Template-dependent DNA ligation for the synthesis of modified oligonucleotides

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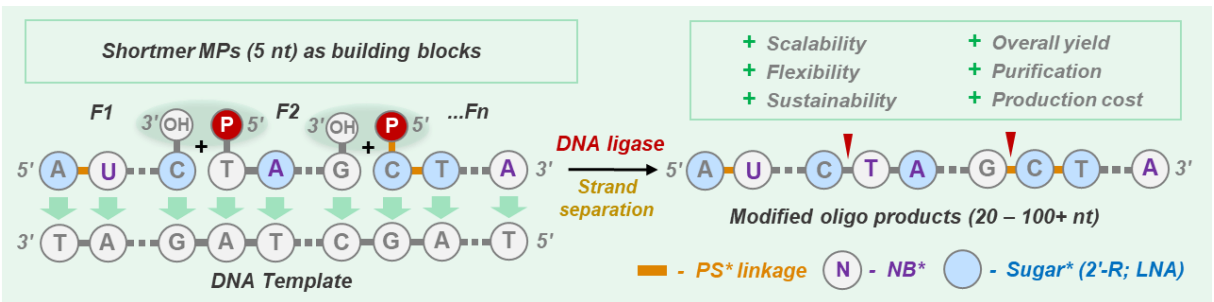
Abstract

Chemical modification of DNA is a common strategy to improve the properties of oligonucleotides, particularly for therapeutics and nanotechnology. Existing synthetic methods essentially rely on phosphoramidite chemistry or the polymerization of nucleoside triphosphates but are limited in terms of size, scalability, and sustainability. Herein, we report a robust alternative method for the *de novo* synthesis of modified oligonucleotides using template-dependent DNA ligation of shortmer fragments. Our approach is based on the fast and scaled accessibility of chemically modified shortmer monophosphates as substrates for the T3 DNA ligase. This method has shown high tolerance to chemical modifications, flexibility and overall efficiency, thereby granting access to an ultimately broad range of modified oligonucleotides of different lengths (20 → 120 nucleotides). We have applied this method to the synthesis of clinically relevant antisense drugs and diversely modified ultramers. Furthermore, the designed chemoenzymatic approach has great potential for diverse applications in therapeutics and biotechnology.

Graphical Abstract



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Main

The intrinsic properties of DNA, particularly the high degree of programmability mediated by the Watson-Crick base pairing, have propulsed synthetic oligonucleotides into the forefront of numerous applications. For instance, the self-assembly of thousands of short DNA sequences can be harnessed to create intricate 2D and 3D nanomaterials[1] and computing[2, 3] devices. In addition, computing digital information into DNA sequences represents a potentially more powerful storage medium than existing silicon-based technologies.[4, 5] When combined with chemical modifications (Fig. 1A), DNA and RNA can be converted into highly potent therapeutic agents as highlighted by the recent advent of mRNA-based vaccines.[6-9] This large and increasing demand for oligonucleotides needs to be supported by robust, efficient, cost-affordable, and sustainable synthetic methods.[10] However, our capacity at reading (i.e. sequencing) still outperforms that of writing (i.e. synthesizing) DNA.[11] This discrepancy mainly stems from the lack of alternative *de novo* DNA synthesis methods. Indeed, most synthetic oligonucleotides are produced by solid-phase DNA synthesis where activated phosphoramidite building blocks are assembled by iterative synthetic cycles on an immobilized nucleoside (Fig. 1B).[12] Application of this versatile and robust method allows for the efficient, large-scale (up to 10 kg batches) synthesis of short to moderately long (5 – 80 nt) and often heavily modified oligonucleotides.[6, 13-17] Alternatively, oligonucleotides can be produced by biocatalytic approaches[18-22] mainly based on the polymerization of temporarily blocked nucleotides catalyzed by polymerases (Fig. 1C).[23-32] However, most current methods are restricted either in terms of sequence length, possibility of site-specific introduction of modified nucleotides, speed of production, and/or sustainability.

To address the limitations of the current synthetic approaches, we present a robust alternative method for the *de novo* synthesis of chemically modified oligonucleotides which can produce short (~20 nt) therapeutic antisense oligonucleotides equally well as longer (> 100 nt) sequences decorated with modifications at virtually *any* position of the nucleotidic scaffold (Fig. 1D).

