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Template-dependent DNA ligation for the synthesis of modified oligonucleotides

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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 a 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 ultramers containing diverse modifications. Furthermore, the designed chemoenzymatic approach has great potential for diverse applications in therapeutics and biotechnology.

The intrinsic properties of DNA, particularly the high degree of programmability mediated by the Watson-Crick base pairing, have propelled 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 nanomaterials¹ 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 and antisense oligonucleotides (ASOs)^{6–9}. This large and increasing demand for oligonucleotides needs to be supported by robust, efficient, cost-affordable, and sustainable synthetic methods¹⁰. However, our capacity for reading (i.e., sequencing) still outperforms that of writing (i.e., synthesizing) DNA¹¹. This discrepancy mainly stems from the lack of alternative de novo DNA synthesis methods. Indeed, most synthetic oligonucleotides

are produced by solid-phase oligonucleotide synthesis (SPOS) in which 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}. Nonetheless, despite these attractive features, SPOS is impinged by low sustainability, truncated byproduct formation, and low overall yields, especially for long sequences. Consequently, its application is restricted to rather shorter sequences of modified oligonucleotides^{11,18,19}. Alternatively, oligonucleotides can be produced by biocatalytic approaches^{20–24} mainly based on the polymerization of nucleotides catalyzed by polymerases (Fig. 1C)^{25–36}. While polymerase-based methods do not display size limitation, control of the position, number, and complexity of modified nucleotides is very limited.

Chemoenzymatic strategies based on the convergent fragment assembly using ligation represent an alternative synthetic approach

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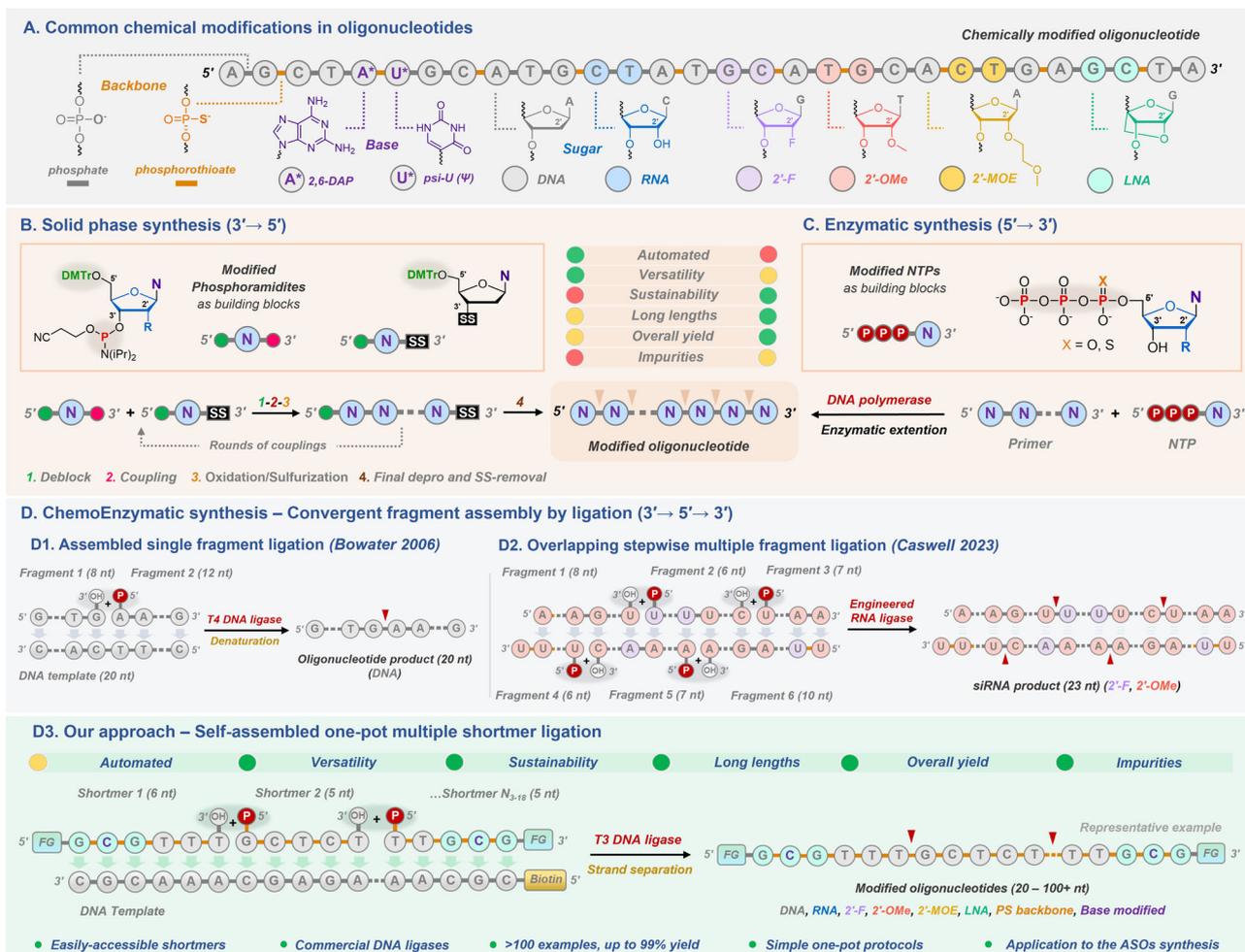


