Chemical DNA Ligation

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DNA with 3′-5′-Disulfide Links—Rapid Chemical Ligation through Isosteric Replacement**

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Abstract: *Efforts to chemically ligate oligonucleotides, without resorting to biochemical enzymes, have led to a multitude of synthetic analogues, and have extended oligomer ligation to reactions of novel oligonucleotides, peptides, and hybrids such as PNA.[1] Key requirements for potential diagnostic tools not based on PCR include a fast templated chemical DNA ligation method that exhibits high pairing selectivity, and a sensitive detection method. Here we report on a solid-phase synthesis of oligonucleotides containing 5′- or 3′-mercapto-dideoxynucleotides and their chemical ligations, yielding 3′-5′-disulfide bonds as a replacement for 3′-5′-phosphodiester units. Employing a system designed for fluorescence monitoring, we demonstrate one of the fastest ligation reactions with half-lives on the order of seconds. The nontemplated ligation reaction is efficiently suppressed by the choice of DNA modification and the 3′-5′ orientation of the activation site. The influence of temperature on the templated reaction is shown.*

Previous studies on template-directed reactions[2] and self-replicating systems[3] based on chemical ligations[4] employed the formation of phosphodiester, pyrophosphate, and phosphoramidate bonds through carbodiimide or imidazolide activation. Phosphoramidate bond formation has been developed further to yield highly efficient and fast templated polymerization syntheses.[5] Several chemical methods based on the autoligation of oligonucleotides for diagnostics are based on phosphorothioate with iodide or tosylate,[6] Diels–Alder, and click reactions.[7] Furthermore, many template-based methods for nucleic acid detection have been reported based upon the Staudinger reaction,[8] native chemical ligation,[9] metal-catalyzed reactions,[10] photocatalyzed reactions,[11] and nucleophilic aromatic substitutions.[12] The template approach has resulted in rate accelerations of up to 1000-fold and half-lives as low as 90 s.[13]

Thiol modifications are widely used in oligonucleotide (ON) synthesis. Thiol modifiers are commercially available and routinely used to immobilize DNA on gold surfaces[14] or on thiolated supports.[15] Disulfide-based crosslinking by means of thiol-modified bases can be used to transform double-stranded DNA into covalently linked species.[16] Disulfide-based templated ligations were developed with phosphorothioates as functional groups.[17] These systems yielded significant amounts of side products through non-templated reactions. Ligations based on the formation of 3′-5′-disulfide linkages were suggested by Schwartz and Orgel as early as 1985.[18] Witch and Cosstick synthesized dithymidine building blocks with 3′-5′-disulfide linkages, aiming to use the latter to generate a novel class of antisense ONs.[19]

Molecular modeling suggested that the replacement of the phosphodiester bridge by a 3′-5′-disulfide bond expressing a favored 90° dihedral angle should result in only little conformational distortion of the B-DNA backbone (Figure 1). Our approach involves the formation of a 3′-5′-disulfide bond through a disulfide-exchange reaction, which is attractive because it is fast and highly selective, and can be directed by control of the redox conditions and pH. The structural similarity between the disulfide bond and the native phosphodiester bond should favor the templated reaction pathway, whereas the application of longer and flexible ligation sites resulted in increased turnover rates.[6b]

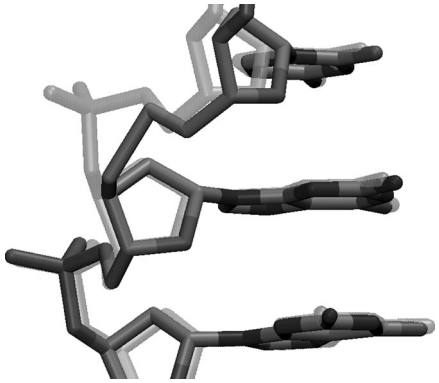

Figure 1. Overlay of two geometry-optimized models for B-DNA (computation: semiempirical, PM3; counterstrand removed), one containing a 3′-5′-phosphodiester, the other a 3′-5′-disulfide linkage. The C(3′)-S-S-C(5′) dihedral angle is close to the optimum of 90°.

