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Enhanced reactivity of amino-modified oligonucleotides by insertion of aromatic residue

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Abstract—We developed novel amino-modifying reagents, of which an amino group was connected with an aromatic residue by aliphatic linker. It was proved that the insertion of the aromatic residue could increase the reactivity of the amino group on oligonucleotides in comparison with conventional amino-modification.

Terminal modification of oligonucleotides is necessary for immobilization of oligonucleotide probes on solid surface. There are many kinds of terminal modifications such as sulfhydryl group,[1] hydrazide,[2] anthraquinone[3] and acrylamide[4] for attachment on the solid surface; among them, the aliphatic amine is most cost effective and produces less side products under standard deprotecting condition after oligonucleotide synthesis.[5] Thus, oligonucleotides modified with aliphatic amine have been frequently used for a gene analysis system requiring diverse oligonucleotide probes.[6] Besides aliphatic amino groups, there are nucleosidic-type amino modifiers to date.[7] However, these nucleosidic amino modifiers are able to form base pairs with opposite nucleotide in binding with target strand. Therefore, these nucleosidic amino modifiers are not suitable for universal terminal-modification for preparation of diverse oligonucleotide probes. On the other hand, polycyclic aromatic residues inserted into oligonucleotides have been well studied.[8] Although, these aromatic groups are known to increase the hybridization affinity for a complementary double strand, the effects of the aromatic residues on the reactivity of primary amino group have not been examined. We expected that the aromatic groups would improve the reactivity of primary amine and assist the purification steps of amino-modified oligonucleotides by their hydrophobic properties.

We designed and synthesized a series of amidite units, which have a primary amino group and an aromatic residue, by connecting with a short (S) or a long (L) linker of various lengths (see Supplementary data). Naphthalene (N) and anthracene (A) were selected as the aromatic groups (Fig. 1). The synthesized amidite units are referred to as SN, SNO, ssN, LNO, and LAO. The large letter 'O' indicates the carbamate structure in the linker between the amino and the aromatic residue. Although ssN consists of a hexamethylene linker and carbamate group, similarly to the structure of SNO, the naphthalene residue of ssN is very proximal to the primary amino group.

Amino-modified oligodeoxynucleotides of 25 bases (ODNs, X-25) were chemically synthesized by introducing each amino modifier at the 5′-ends (Fig. 2b). As control molecules, conventional amino-modified ODN (Con-25) and PL-25 were prepared. In PL-25, the aminohexyl group is tethered to the ODN by a propanediol phosphoric acid (Fig. 2a). PL-25 was synthesized to estimate the effect of linker length on the reactivity of amino group. Photocleavable (PC) amino-modifying reagent, which is commercially available, has an *o*-nitro-benzyl

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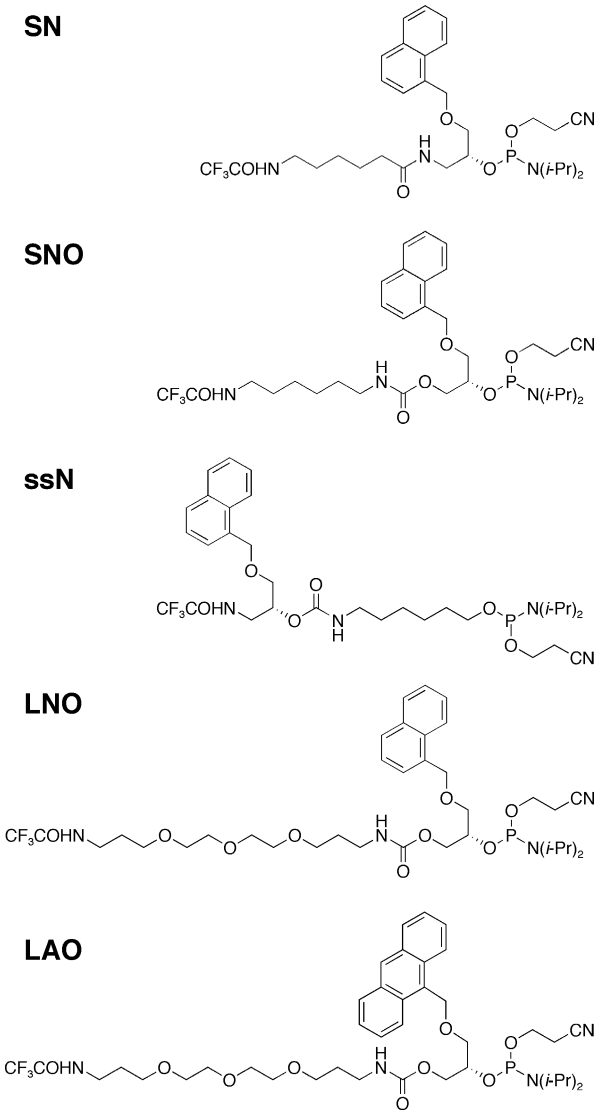

