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# Control of Reaction Chemoselectivity with a Circular DNA Template in the Ligation of Short Oligodeoxyribonucleotides

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Dedicated to Professor Peter Dervan for his influential contributions to Bioorganic Chemistry

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**Abstract**—A study has been conducted to examine the chemoselective control attained in the ligation of short oligodeoxyribonucleotides (ODNs) directed by association on a circular DNA template through triplex formation relative to the same ligation on a single-strand DNA template through duplex formation. The highly efficient, chemospecific ligation achieved under a variety of conditions on the circular DNA template presumably arises from the higher substrate–template association resulting in improved transition state accessibility and protection of the ODNs from side reactions. © 2001 Elsevier Science Ltd. All rights reserved.

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Nonenzymatic oligonucleotide ligation reactions that use a DNA template to direct the covalent bond formation between two oligonucleotides have been reported in duplex[1–4] and triplex[5–8] directed systems.[9] Chemical ligation strategies are advantageous for constructing circular[10–15] and modified oligonucleotides.[16–32] Extension of template-directed oligomerization reactions to non-natural nucleic acid modifications requires improved substrate–template association[33] while still allowing product turnover.[9,28,34,35] One recently reported solution to improving template turnover was accomplished by attaching the template oligonucleotide to a solid support.[36] While we are developing approaches to accomplish both of these objectives, our initial report demonstrated the use of a circular DNA template to improve substrate–template association resulting in near quantitative ligation yields of short ODNs in cyanogen bromide activated ligation reactions.[37] The significant improvement in ligation yields on the circular DNA template relative to the optimized yields on a linear DNA template were a result of the homopurine ligation substrates associating with opposing sides of the pyrimidine-rich circular template through both Watson–Crick and Hoogsteen hydrogen bonding (Fig. 1).[38] Gao and Orgel recently reported a novel reversibly cross-linked template–substrate complex which similarly realized much improved ligation yields. Their results also revealed the advantages of a more restricted template-substrate binding on ligation efficiency.[39]

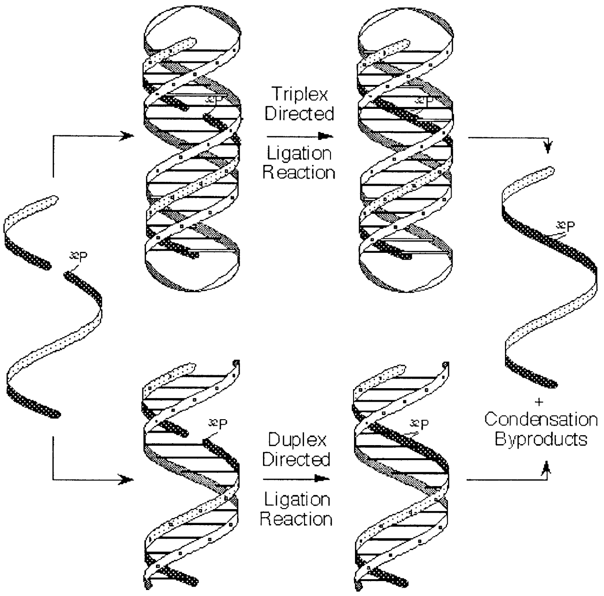

**Figure 1.** Top: A triplex directed ligation reaction resulting from two homopurine ligation fragments hydrogen bound to pyrimidine bases of the circular DNA template. Bottom: Ribbon graphic of the duplex directed ligation reaction of two ODNs directed by a single strand DNA template.

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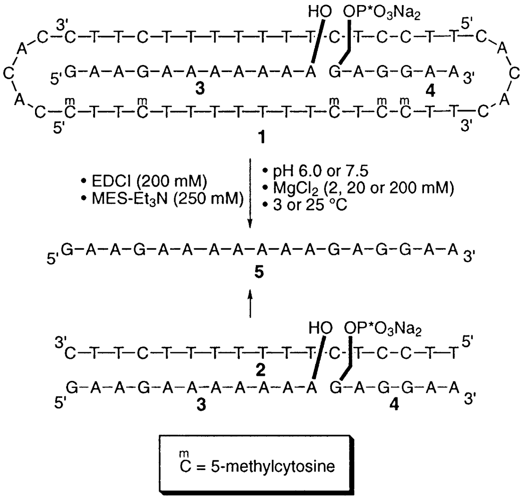
While chemospecific phosphodiester bond formation was achieved with cyanogen bromide to produce ligation products free of any contaminating byproducts in our initial report,[37] we sought to further investigate the effect of the circular template in directing ligations with a less chemoselective activating reagent. Our interest was in probing the degree of nucleobase protection and reaction control under ligation reaction conditions known to give byproducts through nucleobase condensation and pyrophosphate formation. We now report our investigations using the water soluble carbodiimide EDCI (3-ethyl-1,3-dimethylaminopropylcarbodiimide hydrochloride) in ligation reactions of short ODNs directed by a circular DNA template. Carbodiimide promoted ligation reactions are well known for producing various byproducts through non-chemoselective reactivity at the nucleobase and terminal phosphate residues.[40–43] However, it is recognized that hybridized oligonucleotides are substantially protected from modification.[44] This reactivity is advantageously used for mutation detection via unprotected base mismatches in hybridized DNA.[45] Our investigations reveal improved substrate–template association with circular DNA templates based on the production of clean ligation products using a carbodiimide activating reagent.