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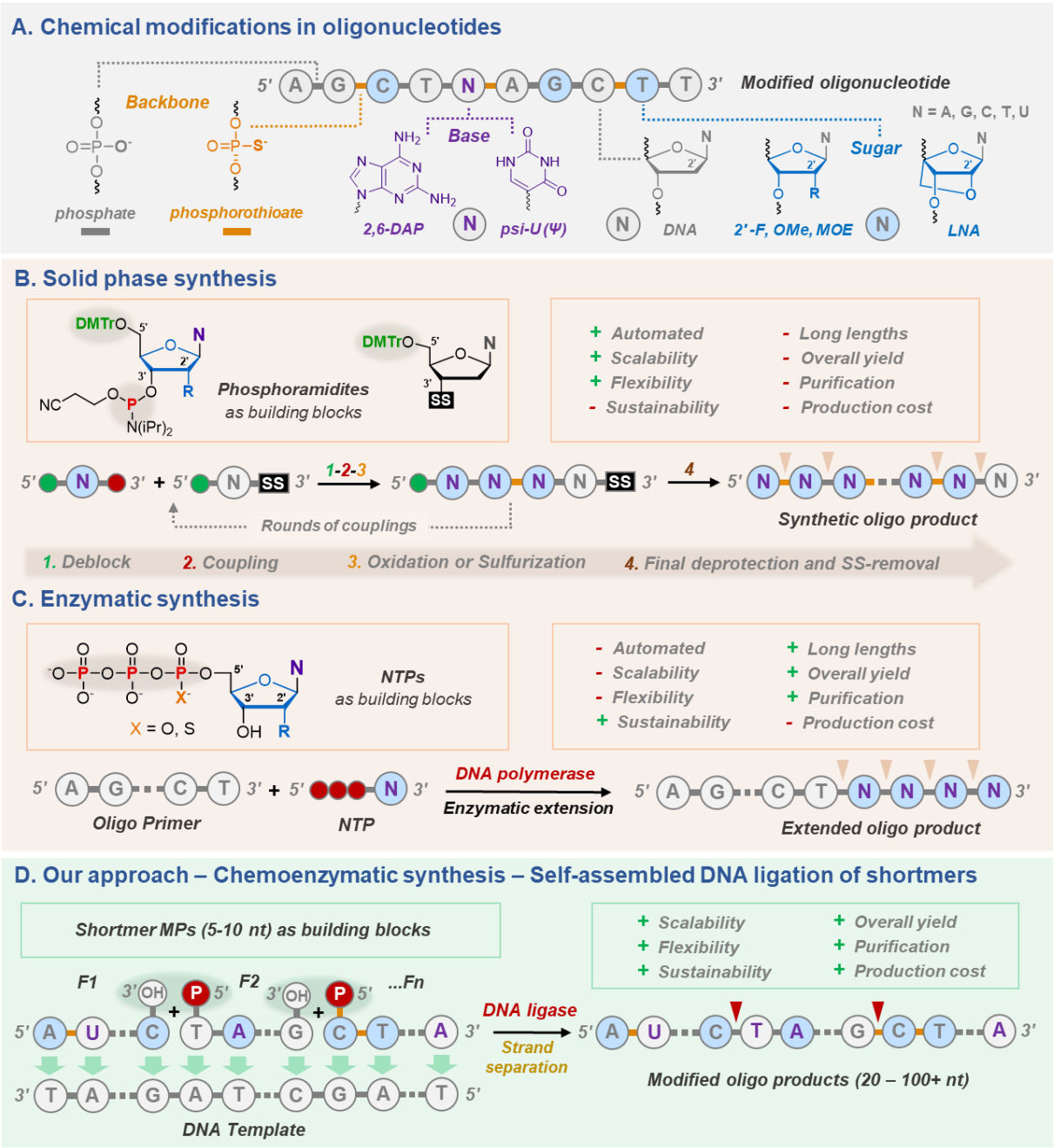

Fig. 1. Overview of existing synthetic protocols and of the co-polymerization of shortmer fragments. (**A**) Common chemical modifications in oligonucleotides; (**B**) – Overview of solid phase synthesis; (**C**) Schematic representation of enzymatic synthesis; (**D**) – Proposed alternative chemoenzymatic approach.

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Results

Design of the method

Despite impressive catalytic efficiencies[33] of around 10 $\mu M^{-1} \cdot s^{-1}$ (equating to the insertion of up to a few hundreds of nucleotides per second[34]), DNA polymerases struggle with substrates consisting of altered sugar motifs which are not only found in most therapeutic oligonucleotides but also commonly used in chemoenzymatic approaches for *de novo* synthesis of DNA. On the other hand, DNA ligases have been reported to be rather tolerant to the presence of both base-[35-39] and sugar-modifications even on short sequences[40-42] which constitutes the basis of our approach. Nonetheless, existing ligation methods rely on using scarecly modified oligonucleotides of moderate size (10 – 12 nt) which are already impinged by some of the aforementioned limitations. Instead, we based our method on the co-ligation of short, 5'-phosphorylated pentanucleotides[43] containing a broad variety of chemically modified nucleotides. These shortmers are readily produced in high yields by solid-phase synthesis and the concomitant templated ligation produces modified oligonucleotides without any size restrictions.

Scope of the ligase-mediated synthesis of modified oligonucleotides

In order to evaluate the possibility of constructing short, chemically modified oligonucleotides with ligases, we designed primer-template duplexes formed by a 5'-FAM-labeled DNA primer **P1** and DNA templates of different lengths (**1x**-22 nt, **2x**-27 nt, **4x**-32 nt, **14x**-87 nt) (Fig. 2). In this design, the duplexes can accommodate either one or multiple shortmers in ligase-catalyzed reactions. The shortmer fragments (5 nt) consisted of the random sequence (5'-TAATT-3')[41] and were equipped with 5'-phosphate moieties (Fig. 2). In order to evaluate the compatibility of ligases with chemical modifications as well as the influence of the position of the modifications on the outcome of the reactions, we prepared a small subset library of pentanucleotides **1-23** containing one or multiple chemical modifications at all positions of the nucleotidic scaffold. More specifically, we considered typical sugar modifications present most short therapeutic oligonucleotides (2'-OH, 2'-F, 2'-OMe, 2'-MOE, LNA) **3-15**, an altered phosphate backbone (phosphorothioate) **16-18**, modified nucleobases (2-amino-dA, 5-octadiynyl-dU) **19**, or a combination of multiple modification patterns **20-23**. The presence of a 5'-FAM label on the DNA primer facilitated the monitoring of the reaction progress by gel electrophoresis PAGE (20%) followed by phosphorimaging and quantification of the resulting products (Fig. 2A-E).

