Nucleic Acid Modifications

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Selenomethylene Locked Nucleic Acid Enables Reversible Hybridization in Response to Redox Changes**

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DNA and RNA play important roles not only for the storage and flow of genetic information but also for the modulation of gene expression within an organism. For example, non-coding RNAs regulate many biological processes such as cell proliferation, cell death, and cell development.[1–3] It has also been suggested that guanine-rich DNA sequences regulate a variety of gene expression through G-quadruplex structures.[4,5] Moreover, epigenetic DNA modifications, including 5-methylcytosine, have recently been reported to participate in various diseases.[6–8] These gene-regulation systems, which are said to be natural nucleic acid switches, are controlled by changes in biochemical environments and gene-expression levels within cells.

Recently, chemically modified nucleotides that can reversibly change their properties by sensing differences in the surrounding environment have attracted attention. Such artificial nucleotides show promise as nucleic acid switches regulated by biological functions, which are impossible for natural nucleic acid switches. Several external-stimulus-responsive nucleic acids have been developed by regulating hydrogen-bonding interactions between nucleobases,[9] stacking within the DNA helices,[10] or the inversion of helicity.[11] Herein, we designed and synthesized a new redox-responsive nucleotide focusing on the reversible oxidation/reduction of selenium, as well as incorporated it into an oligonucleotide (ON) and characterized its properties as a nucleic acid switch.

Locked nucleic acid (LNA)[12]/2′,4′-bridged nucleic acid (2′,4′-BNA)[13] **1** has a methylene bridge between the 2′-oxygen and 4′-carbon atoms of the ribose sugar, which locks it in the C3′-*endo* conformation (Figure 1a). ONs containing LNA show strong binding affinity against complementary DNA and RNA.[12,14] Since the initial synthesis, a number of

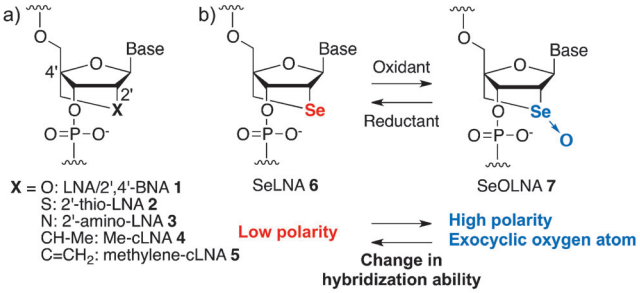

Figure 1. a) Formulas of 2′-substituted LNA analogues. b) Reversible structural change between SeLNA (**6**) and SeOLNA (**7**) by oxidant and reductant.

LNA analogues have been developed.[15] For example, 2′-thio-LNA (**2**)[16,17] and 2′-amino-LNA (**3**)[17,18] have the oxygen atom in the bridge replaced with sulfur and nitrogen atoms, respectively. These analogues show high binding affinity against complementary strands, similar to LNA, and we anticipated that the type of heteroatom at the 2′-position would have little influence on the binding properties of LNA analogues. On the other hand, it is known that the substituent on the bridged structure influences the hybridization property of LNA analogues because it interacts electrostatically and sterically with the minor groove of the duplexes. For example, Chattopadhyaya and co-workers reported that Me-cLNA (**4**), where the 2′-oxygen atom of LNA was replaced with a methyl-substituted carbon atom, shows reduced binding affinity for complementary RNA relative to LNA,[19] while Seth et al. reported that the replacement of the 2′-oxygen atom in LNA with an exocyclic methylene group does not affect the hybridization ability of modified ON [methylene-cLNA (**5**)].[20] These substituents in LNA analogues are located in the minor groove and thus can influence the duplex stability depending on their polarity and orientation.

