報告番号	甲　第　14042　号

主　論　文　の　要　旨

論文題目　Studies on Fluorescent Probe Composed of Artificial Nucleic Acids for Detection of Natural Nucleic Acids
(天然核酸検出に向けた人工核酸を有する蛍光プローブに関する研究)

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論　文　内　容　の　要　旨

1. Introduction

As a conformer of fluorescent probe, DNA has drawn much attention due to its specific and predictable nucleobases interaction and defined length scale. Generally, under a precise design, the DNA probes with modification of signal molecules enable to show different emitting pattern to corresponding conformation with/without target nucleic acids, herein the detection will be implemented by monitoring the emission. However, application of DNA fluorescent probe *in vivo* is limited, mainly caused by two factors: 1. Since DNA suffers low enzymatic resistance, false signal will be observed in the cell due to degradation. 2. Unintended cross-hybridization usually occurs under complicated environments, such as mismatched RNA, indicating an insufficient orthogonality of DNA.

Our laboratory has reported three *acyclic* artificial nucleic acids (XNAs) with high nuclease resistance, which are composed of nucleic acids analogs: acyclic D-threoninol

nucleic acid (D-*a*TNA), serinol nucleic acid (SNA) and *acyclic* L-threoninol nucleic acid (L-*a*TNA) (figure 1a). Conformers carrying natural bases are linked via phosphodiester bonds, enable *a*TNA and SNA oligomers to dissolve in the water easily. Because of suppressed electrostatics repulsion among the acyclic scaffolds, all the *acyclic* XNA conformers form ultra-stable homo duplex, indicating strong base-pairing interaction. Since the acyclic XNAs are well-characterized, the conformers exhibit different usage: for example, D-threoninol modified with fluorophore can serve as a functional part in the DNA duplex, which is supposed to intercalate between nucleobases. Besides, these conformers show totally different recognition patterns due to the helicity: the right handed L-*a*TNA conformer is orthogonal to left handed D-*a*TNA conformer, and vice versa; SNA conformer shows almost non-helical because of its flexible scaffold, herein SNA shows high affinity to both *a*TNA and natural nucleic acids, enabling the informational translation from one system to the other one (figure 1b). In a nut shell, *acyclic* XNAs conformers are desirable for developing fluorescent probes.

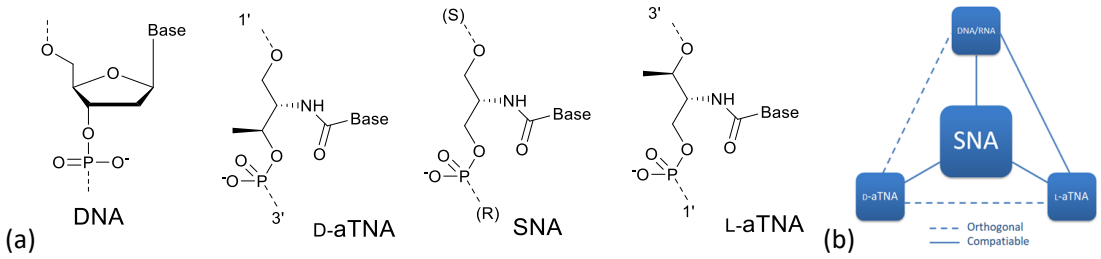

Figure 1. (a) Chemical structures of DNA and acyclic XNAs. (b) Biocompatibility among natural nucleic acids and acyclic XNAs.

This thesis will mainly introduce the developments of the efficient fluorescent probe composed of artificial nucleic acids targeting natural nucleic acids, which is divided in the following two parts.

2. Study on triplex-forming linear probe for duplex DNA detection

In this study, we report a triplex-forming linear probe tethering multiple fluorophores at intervals of native oligonucleotides with D-threoninol as scaffold, which enables simple detection of the double-stranded DNA (dsDNA) without denaturation. The principle of the linear probe is schematically illustrated in figure 2 on the left: At single-stranded state, linear probe self-quenches due to hydrophobic interaction among fluorophores, while it sequence-specifically forms triplex with target dsDNA via Hoogsteen base-pairing. As a result, fluorescence remarkably recovers because weakly stacked fluorophores are separated by their intercalation between the base-pairs. Since multiple fluorophores are incorporated to the fluorescent probe in linear structure, we

assume the linear probe would achieve fast kinetics with high emission efficiency.

