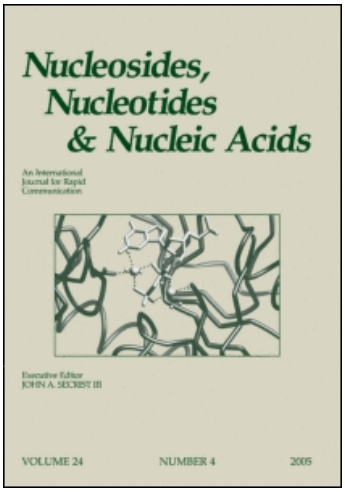

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Cyclic Enzymatic Solid Phase Synthesis of Isotopically Labeled DNA Oligonucleotides

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CYCLIC ENZYMATIC SOLID PHASE SYNTHESIS OF ISOTOPICALLY LABELED DNA OLIGONUCLEOTIDES

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□ *Isotopic labeling of DNA using standard solid phase synthesis requires expensive phosphoramidites that are used in large excess. We have developed a protocol where enzymatic, cyclic, solid phase synthesis of DNA facilitates a more economical use of the less expensive labeled DNA triphosphates (dNTP). In this approach, the DNA template is immobilized on an epoxy-activated solid support. Both the support and the linkage between DNA and resin are inert to high pH conditions which are required for product release in this scheme. Efficient covalent attachment of the DNA to the resin was achieved when the reaction was carried out in $MgCl_2$/CAPS. The enzymatic fill in reaction as well as product release and recycling conditions were optimized for efficient reuse of dNTPs without any purification. The developed protocol was used to generate a selectively [^{13}C, ^{15}N] G labeled 10-mer duplex.*

Keywords DNA; isotopic labeling; solid phase; NMR; enzymatic synthesis

INTRODUCTION

Nuclear magnetic resonance (NMR) spectroscopy is a powerful tool in the study of macromolecular structure and dynamics. However, spectral overlap is a common problem making assignments more difficult, especially for larger molecules. Isotopic labeling [^{13}C, ^{15}N] either nonspecific or, especially, residue specific greatly simplifies spectra and the resonance assignments. While protocols for isotopic labeling of proteins and RNA are well established, labeling DNA has proven to be more cumbersome. Several different approaches have been described that can be classified into chemical or enzymatic methods. From these, solid phase chemical synthesis is conceptually the most straightforward but requires a large

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excess of expensive [^{13}C, ^{15}N] phosphoramidites. Among the enzymatic methods, one approach—called endonuclease sensitive repeat amplification (ESRA)—uses a plasmid or a PCR based strategy.[1] In the plasmid approach, a DNA sequence containing multiple copies of the target oligonucleotide, separated by endonuclease restriction sites, is ligated into a suitable plasmid and then transformed into cells and grown in media with ^{15}N and ^{13}C nutrients. The polymerase chain reaction (PCR) strategy amplifies the target DNA sequence with labeled deoxyribonucleotide triphosphates (dNTPs). In both cases, the final product is obtained by treating the plasmid or PCR amplified DNA with the appropriate restriction endonuclease. A constraint of this technique is that ESRA products are double-stranded complementary sequences; therefore, sequences that contain mismatches or noncomplementary portions require additional procedures and purification. Complications can occur during PCR amplification if the desired product is intended to be partially or completely self-complementary. Furthermore, the ends of the final product may retain a part of the restriction site, which might not be desired and, in addition, the labeled DNA product must be purified from the reaction mixture. Other approaches reported are enzymatic-based synthesis of the target single stranded (ss) oligonucleotide on a DNA template with labeled dNTPs.[2,3] The Zimmer and Crothers[2] technique uses the 3′–5′ exonuclease deficient Klenow fragment (exo- KF) from DNA polymerase I to incorporate (fill in) labeled dNTPs into a partial DNA hairpin (similar to Figure 1) that acts as both primer and template. This partial DNA hairpin contains a ribonucleotide at the 3′ terminus allowing release of the product from the filled in hairpin by alkaline hydrolysis. The method described by Masse et al.[3] differs from the above in that the primer is not covalently attached to the template and Taq polymerase is used instead of exo- KF. Both approaches produce considerable quantities of labeled oligonucleotides, however, the DNA template is utilized only once and the desired labeled product must be purified from a reaction mixture containing template, product, enzyme, etc. Similarly, the dNTPs are not readily reused and must also be purified. Also, in addition, the product must be examined carefully and potentially be trimmed since both Taq polymerase and exo- KF may produce N+1 sequences.[2,3]

