A Covalent Lock for Self-Assembled Oligonucleotide Conjugates

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Oligonucleotide conjugates have potential as components in creating self-assembling supramolecular systems. Since the hybridization reactions are reversible, convenient procedures for locking such systems in place after assembly can be useful. Several chemical procedures have been reported for linking oligonucleotide blocks aligned on a single- or double-stranded oligonucleotide template. The most effective methods generating natural phosphodiester bonds [−OP(O)(O⁻)O−] utilize activation of a phosphoryl group with cyanogen bromide, cyanoimidazole, or a water-soluble carbodiimide.[1] Ligation yields are variable and are sensitive to minor distortions in geometry at a nicked site in a duplex.[1c,d] Efficient chemical ligation has been achieved *via* oxidative coupling of terminal phosphorothioates[2] and displacement of bromide from a bromoacetylamino oligonucleotide by a terminal phosphorothioate.[3] For some applications, however, the distortions introduced by the extended internucleoside bridges formed in these reactions [−OP(O)(O⁻)S-SP(O)(O⁻)O− and −OP(O)(O⁻)SCH$_2$C(O)-NH−] would be disadvantageous. We describe here a hybridization-dependent autoligation that affords a bridge [−OP(O)-(O⁻)S−] very close in geometry and charge distribution to a natural phosphodiester link. The coupling, utilizing displacement of a 5′-*O*-tosyl group by a 3′-phosphorothioate,[4] is shown to be remarkably selective and efficient when the structural units are appropriately organized in solution. We illustrate the approach with three different systems: ligation of a nicked dumbbell oligonucleotide (**1**), cyclization of a conjugate (**3**) possessing a short oligonucleotide overlap at the juncture site, and closure of a cap at the end of a duplex (**5** + **6**) (Chart 1).

Oligomer **1** was prepared by solid phase synthesis using commercial reagents and a 5′-*O*-tosylthymidine 3′-(cyanoethyl)-phosphoramidite reagent.[5] Although it possessed two potentially interactive functional groups, **1** could be isolated readily. It

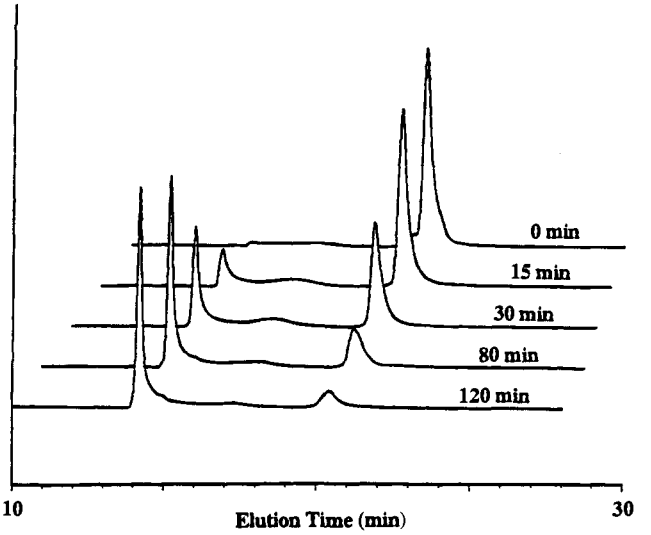
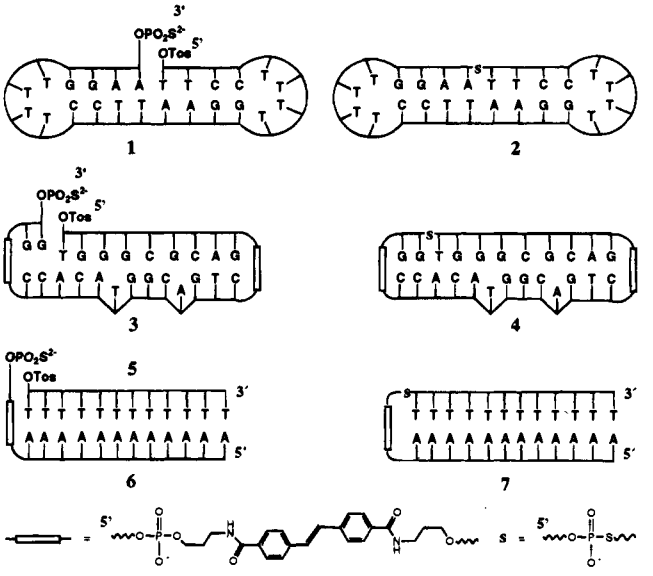


Figure 1. RP HPLC analysis of reaction of **1** at 37 °C after 0, 15, 30, 80, and 120 min. The peak eluting at 14 min corresponds to compound **2**; that at 20 min, to compound **1**.