Fig. 1 | Overview of existing synthetic protocols and of the co-polymerization of shortmer fragments. **A** Common chemical modifications in oligonucleotides. **B** Overview of solid phase synthesis. **C** Schematic representation of enzymatic synthesis. **D** Alternative chemoenzymatic synthesis based on convergent fragment

assembly by ligation. **D1** Reported assembled single fragment ligation. **D2** Reported overlapping stepwise multiple fragment ligation. **D3** Our approach is based on self-assembled one-pot multiple-fragment ligation.

that could lift the limitations present in existing methods leading to better versatility and sustainability, higher yields, and overall purity (Fig. 1D). Even though several ligation methods have been reported they are restricted either: (1) by the length of assembled fragments (there is little advantage of using fragments of moderate size (10–20 nt)); (2) by the nature and diversity of chemical modifications accepted by ligases; (3) by the number and order of shortmers that can be assembled, and hence by the length and complexity of modified oligonucleotides that can be produced; (4) by the strict requirement of using engineered ligases; (5) to the combination of above-mentioned factors limiting the process efficiency and versatility (Fig. 1D1, D2)^{37–40}.

Taken together, the main aim of this work was to address these limitations by developing a general, rather than specific, approach using easily accessible shortmers and DNA ligases. Therefore, we present an approach that permits the co-ligation of multiple chemically modified pentamers in a one-pot manner with commercially available ligases and is thus readily accessible to most laboratories (Fig. 1D3).

Our approach represents an 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 selected positions of the nucleotidic scaffold.

Results

Design of the method

Despite impressive catalytic efficiencies⁴¹ of around $10 \mu\text{M}^{-1}\text{s}^{-1}$ (equating to the insertion of up to a few hundred nucleotides per second⁴²), 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^{43–47} and sugar modifications even in short sequences^{38,39,48} which constitute the basis of our approach. Nonetheless, most existing ligation methods rely on using scarcely modified oligonucleotides of moderate size (10–20 nt), and these are already impacted by some of the aforementioned limitations (*vide supra*). Instead, we based our method on the co-ligation of short, 5'-phosphorylated pentanucleotides⁴⁹ containing a broad variety of chemically modified nucleotides. These shortmers are readily produced in high yields and purities by SPOS, and the concomitant templated ligation produces modified oligonucleotides in high yields, overall purity, and without 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

shortmers **1–23** on DNA templates of different lengths (**1x**, **2x**, **4x**) (Supplementary Fig. 2B–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 phosphatase²⁸); 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 (Supplementary Fig. 2B–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 adenylation⁵⁰. Consequently, by varying the above-mentioned parameters, we identified reaction conditions (4 °C reaction temperature, the addition of PEG8000 (20%), DMSO (10%), $MnCl_2$ (1 mM final), 20x less ATP (50 μM 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. 2B–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 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 large-scale analytical reactions (Supplementary Tables 1–4). 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 synthesizing 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 required⁵¹. 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**, and **30** using the 5′-FAM-labeled DNA primer **P1** (Fig. 3E).