## Results and Discussion

Our investigations of EDCI activated ligation reactions directed on a circular DNA template were compared to the identical ligation reaction directed by the Watson–Crick linear template that would essentially duplicate the directing effects of one side of the circular template. Pyrimidine-rich circular template **1** (Scheme 1) was synthesized in 60% yield by slight modification of an existing procedure,[11] as previously reported.[37] Circular template **1** was designed with partial incorporation of 5-methylcytidine in order to improve selectivity for Hoogsteen hydrogen bonding to one side of the circular template in the triplex directed ligation reactions (Fig. 1 (top) and Scheme 1).[46–49] Linear, homopyrimidine template **2** was prepared[50,51] for relative assessment of the corresponding duplex directed ligation reactions through Watson–Crick hydrogen bonding alone (Scheme 1). Linear template **2** was designed so that Hoogsteen hydrogen bonding to the ligating ODNs would be ineffective, thus triplex directed ligation could not occur with two equivalents of linear template **2**. The ligation substrates and template design were based on our previous report with the intent of giving moderate yields of ligation on the single strand template (51% yield in our previous work) so that yield variations in either direction could be assessed on either template.[37] Template and ligation substrate modifications that improve the yields on the single strand template result in yields which are too high on the circular template to determine relative changes in ligation yields under varied conditions.



Scheme 1.

The ligation reaction was examined on both circular template **1** and linear template **2** using ligating ODNs **3** (an 11-mer) and **4** (a 6-mer) (Scheme 1). ODN **4** was 5′-$^{32}$P-labeled[51] for analysis and quantification of the ligation reactions by autoradiogram densitometry.[37] The ligation reactions of ODNs **3** and **4** (1:1, 50 nM) to produce **5** (a 17-mer) on template **1** or **2** (1.0 or 2.0 equiv) were studied at either 3 or 25 °C with EDCI (200 mM) in MES–$Et_3N$ buffer (250 mM, pH 6.0 or 7.5) in the presence of $MgCl_2$ (2, 20 or 200 mM). Reaction aliquots were analyzed at 1.5, 2.5 and 3.5 days by autoradiogram densitometry. Two primary control reactions performed under the same conditions of each monitored ligation reaction included: (i) **3** and **4** with no template to assess for off template reactions and (ii) **4** alone to assess for any reactions altering the starting ODNs.

A representative denaturing PAGE autoradiogram for analysis of the ligation reactions is shown in Figure 2. The histogram summary of the PAGE autoradiogram densitometry data is shown in Figure 3. A significant amount of data is compiled into the graphs of Figure 3 to provide a concise overview of a variety of experimental parameters and their relative effects on the ligation reaction and accompanying side reactions. The yield (% relative to all radioactivity in the respective gel lane) of all radiolabeled reaction products quantified from the gel densitometries are reported in three categories: (i) ligation product **5** (yellow), (ii) byproducts that migrated slower than **5** (red), and (iii) byproducts that migrated faster than **5** (blue). Ligations reactions run at 3 °C are shown in graphs I (pH 6.0) and III (pH 7.5) (top of Fig. 3). Ligation reactions run at 25 °C are shown in graphs II (pH 6.0) and IV (pH 7.5) (bottom of Fig. 3). Data reported in all bar graphs is grouped in sets of three (front-to-back) $MgCl_2$ concentrations of 2, 20, and 200 mM. At each $MgCl_2$ concentration, the bar graphs show aliquots analyzed from the reaction at 1.5 days (first in each set), 2.5 days (second in each set) and 3.5 days (third in each set). This allows assessment of pH, temperature, and ionic strength and affords some detail regarding relative rates of ligation and side reactions. The graphs in Figure 3 also include experiments run with zero, one and two equivalents of circular template **1** and single strand template **2** to assess the template stoichiometry effect.