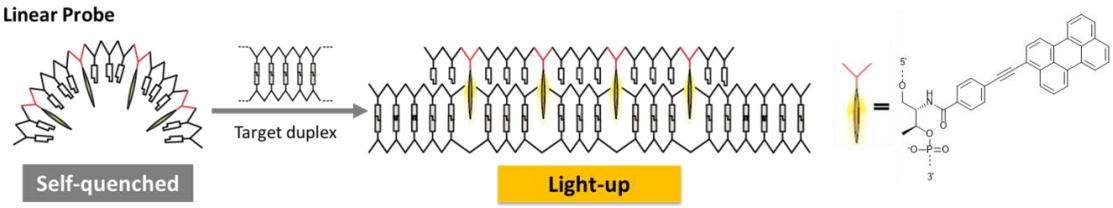

Figure 2. Schematic illustration of the linear probe targeting dsDNA. Chemical structure of fluorophore L was shown on the right.

As a result, by the incorporation of perylene derivative L (figure 2 on the right)and anthraquinone derivative gQ (Quencher, structure not shown), we have successfully designed an optimal probe 3L1gQ, targeting human Androgen Receptor (AR) gene. Signal/background (S/B) ratio of 3L1gQ was as high as 278 (figure 3a). Moreover, because of the intercalation of fluorophore, the melting temperature (T_m) of the triplex with 3L1gQ (T_m= 61.9 °C) was much higher than that of native DNA triplex-forming oligonucleotides (T_m= 40.0 °C), suggesting remarkable improvement on triplex stability. As possible application, plasmids with 0, 2, 5 AR gene sites have been constructed for detection of PCR products: 3L1gQ again showed specific fluorescence emission in the presence of product-dsDNA containing AR target site after PCR (spectra not shown), only by the addition of the probe to PCR solution. Notably, because of high S/B ratio, specific fluorescence emission of 3L1gQ could easily be visualized by naked eyes under hand-held UV lamp (figure 3b). These results demonstrate remarkable ability as a fluorescent probe for nucleic acid test, and its possibility for applying to dsDNA-targeted diagnosis in cell.

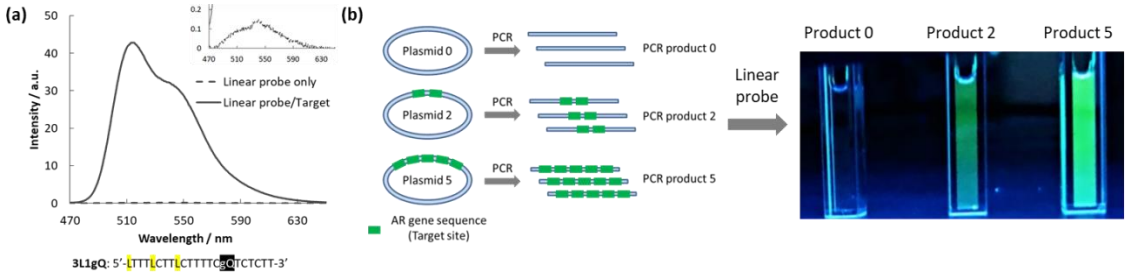

Figure 3. (a) fluorescence spectra of 3L1gQ targeting AR gene duplex. [AR sense]= [3L1gQ]= 1 μM, [AR antisense]= 1.1μM, 90 mM TBM buffer ($MgCl_2$=100 mM), pH 7.0, 20 °C. (b) Illustration of plasmid construction and fluorescence emission for the detection of AR gene after PCR under hand-held UV lamp (254 nm), [3L1gQ]= 1 μM, 50 uL crude PCR products after 30 cycles, 9 vol% glycerol within 90 mM TBM buffer ($MgCl_2$=100 mM), pH 7.0 in a final volume of 200 μL.