Given this background, we designed a new LNA analogue possessing a low polaraity selenium atom at the 2′-position [SeLNA (**6**), Figure 1b]. Micura and co-workers reported the reversible oxidation/reduction of 2′-methylselenoguanosine in RNA;[21] therefore, a reversible structural change between SeLNA and its selenoxide-bridged analogue SeOLNA (**7**) should be possible. SeOLNA would have a highly polar selenoxide group in the bridge and the exocyclic oxygen atom

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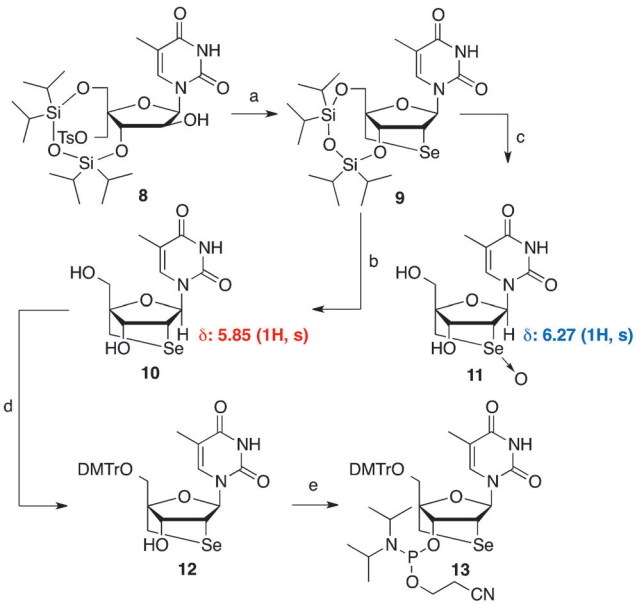
would be located in the minor groove of the duplex, hence the hybridization ability of SeOLNA is expected to be strikingly different from that of SeLNA.

The synthesis of SeLNA phosporamidite is summarized in Scheme 1. An arabino nucleoside derivative **8**[22] was converted into the triflate, which was treated immediately with sodium selenide[23] in EtOH/tetrahydrofuran (THF) to afford



Scheme 1. Synthesis of SeLNA phosphoramidite **13**: a) 1) Tf_2O, pyridine, CH_2Cl_2, 0 °C to RT; 2) Se, $NaBH_4$, EtOH, THF, 60 °C, 44% yield (2 steps); b) TBAF, THF, 0 °C, 67% yield; c) 1) *m*CPBA, CH_2Cl_2, 0 °C; 2) TBAF, THF, 0 °C (2 steps); d) DMTrCl, pyridine, RT, 77% yield; e) 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, DIPEA, MeCN, RT, 82% yield. Tf = trifluoromethanesulfonyl, TBAF = tetra-*n*-butylammonium fluoride, DMTr = 4,4′-dimethoxytrityl, DIPEA = *N*,*N*-diisopropylethylamine.

the desired cyclized product **9**. Desilylation with tetra-*n*-butylammonium fluoride (TBAF) was carried out to give the corresponding nucleoside **10**. Alternatively, **9** was oxidized by *meta*-chloroperoxybenzoic acid (*m*CPBA) and converted into SeOLNA nucleoside **11** as a single diastereomer. The C3′-*endo* sugar conformation of **10** and **11** was confirmed by ^{1}H NMR spectroscopy where the H1′ signal was observed as a singlet.[24] We also examined the stereochemistry of the selenoxide group based on the change in the chemical shifts ($\Delta\delta$ values) induced by oxidation. Hartree–Fock (HF) and density functional theory (DFT) calculations of the (R_{Se})-isomer **S1** and (S_{Se})-isomer **S2** (see Supporting Information for formulas of **S1** and **S2**) clearly indicated that the experimental $\Delta\delta$ values of H1′, H6′α, and H6′β induced by oxidation were in very good agreement with the calculated ones for the (R_{Se})-isomer; therefore, we confirmed the stereochemistry of the selenium center of selenoxide **11** as the (R_{Se})-configuration (Supporting Information, Table S1). Tritylation at the primary hydroxy group of **10** with DMTrCl and phosphitylation at the secondary hydroxy group yielded phosphoramidite **13**. The amidite **13** was incorporated into ONs using conventional solid-phase phosphoramidite synthesis. The ON sequences used in this study are shown in Figure 2.

There have been reports of the oxidation of 2′-methylseleno RNA by iodine treatment during solid-phase synthesis;[21,25–28] however, we did not observe such oxidation of

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14     5'-d(GCGTTTTTTGCT)-3'
15     5'-r(GCGUUUUUUGCU)-3'
16a-c  5'-d(GCGTTTTTTGCT)-3'
17a-c  5'-d(GCGTTTTTTGCT)-3'
18a-c  5'-d(GCGTTTTTTGCT)-3'
19a-c  5'-d(GCGTTTTTTGCT)-3'
20     5'-d(AGCAAAAAACGC)-3'
21     5'-r(AGCAAAAAACGC)-3'
MBa-b  5'-FAM-d(TTTTTTCTTTTGTCCCTTCTT-
                AAAAAA)-BHQ1-3'
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Figure 2. ON sequences used in this study. Underlined bold characters indicate modified residues. Series **a** = SeLNA, **b** = SeOLNA, and **c** = LNA modifications in the ONs.

