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Interstrand cross-link of DNA by covalently linking a pair of abasic sites†

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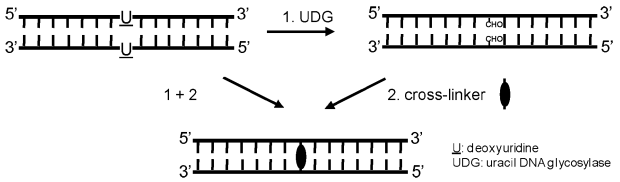
A pair of apurinic/apyrimidinic sites formed in DNA has been covalently connected with bis(aminooxy) derivatives. The efficacy of the interstrand cross-link is associated with the structural tethering of two aminooxy groups. The interstrand cross-link constructed stable DNA scaffolds for enzyme alignment.

Various chemical and environmental agents generate interstrand cross-links (ICLs) in double-stranded DNA. Bifunctional alkylating agents,[1] platinum compounds,[2] psoralen,[3] and unsaturated aldehydes[4] react with the natural bases of double-stranded DNA, resulting in ICL-duplexes. Site-directed ICLs are also formed by incorporating chemically modified nucleosides that allow disulfide bond formation,[5] amination,[6] radicals formations,[7] and photo activation.[8] These ICLs are applicable to some biological studies; however, most of them lead to the breakage of the connective base stacking at ICL-sites or protrude bulky linkers outside helices.

At equilibrium, an AP site resulting from the hydrolysis of the glycosidic bonds of nucleotides exists between a cyclic hemiacetal form and an open-chain aldehyde form. The latter form having an electrophile has the potential to conjugate with exocyclic amines of opposing bases[9] and proteins.[10] Because an aminooxy group readily reacts with the aldehyde group, mono-aminooxy-containing agents are powerful tools for quantification of the AP sites in DNA.[11] We previously reported that an aminooxy compound having a naphthalene residue improves conjugation efficiency toward AP sites.[12] In addition, an AP site becomes a recognition site for a number of small ligands which interact with the nucleobases surrounding the AP site.[13]

By exploiting these chemical properties of AP sites, we have developed a covalent cross-link of a pair of AP sites (AP pair) on the opposite positions of complementary strands (Scheme 1). In the reaction, two aldehyde groups of the AP pair are linked with a bifunctional cross-linker having two aminooxyacetyl groups. In other words, a single molecule such as an aromatic ring is covalently introduced in place of a base pair. Thus, various



Scheme 1 ICL reaction of an AP pair.

cross-linkers can be tested on a single DNA molecule, resulting in the production of ICL-duplexes including different cross-linkers. We synthesized four types of cross-linkers that comprise naphthalene (aoNao), propyl (aoPao), 1,2- and 1,4-substituted benzene (aoOBao, aoPBao) at the spacer tethering aminooxy groups (Fig. 1a). The benzene derivatives were prepared to examine the distance between the two aminooxy groups needed to form ICLs. The AP pair was produced from the hydrolysis of a deoxyuridine pair with uracil DNA glycosylase (UDG) immediately before the cross-linking reactions (Scheme 1).

We first investigated whether bis(aminooxy) derivatives linked complementary oligodeoxynucleotides (ODNs) (I/II, Fig. 1b) after UDG treatment, as shown in Scheme 1 (two-step reactions of 1 and 2). All reactions were conducted in an aqueous solution except for that of aoPBao, which was dissolved in 5% dimethylsulfoxide because of its insolubility in aqueous solutions. Products were analyzed with denaturing polyacrylamide gel.

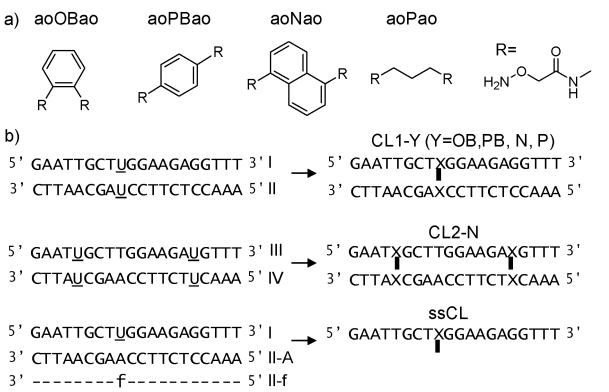


Fig. 1 (a) Structures of the bifunctional cross-linkers used for the ICL reactions. (b) Sequences of ODNs. U, f, X and the bold lines indicate deoxyuridine, the tetrahydrofuran derivative, AP site, and cross-linker, respectively.