3. Study on XNA hybridization chain reaction (HCR) for orthogonal detection of RNA

In this study, by employing the biocompatibility among the *acyclic* XNAs, we developed novel HCR circuits composed of *α*TNA with high orthogonality. L-*α*TNA (LT) and D-*α*TNA (DT) in same sequence were designed in hair-pin conformation, which were supposed to form energy-barrier for XNA oligomerization (DT/LT-H1). Initiator composed of SNA, L-*α*TNA and D-*α*TNA with complementary toehold would kinetically trap hair-pin monomers thereby triggering cascade reaction. Since Cy3, Cy5 and nitro methyl red were modified in the D-/ L-*α*TNA hair-pin (DT/LT-H2), respectively. The kinetics of cascade reaction could be monitored via the fluorescence emission (figure 4).

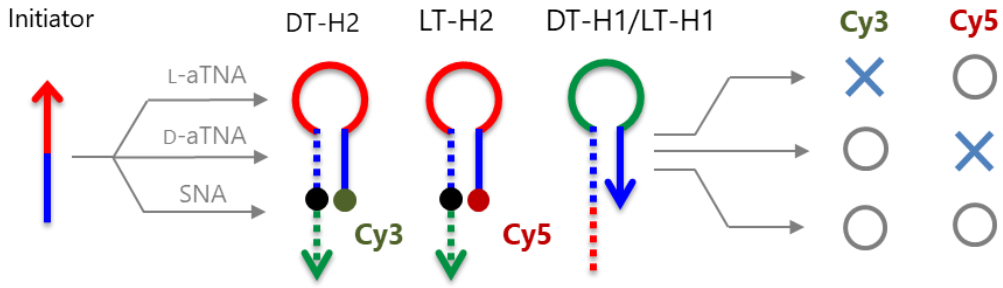

Figure 4. Illustration of orthogonal *α*TNA HCR circuits conducted by initiator composed of different acyclic XNAs.

As a result, not only individual *α*TNA HCR circuits, mixed L-/ D-*α*TNA HCR circuits showed highly independent cascade reactions: circuit was conducted only when corresponding *α*TNA or SNA initiator was added, while orthogonal initiator exhibit minimal leakage (figure 5). Notably, since *α*TNA formed ultra-stable hair-pin structure (T_m> 80 °C), *α*TNA HCR circuits achieved fast kinetics in timescale of minutes due to short stem (7 base-pairs) in the hair-pin.

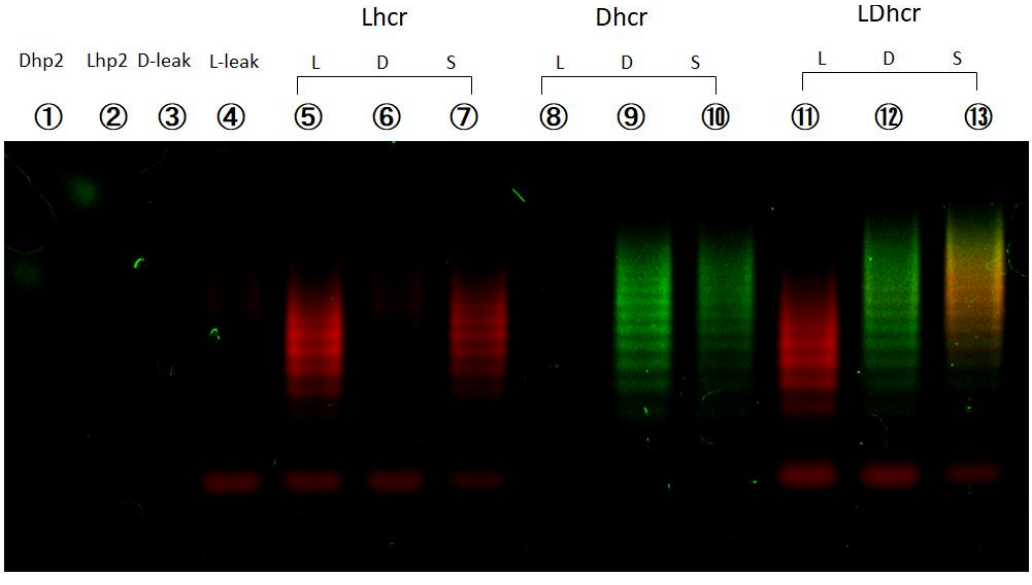

Figure 5. Gel analysis of HCR products. Lane 1-2: DT/LT-H2, Lane 3-4: DT/LT H1+H2, Lane 5-13: L-*α*TNA(Lhcr), D-*α*TNA(Dhcr), mixed L-/ D-*α*TNA HCR(LDhcr) circuit conducted by L-*α*TNA(L), D-*α*TNA(D) and SNA(S) initiator, respectively. [initiator]= 20 nM, [individual hair-pin monomers]= 100 nM, 10 mM phosphate buffer (NaCl= 200 mM) under 2 hours reaction, 37 °C, pH=7. Products were analyzed in 3 w/v% agarose gel under 100V for 30min, ex.= 532 nm for Cy3, 635 nm for Cy5.