In the same manner, we performed the co-ligation of shortmers **12–13** (LNA as first or last nucleotide) and fully RNA and RNA-phosphorothioate shortmers **4b–c** (Fig. 3F). Gratifyingly, all reactions proceeded with high conversions toward the expected full-length products with marginal truncated product formation, suggesting that with a careful design in mixed ligation, even highly modified DNA or RNA, XNA 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 large-scale analytical reactions (Supplementary Table 4).

Having explored the scope and limitations of the method, we next explored different methods for isolation of the single-stranded product stemming from the ligation protocol. Thus, we first tested two methods based on the template digestion using an RNA instead of a DNA template followed by RNase H treatment⁵² and using a 5′-phosphorylated DNA template followed by λ-exonuclease digestion⁵³. Unfortunately, the RNA template was not compatible with the DNA ligase, and the use of a 5′-phosphorylated DNA template resulted in the formation of longer side-products and reduced/absence of λ-exonuclease digestion (Supplementary Fig. 3G). Nevertheless, we then successfully applied the use of a 5′-biotinylated template and magnetic separation using Streptavidin magnetic beads (see the following sections, Supplementary Protocol for magnetic separation, and Supplementary Fig. 3G)⁵⁴.

Synthesis of long and highly modified oligonucleotides

Having established a 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

Template-dependent DNA ligations of diverse sequences. Scope and limitations.