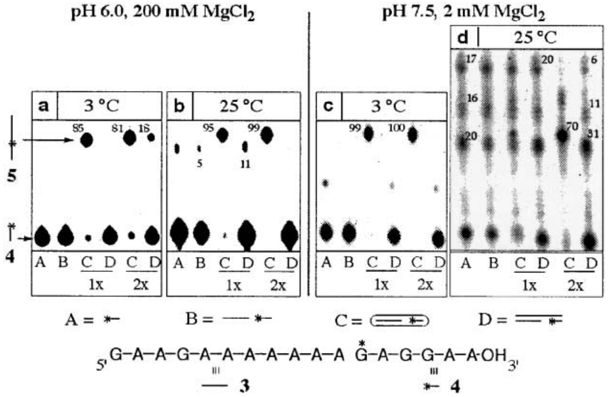


**Figure 2.** Representative autoradiogram scanned images from denaturing PAGE analysis of EDCI activated ligation reaction aliquots taken after 3.5 days under the specified conditions. All reactions contain 50 nM combined ligation ODNs **3** and **4** (1 pmol of each), 200 mM EDCI and 250 mM MES–Et$_3$N. The numbers overlaying the gel images refer to the % yield of the labeled fractions relative to the total radiation densitometry in the given lane. (a) Ligation reactions at 3 °C, pH 6.0, 200 mM MgCl$_2$; (b) ligation reactions at 25 °C, pH 6.0, 200 mM MgCl$_2$; (c) ligation reactions at 3 °C, pH 7.5, 2 mM MgCl$_2$; (d) ligation reactions at 25 °C, pH 7.5, 2 mM MgCl$_2$. Lane designations are graphically defined and refer to: (A) control reaction containing 5-$^{32}$P-**4** with no **3** or template run under the identical ligation reaction conditions specified; (B) control reaction containing ODNs **3** and 5′-$^{32}$P-**4** with no template run under the identical reaction conditions specified; (C) ligation reaction on circular template **1**; (D) ligation reaction on linear template **2**. The 5′-$^{32}$P-**4** and ligation product **5** bands are identified graphically at the left of the figure.

A cursory overview of the ligation reaction results will precede a more detailed analysis. The results in Figure 3 demonstrate that ligation product **5** (yellow) was produced on the circular template to some extent under all temperature and pH's examined. It also reveals that condensation byproducts arising from EDCI (red and blue) were minimal at lower temperature (Fig. 3, graphs I and III), but more significant at 25 °C (Fig. 3, graphs II and IV). The most significant formation of reaction byproducts was observed at 25 °C, pH 7.5 (Fig. 3, graph IV). However, note that even under these conditions, pure ligation product **5** was produced at the exclusion of any detectable by-products in 82% yield at 20 mM MgCl$_2$ after 3.5 days. A further general trend observed in a cursory overview of all the reaction results presented in Figure 3 is the apparent lowered reactivity of EDCI and its condensation intermediates at higher MgCl$_2$ concentrations. This is observed by the general lower production of ligation product **5** and/or any byproducts at 200 mM MgCl$_2$ (Fig. 3, back rows of graphs I–IV) and the slower rates of formation of ligation product **5** over the course of the 3.5 day reactions relative to the corresponding formation of combined ligation product **5** and all byproducts at lower MgCl$_2$ concentrations. These general trends will be more specifically described below.

As realized in our initial investigations,[37] we expected that lower pH, lower temperature and higher ionic

