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Efficient synthesis of oligonucleotide conjugates on solid-support using an (aminoethoxycarbonyl)aminohexyl group for 5′-terminal modification

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ABSTRACT

Solid-support conjugation at the 5′-terminal primary amine of oligonucleotides is a convenient and powerful method for introducing various functional groups. However, conventional aliphatic amines do not necessarily provide conjugates with sufficient yields. To improve the modification efficacy, we used the amino-linker (aminoethoxycarbonyl)aminohexyl group (ssH-linker), for solid-support conjugation. In the ssH-linker terminal modification, reactive free amino group could be easily presented onto a solid-support due to rapid removal of the amino-protecting group, and activated amino acids or cholesterol molecules could be covalently connected more efficiently than to typical 6-aminohexyl-linkers. Based on these results, the ssH-linker can be a useful terminal modification for the solid-support conjugation of functional molecules.

Oligonucleotides (ONTs) are functionalized by attaching fluorophores, hydrophobic molecules[1] or peptides[2] to achieve gene detection or gene delivery. These functional molecules can be covalently connected to a primary amine on the ONTs. An aliphatic amine as typified by the 6-aminohexyl group (C6-linker, Fig. 1a) at the 5′-terminus of ONTs has been used for these functionalizations. The conjugations with the amino terminus of the ONTs are carried out in solution- or as a solid-phase reaction.[3] The reaction method is selected as the functionalization demands. When an anhydrous condition in organic solvent is required for the conjugation reaction, then a solid-support reaction is suitable.

In the solid-phase reactions, the protecting groups are first removed from the 5′-terminal amines on the solid-support after the ONTs are synthesized, and free amino groups are presented onto the solid-support, followed by coupling with target molecules. The monomethoxytrityl (MMT) group has generally been used as the protection for a 5′-terminal aliphatic amine. However, the MMT group is not rapidly removed from the aliphatic amine, even with stringent acidic treatment, which sometimes causes depurination of the ONTs. Furthermore, the reactivity of the primary amine is not sufficient for obtaining conjugates at high yields. Thus, excess amounts of exogenous molecules and longer reaction times are required for conjugations with the conventional amino group of ONTs.[4]

Recently, we developed a new amino-linker with an (aminoethoxycarbonyl)aminohexyl structure (ssH-linker, Fig. 1a).[5] We reported that ssH-linker modified ONTs (ssH-ONTs) reacted more efficiently with active esters in aqueous solution as compared with C6-linker modified ONTs (C6-ONTs). In addition, the MMT group of the ssH-linker could be removed much faster than that of the C6-linker under mild acidic conditions. However, the potential use of the ssH-linker for solid-phase modification has not been investigated. We describe here the utility of the ssH-linker for solid-support reactions.

We first examined the acid labilities of the protecting groups for the C6- and ssH-linkers since 4,4′-dimethoxy-4″-methanesulfinyltrityl (DMS(O)MT, Fig. 1a) was recently reported as a novel protecting group for the C6-linker.[6] C6- and ssH-modified 25-base ONTs synthesized with 'trityl on mode' (X-25; X = C6- or ssH-linker, Fig. 1b) were treated with a mild acidic solution (1% acetic acid), and the observed rate constants (k_{obs}) were determined from the percentage of 'trityl off' ONTs (see Supplementary data for details). The MMT group of ssH-25 was almost completely cleaved from the amine in 20 min (k_{obs} = 0.25 min^{-1}) as reported in a previous study,[5a] whereas C6-25 protected with DMS(O)MT group gave a 60% 'trityl off' product yield (k_{obs} = 0.048 min^{-1}, also see Fig. S1 in Supplementary data). MMT protected C6-25 yielded only a small amount of 'trityl off' ONTs after 20 min. These results show that the ssH-linker is superior to the C6-linker in the removal of the protecting group. The fast deprotection of the MMT group from the ssH-linker is responsible for the aminoethyl carbamate structure, as described in a previous report.[5a] This MMT deprotection was also checked on a DNA synthesizer, and the MMT group was found to be completely removed from ssH-25 synthesized on a controlled pore glass (CPG)-support by a single 'detritylate procedure' step