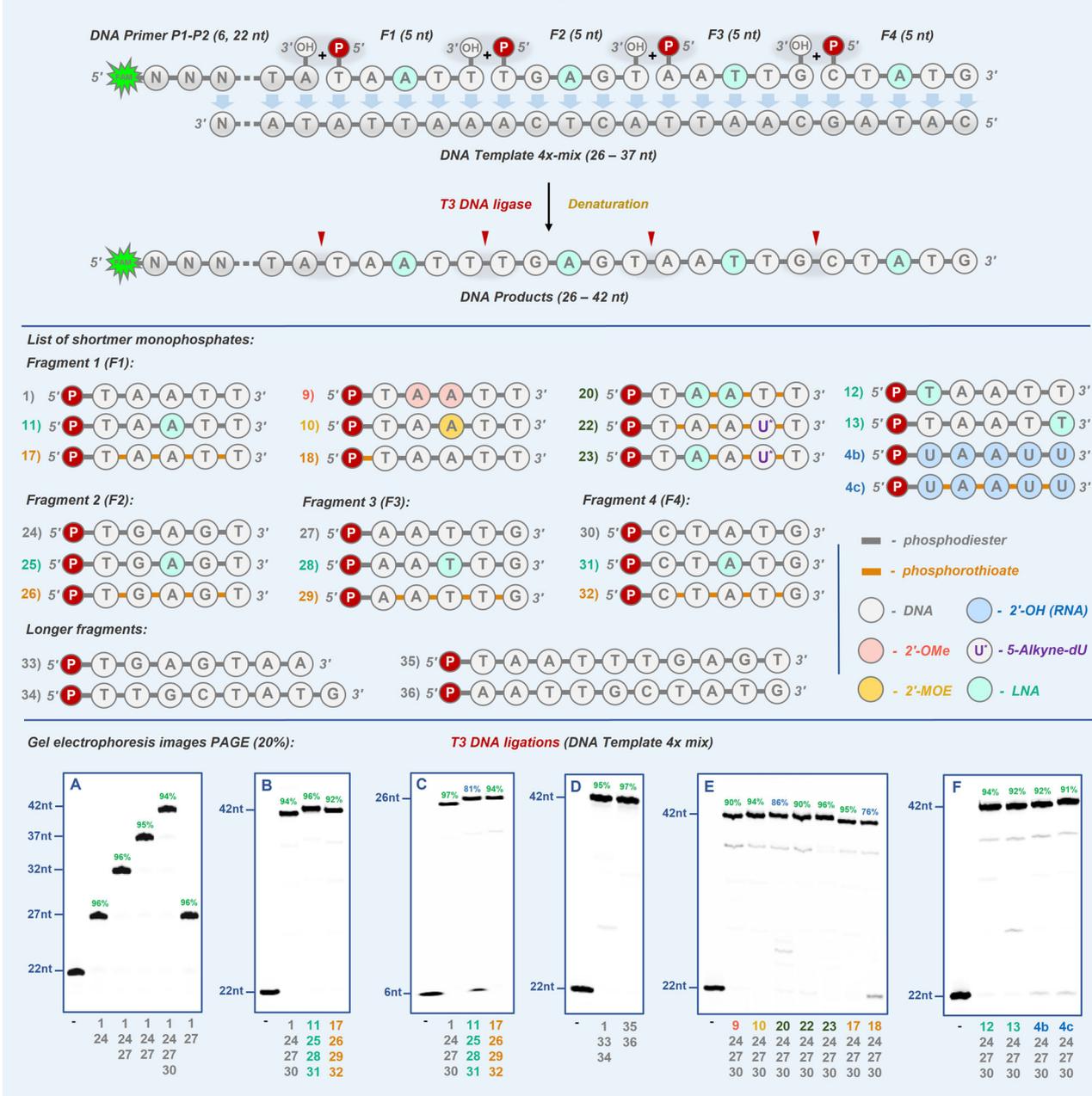


Fig. 3 | DNA ligations of diverse sequences. Scope and limitations. Gel electrophoresis images PAGE (20%): **A** Study of stepwise ligation of natural shortmers (5 nt) to DNA primer **P1** (22 nt) on DNA template **4x-mix** (37 nt). **B** Ligation of diverse natural and modified (LNA, phosphorothioate) 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. **F** Ligation of diverse natural and variously modified (LNA, RNA, RNA-phosphorothioate) shortmers (5 nt); (-) – negative control in the absence of DNA ligase.

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)⁵⁵ (Fig. 4A, B).

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 shortmers 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% isolated yield (Supplementary Fig. 4A). The nature of the product was also confirmed by LCMS analysis (Supplementary Table 6).

At the same time, we wanted to demonstrate that our ligation approach is compatible with longer assembly using the same setup that could lead to the advantageous production of DNA templates (e.g., for ligation, PEX) and ultralong modified sequences (>150 nt, overcoming the limitation of SPOS). Therefore, we ligated two long fragments **VL** and **IL** (79 nt each) on a short DNA template **TL** (31 nt), leading to the formation of ultramer DNA product **PL** (159 nt) with a good 60% conversion (Fig. 4B). Product formation was analyzed by agarose (4%) gel electrophoresis and further confirmed by LCMS