Figure 1. Structures of amino-modifying amidite units. The large letters, N, A, S, and L, indicate naphthalene, anthracene, and short and long linkers, respectively.

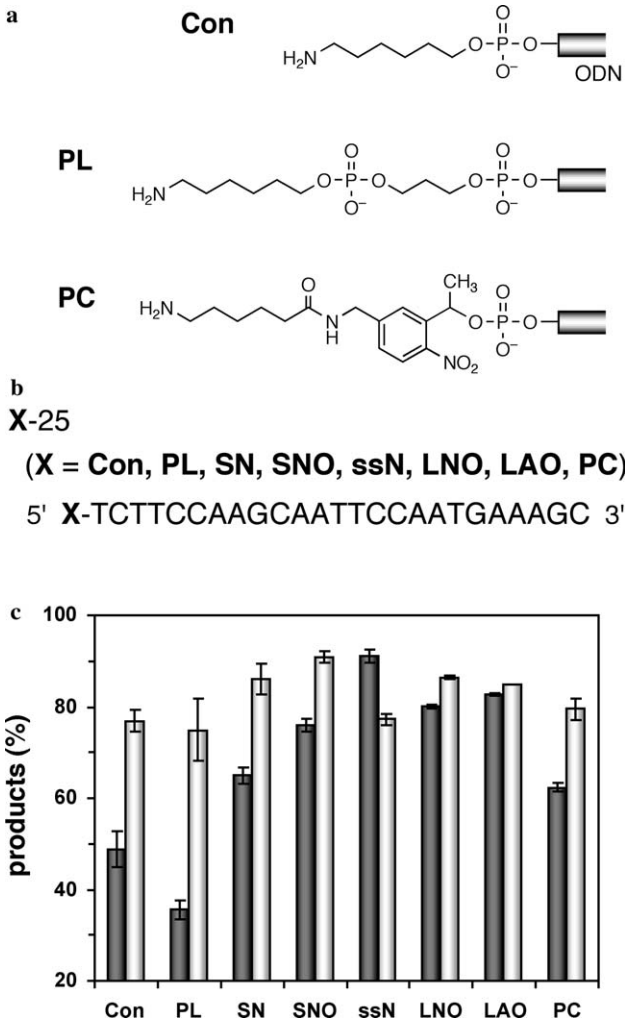

Figure 2. (a) 5′-Terminal structures of Con-, PL-, and PC-modified ODNs. Con is referred to as conventional amino modification. (b) Sequence of the amino-modified ODN X-25, which has a primary amino group at the 5′-terminus (X = Con, PL, SN, SNO, ssN, LNO, LAO, and PC). (c) Percentage of products in coupling reactions of X-25 with FITC. The ratios were calculated from HPLC analyses of 30-min reaction. Black and white bars represent the reactions in 10% and 40% dimethylformamide solutions, respectively.

group as an aromatic residue. Since the reactivity of the amino group has not been studied, PC-25 was also synthesized to evaluate the hydrophobic effect of the phenyl group.

In chemical synthesis of amino-modified ODNs, some amount of incomplete ODNs, which fail to combine with the amino modifier at the final step of the synthesis, are generated. It is generally difficult to remove these impurities from the conventional amino-modified ODNs by a reverse-phase column chromatography because there is little difference between the hydrophobic properties of these ODNs. Analysis of reverse-phase HPLC of the synthesized amino-modified ODNs revealed that all ODNs with the aromatic residue could be easily separated from the impurities due to the hydrophobicity of the aromatic moiety (see Supplementary data). The structures of ODNs were confirmed by MALDI-TOF/MS spectrometry analyses.[9]