Chart 1



was stable to lyophilization conditions and to storage for 10 days at −20 °C. No reaction was observed after **1** was left to stand in concentrated ammonium hydroxide at 37 °C for 2 h.[6] On the other hand, **1** (1.3 μM) afforded an easily isolable product, **2**, in high yield (>94%)[7] on standing for 2 h in aqueous 1 M NaCl (Figure 1).[8] Under the same conditions and at the same concentration (1.3 μM in each oligonucleotide), no coupling was observed within 24 h for a pair of nonhybridizing

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(2) Gryaznov, S. M.; Letsinger, R. L. *Nucleic Acids Res.* **1993**, *21*, 1403−1408.

(3) (a) Gryaznov, S. M.; Letsinger, R. L. *J. Am. Chem. Soc.* **1993**, *115*, 3808−3809. (b) Gryaznov, S. M.; Schultz, R.; Chaturvedi, S. K.; Letsinger, R. L. *Nucleic Acids Res.* **1994**, *22*, 2366−2369. (c) Herrlein, M. K.; Letsinger, R. L. *Nucleic Acids Res.* **1994**, *22*, 5076−5078.

(4) The preparation and some properties of 5′-S analogues of dinucleoside monophosphates and related short polymers obtained by displacement on a 5′-iodo or 5′-*O*-tosyl nucleoside by a nucleoside 3′-phosphorothioate have been reported: (a) Cook, A. F. *J. Am. Chem. Soc.* **1970**, *92*, 190−195. (b) Chladek, S.; Nagyvary, J. *J. Am. Chem. Soc.* **1972**, *94*, 2079−2085. (c) Kresse, J.; Nagpal, K. L.; Nagyvary, J.; Uchic, J. T. *Nucleic Acids Res.* **1975**, *2*, 1−9.

(5) Phosphitilation of 5′-*O*-tosylthymidine and *N*-{[(dimethoxytrityl)oxy]-propyl}-*N*′-(3−hydroxypropyl)stilbenedicarboxamide (ref 15) was carried out by standard procedures (Barone, A. D.; Tang, J.-Y.; Caruthers, M. H. *Nucleic Acids Res.* **1984**, *12*, 4051−4061). Oligonucleotide 3′-phosphorothioates were prepared as described in ref 3c. Expedite phosphoramidite reagents (Millipore Corp.) were employed in synthesizing the oligonucleotides containing a tosyl group, and the products were deprotected with concentrated ammonium hydroxide (30 min at 55 °C, followed by 1.5 h at room temperature).

(6) A reaction of the tosyl derivatives was observed after longer exposure to ammonium hydroxide. Thus, treatment of d-(tosyl-TCCGCTTCTTC-CTGC) (RP HPLC elution time, 20.6 min) with concentrated ammonium hydroxide at 23 °C for 18 h converted ~40% of the oligomer to two faster eluting products (RP HPLC elution times, 15.0 and 15.1 min) in about equal amounts. In a similar reaction carried out at 55 °C for 18 h, conversion to these two products was ~99% complete. Accordingly, base-labile protecting groups that could be removed rapidly under relatively mild conditions were used in synthesizing tosyl oligonucleotide derivatives.

(7) No further reaction occurred on standing at 37 °C for 2 h more. The residual peak at 20.1 min in the HPLC profile (Figure 1) probably stems from a small amount of an oligonucleotide-3′-phosphate in the sample of the 3′-thiophosphate.

(8) Cyclization of **1** and **3** was carried out with 1.3 μM oligonucleotide in 1 mL of aqueous buffer (pH 7.2, 30 mM phosphate, 1 M NaCl) at 37 °C. Samples were removed for analysis by HPLC at the indicated times. The reaction of **5** (3.45 μM, 15% excess) with **6** (3.0 μM) was carried out in the same way at 35 °C.