SeLNA-containing ONs. Moreover, SeLNA was found to be stable in air (Figure S3). This may be because SeLNA is stable against oxidants or that deoxygenation of SeOLNA occurs during DNA synthesis. Therefore, we investigated the redox properties of SeLNA in ONs. Initially, ON **16a** having one SeLNA unit was treated with hydrogen peroxide, and the resulting product was analyzed by reverse-phase (RP) HPLC at several reaction times (Figure 3a). The signal corresponding to ON **16a** completely disappeared within 12 hours and ON **16b** with SeOLNA was generated. Further oxidation to the selenone-bridged analogue was not observed in the MALDI-TOF mass spectra. We then reduced SeOLNA back to SeLNA using dithiothreitol (DTT) as a reducing agent; reduction of SeOLNA was complete within 12 hours. We also evaluated the concentration dependence of the redox reagents and confirmed that other redox systems could also

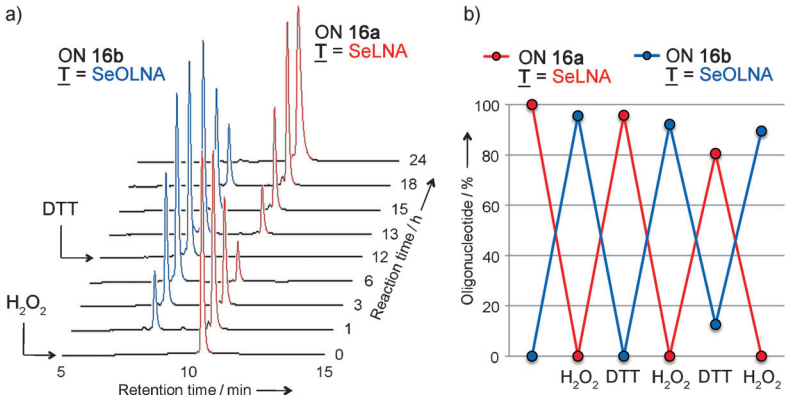

Figure 3. a) Reversible redox reaction of ON **16a** by H_2O_2/DTT observed by RP-HPLC. Conditions: ON **16a** (10 μM), sodium phosphate buffer (25 mM, pH 7.2), H_2O_2 (10 mM), DTT (10 mM), 37 °C. b) Repetitive redox reaction of ON **16a**. The percentages of ON **16a** and ON **16b** were obtained from the HPLC peak areas.

change the oxidation state of SeLNA (Figure S2). Moreover, redox reactions of ON **16a** using H_2O_2 and DTT were repeated, showing that the reaction was reversible at least five times in a row (Figure 3b). Thus, SeLNA has the potential to work as a redox switch for various biomolecules. There have been few reports on nucleosides or nucleotides that can reversibly change their structures and properties in response to the surrounding redox conditions.[29]

The hybridization ability of SeLNA- and SeOLNA-modified ONs to complementary DNA and RNA was evaluated by UV melting experiments and compared with LNA-modified ONs (Table 1). SeLNA- and SeOLNA-modified ONs formed more stable duplexes with an RNA complement than with a DNA complement, similar to other LNA analogues that were previously reported. As the number of modifications increased, the difference in hybridization ability between SeLNA and SeOLNA increased. Notably, ON **19a** with six consecutive SeLNA units showed a T_m value that was over 20 °C higher than that of ON **19b** with six consecutive SeOLNA units (DNA: 71 °C versus 44 °C; RNA: 84 °C versus 64 °C). To clarify the factors affecting the differences in hybridization ability between SeLNA and SeOLNA, we studied the thermodynamic parameters by concentration-dependent melting experiments (Table 2; Tables S4,S5). Thermodynamic data indicated that the introduction of an SeLNA unit to an ON caused gains in entropy and losses in enthalpy compared to the natural duplexes. Especially, the high stability of duplexes formed between ON **19a** containing six consecutive SeLNAs and complementary DNA or RNA was due to the large entropy gain that more than compensated for the loss of enthalpy. On the other hand, duplex formation of ON **19b** with the complementary strand had a heavy disadvantage in terms of entropy. Egli, Rozners, and co-workers revealed that a 2′-fluoro-modified RNA duplex was dramatically dehydrated relative to an unmodified RNA duplex owing to the low hydrogen-bonding ability of the 2′-fluorine atom with water molecules.[30] Consecutive low-polarity selenium atoms in ON **19a** could contribute to partial dehydration of the minor groove during the transition from single strands to duplexes, resulting in a decrease in entropy loss as compared to SeOLNA and LNA. Additionally, decreased Watson–Crick H-bonding strength from the less electronegative 2′-selenium atom might lead to a disadvantage in terms of enthalpy.[31]