In earlier phosphoramidate replication studies, the CG motif was found to be the most effective ligation site because of base-stacking effects.[20] We synthesized DMT- and base-protected thiol-deoxynucleosides 5′-HS-dC and 3′-HS-dG and immobilized them as disulfides on a thiolated controlled pore glass (CPG) solid support. The thiol-ONs were prepared

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from the corresponding 3′- and 5′-phosphoramidites using the solid supports as shown in Figure 2 (for details see the Supporting Information). The solid supports allow the utilization of standard synthesis protocols.

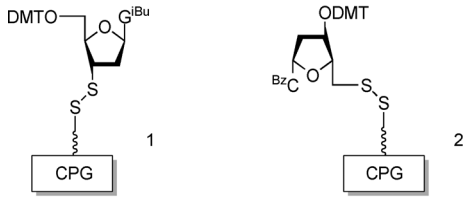


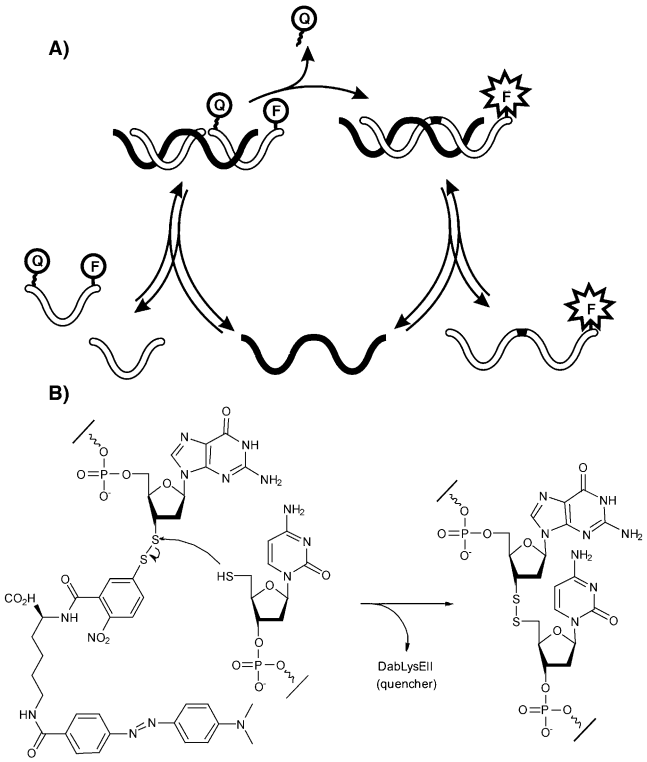
Figure 2. Structure of the solid supports employed in the synthesis of 5′- and 3′-thiol-ONs (details in the Supporting Information). DMT: dimethoxytrityl, ^{iBu}G: N2-isobutyrylguanine, ^{Bz}C: N4-benzoylcytosine.

We chose Ellman's reagent to preactivate one precursor molecule as a mixed disulfide. A further customized modification could be introduced through the carboxyl moiety of Ellman's reagent: a dabcyl unit as a quencher leaving group for FRET detection. This feature, combined with a 6-FAM-labeled ON, which is common in existing probes,[6b–d] made it possible to monitor the reaction directly by fluorescence down to nanomolar concentrations (Scheme 1 B; for details see the Supporting Information). The quencher can be introduced to the thiol-ON at either the 3′- or 5′-end.

On ligation with a thiol-ON as a nucleophile, the quencher (Q) is liberated producing a fluorescence signal (Scheme 1 A). Preliminary ligation experiments with 5′-activation had revealed a very fast ligation reaction,[21] mandating low micromolar concentrations of thiolated DNA precursors to minimize spontaneous nontemplated ligation. Sequences with 9-mer precursor molecules and 18-mer templates were chosen for the ligation to provide a sufficient population of the termolecular complex at such low concentrations. In the 9–18-mer system, two complementary 3′-S-ON, 5′-S-ON pairs and the corresponding templates were synthesized (Table 1). The 6-FAM-labeled ONs were converted into activated and quenched precursors using a 20-fold excess of the dabcyl linker molecule (pH 7.0 buffer, RT, 15 min).

Table 1: Oligonucleotide sequences and temperature effects for non-templated ligation experiments.

Ligation	3′-Activation	5′-Activation
3′-S-ON	^{F}ATTATACCG^{Q} **A**	TACTTCAAG^{S} **E**
5′-S-ON	^{S}CTTGAAGTA **B**	^{Q}CGGTATAAT^{F} **D**
T [°C]	k_1 [$M^{-1}s^{-1}$]	
35	33	391
38	37	547
40	43	718
45	54	844
55	62	1413



Scheme 1. The thio-DNA ligation cycle. A) Overall ligation cycle. B) Ligation reaction with 3′-activation.