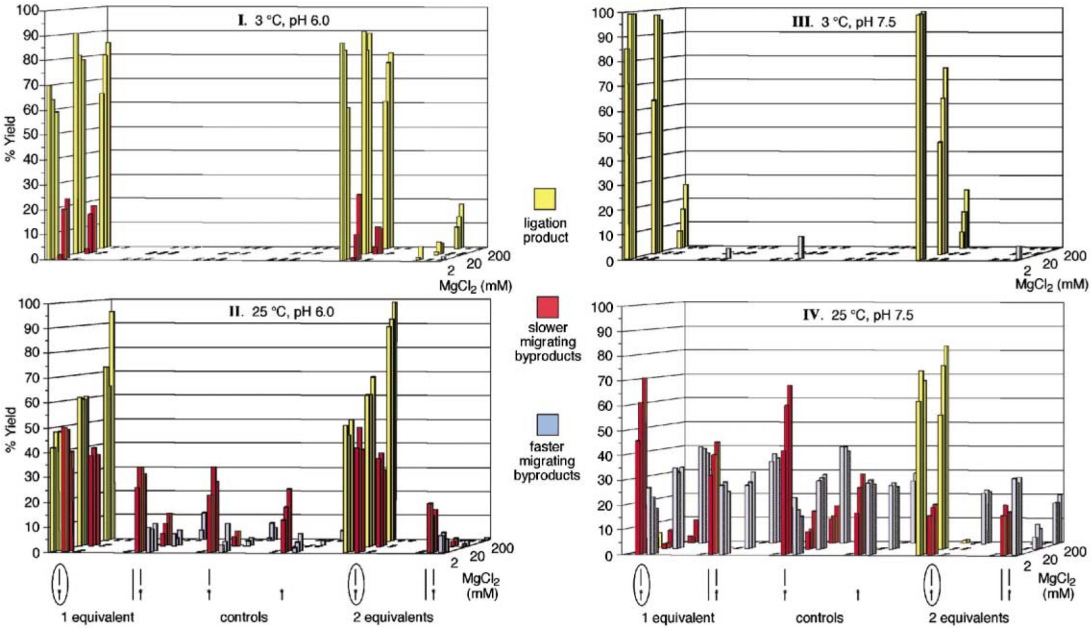


**Figure 3.** 3-D bar graphs showing the yield (%) of ligation product **5** (yellow), byproducts that migrated slower than **5** (red), and byproducts that migrated faster than **5** (blue) as determined by denaturing PAGE autoradiogram densitometry analysis. Graph I shows ligation results at 3 °C, pH 6.0 with MgCl$_2$ concentrations of 2, 20 and 200 mM. Graph II shows the same ligation reactions run at 25 °C, pH 6.0. Graph III shows ligation results at 3 °C, pH 7.5. Graph IV shows the ligation reactions run at 25 °C, pH 7.5. Data is grouped in series of three (front-to-back) within each specified MgCl$_2$ concentration refering to aliquots analyzed from the reaction at 1.5 days (first in each series), 2.5 days (second in each series) and 3.5 days (third in each series). The contents of each reaction are identified graphically at the bottom refering to (from left to right): first, ligation reactions with one equivalent of circular template **1** relative to ODNs **3** and **4**; second, ligation reactions with one equivalent of linear template **2**; third, control reactions containing ODNs **3** and **4** with no template run under the identical reaction conditions specified; fourth, control reactions containing ODN **4** with no **3** or template run under the identical ligation reaction conditions specified; fifth, ligation reactions with two equivalents of circular template **1**; sixth, ligation reactions with two equivalents of linear template **2**. All reactions were reproduced a minimum of two times to afford a % yield error of ±3.

strength would result in optimal substrate–template association[52] to afford the highest yield on linear template **2**. This proved true by analysis of the denaturing PAGE autoradiogram (Fig. 2a). The histogram of our PAGE autoradiogram densitometry is shown in bar graph I, Figure 3. Optimal conditions with template **2** afforded only 18% yield of the ligated product **5** with two equivalents of template **2** relative to ligating ODNs **3** and **4** at pH 6.0, 200 mM $MgCl_2$, 3 °C, requiring 3.5 days to reach maximum yield (Figs 2a and 3, graph I). Note that with one equivalent of template **2** no reaction was observed. The requirement for two equivalents of linear template **2** is not due to the formation of a triple helical complex (i.e., a pyr–pur–pyr triplex with two linear templates: one of each ligation ODN) as the ligating ODN sequences were designed so that a complex requiring parallel Hoogsteen binding of a second equivalent of template **2** to the ligating ODNs would not be expected. This was verified by a non-denaturing polyacrylamide gel shift assay under the reaction conditions where only duplex formation was observed (results not shown). Slow template turnover in the presence of ligation product **5** is not considered a factor in the requirement for excess template in this reaction as *no* ligation product was observed with a single equivalent of template **2**. The low equilibrium concentration of simultaneous occupancy of ODNs **3** and **4** in the presence of excess template **2** is reflected by the slow rate of the ligation reaction from 8% at 1.5 days to 18% at 3.5 days. No sign of any byproducts from non-chemoselective condensations with EDCI were evident under these reaction conditions.