We carried out coupling reactions of the amino-modified ODNs with fluorescein isothiocyanate (FITC) in aqueous solution containing 10% dimethylformamide. All amino-modified ODNs with the aromatic groups reacted with FITC more efficiently than Con-25 (black bars, Fig. 2c). Especially, ssN-25 showed the highest reactivity among all ODNs. LAO-25 also showed higher reactivity than LNO-25. The reactivity of PC-25 was slightly higher than that of Con-25, but less than that of other modifications. PL-25 without an aromatic residue showed the lowest reactivity among all probes. These results suggest that the presence of hydrophobic residue increased the reactivity of the amino-group in coupling reactions. It is thought that the hydrophobic residue of the amino-modified ODNs facilitates the association with FITC by a hydrophobic interaction between the aromatic group and FITC. The amino-reactivity is also related to the distance

between the amino group and the aromatic residue, as suggested from the effective coupling reaction of ssN-25. The labeling reactions were also checked in the solution containing 40% dimethylformamide (white bars, Fig. 2c). In this solution, all amino-modified ODNs reacted quite efficiently, and the effect of the aromatic residue seemed to be diminished. This result indicates that the 'more hydrophobic' condition suppressed the hydrophobic interaction, which becomes more important factor for the efficient coupling reactions in the hydrophilic condition. In practical labeling experiments, reactions are generally carried out in the solution containing dimethylformamide or dimethylsulfoxide to dissolve reporter groups, and as low concentration of organic solvent as possible makes the purification of labeled oligonucleotides successful in gel filtration or reverse-phase column. Therefore, the ssN is effective modification for labeling reactions.

Fluorescence spectra of FITC in conjugation with the ODNs are shown in Figure 3. Significant fluorescent quenching was observed only in LAO-25, which was presumably caused by some kind of interaction of fluorescein with an anthracene residue on the identical ODN. In ssN-25, the fluorescent quenching was not observed.

We also tested coupling reactions with other reporter groups, such as Cy5-succinimidyl ester or FAM-succinimidyl ester, and also with biotin-succinimidyl ester. In all cases, amino-modified ODNs with the aromatic residue reacted more efficiently than the conventional amino group, and ssN-modified ODN showed the most effective coupling reactions with these reporter groups similarly to the reaction with FITC (data not shown).[10]

The primary amino group is able to react with an aldehyde group to form a transient Schiff base, which is stabilized under reducing conditions.[11] We next evaluated the crosslinking rates of these amino-modified ODNs in covalent bond formation between complementary double-stranded ODNs. 5′-Fluorescein-labeled ODNs of 15 or 16 bases (F-AS15rG or F-AS16rG, Fig. 4a) were prepared as the 5′-side fragment (acceptor strand) of the ligated hairpin structure. Both strands had

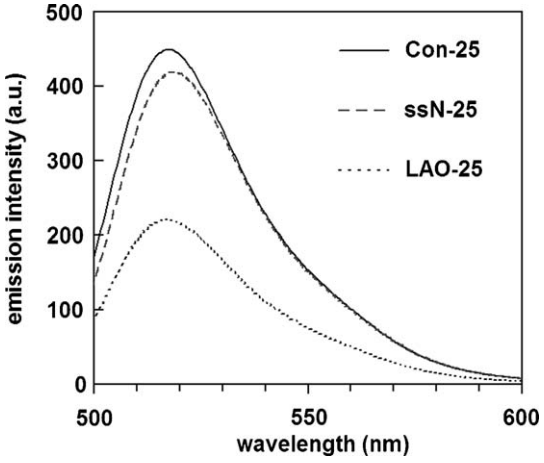

Figure 3. Fluorescence emission spectra of ODNs coupled with FITC. Solid line, Con-25; broken line, ssN-25; dotted line, LAO-25.