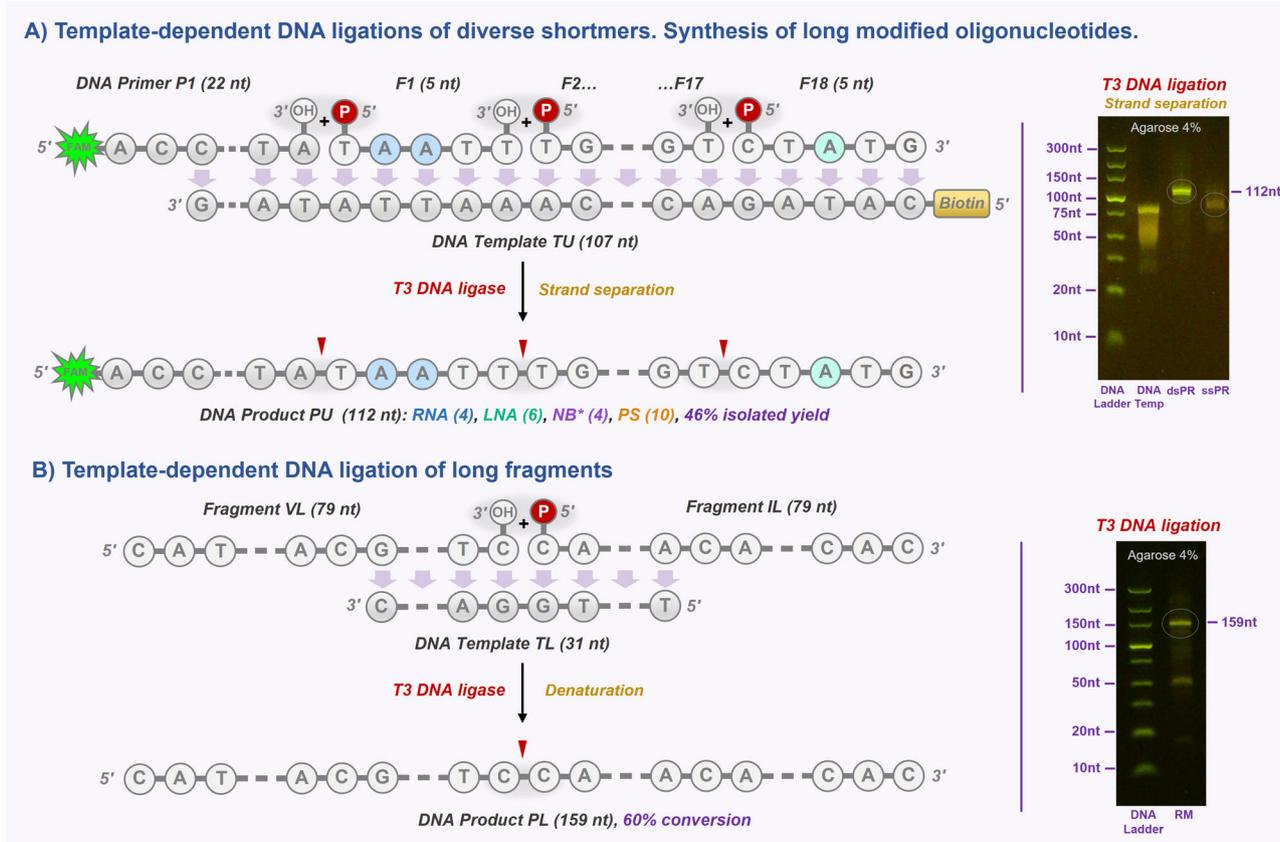


Fig. 4 | Synthesis of long and modified oligonucleotides. Gel electrophoresis images Agarose (4%). **A** DNA ligations of multiple diverse shortmer fragments (5 nt) **F1–F18** to DNA primer **P1** on ultramer DNA template **TU** (107 nt). **B** DNA ligation of long fragments **VL–IL** (79 nt) on shorter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

analysis (Supplementary Fig. 4B and Supplementary Table 7). Efficient product isolation could be achieved by either magnetic separation from biotinylated template⁵⁶, 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. Shortmer approach will be more practical for the production of longmers roughly up to 150 nt in length. Consequently, longer assembly will be more practical to access longer (>150 nt) templates. Moreover, both approaches could be combined towards ultralong modified sequences (e.g., 300–500 nt).

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 analog (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 (Supplementary Fig. 6A). 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 analogs (Supplementary Fig. 6B). This analysis revealed that even by lowering the reaction temperature to 4 °C, conversion of the 5'-monophosphate fragments to products was >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. 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 synthesized 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). In addition, 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 ASOs (Fig. 5D, E). Finally, we also synthesized the Fomivirsen LNA gapmer analog 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

Applications. Synthesis of Fomivirsen antisense drug, its conjugates and analogues.