Here we explore a convenient approach to produce labeled ssDNA oligomers using commercially available reagents. Our method is an

FIGURE 1 Sequence and structure of the 26-mer DNA primer-template. The T_{17} residue in the hairpin loop contains the primary amine linker.

extension of Zimmers and Crothers work, which now permits reuse of template and reagents, and has an advantage over other approaches in that it facilitates purification of the final product after enzymatic synthesis. Furthermore, unused dNTPs can be reused for further rounds of synthesis with the same template or a completely different template without any purification. In order to recycle the dNTPs, we focus on solid phase enzymatic synthesis of the desired oligonucleotide on a DNA hairpin primer-template (Figure 1), which necessitates covalent attachment to a solid support. To prevent the formation of N+1 oligonucleotides, Klenow fragment with 3′–5′ exonuclease (KF) is used. For this synthesis procedure several requirements must be considered and individual steps need to be optimized:

1. linkage between DNA and resin must be resistant to alkaline conditions;
2. polymerase must be able to process the immobilized substrate;
3. product must be readily released from the template nondestructively;
4. simplified purification of the product from the reaction mixture; and
5. reuse of labeled dNTPs.

The optimized procedure was used to produce a [^{13}C ^{15}N] G labeled 10 mer.

RESULTS AND DISCUSSION

Solution Synthesis and Release of 10-mer Product

The DNA template forms a hairpin with an uridine at the 3′ end (Figure 1). This enables the hairpin to function both as a template for synthesis and facilitate subsequent cleavage of the 10-mer product. Complete enzymatic synthesis (fill-in) of the 26-mer primer-template was achieved in 2 hours at 37°C with KF. Two other constructs that were 26 and 36 nucleotides long and produced 10 and 20-mers, respectively, were equally efficient (see Appendix A). This demonstrates that the hairpin constructs are versatile to accommodate different sequences and lengths. We also explored the use of Taq polymerase, however, these reactions did not go to completion even after 48 hours (ca. 60% of the 26-mer filled in; see Appendix A). Adding KF completed the reaction, demonstrating that these small hairpin primer-templates are suitable for use with KF but not Taq polymerase. The filled in primer-templates were analyzed by denaturing PAGE, neither in the case of the filled in templates nor for the released products could we detect the presence of any N+1 products.

The primer-template has a 3′ ribouridine that allows cleavage of the product from the filled in hairpin under alkaline conditions.[2] Optimal conditions to achieve product release were explored using the filled in hairpin in Figure 1 for a range KOH concentrations and temperatures. Complete hydrolysis was obtained in 0.2 M KOH within 2 hours at 55°C,

while less than 4 hours were required at 37°C. During a cyclic process, the 26-mer template will be repeatedly exposed to conditions that could result in base modifications. Cytosine especially, is sensitive to deamination to uracil in alkaline conditions.[8] The extent and rate of deamination was determined by monitoring base protons by NMR. No changes (<1%) were observed for adenosine and guanosine nucleosides in 0.2 M KOH even after 40 hours at 55°C. Degradation of cytidine (21%) and, to a smaller extent, of dCTP (6%) were detected after 10 hours at 55°C indicating that a charged molecule is more resistant. For cytidine in context of an oligonucleotide less than 4% degradation occurred after 10 hours at 55°C. At 37°C the reaction is much slower and only 0.5% degradation of cytidine is detected in 10 hours, while no changes were observed for the 10-mer oligonucleotide. Therefore, a DNA template is able to endure an extended time, or multiple cycles, in alkaline conditions that are needed for repeated product release. For subsequent product release reactions from the resin, 0.2 M KOH at 45°C was used.

Conjugation of Primer-Template to Resin

The modified thymidine in the hairpin loop contains an alkyl primary amine that covalently reacts with the epoxy resin. Using monovalent cations (Na^+ and K^+), less than 40% conjugation was observed even after 75 hours incubation in potassium phosphate buffer, pH = 9.4 (Figure 2). Under similar conditions, complete immobilization of a control ligand, tryptophan, could be achieved within 24 hours. DNA is highly negatively charged and the resin matrix, a branched polysaccharide of varying chain lengths, also carries an overall negative charge under the immobilization conditions (pH = 9.4).[9] Therefore, reducing the electrostatic repulsion by more effective charge screening should improve DNA immobilization.