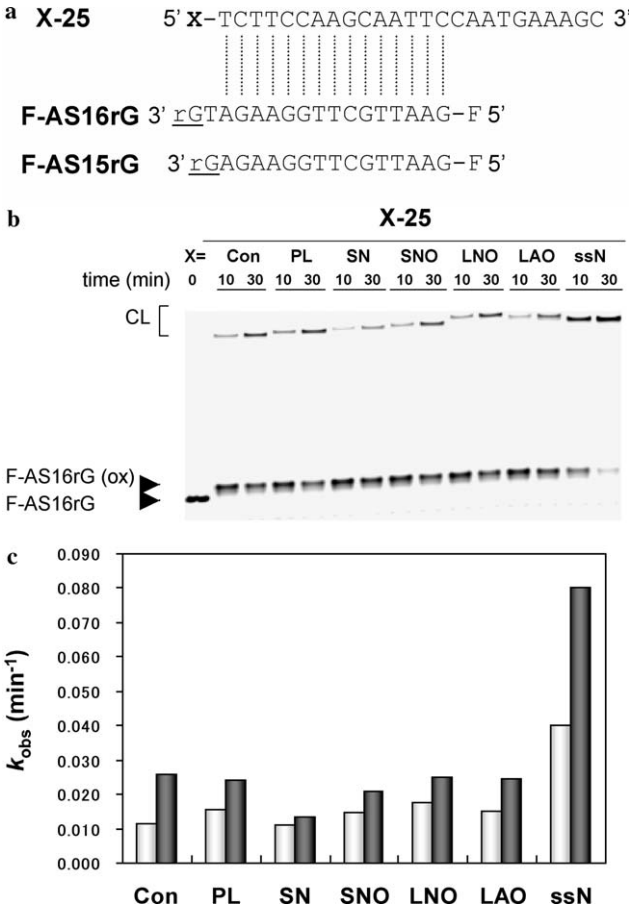

Figure 4. Crosslinking reactions between complementary double-stranded oligonucleotides. (a) Sequences of donor (X-25) and acceptor (F-AS16rG, F-AS15rG) ODNs. 5′-End of the acceptors was labeled with fluorescein (F), and each acceptor had a guanosine (rG) at the 3′-end to produce dialdehyde groups by periodate oxidation. (b) Analysis of the crosslinking reactions of X-25/F-AS16rG by denaturing polyacrylamide gel electrophoresis. The donor X-25 was added after periodate oxidation for 60 min, and each lane indicates the analyses of 10- and 30-min reactions from the additions of donor strand. CL and F-AS16rG (ox) indicate the crosslinked products and F-AS16rG oxidized by periodate ions, respectively. (c) Observed rate constants (k_{obs}) of the crosslinking reactions. White and black bars represent the observed rate constants for F-AS15rG and F-AS16rG, respectively.

a riboguanosine at the 3′-end to generate 2′,3′-dialdehyde group by periodate oxidation.

As the results of crosslinking reactions, all amino-modified ODNs (donor strands) provided crosslinked products, and the oxidized acceptor strands were simultaneously decreased as shown in Figure 4b. Observed rate constants for the crosslinking reactions were calculated from the percentages of the products (Fig. 4c). Interestingly, ssN-25 exhibited the fastest crosslinking rate. The crosslinking rate was about threefold higher than that of Con-25. This result is consistent with that observed in the coupling reaction with FITC under 'hydrophilic' condition. In contrast, other ODNs containing an aromatic residue showed almost the same reaction rate as Con-25. The reason for the significant reactivity of ssN is not clear, but the characteristic structure of ssN might contribute to the effective coupling

Table 1. Melting temperatures (°C) of crosslinked products

	Con-25	ssN-25
AS15rG	66.6 (35.6)[a]	64.0 (35.8)
AS16rG	64.1 (34.5)	64.0 (37.5)

[a] Melting temperatures of double-stranded DNAs before crosslinking reactions are listed in parentheses.

reaction by facilitating the hydrophobic interaction of naphthalene residue with nucleobase at the 3′-end of the oxidized acceptor strand.

Crosslinking products using ssN-25 and Con-25 were purified by HPLC, and the molecular weights and thermal stabilities were examined (see Supplementary data). All crosslinked products showed T_m values of about 30 °C higher than those of the double-stranded ODNs without the covalent linkage (Table 1). The results of the thermal stabilities and the mass spectroscopic analyses proved the formation of the crosslinked products.

We showed that the reactivity of the amino group on the oligonucleotides was increased by the introduction of an aromatic residue. Especially, it was found that the amino group close to the aromatic residue could react very efficiently with FITC and aldehyde groups in comparison with the conventional amino modification. Although anthracene is thought to be more effective than naphthalene residue in respect of hydrophobicity, anthracene yields its own fluorescence, and this character is not appropriate for fluorescence assay using other dyes. Therefore, ssN, which has a naphthalene residue, can be a profitable amino-modification for oligonucleotides and is applicable for various biological studies. For instance, it will be useful for efficient immobilization of probes onto oligonucleotide array because the ssN-modified oligonucleotides are thought to react efficiently with the reactive groups on the array surface. This effect will improve the sensitivity of the oligonucleotide array.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.07.027.

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