Enzyme-Free Ligation of 5′-Phosphorylated Oligodeoxynucleotides in a DNA Nanostructure

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Multicomponent reactions are difficult synthetic transformations. For DNA, there is a special opportunity to align multiple strands in a folded nanostructure, so that they are preorganized to give a specific sequence. Multistrand reactions in DNA origami structures have previously been performed using photochemical crosslinking, 1,3-dipolar cycloadditions or phosphoramidate-forming reactions. Here we report carbodiimide-driven phosphodiester formation in a small origami sheet that produces DNA strands up to 600 nucleotides in length in a single step. The method uses otherwise unmodified oligodeoxynucleotides with a 5′-terminal phosphate as starting materials. Compared to an enzymatic multistrand ligation involving linear duplexes, the carbodiimide-driven ligation gave fewer side products, as detected by gel electrophoresis. The full-length 600mer product was successfully amplified by polymerase chain reaction.

Keywords: Oligonucleotides, DNA, Ligation, Origami, Nanostructure.

Introduction

The parallel formation of several covalent bonds between functional molecules to produce a product of defined length is a synthetic challenge. An area of considerable interest is the synthesis of long DNA strands that can act as genes in the context of synthetic biology. Standard methods of gene synthesis involve the preparation of oligonucleotides via solid-phase DNA synthesis, followed by multiple ligation steps. This is a time-consuming procedure, requiring purification and handling of intermediates that is associated with low overall yield. Recently described methods utilize assembly-based strategies. Important examples are the ‘Golden Gate Cloning/Assembly’ and the ‘Gibson Assembly’ strategies. Instead of using many short splint strands, a template strand aligns the oligonucleotides, reducing errors due to cross-ligation. The ligation involves a one-pot reaction with polymerases, restriction enzymes and exonucleases. This leads to products of greater purity and avoids purification between ligation steps. Still, such methods are limited by the accessibility of the corresponding template strand.

An interesting alternative method to align several oligonucleotides in a desired sequence is the use of a three-dimensional DNA nanoassembly. The origami method has proven to be an enabling technique, with which hundreds of different strands can be successfully hybridized into a single assembly in high yield. Traditionally, the formation of DNA origami is relying on a long single-stranded scaffold strand that is derived from a bacteriophage vector and approximately 200 short synthetic staple strands, but alternative approaches with a larger number of shorter strands also exist. There have been attempts to ligate the staple strands within an origami enzymatically, but this approach is hampered by the steric accessibility of the ligation sites. Instead, covalent bonds between individual strands have been installed using UV-crosslinking with a psoralen derivative, resulting in a thermally stabilized structure. Further, copper-catalyzed 1,3-dipolar cycloadditions between azido- and propargyl-terminated strands, also known as CLICK reactions, have been employed to produce origami structures with increased thermostability and improved stability against degradation by restriction enzymes.

Recently we were able to show that the simultaneous ligation of six 100mer oligonucleotides to form a 600mer can be induced in a preassembled small DNA origami when the 3′-termini of the strands feature 3′-
amino-2',3'-dideoxynucleosides and the 5'-termini are phosphorylated. The resulting linkages between the synthetic strands are phosphoramidates, though, not natural phosphodiesters. Still, this study showed that a series of 100mer strands, replacing the continuous scaffold strand of traditional origami, assemble with the help of approximately 20 staple strands into small nanostructures in which the 100mers are aligned for chemical ligation.

When sufficiently reactive condensing agents are used, chemical ligations producing phosphodiester bonds are known to occur. Studies by Shabarova et al. have used 1-[(3-dimethylamino)propyl]-3-ethylcarbodiimide (EDC) as condensing agent for ligations in aqueous solution. However, such chemical ligations are known to be rather slow and prone to give incomplete conversion. A condensing agent giving fast, but often incomplete reactions is cyanogen bromide (BrCN). Unfortunately, the rapid release of HBr and the potential for side reactions limit its use in large multistrand assemblies. Here we report a method for a parallel phosphodiester ligation of synthetic 5'-phosphorylated oligonucleotides, aligned by 19 staple strands in a small origami nanostructure. In our study, we also compared this approach to other ligation methods, using linear DNA duplexes.

Results and Discussion

The small origami structure employed in our study is shown in Fig. 1. It consists of a planar DNA origami sheet, in which six 5'-phosphorylated oligonucleotides, between 97 and 104 nucleotides in length, act as scaffold strands (1a – f), and 19 shorter oligonucleotides, 21 – 45 nucleotides in length, act as staple strands (2a – s). The sequence chosen is that of the main portion of the scaffold dubbed ‘M1.3’, which was also used in our earlier studies on phosphoramidate ligations. The full sequences of all strands are listed in the Supporting Information (SI). Folding was induced by mixing the strands at a molar ratio of 1:1.2 (scaffold strands/staple strands) and annealing from 85 °C to 22 °C in 36 h. Control experiments showed that short hybridization times were unfavorable, and an annealing time of < 1 h gave no detectable product band at all. After hybridization, the origami nanostructure was freed of excess staple strands by spin filtration, and a sample was analyzed by agarose gel electrophoresis (Fig. S1, Supporting Information).