In contrast to the ligations on linear template **2**, those directed on circular template **1** were significantly improved. Under the same reaction conditions (3 °C, pH 6.0, 200 mM $MgCl_2$), one equivalent of circular template **1** afforded 85% yield of ligation product **5** in 3.5 days (Figs 2a and 3, bar graph I). With 20 mM $MgCl_2$ the yields were observed to increase to 90% in only 1.5 days (Fig. 3, graph I). The presence of excess circular template **1** had little effect on the ligation yields under these reaction conditions, although at low $MgCl_2$ concentrations (2 mM) the yield in going from one to two equivalents of template improved from 70 to 85% in 1.5 days. This improvement in yield and increase in the rate at which **5** was formed appears to have some degree of correspondence with the formation of byproducts characterized as being slower migrating bands than 17-mer ligation product **5** by PAGE analysis (represented quantitatively by red bars, Fig. 3, graph I). As these slower migrating byproducts were only observed under these reaction conditions when ligation product was formed, they likely arise from a modification of **5** through EDCI condensation. According to precedent, all of the condensation byproducts in these ligation reactions are anticipated through reaction at the guanine, adenine and/or terminal phosphate (only **4** carries a 5′-phosphate).[40–43] These higher molecular weight byproducts were not the result of condensation with the template as they were faster migrating than the circular template itself (results not shown). Note that in cases where there appears to be minor improvements in the ligation yields in going from one to two equivalents of template **1**, there also is a slight decrease in the production of these slower migrating byproducts. The decrease in the formation of these slower migrating byproducts also corresponds with increasing concentrations of $MgCl_2$, where only ligation product **5** was observed at 200 mM $MgCl_2$. This is presumed to arise from a combination of increased protection from secondary condensation reactions due to higher association of **3**, **4**, and **5** to circular template **1** and the lower reactivity of EDCI and its condensation intermediates at 200 mM $MgCl_2$, pH 6.0. The lowered reactivity of EDCI at 200 mM $MgCl_2$ is not only observed by the lack of byproduct formation, but also in the slower, but steady yield increase from about 60% at 1.5 to 80–85% by 3.5 days. At lower $Mg^{2+}$ concentrations (2 and 20 mM), the highest ligation product **5** was recorded at 1.5 days (Fig. 3, graph I). Further studies are required in order to parse out template–substrate association and dissociation rates and the corresponding rates of ligation and secondary condensation reactions. However, note that the formation of the slower migrating byproducts is nearly undetectable at 1.5 day analysis times, but increases as the reaction proceeds. The formation of these byproducts is obviously a much slower reaction than the corresponding ligation reaction. Lastly, note that the increased production of slower migrating byproducts at the lower $Mg^{2+}$ concentrations build up with a corresponding lowering of the ligation product yield over the course of the reaction. This strongly correlates to a condensation modification of the already formed ligation product **5** over the reaction course that appears to be accentuated at lower $Mg^{2+}$ concentrations (vide infra). Therefore, at pH 6.0, 3 °C, the circular template does not appear to fully protect the ligation product from further condensation reactions at lower $Mg^{2+}$ concentrations, but complete chemoselectivity for ligation reaction is observed at higher $Mg^{2+}$ concentrations.