In addition to various chemical modifications, we also evaluated the capacity of various, commercially available DNA ligases (i.e. T4, Hi-T4^{TM}, T3, and T7) to catalyze the ligation reactions of natural shortmer **1** with the primer using DNA templates **1x** (22 nt) and **2x** (27 nt) (Fig. 2A and Supplementary Fig. SI2A). Most of the DNA ligases (T4, Hi-T4^{TM} and T3) showed

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excellent activity yielding the desired products (27 – 32 nt) with high conversion efficiencies (82-96%) except for the T7 DNA ligase which was reluctant at catalyzing the reaction (7-10% of product formation). Considering the high compatibility of the T3 DNA ligase with base-modified fragments and the high conversion yields obtained with fragments **1-23**,[35-39] we decided to further explore the substrate scope of the reaction with this enzyme.

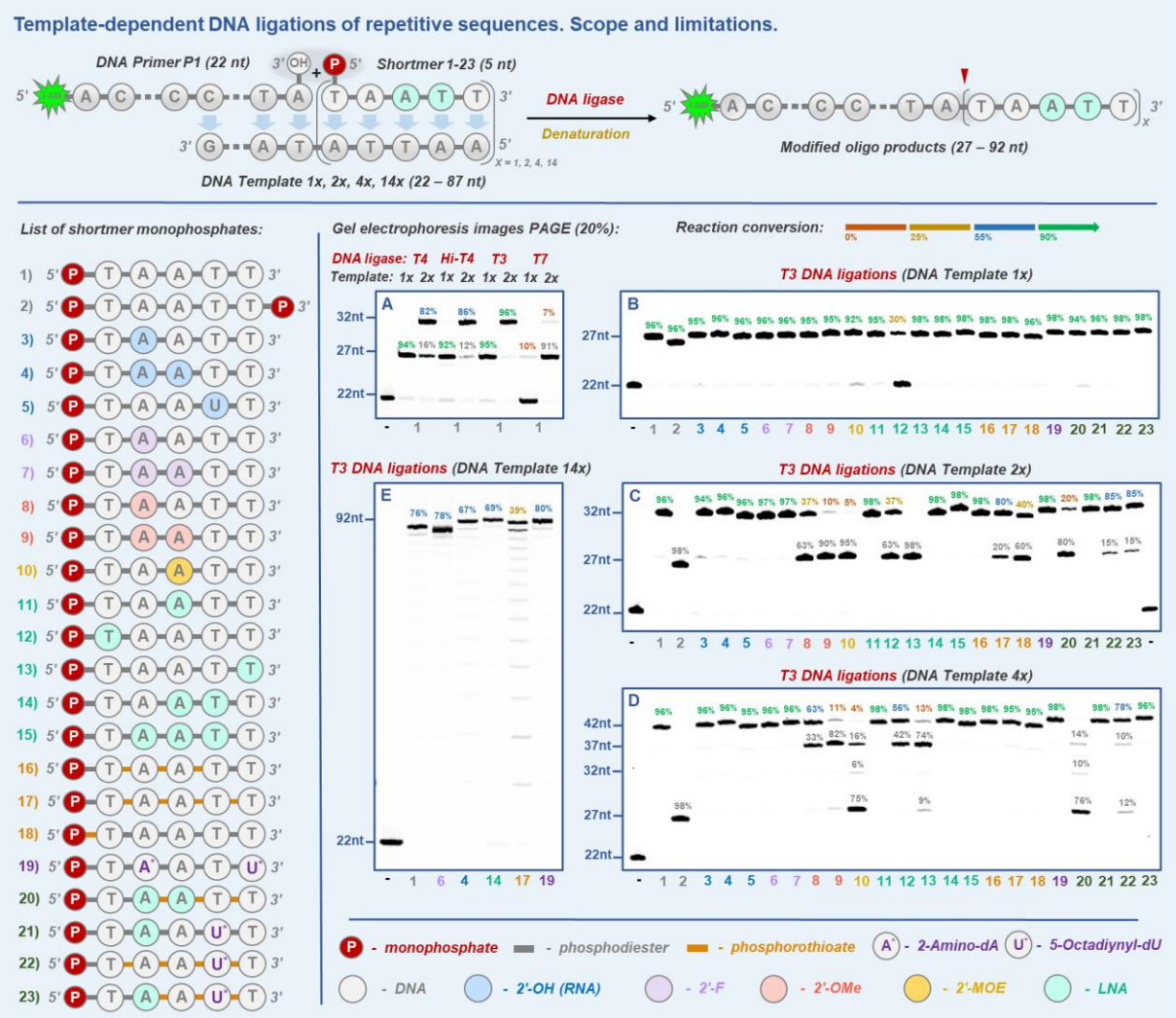

Fig. 2. DNA ligations of repetitive sequences. Scope and limitations. Gel electrophoresis images PAGE (20%): (**A**) – Screening of commercial DNA ligases; (**B**), (**C**), (**D**), (**E**) – DNA ligation reactions of shortmer (5 nt) fragments **1-23** using T3 DNA ligase, 5'-FAM labeled DNA primer **P1** (22 nt) on complementary DNA templates of different length (**1x**-22 nt, **2x**-27 nt, **4x**-47 nt, **14x**-87 nt). (**-**) – negative control in the absence of DNA ligase.

To do so, we first optimized the reaction conditions for ligation reactions with the unmodified shortmer **1** by modulating different parameters such as ratios of oligonucleotide components (i.e. primer, template, shortmer), temperature, and reaction time (see Supplementary Fig. SI2O). Suitable conditions (resulting in high conversions) were obtained by using a slight excess of DNA template and shortmer compared to primer, with reaction times of over 6 hours at 16°C. With these conditions, we evaluated the scope and limitations of the

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method by running the ligation reactions of all monophosphate shortmers **1-23** on DNA templates of different length (**1x**, **2x**, **4x**) (see Supplementary Fig. SI2B-D).

