μL and incubated for 1 h at 25 °C. Then, 1 μL of ABTS (final, 2 mM) was added to the reaction mixture before transferring the reaction mixture to a 96-well clear-bottomed plate. A colorimetric reaction was started by the addition of 10 μL of H_2O_2 (final, 2 mM) to a final volume of 100 μL. The absorbance at 420 nm was measured immediately at 10 s intervals using a Spectra Max M5 (Molecular Devices Japan, Tokyo, Japan).

3. Results and Discussion

Split Gq-based DNA-NTs recognize target nucleic acids with target recognition sites encoded in both arms and exhibit peroxidase activity. To discriminate miRNAs with a single-base mismatch, it is necessary to control the peroxidase activity between the "on" and "off" states according to the single-base difference. Therefore, we first determined the Tm condition at target recognition sites to eliminate their peroxidase activities by shortening the length of one arm in the split Gq-based DNA-NTs for Hes-1 as a model. As shown in Fig. 2, the split Gq-based DNA-NTs did not exhibit peroxidase activity when the Tm of the target recognition site in one arm was lower than 30 °C. This temperature was slightly higher than room temperature. It was suggested that split Gq-based DNA-NTs tend to maintain an open status due to the steric obstacle of the hinge and/or the repulsion of their arms because of the negative charge of the DNA backbone.[10] Thus, a higher Tm, implying a higher affinity, may be needed to bind to a target to maintain the closed status at temperatures exceeding room temperature.

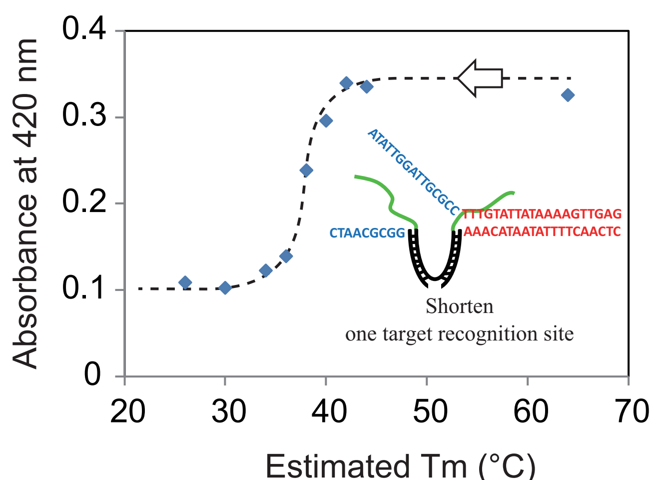

Fig. 2. (Color) Peroxidase activities of split Gq-based DNA-NTs for Hes-1 with target recognition sites of various lengths. Their melting temperatures (Tms) were estimated as described in Sect. 2.3 in Materials and Methods. Peroxidase activity was measured by determining the change in absorbance at 420 nm at 100 s after the start of the reaction.

Typically, miRNAs are approximately 20–30 nucleotides in length. Therefore, it is difficult to guarantee that the Tm at the target recognition sites of DNA-NTs will exceed 30 °C because the target should be divided into two sequences that are approximately 10–15 nucleotides in length. In fact, the Tm of the front halves of let-7a and let 7c, which have identical 10-nucleotide sequences, was estimated to be 28 °C. Therefore, we added one BNA to increase the Tm of the target recognition sites of both chimeric DNA-NTs (Fig. 3). Then, we determined whether the DNA-BNA chimera NTs with a BNA at the mismatched point respond to the corresponding target but not to a target with a single-base mismatch. Namely, the DNA–BNA chimera NTs would alter their structure through the hybridization of specific miRNAs with the target recognition site, leading to a closed status that exhibits peroxidase activity ("on"). Moreover, an open status lacking peroxidase activity ("off") would be maintained against other miRNAs with a single-base mismatch because of the lower affinity. In fact, as shown in Figs. 3 and 4, the DNA–BNA chimera NTs for let-7a exhibited peroxidase activity only in the presence of let-7a, whereas no activity was observed in the presence of let-7c [Figs. 3(a) and 4(a)]. Similarly, the DNA–BNA chimera NTs for let-7c responded only in the presence of let-7c but not let-7a [Figs. 3(b) and 4(b)].