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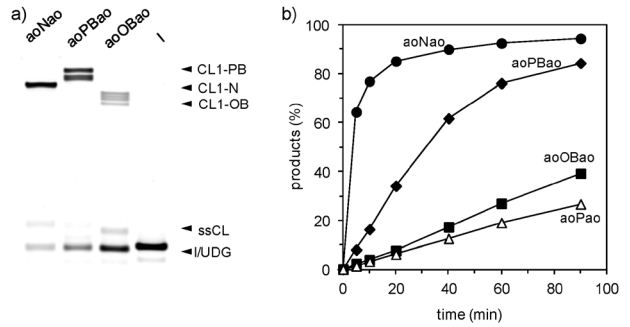

Fig. 2 Analysis of the ICL reactions. (a) Denaturing polyacrylamide gel analysis of the reactions using aoNao on single- and double-stranded ODNs. ssCL and I/UDG indicate the conjugate to the single stranded ODN-I and UDG-treated ODN-I. (b) Plots of the percentages of ICL products *versus* time.

All cross-linkers produced ICL-duplexes (CL1-OB, CL1-PB, CL1-N, and CL1-P), which showed retarded migration in the denaturing gel (Fig. 2a). aoNao provided most efficiently ICL-duplexes and aoPao exhibited the lowest activity (Fig. 2b). Interestingly, the yields and the migration speeds between aoOBao and aoPBao were quite different. CL1-OB and CL1-PB mediated by the benzene cross-linkers were observed as multiple bands only in the denaturing gel analysis, whereas by treatment with sodium cyanoborohydride, they were converted to a single band (Fig. S3 in ESI†). Thus, we concluded that the multiple bands were derived from the isomers of two oxime linkages.

The ICL reactions largely depended on the structures of the cross-linkers. In particular, the hydrophobic residue contributed to the cross-link of the AP pair as shown in our previous results.[12] Because the AP pair provides a space flanked by base pairs, the stacking interaction between the aromatic ring and the base pairs might facilitate cross-linker binding into the pocket in the helix.

All cross-linkers efficiently reacted with double strands in comparison with a single-stranded ODN (ssCL) (Fig. S1 in ESI†). To perform quantitative analyses, we determined the observed rate constants of the reactions to a single reaction site (monoAP site) in the presence or absence of a nonreactive template (II-A or II-f, Fig. 1b), which contained an adenosine or tetrahydrofuran derivative opposite an AP site. The I/II-f duplex is supposed to offer a space similar to that of an AP pair in the helix. aoNao efficiently reacted with the monoAP site in the double-stranded forms, and the rate constants of I/II-f (7.4×10^{-2} min^{-1}) and I/II-A (4.8×10^{-2} min^{-1}) were approximately five- and three-fold higher than that of the single strand (I, 1.4×10^{-2} min^{-1}), respectively. On the other hand, aoPao, aoOBao, and aoPBao only slightly increased the yields in the double-stranded forms containing nonreactive templates; however, the rate constants could not be determined because only few conjugates were formed. Whereas aoPBao and aoOBao exhibited different reaction efficiencies in the ICL containing double reaction sites, they showed equal potential for reacting with the monoAP site. This might be because the insufficient spatial distance between the double aminooxy groups of aoOBao did not allow the ICL of the AP pair.

The aoNao showed the potent activity for the ICL, and we synthesized an alternative naphthalene derivative (aNa) containing bis-primary amine instead of aminooxy groups. However, similar to aoPao, aNa exhibited few conjugates in the presence of sodium cyanoborohydride (Fig. S5 in ESI†). This result confirmed the importance of the combination of the aminooxy group and aromatic residue in this reaction.

Using aoNao, we examined the effects of the reaction temperature and the mismatched base pairs adjacent to the AP pair on the ICL reaction. The ICL duplex was dominantly obtained at 27 °C whereas the yields of the single stranded conjugates increased at the higher reaction temperature (Fig. S4a in ESI†). Similarly, the ICL formation was inhibited by introduction of the mismatched base pairs adjacent to the AP pair (Fig. S4b in ESI†). These results indicated that it is essential for the ICL reaction to form a stable AP pair pocket. However, it should be noted that the formation of the AP pair destabilizes duplexes. Thus, we performed UDG reaction in the presence of aoNao, as shown in Scheme 1 (the reaction of 1 + 2), to promote a simultaneous reaction. We found that the ICL-duplex could be obtained with the same efficiency as in the two-step reactions. It means that the ICL can be prepared more conveniently from hybridized ODNs.

aoNao could mediate double ICL formations in duplex III/IV (Fig. 1b). We examined thermal melting curves (T_m) of the ICL-duplexes containing single (CL1-N, CL1-P) and double ICL sites (CL2-N). CL2-N did not show a denaturing profile because both its terminals were constrained by ICLs. In contrast, CL1-N and CL1-P linked at the center of the sequence showed standard sigmoidal curves (Fig. S6b in ESI†), and their T_m values were found to be 75.7 °C (CL1-N) and 70.5 °C (CL1-P), respectively. These values were much larger than those of I/II (dU:dU mismatched duplex, 40.5 °C) and I/II-A (dA:dU perfectly matched duplex, 47.1 °C). Note that CL1-N showed a larger T_m value than CL1-P. We believe that the increase was due to the stabilizing effect of the naphthalene residue conjugated in the inside helix.