Ligation reactions were carried out to investigate the nontemplated background reaction (for details see the Supporting Information). Surprisingly, the 3′- and the 5′-electrophiles underwent the nontemplated background reactions at very different rates. For a 3′-electrophile (**A**) and a 5′-nucleophile (**B**) we observed significantly lower rates than with a 5′-electrophile (**D**) and a 3′-nucleophile (**E**) (Table 1).

The nontemplated reactions show conversions at pH 7 (on a timescale of 30 min) of 6–11 % for the 3′-activation versus 60–90 % for 5′-activation. These results cannot be explained by nucleophilicity, since the 5′-thioate is expected to be the stronger nucleophile. Experiments with interchanged nucleophiles, leading to 3′-3′ and 5′-5′ ligations, also gave relatively low yields with the 3′-electrophile (see data in the Supporting Information). The effect apparently originates from steric hindrance. 3′-Activation (see Table 1) showed only minor temperature dependence (over this range). A template effect was observed for both activation pathways, but for the 5′-activation the nontemplated reaction is dominant. Consequently, we restricted our examination of template and temperature effects to the 3′-activated system.

To prove that the products are pure and that the ligations are unambiguous we carried out qualitative gel analyses of the templated ligation reactions (Figure 3). The two fluorescent bands were identified as the precursor molecule and the product by MALDI-TOF mass spectrometry (see the Supporting Information).

In the ligation experiments, we observed a clean and fast ligation reaction as well as strong template and temperature effects. At 25 °C and 35 °C we observed a significant template-dependent conversion already after a reaction time of 30 s. After 5 min the templated ligation (1 equiv) is almost complete. Furthermore, for the nontemplated ligation, no product formation was observed after 5 min. At 45 °C, which

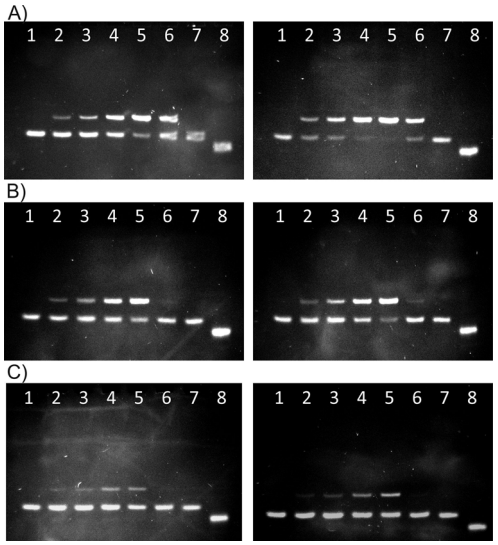


Figure 3. Gel mobility analysis of the templated ligation reaction at 25 °C (A), 35 °C (B), 45 °C (C). Left: Reaction after 30 s. Right: Reaction after 5 min. Lane 1: nontemplated reaction, lanes 2–5: 0.1 equiv, 0.2 equiv, 0.5 equiv, and 1 equiv of template TACTTCAAGCGGTATAAT, lane 6: 1 equiv of template TACTTCAAGC**T**GTATAAT, lane 7: 6-FAM precursor ON, lane 8: reduced 6-FAM precursor ON. All reactions were performed in 0.1 M phosphate buffer (pH 7) containing 100 mM NaCl with 0.5 μM precursor concentration. Template concentration was varied from 0 to 0.5 μM. Ligation reactions were stopped by addition of a 50-fold excess of Ellman's reagent in a saturated urea solution after 30 s and 5 min, and analyzed by denaturing polyacrylamide gels by fluorescence detection.