From these first studies, we learned that: 1) 5'/3'-bisphosphorylated shortmers (substrate **2)** are only added once since the 3'-phosphate moiety acts as an efficient blocking group (but could be deprotected with alkaline phosphatase[26]); 2) substrates containing either 2'-OH or 2'- F sugar modifications (substrates **3-7**), LNA units in the internal positions (substrates **11, 14-15**), phosphorothioate modifications at all but the last phosphodiester or at all linkages, respectively (substrates **16-17**), nucleobase-modified substrate **19**, substrates **22-23** (containing all modification types), are generally well-tolerated by the T3 DNA ligase, leading to full-length products with good to high conversions on either of the DNA templates; 3) substrates containing internal 2'-OMe and 2'-MOE modifications (**8-10**), LNA as first or last nucleotide (substrates **12-13**), equipped with a 5'-α-monophosphorothioate unit (substrate **18**), substrates **20-21** (with different modification patterns) showed limited substrate tolerance by the T3 DNA ligase leading to mostly lower reactivities and only truncated products (see Supplementary Fig. SI2B-D). Hence, due to the low reactivities of some of the substrates, we undertook another optimization campaign. To do so, we had to take into more detailed consideration the properties of such short DNA sequences (namely, base pairing efficiency, low T_m values, bulky modifications) and we considered several parameters to be further optimized: 1) lower reaction temperatures to ensure more efficient annealing on the template; 2) addition of crowding agents like polyethylene glycol (PEG) and DMSO in order to improve the close proximity and efficiency of the alignment of the shortmer fragments on templates; 3) addition of other metal cofactors (e.g. Mn^{2+}, Ca^{2+}, or Co^{2+}) along with Mg^{2+} (present in the reaction buffer) to increase flexibility and tolerance of bulkier modifications; 4) optimization of the amount of ATP cofactor (present in the reaction buffer) to avoid potential side reactions such as adenylation.[44] Consequently, by varying the above-mentioned parameters, we identified reaction conditions (4°C reaction temperature, the addition of PEG8000 (20%), DMSO (10%), $MnCl_2$ (1mM final), 20x less ATP (50µM final)) which significantly improved the reactivity and substrate tolerance. Indeed, under these optimized reaction conditions, most of the evaluated chemical modifications were well-tolerated with excellent conversions to the desired full-length products by ligating multiple, consecutive shortmer substrates **1-23** on DNA templates **1x**, **2x**, **4x** (Fig. 2B-D and Supplementary Fig. SI2B-D). Nonetheless, some of the substrates (**8-10**) bearing bulkier 2'-sugar modifications (2'-OMe, MOE) struggled to yield full-length products, as well as those (**12-13**) having unfavorable C3'-*endo* sugar conformations at the beginning and the end of the sequence. It is also worth mentioning that even though reactions with substrate **18** led to high conversions, some desulfurization (PS to PO) could be detected by LCMS analysis. Importantly, shortmer substrates **21-23** containing all

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combinations of chemical modifications (i.e. sugar, phosphate, and nucleobase) in a single fragment acted as excellent substrates for the DNA ligase (Fig. 2B-D).

Next, we evaluated the possibility of producing longer modified oligonucleotides by this method and we tested the repetitive DNA ligation of several substrates bearing various modifications on a longer DNA template **14x** allowing for the successive, one-pot incorporation of up to 14 pentanucleotide fragments. Under the optimized conditions, we could produce full-length (92 nt) products with good conversions (Fig. 2E). Finally, the chemical nature of all reaction products (Fig. 2A-E) was confirmed by LCMS analysis after running the preparative scale reactions, and the results are summarized in the Supplementary information (Tables SI1-4). Taken together, DNA ligation of repetitive sequences offers the possibility of efficiently introducing multiple chemical modifications at user-defined positions by simply changing the nature of the pentanucleotide fragments hence overcoming the challenges associated with classical strategies such as primer extension (PEX) reactions.

We then explored the possibility of synthetizing oligonucleotides with more diverse sequence compositions ligating the shortmers **F1-F4** to the 5'-FAM-labeled DNA primers **P1-P2** on the complementary DNA templates **4x-mix** (Fig. 3). First, we used the optimized conditions to add the unmodified shortmers **1, 24, 27, 30** to the primer **P1** in a stepwise manner to show the possibility of controlled synthesis of desired lengths with one single template, and to exclude the possibility of template-independent cross-ligation reactions (Fig. 3A). Next, we carried out mixed ligation experiments with the unmodified substrates **1, 24, 27, 30,** the LNA-containing substrates **11, 25, 28, 31,** and substrates **17, 26, 29, 32** equipped with phosphorothioates along with longer (**P1**, 22 nt) and shorter (**P2**, 6 nt) DNA primers. All these T3 DNA ligation reactions resulted in excellent conversions to the desired products (Fig. 3B-C). Our method is mainly based on the ligation of pentanucleotide fragments, nonetheless, for the construction of specific oligonucleotides of lengths differing from multiples of five, fragments of other lengths would be required.[45] Hence, we evaluated the possibility of adjusting our method to slightly longer oligomers. To do so, we carried out two separate DNA ligation reactions with primer **P1** using shortmers **1, 33, 34** (5 nt, 7 nt, 8 nt, respectively) and **35, 36** (10 nt each) (Fig. 3D). All reactions proceeded with excellent conversions and full-length products could be identified without the formation of truncated products, suggesting that the length of the shortmer does not negatively impact the outcome of the reaction. Since these reactions with unmodified shortmers proceeded very well, we rationalized that a mixture of unmodified and modified fragments could be employed, to incorporate the least reactive of the modified pentanucleotide substrates identified previously. Hence, we carried out co-ligation reactions with modified pentanucleotides **9, 10, 17, 18, 20, 22, 23** along with unmodified substrates **24, 27, 30** using the 5'-FAM-labeled DNA primer **P1** (Fig. 3E). Gratifyingly, all reactions proceeded with high conversions towards the expected full-length products with