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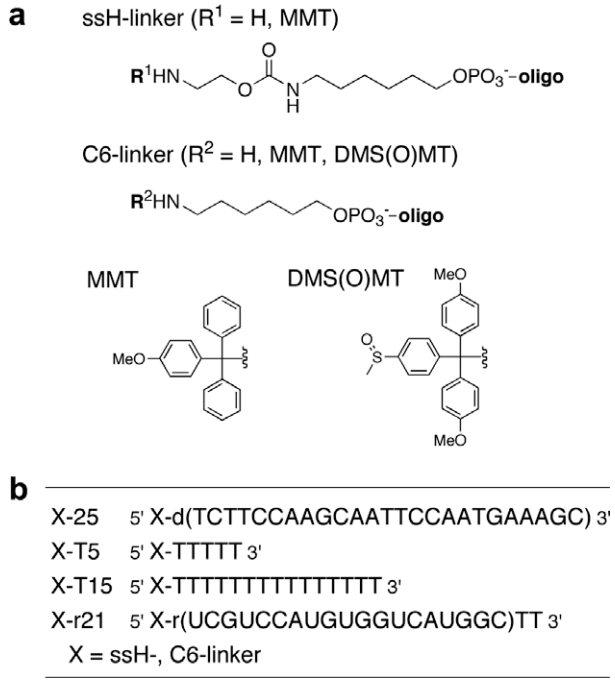

Figure 1. Amino-modified ONTs. (a) 5′-terminal structures of the amino-modified ONTs. The protecting group of the ssH-linker is MMT, and the C6-linker is MMT or DMS(O)MT. (b) Sequences of the oligonucleotides. X indicates the ssH-linker or C6-linker.

(3% trichloroacetic acid in dichloromethane for 60 s for a 0.2 μmol scale synthesis), and could be detected by the trityl-monitor of a DNA synthesizer. On the other hand, repetitive treatments (usually 3–4 times) were necessary to remove the MMT group from C6-25.

Next, we investigated the coupling reactions with ssH- and C6-modified ONTs on a CPG-support. First, an amino acid conjugation was performed, because the total synthesis of peptide-ONT conjugates depends on the coupling efficiency between a carbonyl group of the amino acid and a terminal amine of the ONTs.[7] Initially, C6- or ssH-modified penta-thymidylic acids (X-T5; X = C6- or ssH-linker, Fig. 1b) were used for the coupling reactions on the CPG-supports. After the MMT groups were removed on a DNA synthesizer, the CPG-supports were washed with diisopropylethylamine (DIEA) in DMF in order to neutralize the 5′-terminal amino groups.[8] Next, N-protected phenylalanine (*N*-Fmoc-Phe) or its pentafluorophenyl ester (*N*-Fmoc-Phe-OPfp) were added to the support by combining (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP®),[8] *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU)[9] or 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide hydrochloride (EDC)–1*H*-benzotriazole (HOBt) in the presence of DIEA.[10] The reactions were then analyzed by HPLC after deprotection of the Phe-X-T5 (X = C6- or ssH-linker) with aqueous ammonia. When PyBOP was used as a coupling reagent for *N*-Fmoc-Phe conjugation, ssH-T5 gave a 74.1% yield of Phe-conjugates after 2 min of reaction, whereas C6-T5 gave a 54.1% yield (Fig. 2a; also see Fig. S2 and Table S1 in the Supplementary data).[11] Similarly, ssH-T5 conjugated efficiently with *N*-Fmoc-Phe in the presence of HBTU. Although the reactions using EDC–HOBt or *N*-Fmoc-Phe-OPfp gave products with lower efficiencies in comparison with PyBOP or HBTU, ssH-T5 showed about 10% higher coupling yields than C6-T5. These results indicate that the ssH-linker is able to react with a target molecule on a solid-support as well as in solution.[5a]

We then carried out the same coupling reactions (*N*-Fmoc-Phe/PyBOP) with amino-modified ONTs containing all four nucleobases (X-25; X = C6- or ssH-linker). However, reactions using ssH-25 and C6-25 resulted in the predominant formation of 5′-N-acetylated ONTs, and their molecular weights were confirmed by MALDI-TOF/MS measurements (Fig. 2b). Strömberg and co-workers reported a similar 5′-N-acetylation as a serious side reaction during amino acid coupling with amino-modified ONTs on a CPG-support.[9] They explained that the acetyl groups were derived from acetylated nucleobases formed by the capping reagent for ONT synthesis, and eliminated these reactive acetyl species by washing