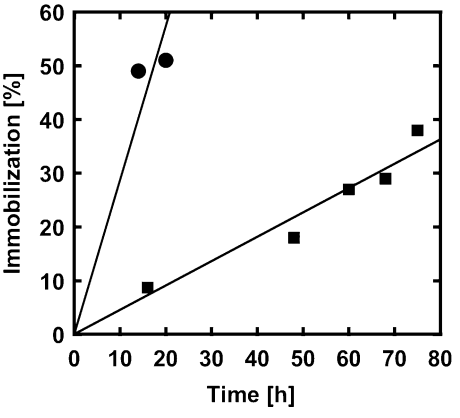

FIGURE 2 Immobilization time course of amine functionalized DNA on epoxy resin for 3 M potassium phosphate (squares) and 800 mM $MgCl_2$/200 mM CAPS buffer (circles).

Using $MgCl_2$/CAPS buffer a 6-fold faster immobilization rate was achieved, allowing ~49% template conjugation to resin within 14 hours (Figure 2). However, after 14 hours, immobilization of the template halted, even after washing and adding fresh template to the resin, suggesting a loss of reactive sites due to hydrolysis.[10] This notion was confirmed by immobilization of tryptophan after pre-incubating the resin with $MgCl_2$/CAPS for 24 hours, which resulted in only ~50% binding. The DNA immobilization was indirectly monitored by quantifying the remaining free ligand in solution by UV spectroscopy. The amount of resin bound template was also confirmed by conjugating the DNA hairpin-template containing the 5′ DMT group and quantifying DMT (A_{498nm}) after release with TCA/dichloromethane.[11] This method showed that 47% of the total DNA was bound to the resin, in good agreement with the indirect approach. The loading capacity of the resin in $MgCl_2$/CAPS buffer was thus determined to be ~0.48 OD_{260nm} template/mg of resin for a reaction with 0.98 OD_{260nm} template/mg resin. Higher amounts of DNA can be conjugated for scale up using a larger excess of DNA. It should be noted that DNA that did not bind is readily recovered and can be reused for subsequent reactions.

Optimization of Product Synthesis on Resin and Recycling

Initial fill in reactions followed by cleavage only produced a modest amount of the desired 10-mer product, prompting us to optimize the reaction conditions. For 10 U KF, 0.45 OD_{260nm} bound template, (1.88 nmol DNA, ~1 mg resin) and 6 mM of each dNTP in 400 μl for a 4 hour-reaction, 1.35 nmol of 10-mer was produced, that is, 72% of the of template was utilized (Figure 3). Using 20 U KF 10-mer increased the yield to 85% (4 hours), while higher amounts of KF as well as extended reaction conditions (24 hours) resulted in lower yields (data not shown). In this reaction, the dNTP concentrations (6 mM each) are in 300–600 fold excess of the required amount to fill-in the 26-mer template. By decreasing the dNTP concentration to 0.5 mM and using 10 or 2 U KF the 10-mer yields are 60% and 28%, respectively. These results demonstrate that 0.5 mM dNTP (25- to 50-fold excess), and $\geq$10 U KF give acceptable yields for a single fill in reaction (Figure 3).

In order to make the reaction cyclic, the supernatant from the 20 U KF reaction was used for a second and third synthesis round. After each fill in reaction, the supernatant was removed and added to a fresh batch of resin bound template (1.88 nmol DNA). No additional dNTPs were added. In round 2, only 41% of the 10-mer obtained in round 1 was produced, while in round 3 the production decreased further to just 6%. The decrease in 10-mer production between successive rounds could be due to several factors: a) enzyme inhibition due to pyrophosphate produced during the fill in reaction; b) loss of dNTPs due to KF activity;