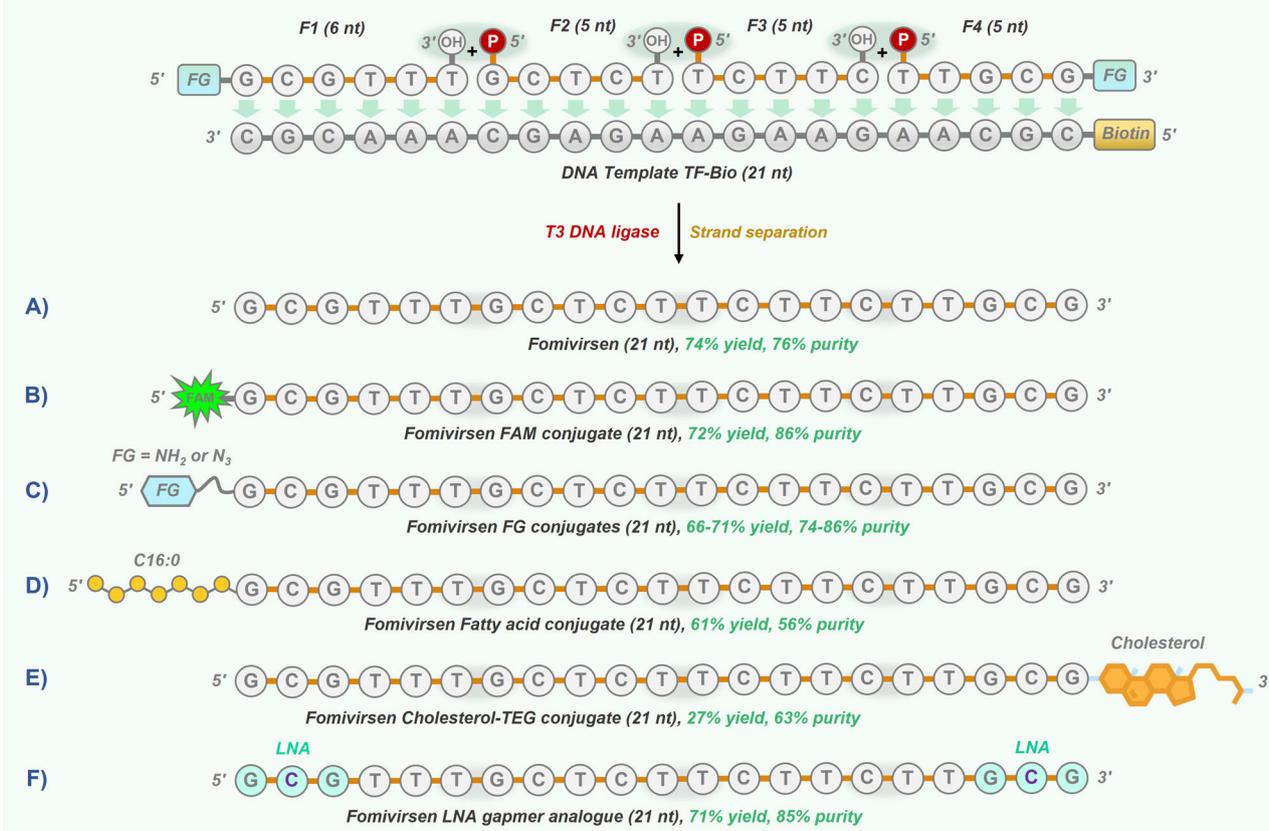


Fig. 5 | Synthesis of therapeutic antisense oligonucleotide and its conjugates. **A** Synthesis of Fomivirsen. **B** Synthesis of Fomivirsen FAM conjugate. **C** Synthesis of Fomivirsen FG (NH₂, N₃) conjugates. **D** Synthesis of Fomivirsen Fatty acid (C16:0)

conjugate. **E** Synthesis of Fomivirsen Cholesterol-TEG conjugate. **F** Synthesis of Fomivirsen LNA gapmer analog.

confirmed by LCMS analysis (Supplementary Table 8). The majority of the impurities stem from a PS to PO conversions which have many origins including small PO impurities (~5%) in starting fragments after phosphoramidite synthesis^{57,58}, conversion during LCMS analysis, and potentially due the ligation reaction conditions.

In addition, using 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. 8A, B). By taking advantage of the size difference (63 nt for template *vs.* 21 nt for the product) this approach could be applied 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 polymerases²⁶.