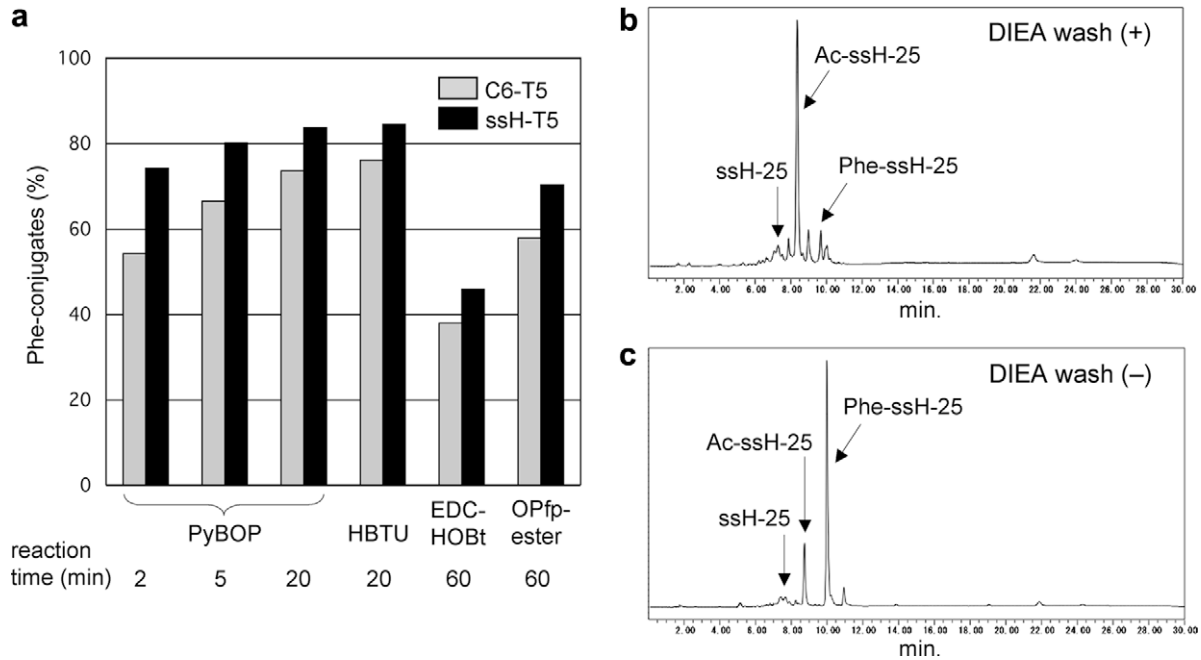

Figure 2. Conjugations of the amino-modified ONTs with *N*-Fmoc-Phe. (a) Reactions of X-T5 with *N*-Fmoc-Phe or *N*-Fmoc-Phe-OPfp.[10] The gray and black bars indicate C6-T5 and ssH-T5, respectively. The coupling reagents used in each reaction are listed in the horizontal bar, and the reaction times are shown below the graph. HPLC analyses of the conjugation reactions of ssH-25 with *N*-Fmoc-Phe after the DIEA wash (b), and after omitting the DIEA wash (c). Ac-ssH-25 indicates the 5′-N-acetylated ONT. See the Supplementary data for the HPLC conditions.