Table 1: Melting temperatures of SeLNA-, SeOLNA-, and LNA-modified duplexes.[a]

Duplex		T_m (ΔT_m per modification) [°C][b]		
	T =	SeLNA	SeOLNA	LNA
16a–c/20		49 (−2.0)	46 (−5.0)	52 (+1.0)
17a–c/20		50 (−0.5)	43 (−4.0)	53 (+1.0)
18a–c/20		53 (+0.7)	44 (−2.3)	55 (+1.3)
19a–c/20		71 (+3.3)	44 (−1.2)	66 (+2.5)
16a–c/21		50 (+4.0)	48 (+2.0)	52 (+6.0)
17a–c/21		56 (+5.0)	50 (+2.0)	56 (+5.0)
18a–c/21		65 (+6.3)	56 (+3.3)	63 (+5.7)
19a–c/21		84 (+6.3)	64 (+3.0)	79 (+5.5)

[a] UV melting profiles were measured using a solution containing each oligonucleotide at a concentration of 4.0 μM in 100 mM NaCl and 10 mM sodium phosphate buffer at pH 7.2. [b] The T_m value given is the average of three independent measurements. ΔT_m values are calculated relative to the T_m values of unmodified DNA **14**/DNA **20** (51 °C) or DNA **14**/RNA **21** (46 °C) duplexes.

Table 2: Thermodynamic data for duplexes.[a]

Duplex	$\Delta H°$ [kcal mol^{-1}]	$\Delta S°$ [cal K^{-1} mol^{-1}]	$\Delta G°_{310K}$ [kcal mol^{-1}]
14/20	−84.6	−235	−11.6
19a/20	−65.2	−164	−14.5
19b/20	−96.0	−278	−9.9
19c/20	−80.3	−211	−15.0
14/21	−98.4	−282	−10.9
19a/21	−76.3	−187	−18.2
19b/21	−125.1	−346	−17.9
19c/21	−101.5	−262	−20.3

[a] These values were determined by van't Hoff plots with six data points (0.89–10.9 μM).

Interestingly, ON **19a** containing six consecutive SeLNA modifications showed much higher hybridization ability than ON **19c** containing six consecutive LNAs (DNA: 71 °C versus 66 °C; RNA: 84 °C versus 79 °C). Such excellent hybridization was also observed in ONs modified with six consecutive 2′,4′-BNA^{NC} modifications, which have a six-membered bridging structure.[22] Modeling studies suggest that the torsion angle (δ) and the maximum out-of-plane pucker (ν_{max}) of the SeLNA nucleoside was closer to that of 2′,4′-BNA^{NC} than that of LNA (Table S2). SeLNA has a five-membered bridge; however, the large selenium atom expands the size of the bridge and affects the hybridization properties.

Circular dichroism (CD) spectroscopy of SeLNA- and SeOLNA-modified duplexes were also performed to investigate their structural preferences (Figure S7). The spectrum of SeLNA-modified ON **19a** with complementary DNA **20** was very similar to that of a natural RNA/DNA (**15**/**20**) duplex. Moreover, minimal spectral differences were observed in the duplex formed between ON **19a** with RNA **21** compared with a natural RNA/RNA (**15**/**21**) duplex. These observations indicate that SeLNA formed duplexes with DNA and RNA in the same manner as natural RNA did. On the other hand, the CD spectra of SeOLNA-modified duplexes were different from that of natural DNA/DNA (**14**/**20**), DNA/RNA (**14**/**21**), RNA/DNA (**15**/**20**), and RNA/RNA (**15**/**21**) duplexes, suggesting that the duplexes modified with SeOLNA might not be typical A- and B-form duplexes, likely owing to local conformational alterations. However, it is possible that the CD spectra do not reflect the structure of SeOLNA-modified duplexes, because CD spectra may be affected by the existence of the UV-absorbing selenoxide group.