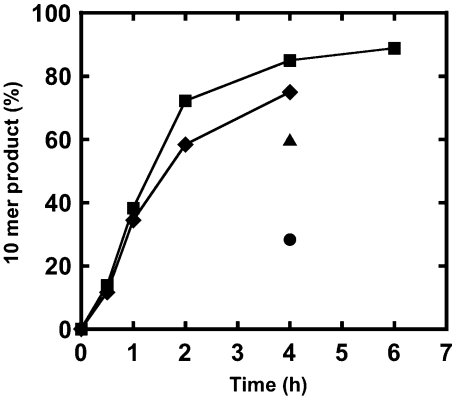

FIGURE 3 Solid phase enzyme/dNTP optimization. dNTP concentrations (each) were lowered from 6 mM to 0.5 mM. Each reaction run at 37°C used 1 mg resin with 0.5 OD bound template and 2–20 U KF in 400 μl. Combinations of enzyme units and dNTP concentrations are as follows: 10 U KF, 6 mM dNTP each (square), 20 U KF, 6 mM dNTP each (square), 10 U KF, 0.5 mM dNTP each (triangle), 2 U KF, 0.5 mM dNTP (circle). Percentage of product is based on template utilization.

c) inactivation of the enzyme. However, the presence of a large excess of sodium pyrophosphate (1 mM) had no effect on the reaction. Similarly, the dNTPs are not degraded in presence of KF and template as determined by ^{31}P NMR spectra of the reaction before and after enzymatic synthesis (24 hours). The lowered product synthesis between two rounds could then be attributed to inactivation of the enzyme, which was confirmed by adding a fresh aliquot of KF (20 U) in round 2, which restored the 10-mer yield to 80% of round 1. The drop in the yield for round 2 compared to round 1, despite addition of fresh enzyme, may be due to the inactive enzyme interfering with the polymerase reaction. To address scale up and recycling, we have also explored the effect of reaction volumes. Fill in reactions were run at constant volume (400 μl) or by increasing the reaction volume with increasing amounts of template and enzyme (Figure 4). The fixed volume reactions produced significantly less product than corresponding reactions where the increase in template and enzyme was accompanied by an increase in the reaction volume. Consequently, in a cyclic reaction the volume should be increased with each subsequent addition of KF such that the total enzyme concentration, including the inactive enzyme fraction, is below 25 U/ml. The product release by alkaline hydrolysis leaves a phosphate group on the 3′ ribouridine, that must be removed so that the template can be reused again. This is readily accomplished by treating the template after product release with Antarctic phosphatase[2] (see Appendix A).

These optimizations are collected in the protocol outlined in Figure 5 and were used to produce a [^{13}C, ^{15}N] G labeled 10-mer. After each cycle, the template was regenerated with phosphatase and new KF was added to

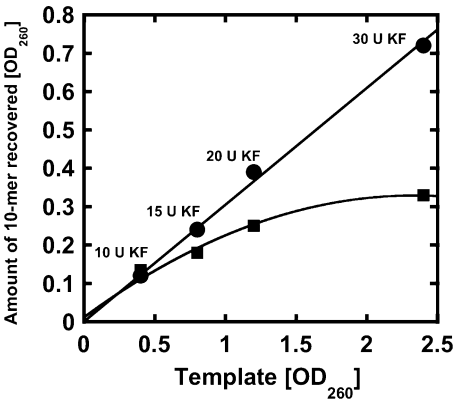

FIGURE 4 Effect of reaction volumes on 10-mer production. Increasing amounts of template were filled in with 10, 15, 20, and 30 U of KF enzyme at 37°C in 0.4 ml reaction volumes (circle) or increasing volumes, 0.4, 0.8, 1.4, and 2.0 ml (square). For a predictable linear relationship between template and 10-mer production the reaction volume must be adjusted. 1 OD_{260} of template = 4.2 nmols and 1 OD_{260} of 10 mer = 10 nmols.

the dNTP supernatant. No additional dNTPs were added between rounds. Using 4.8 OD_{260nm} (20 nmol) template, 1.28 (12.9 nmol), 1.12 (11.3 nmol), and 0.94 (9.4 nmol) OD_{260nm} of 10-mer were obtained for rounds 1, 2, and 3, respectively.