Discussion

We have developed a general chemoenzymatic approach for the synthesis of natural and modified oligonucleotides based on the assembly of easily accessible short, 5'-phosphorylated DNA oligomers that does not require engineered enzyme or specific equipment and hence is readily available to most laboratories. 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 under thoroughly optimized conditions. Overall, we have found

that the DNA ligation method displays a high tolerance for chemical modifications at all three levels of the nucleotidic scaffold, including the reactive phosphate group, as first and terminal nucleotides are directly involved in ligation. Moreover, even fully modified shortmer fragments can be incorporated into DNA.

Importantly, this method not only demonstrated high flexibility in terms of the nature of chemical modifications but also in terms of the product size that can be achieved, since we were capable not only of producing shorter oligonucleotides similar to that of clinically relevant ASOs but also larger fragments (>100 nt) which might be directly amenable to address biological questions such as the three-dimensional structure and dynamics of nucleosomes⁵⁹. It is also worthwhile mentioning that longer (>100 nt) modified sequences are difficult to obtain by standard phosphoramidite-based methods, especially with high overall yield and purity. In addition, our ligation method demonstrates great flexibility in terms of the length and sequence combinations of the shortmers, which is an additional important prerequisite for the construction of a wide range of sequences of interest (shortmers from the portfolio can be easily manipulated).

This synthetic methodology also displays a high atom and enzyme economy as fewer functional groups per nucleotide are involved in the ligation reactions. This atom and cost economy can yet be further improved in the future by using optimized automated SPOS or employing manual solid-phase synthesis of shortmers, which requires fewer equivalents of phosphoramidites and shunts the need for some synthetic steps leading to better overall sustainability^{18,33}. The enzyme economy and atom economy are also in stark contrast to the classical enzymatic extension reactions in which nucleoside triphosphates are used in (often large) excess together with DNA polymerases, whereas

in our approach, every fifth nucleotide is ligated, and we use nearly stoichiometric amounts of pentamers. Importantly, extending DNA primers with five nucleotide long blocks rather than single nucleotides (triphosphates or phosphoramidites) also facilitates purification efforts since truncated side-products, if they arise, will be easier to remove. However, a more comprehensive technical study will be crucial in the future to compare the sustainability of our ligase-based method, traditional SPOS, and polymerase-catalyzed enzymatic synthesis and to identify parameters that can be improved.

It is important to mention that this approach does not represent a substitute for SPOS but rather a complementary alternative method. Also, as for most methods, there are some limitations to this approach that will need to be improved in the future. For instance, we have not evaluated the possibility of producing long (>100 nt) fully-modified oligonucleotides, and some modifications (e.g., 2'-MOE) are only moderately well-tolerated. In this context, the use of alternative⁶⁰ or engineered ligases⁶¹ will certainly represent a necessary future step to further improve our methodology. For instance, by further increasing the reactivity and flexibility while ligating the fragments containing terminal 5'-phosphorothioate shortmers, fully or highly modified shortmer sequences towards short or yet longer (>150 nt) target sequences. Another necessary future step to further improve the practical aspect of the method lies in template recovery and recycling. As we demonstrated, longer repetitive templates can be efficiently used and we are currently working on that in our laboratory as well as on the use of self-priming templates. Lastly, engineered ligases will be paramount for the production of fully modified oligonucleotides containing more complex, XNA-like modifications such as Hexitol Nucleic Acids (HNA), Peptide Nucleic Acids (PNA), or Phosphorodiamidate Morpholino Oligomers (PMO).

Overall, we envision that the ligation of short, chemically modified oligonucleotides will improve our capacity to produce short therapeutic and longer oligonucleotides in a high yield and purity, easy-to-implement, and versatile one-pot approach that represents a valid alternative to existing methods.

Methods

Chemicals, oligonucleotides, and ligases

All modified and unmodified oligonucleotides including primers and templates were purchased from Integrated DNA Technologies and Microsynth. All DNA ligases (T4, Hi-T4TM, T3 and T7) were purchased from New England BioLabs.