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marginal truncated product formation, suggesting that with a careful design in mixed ligation, even highly modified shortmers can be incorporated into DNA with good efficiency. The integrity of all ligated DNA products (Fig. 3A-E) was confirmed by LCMS analysis after running the preparative scale reactions, and the results are summarized in Supplementary information (Table SI4).

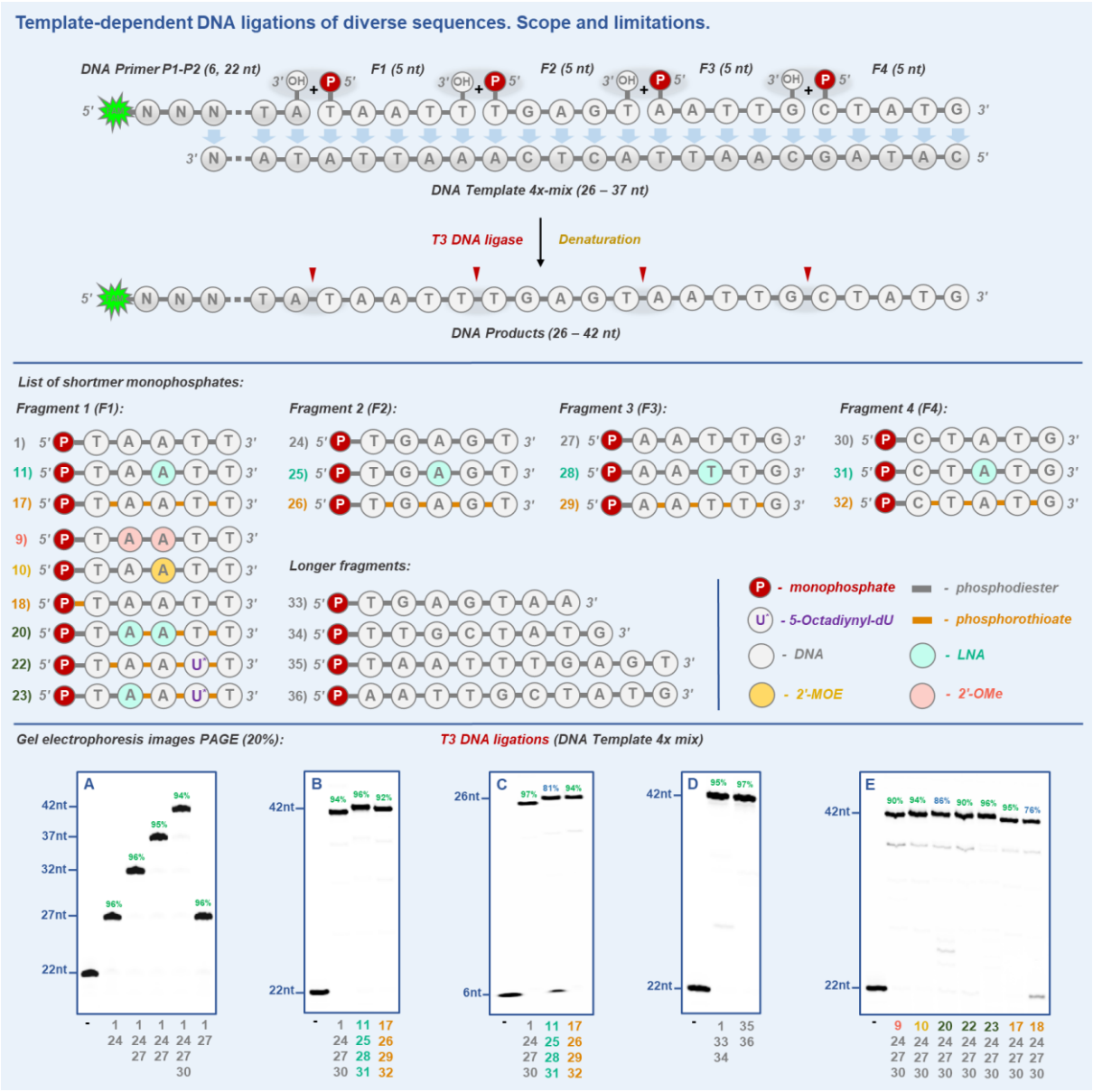

Fig. 3. DNA ligations of diverse sequences. Scope and limitations. Gel electrophoresis images PAGE (20%): (**A**) – Study of stepwise ligation of natural shortmers (5 nt) to DNA primer **P1** (22 nt) on DNA template **4x-mix** (37 nt); (**B**) – Ligation of diverse natural and modified (LNA, phosphorothioate) shortmers (5 nt) to DNA primer **P1** (22 nt); (**C**) – Ligation of diverse natural and modified (LNA, phosphorothioate) shortmers (5 nt) to DNA primer **P2** (6 nt) on DNA template **4x-mixS** (26 nt); (**D**) – Ligation of diverse natural shortmers of different length (5-10 nt); (**E**) – Ligation of diverse natural and variously modified (2'-OMe, 2'-MOE, phosphorothioate, NB) shortmers (5 nt); (**-**) – negative control in the absence of DNA ligase.