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oligonucleotides in which one was terminated by a 3′-thiophosphoryl group and the other by a 5′-*O*-tosyl group. Strong evidence that the product from the reaction of **1** is a cyclic oligonucleotide formed by displacement of the tosyl group was provided (1) by its resistance to hydrolysis by alkaline phosphatase and T4 DNA polymerase,[9] (2) by the large decrease in retention time on RP HPLC,[10] indicative of loss of the hydrophobic tosyl group during the reaction, (3) by the relatively short elution time on ion exchange HPLC at pH 12 (33.9 min for **2** compared to 35.6 min for **1**),[10] which is consistent with loss of negative charge on cyclization and inconsistent with polymerization, (4) by the relatively high mobility on PAGE (R_m = 1.31 for **2** as compared to 1.15 for **1**, both relative to xylene cyanol),[11] which likewise is consistent with loss of charge on cyclization, and (5) by thermal denaturation curves[12] showing that **1** (T_m 45 °C) had been converted to a much more stable base-stacked structure ($T_m \approx 86$ °C). The gel data and the shift in T_m for the nicked relative to the dumbell structure correspond to values reported by Ashley and Kushlan (ΔT_m 41°C) for the related cyclization of d-TTCCTTTTGGAATTCCTTTTGGAAp induced by a carbodiimide.[1f,13] Experiments with isolated samples showed that the phosphothio link in **2** resists attack by concentrated ammonium hydroxide (4 h at 55 °C) or 80% aqueous acetic acid (1 h at room temperature).

A less favorable system for ligation is presented by compound **3**, which may be considered a deoxyribonucleotide mimic of a section of a Rev binding element in the HIV-1 genome.[14] The organized conformation for this conjugate would be expected to have two nucleotide bulges (dT and dA) and two unusual base pairs (dG:dA and dG:dG) as well as short base-paired stem regions. Although a stilbenedicarboxamide bridge helps stabilize a folded conformation,[15a] the broad melting curve for **3** (Figure 2) suggests considerable fraying of the hybridized strands. Nevertheless, ligation in 1 M aqueous NaCl proceeded efficiently, affording oligonucleotide **4** (96%) within 8 h.[8] As for cyclization of **1**, the product eluted faster than the reactant on RP HPLC (19.1 min for **4**, 21.0 min for **3**) and on ion exchange HPLC at pH 12 (36.2 min for **4**, 38.3 min for **3**) and exhibited much greater stability than the reactant on thermal denaturation (Figure 2). The extraordinarily high melting temperature for **4** ($T_m \approx 84$ °C, 0.1 M NaCl), a compound with a short nucleotide sequence containing unmatched bases, may be attributed to the stabilizing influence of stilbenedicarboxamide groups that *covalently cap both ends* of a duplex segment.

The third system (**5** + **6**) was selected to see if the tosyl displacement reaction could be used to close an organic bridge across the end of an oligonucleotide duplex. The expected product, **7**, is closely related to an oligonucleotide conjugate, dT_{12}-□-dA_{12} (**8**), prepared by stepwise synthesis and found to fold to a hairpin structure (T_m 64 °C).[15a] The reaction of **5** with **6** was somewhat slower than cyclization of **1**; however, it proceeded smoothly to give a single product. As indicated in Figure 3, the conversion of the limiting reagent, compound **6** (elution time, 17.7 min), to product **7** (elution time, 18.3 min) was complete within 8 h as judged by analysis by HPLC. In accord with a covalent hairpin structure, the product exhibited a high T_m value (69 °C as compared to 35 °C for **5** + **6**), elution times on RP HPLC and IE HPLC indistinguishable from values for reference compound **8**, and a fluorescence band at 386 nm characteristic of an unperturbed stilbene chromophore. Absence of a band in the 445 nm region indicated absence of ligation products associated as a bimolecular duplex.[15]

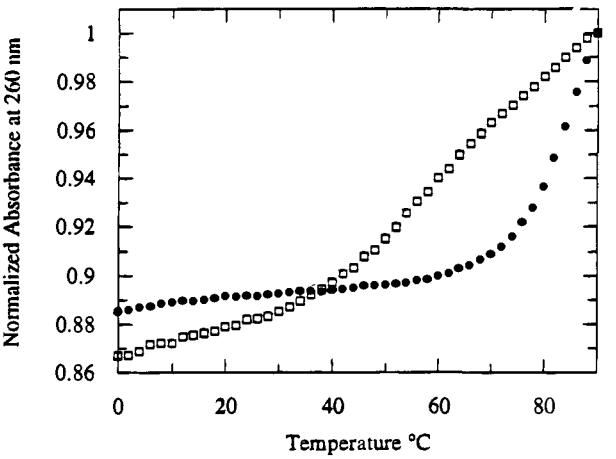

Figure 2. Melting curves for oligonucleotides **3** (□) and **4** (●) in 0.1 M aqueous NaCl (see note 10).