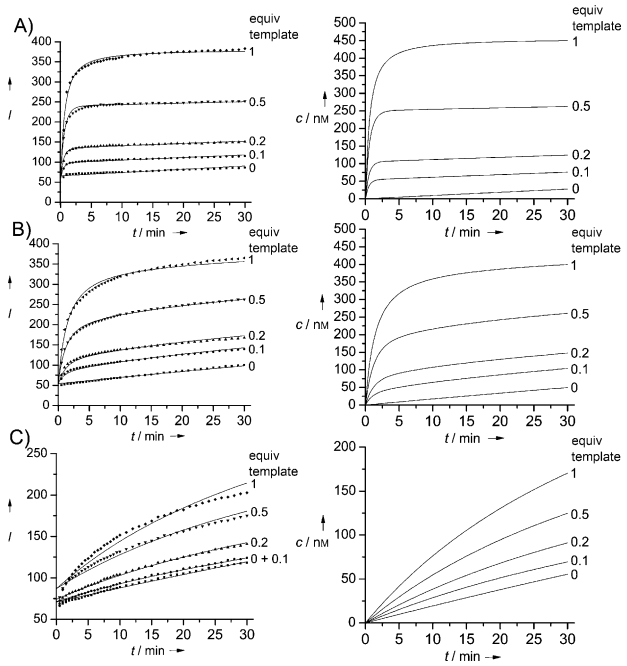
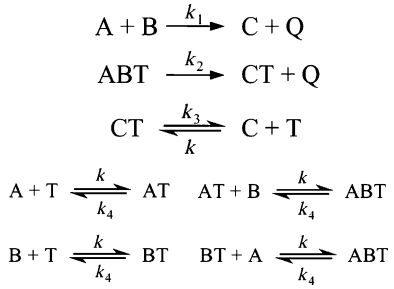


Figure 4. Kinetics of 3′-activated template-directed ligation reaction at 25 °C (A), 35 °C (B), 45 °C (C). The template concentration was varied from 0 μM to 0.5 μM and the influence of template on the ligation was investigated for sets of measurements with varying template concentrations at 25 °C, 35 °C, and 45 °C. Left: Fluorescence data and theoretical curves obtained with the FRET detection method making use of quencher release during ligation. For each set, the rate constants for the nontemplated (k_1) and the templated reaction (k_2) were estimated, as well as the constants for product–template dissociation (k_3) and precursor–template dissociation (k_4) using the SimFit[22] program and Scheme 2. In each series, the measurements were normalized by calibration for 0% and 100% conversion.



Scheme 2. Reaction model for template-directed ligation; A, B: precursors, C: product, Q: leaving group, T: template, *k*: fixed rate constants.

is the melting temperature of the product–template complex (see the Supporting Information), product formation is lowered in accordance with the reduced template effect. Despite the elevated temperature, the nontemplated product formation is still low after 5 min. The formation of side products with the fluorescent probe was not observed.

Selectivity for the templated ligation was also evidenced by ligation experiments with a mutated template (Figure 3 A–C, lanes 6). A change in the sequence of the template by one base, in close proximity to the ligation site, leads to lower product formation than that achieved with the matching template. This effect intensifies with elevated temperature. In this context several template variations were tested, and all exhibited a reduced template effect (see the Supporting Information).

To quantify the kinetics, templated ligation was also monitored directly by fluorescence measurements (Figure 4). An excess of thiol-ON was used to increase the reproducibility and reduce the effects of auto-oxidation within a series of measurements, without inducing side reactions. An additional quenching influence of the template on the product and the precursor was observed (hybridization quenching) and quantified by calibration measurements (for details see the Supporting Information).

The magnitudes of k_3 and k_4 (Scheme 2) were estimated based on dissociation constants obtained from melting curves and the predicted association rate constants k and were employed in the fit. The melting temperatures for the precursor–template and product—template complexes were determined to be 29 and 45 °C, respectively (see the Supporting Information), the latter being about 7 °C lower than that for the natural phosphodiester sequence. The background fluorescence as well as the contribution of hybridization quenching to the fluorescence signal were taken into account as correction factors in the fit. In this way, rate constants and product concentrations (Table 2, Figure 4, right) were directly derived from the observed fluorescence measurements (for details see the Supporting Information).

Table 2: Rate constants and estimated errors for templated ligation experiments of A and B with 3′-activation as a function of temperature.[a]

T [°C]	25 °C	35 °C	45 °C
k_1 [$M^{-1}s^{-1}$]	17.5 ± 2	29.3 ± 0.1	39.1 ± 0.7
k_2 [s^{-1}]	$(2.7 \pm 2) \times 10^{-1}$	1.69 ± 0.01	6.6 ± 0.1
k_3 [s^{-1}]	$(4.7 \pm 6) \times 10^{-6}$	$(3.7 \pm 0.2) \times 10^{-3}$	$(1.3 \pm 0.1) \times 10^{-2}$
k_4 [s^{-1}]	5.3 ± 3	39.7 ± 0.1	820 ± 5
r.m.s.[b] [%]	1.9	2.2	4.3

[a] A significant temperature variation for both the templated ligation and the rate constants of dissociation can be deduced. [b] r.m.s. is the (percentage) ratio root mean square for all species in the overall fit.