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Synthesis of long and highly modified oligonucleotides

Having established a new method for the production of modified DNA oligonucleotides by co-ligating short fragments to a primer on a template, we next set out to evaluate its compatibility with practical applications. In this context, we wanted to assess the possibility of applying this method to the synthesis of ultralong (>100 nt), natural and modified oligonucleotide sequences. To fulfill this goal, we considered two different yet complementary general strategies: 1) ligating multiple diverse shortmer fragments **F1-F18** (5 nt) directly on an ultramer DNA template **TU** (107 nt) produced by phosphoramidite chemistry; 2) ligation of two long fragments (**VL** and **IL**, each of 79 nt) with a shorter DNA splint (**TL**, 31 nt)[46] (Fig. 4A-B).

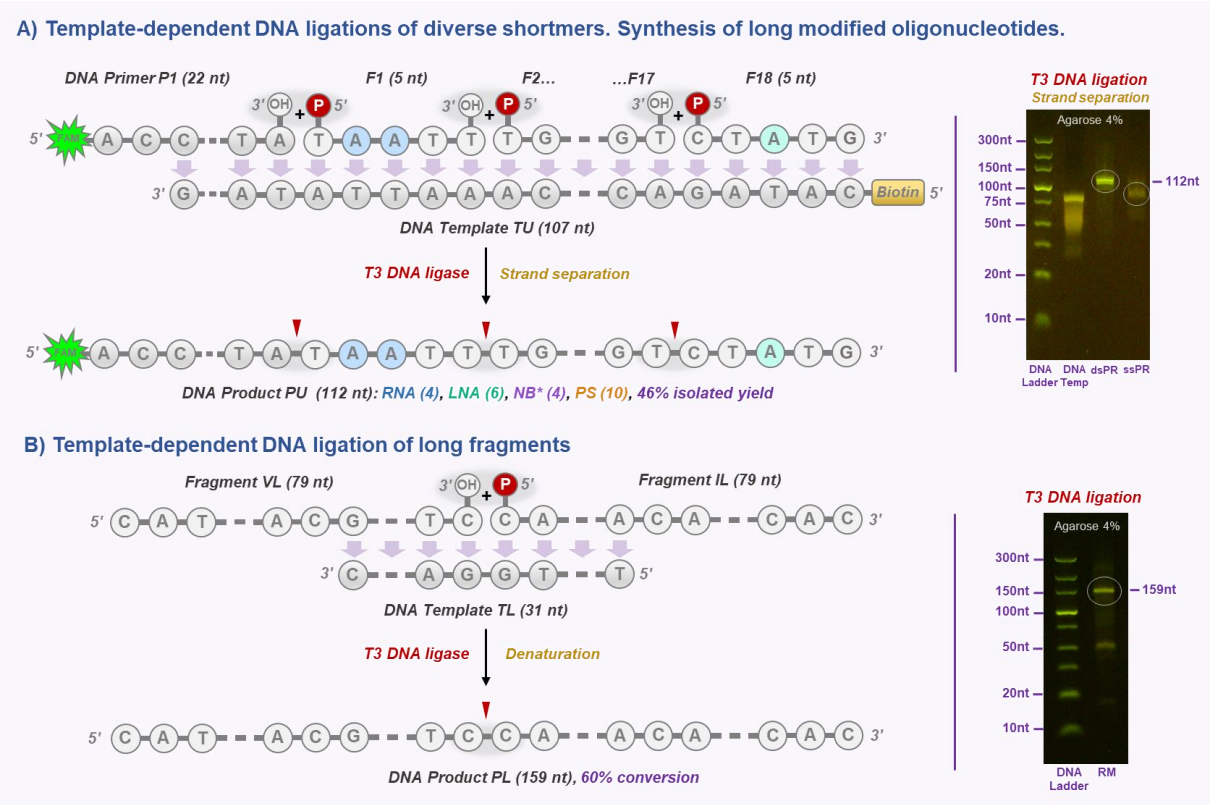

Fig. 4. Synthesis of long and modified oligonucleotides. Gel electrophoresis images Agarose (4%): **(A)** – DNA ligations of multiple diverse shortmer fragments (5 nt) **F1-F18** to DNA primer **P1** on ultramer DNA template **TU** (107 nt). **(B)** – DNA ligation of long fragments **VL-IL** (79 nt) on shorter DNA template **TL** (31 nt).

We started with programmed ligation of multiple unmodified and modified shortmer fragments **F1-F18** (5 nt) on ultramer DNA template **TU** (107 nt). We wanted to demonstrate that our approach is compatible with the diversity of chemically modified shormers as well as to show that the desired product could be recovered using biotinylated template **TU** (Fig. 4A). Using gel electrophoresis (Agarose 4%) we monitored the formation of the product **PU** (dsDNA, 112 nt) before and after the strand separation (ssDNA, 112 nt) that resulted in satisfactory 46%

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isolated yield (see Supplementary Fig. SI4A). The nature of the product was also confirmed by LCMS analysis (see Supplementary Supplementary Table SI6).

Next, we explored the possibility of ligating two long fragments **VL** and **IL** (79 nt each) on short DNA template **TL** (31 nt) leading to the formation of ultramer DNA product **PL** (159 nt) with good 60% conversion (Fig. 4B). Product formation was analyzed by agarose (4%) gel electrophoresis and further confirmed by LCMS analysis (see Supplementary Fig. SI4B and Supplementary Table SI7). This approach could serve as a robust chemoenzymatic alternative for the production of ultramers by assembling two (or several) long fragments previously prepared by phosphoramidite chemistry, in turn leading to higher overall yield and purity. Efficient product isolation could be achieved by either magnetic separation from biotinylated template, denaturing HPLC, electrophoresis, or membrane filtration.