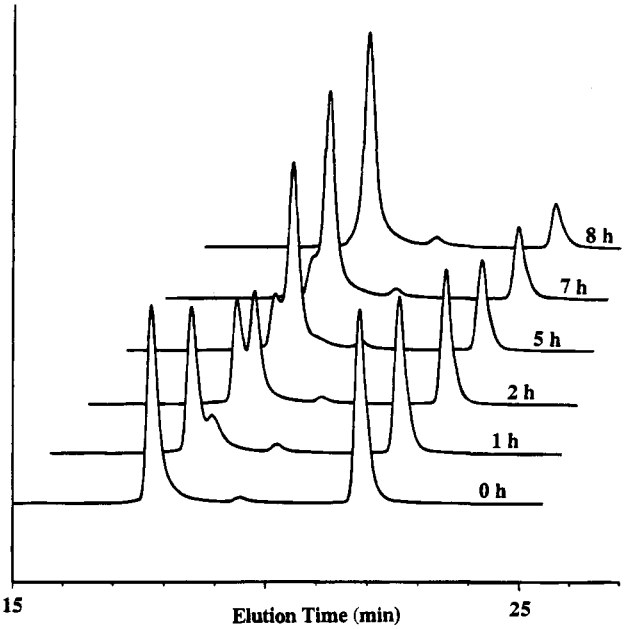

Figure 3. RP HPLC analysis of the reaction of oligonucleotides **5** and **6** at 35 °C after 0, 1, 2, 5, 7, and 8 h. The peak eluting at 18.3 min corresponds to product **7**; peaks eluting at 22 and 17.7 min correspond to reactants **5** and **6**, respectively. The residual peak at 22 min in the profile for the 8 h reaction is due to excess **5** in the original reaction mixture.

These examples demonstrate that the tosyl displacement reaction provides an effective means for covalently linking self-assembled oligonucleotide domains. Coupling depends strongly on organization of the component blocks, but the latitude in the geometrical constraints is sufficient to permit efficient coupling in oligonucleotide systems that differ substantially from those conventionally employed in ligation.

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(9) A control oligonucleotide 3′-phosphorothioate was degraded under the same conditions. For a similar assay for cyclization, see ref 1e and the following: Erie, D. A.; Jones, R. A.; Olson, W. K.; Sinha, N. K.; Breslauer, K. J. *Biochemistry* **1987**, *26*, 7150–7159.

(10) RP HPLC was carried out on a Hewlett Packard Hypersil ODS column, 4.6 × 200 mm with a 1%/min gradient of 95:5 acetonitrile/0.03 M triethylammonium acetate buffer in 0.03 M triethylammonium acetate buffer. For IE HPLC, a Dionex NucleoPac PA-100, 4 × 250 mm column and a Dionex NucleoPac PA-100 guard column at pH 12 (10 mM NaOH) were used with a 2%/min gradient of 1.0 M NaCl in 10 mM NaOH.

(11) Polyacrylamide gel electrophoresis (PAGE) was carried out using a 16% cross-linked denaturing polyacrylamide gel with xylene cyanol as a tracking dye as described in the following Maniatis, T.; Fritsch, E. F.; Sambrook, J. *Molecular Cloning, a Laboratory Manual;* Cold Springs Harbor Laboratory Press: Cold Springs Harbor, NY, 1982; p 185. Samples were visualized using a fluroescent TLC plate as background.

(12) Melting curves were recorded using solutions 0.1 M in NaCl, 10 mM in phosphate buffer (pH 7), and 3 mM in oligonucleotide by monitoring changes in absorbance at 260 nm while ramping the temperature at the rate of 1 °C/min.

(13) In their case, a 30% yield of the cyclic product was isolated from a 4 day reaction.

(14) Giver, L.; Vartel, D.; Zapp, M.; Pawul, A.; Green, M.; Ellington, A. D. *Nucleic Acids Res.* **1993**, *21*, 5509–5516.

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