For the remainder of the material, the adsorbed origami was washed with, and then eluted with buffer containing 500 mM MES at pH 7.5 and 10 mM MgCl2. This buffer was chosen to limit the pH shift expected for ligations induced by cyanogen bromide, which rapidly releases HBr upon addition to the aqueous solution. Cyanogen bromide was tested first, as this reagent is known to induce fast reactions. In the event, the solution was cooled to 0 °C, and BrCN was added to give a 100 mM solution of the condensing agent. Samples were then drawn at 1, 10 and 60 min reaction time, followed by analysis by denaturing PAGE (Fig. 1b).

Figure 1. a) Assembly of a small DNA origami and BrCN-induced ligation of the scaffold strands in the resulting assembly. b) Detection of ligation products by 8% PAGE (19:1 crosslinking, 8 M urea, GelRed-staining). Conditions: 100 nM origami, 100 mM BrCN, 500 mM MES buffer, pH 7.5, and 10 mM MgCl2 at 0 °C. Lane 1 – 3: ligation products after 1, 10 or 60 min reaction time; Lane 4: molecular-weight size marker; 100 nt RNA ladder. Ligation products up to a size of 400 nucleotides are detected.
Even after 1 min, ligation was observed, but the conversion of strands was incomplete, and 400mers were the longest products that were detectable with our method. Further, the reaction stalled after the first time point sampled. This prompted us to test other ligation methods. For this, only five 5′-phosphorylated 100mer oligonucleotides were used, and the sixth scaffold strand was left unphosphorylated (Fig. 2a).

Through this modification, the formation of a cyclic 600mer was avoided, which can be hard to differentiate from a linear 600mer in gels. First, enzymatic ligation was tested using T4 ligase. The pre-assembled origami (30 nM) was allowed to react in ligase buffer with 0.5 mM ATP, 10 mM MgCl₂ and 0.4 U/µl T4 ligase for 24 h at 22 °C. After ethanol precipitation, the products were analyzed by PAGE (Fig. 2b). Little conversion was detected, with a weak band for 300mers as the most advanced products. This confirmed that the ligation sites are not well accessible to the enzyme in the folded origami nanostructure.

Next, we tested EDC as activator. When the strands of the pre-assembled small origami were allowed to react in 100 mM MES buffer, pH 6 at 10 mM MgCl₂ concentration and 400 mM EDC, simultaneous chemical ligation of the phosphorylated strands did occur. Gel electrophoretic analysis after 3 days reaction time at 8 °C gave a series of product bands up to the 600mer expected for ligation of all scaffold strands (Fig. 2c).

Quantitative analysis of the gel indicated that the conversion yield to the 600mer was approximately 7%, as judged by the intensity of the bands. The series of product bands ended at the expected maximum size of the ligation product, indicating that ligation did indeed occur in the origami assembly and not through oligomerization of free strands. Table 1 lists the results from our exploratory optimization study. The use of a higher EDC concentration, reaction times longer than 3 days, or the addition of a possible organocatalyst did not increase the yield of the 600mer ligation product detectably. A pH shift to a value of 5 also had no significant effect. We note that sequence dependence in

Table 1. Results of ligation of the 5′-phosphorylated 100mer oligonucleotides in a DNA origami, as shown in Fig. 2.

| Entry | EDC [mM] | t [days] | Additive | Conversion [%] |
|-------|----------|----------|----------|---------------|
| 1     | 800      | 3        | –        | 7             |
| 2     | 400      | 4        | –        | 7             |
| 3     | 400      | 3        | 2-methylimidazole.b | 0         |
| 4     | 400      | 3        | 1-ethylimidazole.b | 0         |
| 5     | 400      | 3        | –        | 7             |

.a Conditions: 0.1 µM strands, 100 mM MES buffer, 10 mM MgCl₂, pH 6.0; 8 °C. b Organocatalytic additive, if any, at 150 mM, conversion to full length 600mer, as determined by integration of bands in denaturing polyacrylamide gel after staining with GelRed. See Supporting Information for sequences.
chemical ligations has been reported for other systems,[20][26] and we expect ligation rates to vary slightly from one reaction site to another, so that sufficiently long reaction times are recommended.