As a possible application, we next implemented orthogonal RNA detection via SNA-interfaced D-*α*TNA HCR circuits. SNA interface (S-I0s) which was partly complementary to target RNA was designed in hair-pin conformation to ensure the detection *in vitro*. After annealing with target RNA, S-I/target RNA duplex with SNA toehold would serve as duplex initiator for subsequent cascade reaction (figure 5a). Because of the helicity transfer, SNA overhang was assumed to pre-organized in right-handed motif, which greatly suppressed the rate of signal amplification in D-*α*TNA HCR circuits. Subsequently, incorporation of 2 C3-spacers (S-I2s) has successfully blocked the helicity propagation and finally achieve equilibrium signal within 20 mins. Since D-*α*TNA could not hybridized to natural nucleic acids, we assume unspecific emission of HCR would be minimized in the cell imagination. Furthermore, limit of detection (LOD) for D-*α*TNA HCR circuit has been investigated. Similar LOD (500 pM, data not shown) were found for detection of RNA and D-*α*TNA, which indicated information-lossless translation of SNA as the interface in the acyclic XNAs platform.

To the best of our knowledge, this is the first time for conducting heterochiral nucleic acid circuits with one dimension products. Therefore, the localization of readout chain on the target RNA after HCR can facilitate a direct visualization, which is desirable for RNA imagination in vivo. We have reason to believe its potential for the future RNA detection in the cell.

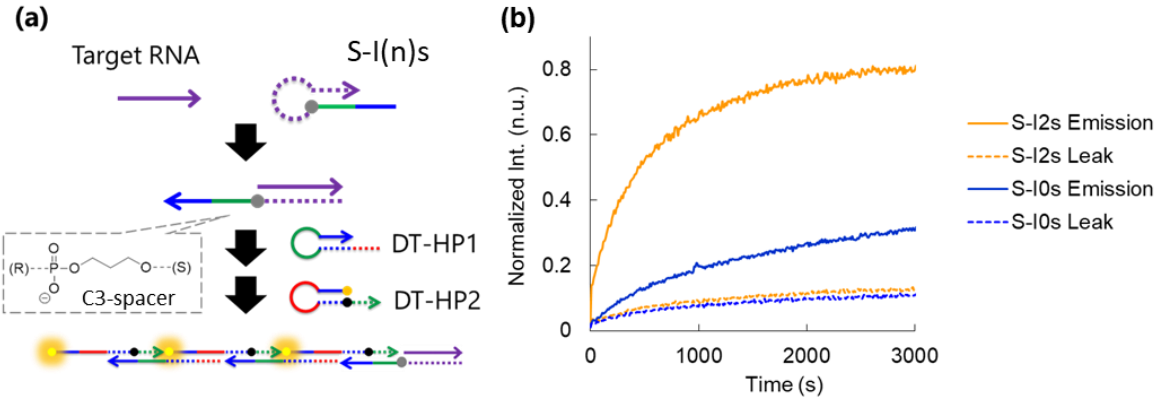

Figure 5. (a) Illustration of SNA-interfaced D-*α*TNA HCR circuit with incorporation of SNA spacer (number n). (b) Kinetic of D-*α*TNA HCR targeting RNA. [S-I(n)s/Target RNA]= 20 nM, [individual hair-pin monomers]= 100 nM, 10 mM phosphate buffer (NaCl= 1 M), 37 °C, pH=7. ex.= 546 nm,

em.= 562 nm.

Related Literatures

1. Chen, Y.; Murayama, K.; Kashida, H.; Kamiya, Y.; Asanuma, H. *Chem. Commun.*, 2020, ***56***, 5358-5361. (Selected as **Back Cover**)
2. Chen, Y.; Murayama, K.; Asanuma, H. *Chem. Lett.* 2020, **Accepted**