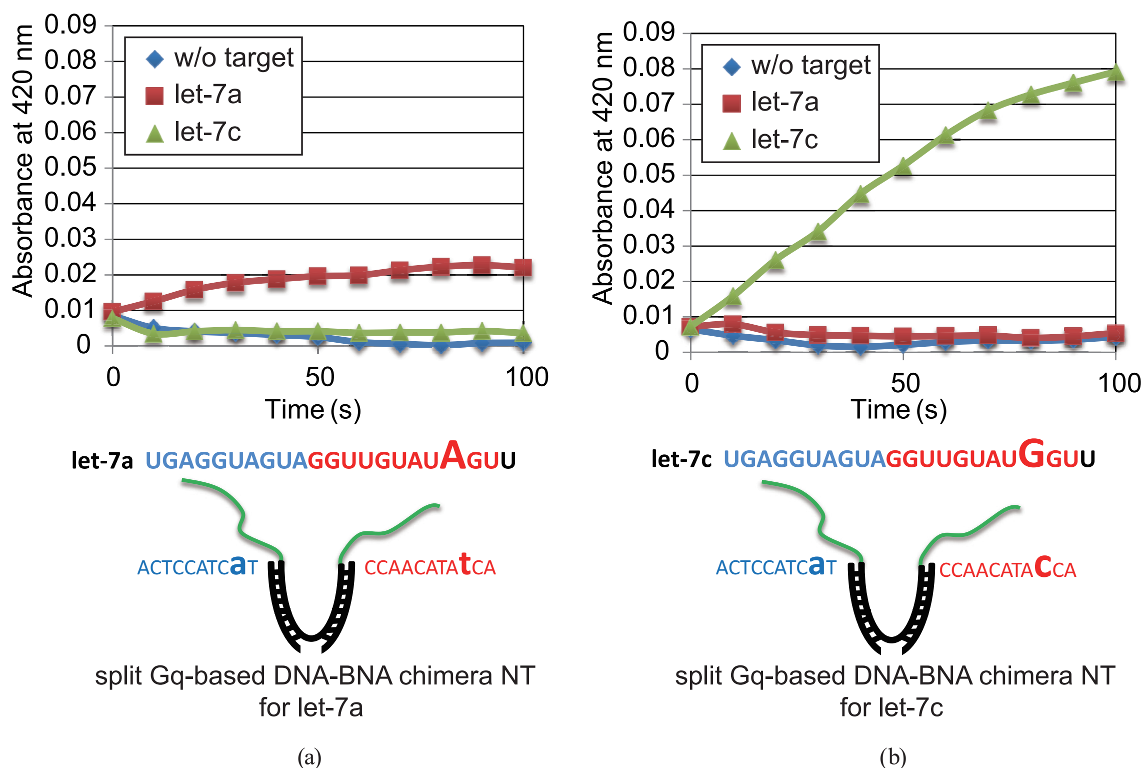

Fig. 3. (Color) Examples of the time course of peroxidase activity induced in response to miRNAs with a single-base mismatch. Split Gq-based DNA–BNA chimera NTs for let-7a (a) and let-7c (b). Bold uppercase letters in the sequences of miRNAs were the mismatched sites. Bold lowercase letters in the target recognition sites were BNAs. The absorbance measured with the buffer solution with only hemin instead of DNA-BNA chimera NTs was subtracted from each data point as a baseline.

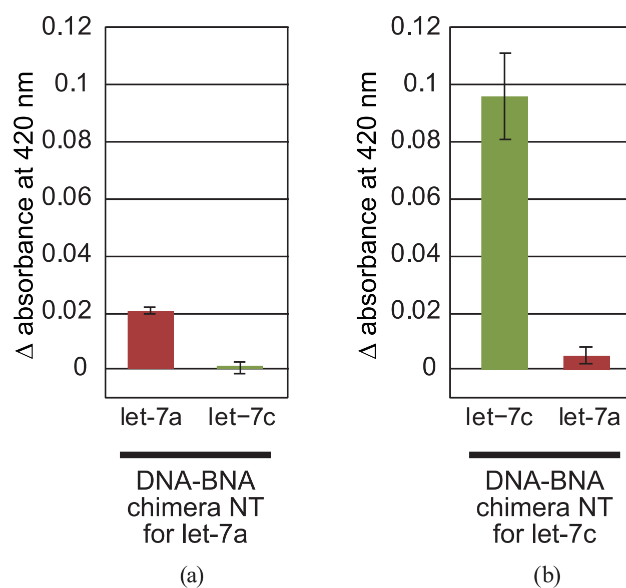

Fig. 4. (Color online) Increases in absorbance during 100 s measured with the (a) split Gq-based DNA–BNA chimera NT for let-7a and (b) split Gq-based DNA–BNA chimera NT for let-7c (n = 3). The absorbance measured with the DNA-BNA chimera NTs in the absence of the target miRNA was subtracted from each data point.

The two DNA–BNA chimera NTs exhibited a difference in peroxidase activity. This might be caused by the difference in the type of inserted BNA base that recognizes each mismatched point in the targets. The 19th base of let-7a is adenine (A), whereas that of let-7c is guanine (G). Accordingly, the BNAs introduced into the target recognition sites were thymine (T) and cytosine (C), respectively. Generally, the affinity between G and C is higher than that between A and T in the case of Watson–Crick base pairing. Thus, the Tm against let-7c is higher than that against let-7a in the case of the DNA–BNA chimera NTs. This difference in Tm was considered to explain the difference in peroxidase activity against the corresponding targets. Nevertheless, it has been successfully demonstrated that split Gq-based DNA–BNA chimera NTs have a potential to discriminate miRNAs with a single-base mismatch.

4. Conclusions

In this study, we have conceptually proved the feasibility of detecting miRNAs with a single-base mismatch using split Gq-based DNA–BNA chimera NTs that offer a convenient method for detecting nucleic acids using peroxidase activity in a homogeneous assay format. Although the detection sensitivity should be further considered, many methods that are capable of amplifying the number of specific nucleic acids under isothermal conditions are now available.[25–28] Also, in addition to a colorimetric method, other methods such as electrochemical detection with split Gq-based DNA-NTs were reported.[19–21] Therefore, by the combination of these methods, it is expected that split Gq-based DNA–BNA chimera NTs will be applicable to the development of POCT devices for the detection of a wide variety of nucleic acids as target recognition and signal generation elements.

Acknowledgments

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