Overall, these results demonstrate the versatility of the DNA ligation strategy and its applicability for the synthesis of long (> 100 nt), natural and modified oligonucleotides, a daunting task for most existing chemical or biocatalytic methods.

Synthesis of therapeutic antisense oligonucleotides

Finally, we set out to demonstrate the compatibility of our chemoenzymatic approach with the preparation of antisense oligonucleotides (ASOs) and their conjugates. As a proof-of-principle, we evaluated the possibility of synthesizing the first FDA-approved antisense drug Fomivirsen (21 nt, all linkages are phosphorothioates), as well as various conjugates and LNA gapmer analogue (Fig. 5A-F).

In this context, our synthetic strategy was based on the simultaneous ligation of four shortmer fragments **F1-F4** on the complementary 5'-biotinylated DNA template **TF-Bio** (21 nt) to isolate the ssDNA Fomivirsen products by magnetic separation on Streptavidin beads. We started the synthesis of Fomivirsen by searching optimal reaction conditions using 5'-FAM-labeled fragment **F1** which enables a facile visualization and quantification of the reaction products by gel electrophoresis. Once again, as demonstrated above, the addition of crowding agents (PEG, DMSO) was essential to improve the initial yield of 30% obtained with standard conditions to 85%. Finally, when the reaction mixtures were supplemented with Mn^{2+} as an additional cofactor, the ligation reaction led to >95% conversion of the expected products (see Supplementary Fig. SI6A). In addition, since all phosphodiester linkages were modified to the corresponding phosphorothioates, we investigated the reactivity of the 5'-monophosphorothioate-containing fragments **F2-F4**. To do so, we carried out a competitive reactivity study between these modified fragments and the corresponding monophosphate analogues (see Supplementary Fig. SI6B). This analysis revealed that even by lowering the reaction temperature to 4°C, conversion of the 5'-monophosphate fragments to products was

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>95% while only 30% for the corresponding 5'-monophosphorothioate shortmers reacted. Nonetheless, even though the reactivity of 5'-monophosphorothioated fragments was lower compared to that of unmodified fragments, we demonstrated that it was still possible to reach high conversions to the expected fully modified oligonucleotide products by comprehensive multi-parametric optimization without requiring the assistance of enzyme engineering.

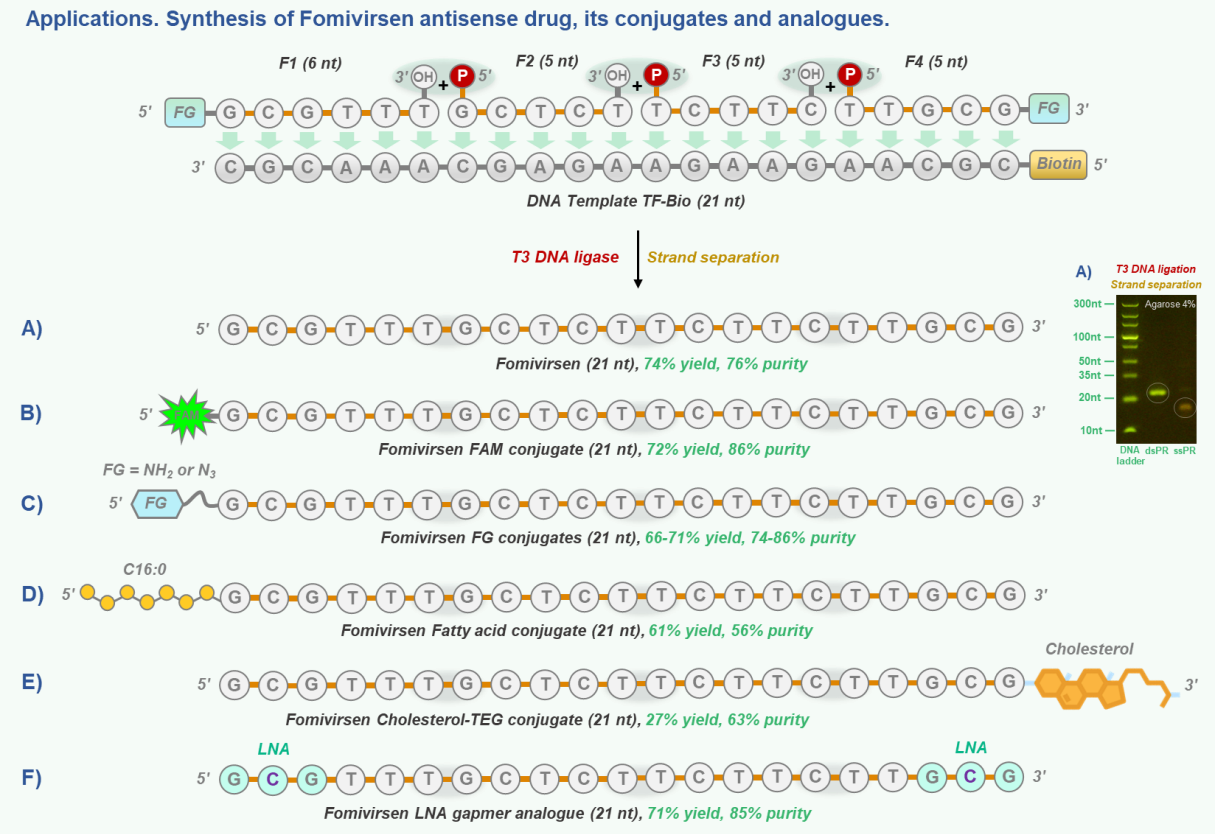

Fig. 5. Synthesis of therapeutic antisense oligonucleotide and its conjugates. (A) – Synthesis of Fomivirsen; **(B)** – Synthesis of Fomivirsen FAM conjugate; **(C)** – Synthesis of Fomivirsen FG (NH_2, N_3) conjugates; **(D)** – Synthesis of Fomivirsen Fatty acid (C16:0) conjugate; **(E)** – Synthesis of Fomivirsen Cholesterol-TEG conjugate; **(F)** – Synthesis of Fomivirsen LNA gapmer analogue.