There were no other reaction conditions found where ligation product **5** was directed by linear template **2**. However, the ligation yields on circular template **1** improved on increasing the temperature to 25 °C while maintaining the pH at 6.0. A 95% yield of **5** was produced with one equivalent of circular template **1** after 3.5 days, 200 mM $MgCl_2$ (Figs 2b and 3, and bar graph II). The presence of two equivalents of circular template **1** afforded slightly higher yields of **5** increasing from 72 to 88% at 1.5 days and from 95 to 99% by 3.5 days (one and two equivalent of template **1**, respectively). This slight yield increase with two equivalents of template is likely due to poor template turnover with a single equivalent of template **1**. Notice at higher temperature more reaction byproducts were observed at lower $Mg^{2+}$ concentrations and at 1.5 day reaction times. The presence of both slower migrating byproducts and faster migrating byproducts (red bars and blue bars, respectively, Fig. 3, graph II) were observed in many of the control reactions. The faster migrating byproducts presumably arose from EDCI condensation reactions with radiolabeled 6-mer **4** (control reaction lanes in Figs 2b and 3, graph II). As no ligation product **5** was observed in any control reaction, the slower migrating byproducts in the control reactions are expected to have arisen from nonchemoselective EDCI homocondensations with **4** or heterocondensations with **3** and **4**. In the 25 °C reactions (Fig. 3, graph II), the formation rate of the slower migrating byproducts is competitive with the formation of ligation product **5** on the circular template at low $Mg^{2+}$ concentrations, and is accentuated by the presence of the circular template. However, as the conditions improve template–substrate association and lower the EDCI reactivity, the amount of ligation product increases and the slower migrating byproducts decrease (i.e., in going from one to two equivalents of template and in going from lower to higher $MgCl_2$ concentration). The increased reactivity of EDCI at lower $MgCl_2$ concentrations is again observed in the near complete reactions affording both **5** and the slower migrating byproducts at the 1.5 day data points at lower $Mg^{2+}$ concentrations, while at 200 mM $MgCl_2$, the reaction continues to progress over the 3.5 day reaction course. Again, at pH 6.0, an increase of the slower migrating byproducts arising from condensation modifying reactions with ligation product **5** on circular template **1** correlates with lower formation of the ligation product. While the exact nature of the slower migrating condensation byproducts (red bars in graph, Fig. 3) requires further analysis, all of the results point to the fact that much of the byproducts produced when ligation product is formed are a result of secondary condensation reactions of EDCI with ligation product **5** under these conditions. This is confirmed by the observation that the condensation byproducts formed when ligation product **5** is also formed clearly migrate as different bands on the gel than the slower migrating bands produced in reactions where no ligation product **5** was formed (e.g., see the gel image in Fig. 2d). It is also apparent that these slower migrating condensation byproducts are uniquely formed only when the circular template is present, as they are not observed in control reactions of **5** under the ligation reaction conditions in the absence of circular template **1** (results not shown). It is apparent that the condensation side reactions on ligation product **5** occur at a faster rate at 25 °C than at 3 °C at pH 6.0, and are less prevalent as the $Mg^{2+}$ concentration increases. As observed at pH 6.0, 3 °C, at 25 °C the circular template does not appear to fully protect the ligation product from further condensation reactions at lower $Mg^{2+}$ concentrations, but complete chemoselectivity for ligation reaction is observed at higher $Mg^{2+}$ concentrations.

Ligation reactions conducted at pH 7.5, 3 °C were significantly cleaner and higher yielding in the presence of circular template **1** at low $MgCl_2$ concentrations (Figs 2c and 3, bar graph III). At 20 mM $MgCl_2$, a 99% yield of ligation product **5** was formed within 2.5 days with one equivalent of template **1**. A 99% yield of **5** was also produced within 1.5 days at 2 mM $MgCl_2$ with two equivalents of the circular template (Fig. 3, graph III). As the amount of $MgCl_2$ was increased, the ligation yields significantly declined at pH 7.5, 3 °C. At 200 mM $MgCl_2$ the ligation reaction progressed much slower, affording approximately 7% yield of **5** within 1.5 days and reaching a maximum of only 25% within 3.5 days in the presence of either one or two equivalents of template. Only a minimal amount of faster migrating byproducts were produced in any of the reactions under these conditions, and only at 2 mM $MgCl_2$ after 3.5 days (Figs 2c and 3, graph III). Less than 5% was observed in the ligation reactions containing linear template **2** or the control reaction containing ODNs **3** and **4** with no template. These results at pH 7.5, 3 °C are consistent with the higher reactivity of EDCI at lower $MgCl_2$ concentration; however, the chemospecificity observed for sole formation of ligation product **5** at higher pH was surprising, as triplex formation favors lower pH for protonation of cytidine. While further studies are required to allow a thorough understanding of the differences in the EDCI activated ligation at pH 7.5 versus pH 6.0, it appears that there may be a mechanistic change involving the role of $MgCl_2$ in the reactions at higher pH.