We then asked whether similar results can be obtained with a linear assembly of the long oligonucleotides, held together at the ligation sites by conventional short splint strands. For this, the system shown in Fig. 3a was used. It consists of the six long 5′-phosphorylated oligonucleotides 1a–f and the five short splint strands 3a–e. The linear assembly was subjected to the same ligation conditions as used for the DNA origami (vide supra). Analysis by denaturing gel showed a series of bands up to 400mers for the EDC-mediated ligation (Lane 1, Fig. 3b). This confirms that the tightly packed structure of the origami, in which the reaction partners are held in close proximity, is favorable for the chemical ligation. On the other hand, the enzymatic ligation gave longer products, up to the expected 600mer, but there was a multitude of bands, suggesting that side reactions occurred, probably induced by cross-hybridization and other phenomena that allow for ligation in other sequences than the desired one (Lane 2, Fig. 3b).

Having confirmed that the successful chemical ligation in the origami assembly is not a trivial result, we finally turned to the product of this EDC-driven reaction again. As shown in Fig. 2c, chemical ligation with EDC had given a well resolved product band. To confirm the integrity of the product, a further characterization was called for. First, the products were analyzed by RP-HPLC. Without proper desalting, there was no baseline separation of the ligation products, but the peak pattern confirmed the assignment obtained by PAGE (Fig. S2, Supporting Information). Then, the full-length oligonucleotide product was amplified by polymerase chain reaction (PCR). The resulting ampli-con was analyzed with an agarose gel, which showed a single, intense band of the correct size (Fig. 4). The starting material was completely consumed. Together, this confirmed the identity of the ligation product as a 600mer DNA strand.

**Conclusions**

The phosphodiester ligation in an origami nanostructure most probably benefits from the cooperativity of the assembly process, which, like crystallization, favors a well-packed structure containing all strands. Linear assemblies with longer single-stranded stretches do not, so that the linear combination of equilibrium processes gives lower yields in correct assemblies. The small molecule reagents appear to penetrate the structure enough to induce ligation. The overall yields are low, but clean bands in the gel allow for facile product isolation and subsequent PCR amplification. The cost of a single multicomponent ligation should be lower than that of a series of convergent ligations, as fewer handling and purification steps are required. The reagents for chemical ligation are also less expensive than the enzymes and substrates used in enzymatic protocols. Since the band pattern of phosphodiester products observed here are quite similar to those observed for phosphoramidate ligation,[16] we suspect that local folding[27] is as important as chemical reactivity in the ligation reaction. The phosphodiester products reported here have the advantage of being the natural substrates of
polymerases and other enzymes of molecular biology, so that standard procedures can be used to process the products of the long strands obtained by chemical 'in-origami' ligation.

**Experimental Section**

**Assembly of the DNA Origami**

The folding of the DNA origami was induced by mixing the six long oligonucleotides 1a – f (100 nm each) and the staple strands 2a – s (120 nm, 1.2 equiv. each) in 100 µL aqueous buffer (10 mM Tris-HCl, 1 mM EDTA, 10 mM MgCl₂, pH 8.0). The solution was heated to 85 °C and cooled to 22 °C within 36 h in a thermocycler to hybridize the strands, using a linear temperature gradient. The removal of excess staple strands was achieved by spin filtration with a 100 kDa molecular weight cut-off (MWCO) filter (Merck Millipore, Billerica, MA, USA). The DNA-containing solution was diluted to 500 µL with MES buffer (100 mM, pH 6) and MgCl₂ (10 mM), and the resulting solution was spin filtered by centrifugation for 7 min at 6'000 g. A second filtration with 500 µL MES buffer (100 mM) and MgCl₂ (10 mM) was done to ensure complete removal of the staple strands and exchange of the buffer. The DNA was recovered in approximately 30 µL buffer by reversing the filter and centrifugation for 3 min at 3000 g. For the subsequent ligation experiments, the solution was diluted to 100 µL.

**Ligation with EDC**

The solution of the DNA origami (100 µL, 100 nm final concentration) was treated with solid EDC hydrochloride to give a concentration of 400 mM in buffer (100 mM MES, pH 6.0, 10 mM MgCl₂), and incubated for 3 days at 8 °C. Afterwards, the DNA was precipitated by adding ethanol (200 µL) and NaOAc solution (10 µL, 0.3 M final concentration). The mixture was incubated for 12 h at –20 °C and then centrifuged at 21’500 g for 30 min. The supernatant was removed, and the DNA pellet was washed once with ethanol (70% in H₂O), followed by a second centrifugation step of 5 min at 21’500 g. The ligation products were analyzed by denaturing PAGE.

**Supplementary Material**

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbdv.201700315.

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**Author Contribution Statement**

Both authors planned the experiments. M. K. performed the experiments and analyzed the data. Both authors contributed to the writing of the manuscript.

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