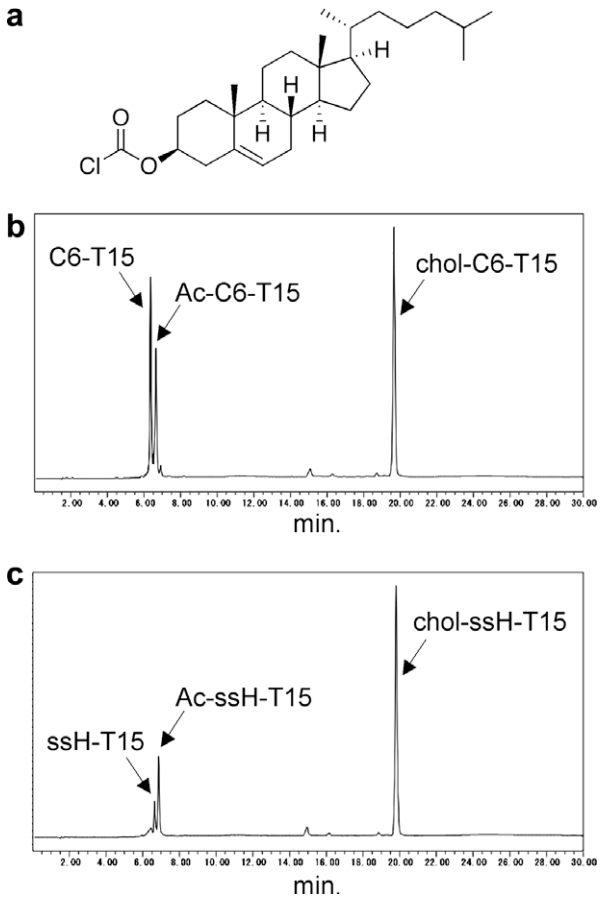

Figure 3. HPLC analyses of the conjugation reactions with cholesteryl chloroformate.[13] (a) Structure of cholesteryl chloroformate. (b) Reaction of C6-T15, and (c) reaction of ssH-T15. These reactions were performed without DMAP. Ac-C6-T15 and Ac-ssH-T15 indicate the 5′-N-acetylated ONTs. See the Supplementary data for HPLC conditions.

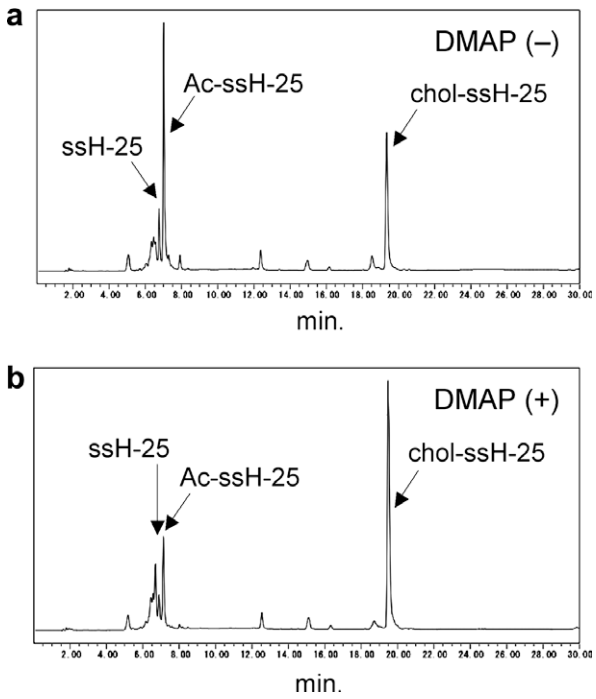

Figure 4. HPLC analyses of the conjugation reactions of ssH-25 with cholesteryl chloroformate. (a) Reaction in the absence of DMAP,[13] and (b) reaction in the presence of DMAP.[14] Ac-ssH-25 indicates the 5′-N-acetylated ONT. See the Supplementary data for the HPLC conditions.

the CPGs with 2% morpholine solution prior to the MMT removal. We simply omitted the neutralization process after the acid treatment for MMT removal. Interestingly, the formation of 5′-N-acetylated ONTs was drastically suppressed, and the yields of the Phe-conjugates increased to 73.7% for ssH-25 and 68.6% for C6-25 (Fig. 2c). These results suggested that 5′-N-acetylated ONTs are probably produced by intermolecular or intramolecular transfer of the acetyl groups from nucleobases (trans-acetylation), via the neutralization of the 5′-terminal amino-groups on the CPG-support. In addition, this acetylation was strongly dependent on the sequence of the amino-modified ONTs (data not shown), and this is consistent with a previous report.[9]