Because the ICL sites of both CL1-N and CL2-N contained the naphthalene ring that absorbs ultraviolet light at 290 nm, their structures were analyzed by complete digestions with nucleases. Both ICL duplexes showed high nuclease resistance because of the stabilization by ICLs, and the reaction times of nuclease digestions were extended for their complete digestions. As expected, both ICL sites were identified as aoNao-conjugates with two deoxyriboses (dR_2-aoNao). Similar to the standard sample, the ICL sites exhibited three peaks in the HPLC analyses (Fig. S7b, d and f in ESI†). They correspond to three structural isomers based on the two oxime linkages, which could be reduced to a single bond by sodium cyanoborohydride under the high acidic conditions (Fig. S7c and e in ESI†).

DNA can offer nanosized platforms for protein arrays[14] and enzyme reactions;[15] however, standard duplexes are reversible and are subject to dissociation. We applied the ICL formation to construct stable DNA scaffolds for enzyme reactions. 5′-Biotin- or 5′-fluorescein-modified ODN of 20 bases was linked with each complementary strand by using aoNao to anchor horseradish peroxidase-streptavidin (HRP-SA)[16] or alkaline phosphatase-anti-fluorescein antibody (ALP-Ab)[17] on DNA,[16] resulting in two-stranded ICL-duplexes (HRP-CL1-N, ALP-CL1-N, Fig. S8 in ESI†). In addition, we constructed a three-stranded duplex, HRP/ALP-CL2-N, that involved both 5′-biotin- and 5′-fluorescein-modified ODNs arranged in tandem on the

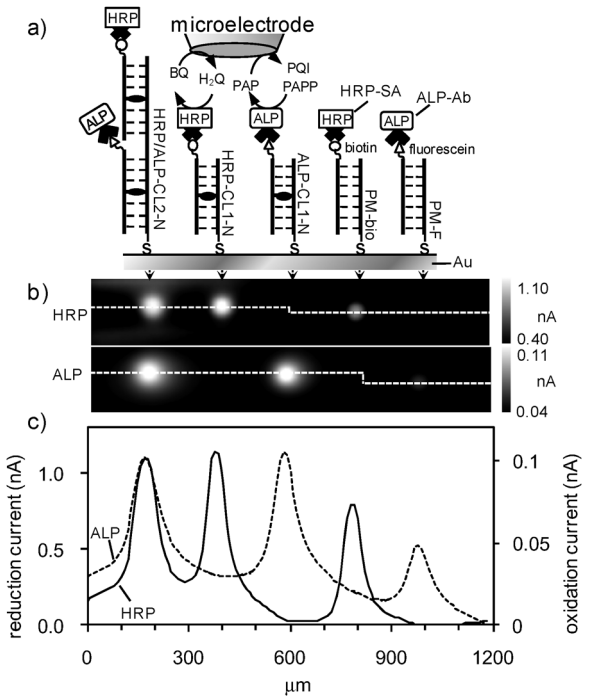

Fig. 3 (a) Schematic drawing of enzyme reactions on ICL-duplexes and the SECM analyses. The bold lines indicate ICL-sites. (b) SECM images of HRP and ALP reactions on each ICL duplex immobilized on a gold chip. (c) Plots of the current intensities of the HRP (solid line) and ALP (dotted line) reactions. These currents were obtained from line scans shown in the white dotted lines of (b).

43-base template. HRP/ALP-CL2-N equivalently offers both the enzyme binding sites on a single DNA molecule. Perfectly matched standard duplexes (PM-bio and PM-F) were also prepared to evaluate the effects of ICL (Fig. 3a). All duplexes were immobilized on a gold surface through a thiol tether at 5′-ends. After the enzymes were bound with the DNA duplexes, each enzyme reaction was conducted in the presence of substrates specific for these enzymes.

HRP oxidizes hydroquinone (H_2Q) to benzoquinone (BQ) and ALP produces *p*-aminophenol (PAP) from *p*-aminophenol phosphate (PAPP). Both catalytic actions were evaluated by reducing BQ and oxidizing PAP with the microelectrode of a scanning electrochemical microscope (SECM) (Fig. 3a).[16,18] As shown in Fig. 3b and c, these enzymes were found to actually promote reactions at exact DNA spots. In particular, HRP/ALP-CL2-N succeeded in accommodating both the enzyme binding sites on the single molecule. On the other hand, the enzymatic productions from the PM-bio and PM-F were significantly decreased to 67% and 38% of the corresponding ICL duplexes (Fig. S10 in ESI†). This result suggests that a part of standard duplexes was subject to dissociation into single strands during the chip preparation or enzyme reactions. The cross-linked duplexes provided stable DNA scaffolds for enzyme reactions.

In conclusion, a pair of AP sites formed in double-stranded DNAs can be linked with a single bis(aminooxy) molecule. This ICL distinctly stabilizes double strands by covalently implanting various molecules in the DNA helix. Hence, this site-specific ICL reaction is applicable for the construction of stable DNAs with different functionalities.

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