The plots and the rate constants reveal a marginal turnover for the ligation system, a very fast templated ligation pathway, and a moderate background reaction. Turnover numbers (TONs) were determined by fluorescence measurements in a series of experiments at various temperatures (see the Supporting Information). In the presence of 0.01 equivalents of template (5 nM) we observed a TON of up to 6 after 100 min. Other groups have reported TONs of up to 402 over 24 h with the addition of 0.01 mol% template and recently a TON of 1500 over 15 h was demonstrated with 0.001 mol% added template.[9c,12]

At 25 °C we observed a rapid ligation with 50% yield within the first 60 s and an almost quantitative conversion after 5 min. The nontemplated ligation accounts for about 5% conversion after 30 min. Due to the rate of the ligation, the initial rate acceleration can be estimated to be at least 300-fold.

With increasing temperature, up to the melting temperature of 45 °C, the template effect is weakened: At 50 °C no template effect was observable. Since the background reaction is apparently only marginally affected by temperature, temperature can be used to reduce the overall ligation rate and to control the templated ligation by influencing hybridization.

We showed that the disulfide-exchange reaction is one of the fastest ligation reactions, exhibiting half-lives for the template-directed synthesis on the order of seconds. It is comparable with enzymatic reaction with ligases, with nucleophilic substitution reactions of thiolated ONs, and with the DNA-templated native chemical ligation of PNA.[13,23] The choice of 3′-activation can very effectively reduce background reactions, whereas in the templated case the proximity and proper orientation of the reactive sites promotes the ligation speed. The ligation reaction is also clean—side products were not observed—and is compatible with elevated temperatures.

Interesting applications could arise from the strong pH dependency of the disulfide-exchange reaction as well as from its reversibility. In the context of self-replication[24] and molecular evolution a fast and externally controllable ligation chemistry can be highly valuable. The reversibility of the ligation could be used to create dynamic combinatorial libraries of disulfides on an ON basis.[25] Significant turnover could be induced by temperature cycling. There is evidence for sequence selectivity, especially at elevated temperatures, which could lead to applications in SNP detection. The breadth of the ligation method needs to be demonstrated by sequence variation to natural samples (e.g. Ras gene). Applications to electronically controlled replication systems and synthetic systems coupling replication and redox metabolism are being investigated.[26]

Experimental Section

Synthesis of 5′-thiol-ON**:** Reverse amidites (ChemGene) were reacted for 5 min on a DNA synthesizer (Gene Assembler Plus) with 5-benzylmercaptotetrazole (emp Biotec) as activator. Standard protocols for detritylation, capping, and oxidation were applied.

ON purification**:** After cleavage from the solid support (conc. ammonia, 55 °C, 16 h) products were reduced with a 100-fold excess of TCEP in buffered solution (100 mM in 0.1M HEPES; pH7) for 15 min. ONs were purified by RP-HPLC.

Synthesis of dabcyl-linker-ON**:** A 20-fold excess of the dabcyl-Lys-DTNB linker **13** was added to the reduced thiol-ON (with a terminal 6-fluorescein-phosphoramidite modification (Glen Research)) (0.1M HEPES; pH 7). After 30 min, the product was isolated by RP-HPLC and desalted with OASIS-HLB cartridges (Waters). The product was characterized by MALDI-TOF MS with 3-hydroxypicolinic acid (3-HPA) as matrix.

Ligation reactions**:** Ligations were performed in 0.1M degassed phosphate buffer (pH 7) containing 100 mM NaCl. The fluorescence increase on reaction was monitored using a Varian Cary Eclipse Spectrometer (excitation 495 nm; emission 515 nm). To start the reaction, 300 μL of buffered and temperature-annealed ON solutions were mixed in a 500 μL fluorescence quartz cuvette (path length 10 mm). Ligation reactions for gel analysis were performed with a reaction volume of 20 μL. To halt the reaction, 10 μL aliquots were quenched with a 50-fold excess of Ellman's reagent in 10 μL of a saturated urea solution. Samples were analyzed by denaturing PAGE (16%, 120 V, 1.5 h, TBE buffer).

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