Consequently, with optimized reaction conditions at hand, we performed the synthesis and isolation of Fomivirsen and its corresponding 5'-FAM conjugate that could be used for imaging and cellular uptake studies (Fig. 5A-B). We then synthetized two Fomivirsen conjugates bearing 5'-terminal reactive amino and azide functional groups that are readily available for post-functionalization *via* amide coupling or click chemistry (Fig. 5C). Additionally, we demonstrated that our ligation approach is also compatible with pre-functionalized shortmers at both 5' and 3' ends. Thus, we prepared two examples of lipid conjugates (with a fatty acid at the 5'- and cholesterol-TEG at the 3'-position) for the improved cellular uptake of

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ASOs (Fig. 5D-E). Finally, we also synthetized the Fomivirsen LNA gapmer analogue to show the compatibility of our ligation approach with the production of gapmers (Fig. 5F).

The isolated yields for the products after magnetic separation were mostly in the 60-70% range with purities around 60-90% as confirmed by LCMS analysis (Supplementary Table SI8). The majority of the impurities stem from a PS to PO conversions which has many origins including small PO impurities (~5%) in starting fragments after phosphoramidite synthesis,[47, 48] conversion during LCMS analysis, and potentially due the ligation reaction conditions.

Alternatively, on the example of Fomivirsen we also demonstrated that our shortmer co-ligation approach is suitable for using longer templates. Therefore, we performed ligation on long natural DNA template **TF-3x** (63 nt) with repetitive sequence leading to 3 equivalents of Fomivirsen with good 77% conversion as detected by gel electrophoresis analysis (Supplementary Fig. SI8A-B). By taking advantage of size difference (63 nt for template *vs*. 21 nt for the product) this approach could be practically used for qualitative product isolation by either denaturing HPLC, electrophoresis, or membrane filtration. It is also worth mentioning that the synthesis of antisense drugs by ligation strategy could be potentially compatible with the use of self-priming templates and restriction endonuclease as demonstrated by the Lovelock group for polymerases.[24]

Discussion

We have developed a general chemoenzymatic approach for the synthesis of natural and modified oligonucleotides based on the assembly of short, 5'-phosphorylated DNA oligomers. In order to evaluate the scope and limitations of this method, we have tested a large variety of chemically modified shortmer monophosphate (5 nt) fragments in template-dependent DNA ligation reactions using commercial T3 DNA ligase. Overall, we have found that the DNA ligation method displays a high tolerance for chemical modifications at any level of the nucleotidic scaffold, and even fully modified shortmer fragments can be incorporated into DNA. Importantly, this method is not only flexible in terms of the nature of chemical modifications but also in terms of size of the sequences that can be achieved since we were capable of producing shorter oligonucleotides similar to that of clinically relevant ASOs and siRNAs, but also larger fragments (> 100 nt) which might be directly amenable to address biological questions such as the three-dimensional structure and dynamics of nucleosomes.[49] Additionally, our ligation method demonstrated great flexibility in terms of the length and sequence combinations of the shortmers, which is an additional important perquisite for the construction of literally any given sequence of interest. This variant synthesis protocol also displays a high atom and enzyme economy as fewer functional groups per nucleotide are involved in the ligation reactions. This is in stark contrast to the classical enzymatic extension reactions where nucleoside triphosphates are used in (often large) excess together with DNA

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polymerases. The use of engineered ligases[50] will certainly represent a necessary future step to further improve our methodology, for instance by increasing the yields of ligation of fragments containing terminal 5'-phosphorothioate shortmers or yet longer (> 150 nt) target sequences.

Overall, we envision that the ligation of short, chemically modified oligonucleotides will improve our capacity at producing short therapeutic oligonucleotides as well as longer systems in a high yielding, easy to implement, and versatile one-pot reaction and represents a valid alternative to exisiting methods.

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Acknowledgments

The authors acknowledge Institut Pasteur, CNRS grant PEPR REV CNRS MOLECULARXIV (M.H.), and Roche for financial support *via* its Technology, Innovation and Science program. Yannick Rondelez (CNRS/ESPCI Laboratory Gulliver) and Anthony Genot (CNRS/University of Tokyo) for fruitful discussions.

Conflicts of interest

There are no conflicts to declare.

Contributions

Conceptualization was done by M.H., K.P., F.S., S.H., and N.S. Methodology was done by N.S. Validation was carried out by N.S. Formal analysis was done by all authors. Investigation was carried out by N.S. and A.S. Resources were provided by K.P., S.H., and M.H. Data were curated by N.S., A.S., and M.H. The original draft was written by N.S. and M.H. Review and editing of the draft were done by all authors. Supervising the project were M.H., S.H., and K.P. Project administration was done by M.H. Funding was acquired by K.P., S.H., and M.H.

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