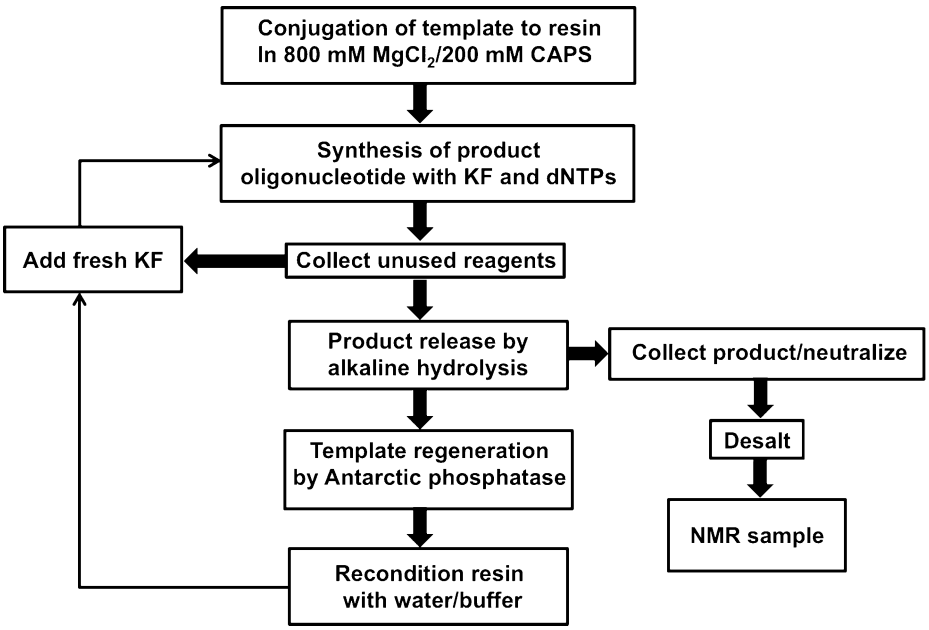

FIGURE 5 Scheme for cyclic enzymatic solid phase synthesis of isotopically labeled oligodeoxyribonucleotides.

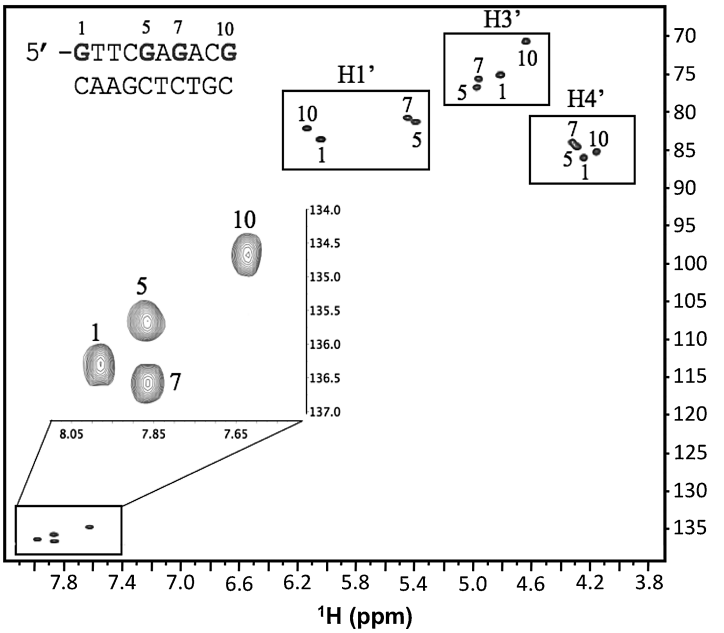

FIGURE 6 ^{13}C-^{1}H HSQC spectra of 30 μM decamer duplex in D_2O, (10 mM sodium phosphate, 100 mM NaCl, 5 mM EDTA, pH* = 6.7) recorded at 298K. H1′, H3′, H4′, and H8 protons are marked. The numbering scheme of the duplex is shown in the inset. Only the 4 G residues in the top strand are labeled.

NMR Experiments

The [^{13}C, ^{15}N] G labeled 10-mer was annealed to its complementary, unlabeled strand and examined by NMR. The imino proton spectra of this duplex, with and without ^{15}N decoupling, were compared to verify the successful labeling of the 10-mer (data not shown). As expected, out of the 10 imino proton resonances, only 4 displayed ^{15}N coupling corresponding to the 4 G′s in the top strand. A HSQC spectrum of the duplex further corroborates the labeled guanosine positions (Figure 6). Assignments were obtained from an unlabeled duplex with the same sequence.