Measuring the T_m values revealed that there were great differences in hybridization ability between the SeLNA-modified ON and SeOLNA-modified ON when six consecutive modifications were introduced. This observation prompted us to use SeLNA in a nucleic acid switch to sense changes in the surrounding redox environment. We designed and synthesized a molecular-beacon-type DNA probe bearing

six consecutive SeLNA modifications in the stem region labeled with FAM at the 5′ end and BHQ1 at the 3′ end (Figure 4a, **MBa**).[32] We measured the change in the fluorescence intensity over time of **MBa** following addition of H_2O_2 (Figure 4b). The fluorescence intensity of **MBa** was gradually recovered in accordance with the reaction time, showing

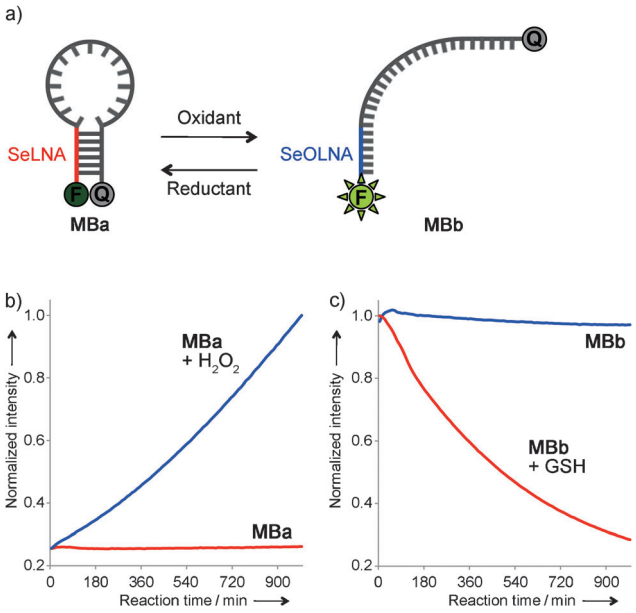

Figure 4. a) Scheme of the structure and fluorescent changes of an SeLNA-modified molecular-beacon-type probe (**MB**) in response to oxidant and reductant. b) Changes in the fluorescence intensity of **MBa** without (red) and **MBa** with (blue) H_2O_2. c) Changes in fluorescence intensity of **MBb** without (blue) and **MBa** with (red) GSH. Conditions: **MB** (0.1 μM), H_2O_2 or GSH (1000 μM), NaCl (100 mM), sodium phosphate buffer (10 mM, pH 7.2), 37°C.

dissociation of the hairpin structure. On the other hand, the addition of a reductant, glutathione (GSH), to the solution of **MBb** caused a decrease in the fluorescence intensity (Figure 4c). H_2O_2 is known as a marker for oxidative stress[33] and GSH as an important redox scavenger of reactive oxygen species.[34] Thus, **MB** could be used as a method for sensing the redox environment within a cell.

In conclusion, we synthesized a novel LNA analogue with a selenomethylene-bridged moiety, SeLNA. The selenium atom in the bridge of SeLNA could be converted into a selenoxide moity by treatment with an oxidant and reverted back to the selenide by treatment with a reductant. SeLNA-modified ONs showed high duplex-forming ability. Six consecutive SeLNA modifications imparted superior hybridizing ability compared to normal LNA modifications. This duplex-forming ability was disrupted by oxidation to SeOLNA. Finally, we demonstrated that a SeLNA-modified molecular-beacon-type probe could be used to sense changes in the surrounding redox environment. Recently, we reported the synthesis of SeLNA triphosphate and the enzymatic incorporation of an SeLNA nucleotide.[35] Further applications, such as the development of SeLNA-modified antisense agents targeting RNA overexpressed in oxidative environments and the evolution of SeLNA-modified aptamers towards the elucidation of aptamer structures by X-ray crystallography, are now in progress in our laboratory.

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