Examination of the ligation reaction at pH 7.5, 25 °C reveals a significant increase in the nonchemoselective production of both the slower and faster migrating byproducts in all of the reactions (Figs 2d and 3, bar graph IV). Any significant amount of ligation product **5** was produced only when two equivalents of circular template **1** were present with optimal conditions found at 20 mM $MgCl_2$ (Fig. 3, graph IV). Under these conditions 82% yield of **5** was observed after 3.5 days. Note that these were the only conditions that afforded chemospecific ligation product **5** free of any byproducts. A general trend in the nonchemoselective formation of byproducts at pH 7.5, 25 °C appears as the slower migrating byproducts dominate at 2 mM $MgCl_2$ and rapidly decrease in going to higher $MgCl_2$ concentrations. There is a significant amount of many different higher order bands observed in the gels that make up these slower migrating byproducts in reactions where **5** is not produced (see Fig. 2d). However, the slower migrating byproducts produced when ligation product **5** is produced are mostly unique relative to the byproducts produced when **5** is not present, and appear to be the same as those byproducts produced at pH 6.0 when **5** is generated (Fig. 2d). In contrast, the amount of nonchemoselective faster migrating byproducts in most reactions remains fairly constant or increases as the $MgCl_2$ concentration increases from 2 to 200 mM (Fig. 3, graph IV). The reason for the minimal 5% yield of **5** at 20 mM $MgCl_2$ with one equivalent of circular template **1**, while 82% of **5** was produced when a second equivalent of template **1** was added is unclear. Further studies are assessing this aspect of the reactions; however, there are no signs of higher order complexes with ligation substrates **3** and **4** or product **5** and circular template **1** beyond the designed triple helical complex in any gel shift assays or UV denaturing experiments conducted to date (results not shown). Once again, even under conditions where the EDCI reactivity appears to be highly nonchemoselective in general, specific conditions were defined with circular template **1** that afforded chemospecific formation of ligation product **5** (two equivalents of **1** at 20 mM $MgCl_2$).

These investigations clearly reveal the propensity of EDCI to produce a variety of reaction byproducts through non-chemoselective condensation reactions, particularly at higher reaction temperatures. EDCI and the reactive intermediates generated were observed to be generally more reactive at lower $MgCl_2$ concentrations. However, under each pH and temperature combination studied, chemospecific ligation product **5** could be produced free of any contaminating byproducts on circular DNA template **1**. At lower pH (6.0), chemospecificity for ligation product is accomplished at higher $Mg^{2+}$ concentrations (200 mM), and the ligation reaction is faster at higher temperature (25 °C). However, at higher pH (7.5), the ligation reaction is faster and chemospecificity is favored at lower temperature (3 °C) and lower $Mg^{2+}$ concentrations (2–20 mM). It should also be noted that these ligation reactions between such small substrates (a 6-mer and an 11-mer) would normally be anticipated to give poor ligation yields, but conditions were realized at both pH 6.0 and pH 7.5 to afford near quantitative ligation product.

Our results in these carbodiimide activated ligation reactions suggest that enhanced ODN association on the circular DNA template through both Watson–Crick and Hoogsteen hydrogen bonding might be beneficial in two important regards: (i) to help protect the ligation substrates from side reactions under certain reaction conditions, and (ii) to improve the transition state geometry for increased ligation rates relative to a single strand template under a range of reaction conditions.[53,54] Further extensions of this template directed methodology are being investigated to expand the scope of its applications to a variety of other substrates, reactions, and to achieve template turnover.

## Experimental

### General

Phosphoramidites including 5-methylcytosine, solid supports and chemicals for DNA synthesis were obtained from Cruachem, Inc. (now Anovis, Inc., Ashton, PA, USA) or Peninsula Laboratories (San Carlos, CA, USA). All enzymes were purchased from Boehringer-Mannheim (now Roche Molecular Biochemicals, IN, USA), New England Biolabs (Beverly, MA, USA) or Gibco BRL Products (now Invitrogen, Grand Island, NY, USA). The radiolabeled 5′-[$\gamma$-$^{32}$P] ATP (> 6000 Ci/mmol) was obtained from Amersham (Piscataway, NJ, USA). All other chemicals were of analytical or HPLC grade. Standard molecular biology techniques were used, if not mentioned otherwise. Analytical and preparative HPLC was performed with a Shimadzu LC-600 liquid chromatograph with SPD-6A UV using Varian 150×4.6 mm, 5 μm, C18, 90 Å column [acetonitrile–0.1 M triethyl ammonium acetate (TEAA) buffer (pH 7.0) as gradient]. Sep-Pak™ was purchased from Waters (Milford, MA, USA). UV measurements were run with a Hitachi U-2000 spectrophotometer. Melting studies were carried out in Teflon capped 1 cm path length quartz cells under nitrogen atmosphere on a Jasco-710 spectropolarimeter equipped with PTC-343 temperature control. Scintillation counting was done on a Wallac 1410 Liquid Scintillation Counter. Gel images were scanned using AGFA-ARCUS II scanner at high resolution, low contrast and imported using Adobe Photoshop 4.0 software. Quantitative analyses of gel images were done with IP Lab Gel using scanned images.