CONCLUSION

Cyclic enzymatic synthesis and chemical cleavage of a [^{13}C, ^{15}N] G labeled 10-mer was carried out successfully using a DNA template bound to solid support. Several reactions steps were examined and optimized. Efficient immobilization of the DNA template was achieved using Mg^{2+} as the counterion. Hydrolysis conditions for release of product were selected to minimize damage to the DNA template, even after repeated exposure to alkaline conditions. Following hydrolysis in KOH, the template was reconditioned with phosphatase followed by washing with buffer to be used

in the next round of synthesis. At each step, a simple water rinse of the resin bound template removed all unwanted reaction components. The dNTPs, and to some extent the polymerase, can be used multiple times, although new enzyme should be added at each cycle. Addition of new enzyme has to be accompanied by an appropriate increase in reaction volumes. Alternatively, removal of enzyme from the supernatant before addition of fresh enzyme and reuse may be beneficial. Recovery or reuse of dNTPs is greatly facilitated because they are not contaminated by product or template and can be used directly for subsequent synthesis of the same or even different oligonucleotides without treatment. The purification of the synthesis product is straightforward. Following cleavage, the product is simply neutralized followed by desalting, which produces NMR pure oligonucleotides.

MATERIALS AND METHODS

DNA Primer-Template: The DNA Primer-Template (26-mer)

d(CGTCTCGAACAGATCCTTXTGGATC)rU (Figure 1) and 10-mer d(CGTCTCGAAC), were synthesized using cyanoethyl phosphoramidites on a Applied Biosystems 391 DNA synthesizer (Applied Biosystems, Foster City, CA, USA). X indicates the location of C6 linker dT phosphoramidite (Glen Research, Sterling, VA, USA), which provides a primary amine functional group in the hairpin loop. Following synthesis, the support was incubated with 1 ml 10% diethylamine/acetonitrile (Glen Research) to remove acrylonitrile (http://www.glenresearch.com/Technical/TB_avoidaminealkylation.html). Products were cleaved from the column with ammonium hydroxide (Fisher Scientific, Pittsburgh, PA, USA) and deprotected at 55°C overnight. The 26-mer and the 10-mer were purified by gel electrophoresis on 15% polyacrylamide 8 M urea denaturing gels, extracted and desalted on G-25 Sephadex column (Amersham Pharmacia Biotech/GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA).

Deamination Studies

Deamination of 20 mM adenosine, guanosine, and cytidine solutions were monitored by NMR spectroscopy (Bruker Avance 600, Bruker, Bio-Spin Corp., Billerica, MA, USA) in 0.2 M KOD in D_2O at 37°C and 55°C, focusing on the aromatic resonances (e.g. H5 and H6 of cytidine). Additionally, deamination of dCTP and a DNA decamer d(GCGAATTCGC) was also investigated similarly.

Solution Phase Synthesis and Release of Product

Enzymatic synthesis (fill-in) of the target 10-mer was carried out using 1.0 μM 26-mer primer-template, 5 U of Klenow fragment with 3′–5′

exonuclease activity (KF) (New England Biolabs, Ipswich, MA, USA) and 3 mM of each dNTP in 50 μl solution (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol). The reaction was run for 2 hours at 37°C and analyzed by denaturing 15% PAGE in 8 M urea. Cleavage of filled-in 36-mer back to 26-mer primer-template and 10-mer product was done in 0.2 M KOH. To optimize the cleavage protocol, reactions at different temperatures and KOH concentrations were investigated.

Conjugation of the DNA Template to the Epoxy Resin

An amount of 4.9 OD_{260nm} (20.5 nmol) of purified 26-mer was incubated with 5 mg Toyopearl AF-Epoxy 650 resin (Tosoh Bioscience LLC, Montgomeryville, PA, USA) in 3 M Na_2HPO_4 or K_2HPO_4, pH = 9.4 at 45°C for 72 hours with slight agitation in a 400 μl solution.[4,5] Progress of DNA conjugation to resin was monitored by determining the UV_{260} absorbance of the free DNA ($\varepsilon_{260nm} = 239{,}500\ M^{-1}\ cm^{-1}$) in solution at regular intervals. A similar reaction was carried out using 800 mM $MgCl_2$ in 200 mM *N*-cyclohexyl-3-aminopropanesulfonic acid (CAPS) pH = 9.4, as the buffer for 14 hours. The amount of DNA bound to resin was also verified by using a 26-mer for conjugation in which the DMT (dimethoxy trityl) protective group was left attached to the 5′ end of the strand. After the conjugation reaction of this 26-mer to resin (800 mM $MgCl_2$/200 mM CAPS for 14 hours) DMT groups were released with 4 × 200 μl 3% TCA/dichloromethane, quantified at 498 nm ($\varepsilon_{498nm} = 70{,}000\ M^{-1}\ cm^{-1}$) and compared to the trityl output from the same amount of free DNA in solution. DNA conjugation efficiency was compared to tryptophan conjugation (45°C for 24–52 hours) in the same buffers (Na_2HPO_4, K_2HPO_4, $MgCl_2$/CAPS, 400 μl). Tryptophan conjugation was determined by quantifying the free tryptophan in solution by UV spectroscopy ($\varepsilon_{280nm} = 5{,}500\ M^{-1}\ cm^{-1}$).