The conjugation of lipophilic groups to synthetic ONTs, such as antisense or siRNA molecules, is shown to mediate the cellular uptake of the conjugates.[1a,12] We next tried to conjugate ssH-ONTs with cholesterol (chol) on a CPG support. Although chol-ONT conjugates are generally synthesized using chol-phosphoramidite units, cholesteryl chloroformate (Fig. 3a) is inexpensive and thus a solid-phase reaction using cholesteryl chloroformate is cost-effective. Amino-modified pentadeca-thymidylic acids (X-T15; X = C6- or ssH-linker, Fig. 1b) were treated with cholesteryl chloroformate in the presence of DIEA.[13] As a result of these reactions, ssH-T15 could provide about 1.5-fold more chol conjugate than C6-T15, as shown in Fig. 3 (chol-ssH-T15, 76.0%; chol-C6-T15, 51.7%, also see Table S2 in the Supplementary data). Subsequently, we carried out these conjugation reactions to X-25 (X = C6 or ssH-linker) without the neutralization process to prevent trans-acetylation within the solid-support. In each reaction, chol-X-25 was produced to some extent, but the 5′-N-acetylated form was the major product (Fig. 4a). Although the trans-acetylation within the solid-support was suppressed by this method, we thought that acetylation to the 5′-terminal amine competed with the coupling reaction due to the low reactivity of cholesteryl chloroformate. Thus, to activate the cholesteryl chloroformate, dimethylaminopyridine (DMAP) was added to the reaction mixture. Under these reaction conditions,[14] the chol conjugates increased successfully to a 73.5% yield for ssH-25, whereas C6-25 gave a 54.6% conjugate yield (Fig. 4b). We also used the ssH-linker for the conjugation reaction of RNA (X-r21; X = ssH-linker, Fig. 1b) with cholesteryl chloroformate in the presence of DMAP on a CPG-support.[15] Expectedly, the ssH-linker gave chol-RNA conjugates at a 65.9% yield (Fig. S3 in the Supplementary data). These results showed that the ssH-linker is a promising terminal modification for the easy and cost-effective preparation of chol-RNA conjugates.

In summary, we have demonstrated efficient solid-phase conjugations with the 5′-terminus of ONTs using the ssH-linker. The ssH-linker is superior to the C6-linker not only for the removal of the protecting group from the 5′-terminal amine, but also for the conjugation efficiency on a solid-support. Although the 5′-terminal amine of ONTs with a hetero-sequence was acetylated during the conjugation reactions, we found that the acetylation could be suppressed by keeping the terminal amino groups protonated before the conjugation. Furthermore, it was found that the activation of exogenous molecules could reduce the acetylation in the case of cholesterol couplings. From these results, we conclude that the ssH-linker can be a very useful terminal modification for the functionalization of oligonucleotides.

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Supplementary data

Detailed experimental protocols of the solid-phase conjugation reactions, list of results, and HPLC chromatograms of the reactions. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.02.121.

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10. The 5′-amino-modified ONTs on the CPG-support (0.1 μmol) were treated with *N*-Fmoc-Phe (10 equiv), the coupling reagent (10 equiv) and DIEA (20 equiv) in DMF (0.5 mL), or *N*-Fmoc-Phe-OPfp ester (10 equiv) and DIEA (20 equiv) in DMF (0.5 mL). See the Supplementary data for more details.
11. The products were confirmed by MALDI-TOF/MS measurements. See the Supplementary data.
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13. The 5′-amino-modified ONTs on the CPG-support (0.1 μmol) were treated with cholesteryl chloroformate (40 equiv) and DIEA (80 equiv for X-T15, 20 equiv for X-25) in dichloromethane (0.5 mL) at room temperature for 15 min. See the Supplementary data for more details. The longer ONTs were used in the cholesterol coupling reactions for the purpose of increasing the solubility of the chol-ONT conjugates in water.
14. The 5′-amino-modified ONTs on the CPG-support (0.1 μmol) were treated with cholesteryl chloroformate (40 equiv), DMAP (20 equiv) and DIEA (20 equiv) in dichloromethane (0.5 mL) at room temperature for 15 min. See the Supplementary data for details.
15. The 5′-amino-modified ONT on the CPG-support (0.1 μmol) was treated with cholesteryl chloroformate (150 equiv), DMAP (20 equiv) and DIEA (20 equiv) in dichloromethane (0.5 mL) at room temperature for 5 min. See the Supplementary data for details.