### Oligonucleotide synthesis

Oligodeoxyribonucleotides were synthesized on an Applied Biosystems 392 synthesizer using β-cyanoethyl phosphoramidite chemistry.[50] Phosphorylation at the 3′-end of the pre-circle oligomer was carried out using modified solid support purchased from Peninsula Laboratories. Cleavage of the solid supports along with the base protecting groups and the phosphate protecting groups were achieved using concentrated ammonium hydroxide. Oligomers were purified by electrophoresis on 20% polyacrylamide gels with 8 M urea and Tris–borate–EDTA (TBE) buffer. The oligonucleotides were isolated from the gels by excision and elution with Tris–EDTA–NaCl (TEN) buffer. The resulting solution was desalted (using Sep-Pak™) and quantified by absorbance at 260 nm using extinction coefficients that were calculated by the nearest neighbor method.[55]

### Circularization procedure

Linear 3′-phosphorylated oligonucleotide and the complementary homopurine template were combined in a 1:1 ratio (50 mM) with $MgCl_2$ (20 mM) in morpholinoethanesulfonic acid–$Et_3N$ buffer (MES) (250 mM, pH 7.5). After heating to 95 °C, the solution was cooled to 3 °C.[37] The reaction was initiated by adding BrCN (50 mM) and allowed to proceed for 24 h at 3 °C. The reaction mixture was quenched with EDTA, lyophilized and redissolved in the loading buffer, and purified by 20% denaturing polyacrylamide gel electrophoresis (PAGE). The circular products migrated at 72% the rate of the linear precursor. Circular oligonucleotide **1** was isolated in 60% yield. To verify the product, melting studies were performed on circular oligonucleotide **1** with the complementary single-strand oligonucleotide and compared with the corresponding precircularized, linear oligonucleotide and the complementary single-strand oligonucleotide under identical conditions. Oligonucleotides were diluted to 3 μM each with 100 mM NaCl, 10 mM $MgCl_2$ in 10 mM MES–$Et_3N$, (pH 7.5). Solutions were heated to 90 °C and allowed to cool slowly to room temperature prior to the melting experiments. Absorbance (260 nm) was monitored while temperature was raised at a rate of 0.5 °C/min. In all cases the complexes displayed sharp, two-state transitions. Melting temperatures ($T_m$) were taken to be the temperature of half-dissociation and were obtained from a plot of temperature versus absorbance at 260 nm. Precision in $T_m$ values were estimated from variations of a minimum of two repeated experiments was ±0.5 °C. An increase of 11 °C in $T_m$ was obtained from the closure of the linear to the circular oligonucleotide **1**. Further verification that **1** was circularized was realized by showing the complete resistance of **1** to cleavage by exonucleases.

## Radiolabeling

The gel purified oligomer **4** (10 pmol, without 5′-phosphate) was dissolved in 10.4 μL of sterilized, double deionized water. To this was added 2 μL of 10×kinase buffer, 6 μL of [$\gamma$-$^{32}$P] ATP (60 mCi), and 2 μL of T4 polynucleotide kinase (10,000 units/mL) for a total volume of 21.4 μL. Following incubation at 37 °C for 3 h, the reaction mixture was heated at 70 °C for 10 min and the radiolabeled oligomer purified by chromatography.[51]

## Ligation reactions

A 1:1 mixture of radiolabeled oligomer **4** (1 pmol) and ligating fragment **3** (1 pmol) along with template **1** or **2** (1.0 or 2.0 equiv) were dissolved in 10 μL of MES–$Et_3N$ buffer (500 mM) and 4 μL of the appropriate concentration of $MgCl_2$ (1.0, 0.10 or 0.01 mM) was added. The Eppendorf tube was heated to 95 °C and cooled to the reaction temperature. A solution of EDCI (2 μL, 5.0 M in $CH_3CN$) was added, vortexed, briefly centrifuged and the reaction was allowed to proceed at the desired temperature (3 or 25 °C), (20 μL total volume). The final concentrations of the ligation reaction components were: MES–$Et_3N$ buffer (250 mM), $MgCl_2$ (2, 20, or 200 mM) and EDCI (200 mM). At precise intervals, an aliquot (1 μL) of the solution was taken and transferred to a tube containing 48 μL of water and 2 μL of 0.5 M EDTA (pH 7.5). An aliquot (1 μL) of that resulting solution was evaporated to dryness, redissolved in the loading buffer, normalized and subjected to electrophoresis on polyacrylamide gel (8%, 8 M urea). Autoradiography was used to analyze the reaction progression. Product yields were determined from the scanned images of the autoradiograms. The yield was calculated from the densitometry ratio of ligated product **5** relative to the total radioactivity per lane.

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