Optimization of DNA Synthesis and Product Release From Resin Bound Primer-Template

Several reactions using resin bound primer-template DNA were used to determine optimal quantities of enzyme and dNTPs required for product synthesis. The reactions were carried out in 400 μl Klenow buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol) with 1 mg resin bound DNA (0.5 OD_{260nm}/mg resin) using 2–20 U KF at 37°C with dNTP concentrations ranging from 0.5 to 6 mM. Alkaline hydrolysis to release 10 mer product was carried out in each case by incubating the resin, after removal of the supernatant (reagent mixture), and a wash step, in 50 μl of 0.2 M KOH for 2 hours at 45°C. Isolated 10-mers ($\varepsilon_{260nm} = 99{,}600\ M^{-1}\ cm^{-1}$) were neutralized with 100 mM HCl and desalted on G-25 sephadex resin and analyzed by 15% denaturing PAGE. The resins were then washed with water and treated with with 5 U Antarctic phosphatase (New England

Biolabs) in 400 μl phosphatase reaction buffer for 1 hour at 37°C (50 mM Bis-Tris-Propane-HCl, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$) for regeneration of the template and subsequent use in the next round of synthesis.

Reaction volumes were optimized by quantifying the product by UV spectroscopy after desalting, from two sets of reactions. In one set, the product synthesis was performed in a fixed volume of 400 μl on resin bound with 0.4, 0.8, 1.2, and 2.4 OD_{260nm} template and 10, 15, 20, and 30 U of KF respectively (37°C for 4 hours, 0.5 mM dNTP each). In the second set, for the above-mentioned combinations of template and enzyme, the reaction volumes were 0.4 ml, 0.8 ml, 1.5 ml, and 2 ml, respectively (37°C for 4 hours, 0.5 mM dNTP each).

Labeled 10-mer Synthesis/Cleavage on Resin

A fill-in reaction was carried out for 4 hours at 37°C using 10 mg resin containing 4.8 OD_{260nm} DNA template, 60 U KF, 0.5 mM each dATP, dTTP, dCTP, and [^{13}C, ^{15}N] dGTP (Cambridge Isotopes, Andover, MA, USA) KF reaction buffer in a total volume of 4 ml. After the reaction the supernatant was recovered and stored. The 10-mer product was released with 800 μl 0.2 M KOH, 2 hours at 45°C and the resin was treated with Antarctic phosphatase (40 U in 800 μl phosphatase reaction buffer for 1 hour at 37°C) and then washed with water. The reconditioned resin was used for a second cycle by reintroducing supernatant from the previous reaction with the unused dNTPs along with 40 U of KF in a total volume of 5 ml. Similarly, a third repeat cycle was run with 40 U of fresh enzyme in 6 ml. No additional dNTPs were added to the reaction supernatant at any stage. All 10-mer products obtained by alkaline hydroysis were neutralized and desalted on Sephadex G-25 resin by gravity flow.

All solid phase enzymatic synthesis reactions mentioned above were done in a 1.5 ml eppendorf tube or a 15 ml falcon tube depending on the reaction volume. The reaction container was placed on a shaker for constant agitation to keep the resin in suspension. Reaction products were removed by pipetting.

NMR Experiments

The NMR sample was prepared by combining all fractions of 10-mer obtained and hybridization with its complimentary unlabeled strand in 300 μl (99.996% D_2O), 10 mM NaH_2PO_4, 100 mM NaCl, 5 mM EDTA (pH = 6.7). Spectra were acquired on a Bruker Avance 600 using a 5 mm quad band Z-gradient probe. The phase sensitive ^{13}C-^{1}H HSQC spectra[6] were recorded at 298 K using echo-antiecho gradient selection and ^{15}N decoupling in the F1 and F2 dimension. Proton chemical shifts are referenced to internal DSS and ^{13}C chemical shift was calibrated indirectly.[7]

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