Protocols for T3 DNA ligations of repetitive sequences

Optimized conditions for T3 DNA ligations on DNA templates 1x, 2x, 4x, 14x (analytical scale, 10 µL). DNA Primer **P1** 10 µM (1.0 µL), *DNA ligase buffer^{OPTI}* 10X (1.0 µL), PEG8000-50% (4.0 µL), DMSO (1.0 µL), T3 DNA ligase (0.5 µL). For DNA template **1x** 10 µM (1.5 µL), DNA shortmers **1–23** 10 µM (1.5 µL) were used. For DNA template **2x** 10 µM (1.5 µL), DNA shortmers **1–23** 20 µM (1.5 µL) were used. For DNA template **4x** 10 µM (1.5 µL), DNA shortmers **1–23** 40 µM (1.5 µL) were used. For DNA template **14x** 10 µM (1.5 µL), DNA shortmers **1, 4, 6, 14, 17, 19** 100 µM (2.0 µL) were used. *DNA ligase buffer^{OPTI} IX composition:* 66 mM Tris-HCl, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM DTT, 50 µM ATP, 7.5% polyethylene glycol (PEG8000), pH 7.6 at 25. Reaction mixtures were incubated at 4 °C for 12 h. Reaction mixtures were then subjected to denaturing gel electrophoresis (PAGE 20%), followed by phosphorimaging and quantification analysis using Typhoon phosphorimager with the ImageQuantTL software (v.10.2) from CytivaTM.

Optimized conditions for T3 DNA ligations on DNA templates 1x, 2x, 4x, 14x (large scale analytical, 100 µL). DNA Primer **P1** 100 µM (5.0 µL), *DNA ligase buffer^{OPTI}* 10X (10.0 µL), PEG8000-50% (40.0 µL), DMSO (10.0 µL), T3 DNA ligase (10.0 µL). For DNA template **1x** 100 µM (7.5 µL), DNA shortmers **1–23** 100 µM (7.5 µL) and water (10.0 µL) were

used. For DNA template **2x** 100 µM (7.5 µL), DNA shortmers **1–23** 100 µM (15.0 µL) and water (2.5 µL) were used. For DNA template **4x** 100 µM (7.5 µL), DNA shortmers **1–23** 200 µM (15.0 µL) and water (2.5 µL) were used. For DNA template **14x** 100 µM (7.5 µL), DNA shortmers **1, 4, 6, 14, 17, 19** 700 µM (15.0 µL) and water (2.5 µL) were used. *DNA ligase buffer^{OPTI} IX composition:* 66 mM Tris-HCl, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM DTT, 50 µM ATP, 7.5% polyethylene glycol (PEG8000), pH 7.6 at 25. Reaction mixtures were incubated at 4 °C for 12 h. The large-scale analytical reaction mixture was diluted with water to 500 µL final volume. Then washed and concentrated using an Amicon[®] Ultra centrifugal filter (3 kDa). Resulted DNA concentrate was additionally precipitated with ethanol, dried (typically 80–90% overall recovery), and used in LCMS analysis.

Protocols for T3 DNA ligations of diverse sequences

Optimized reaction conditions for T3 DNA ligation on DNA template 4x-mix and 4x-mixS (analytical scale, 10 µL). DNA Primer **P1/P2** 10 µM (1.0 µL), DNA template **4x-mix/4x-mixS** 10 µM (1.5 µL), *DNA ligase buffer^{OPTI}* 10X (1.0 µL), PEG8000-50% (4.0 µL), DMSO (0.5 µL), T3 DNA ligase (0.5 µL), the mix of DNA shortmers **1, 24, 27, 30** 30 µM (0.5 µL each), or shortmers **11, 25, 28, 31** 30 µM (0.5 µL each), or shortmers **17, 26, 29, 32** 30 µM (0.5 µL each), or shortmers **1, 33, 34** 30 µM (0.5 µL each), or shortmers **35, 36** 15 µM (1.0 µL each), or shortmers (**9** or **10, 17, 18, 20, 22, 23**), **24, 27, 30** 30 µM (0.5 µL each). *DNA ligase buffer^{OPTI} IX composition:* 66 mM Tris-HCl, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM DTT, 50 µM ATP, 7.5% polyethylene glycol (PEG8000), pH 7.6 at 25. Reaction mixtures were incubated at 4 °C for 12 h. Reaction mixtures were then subjected to denaturing gel electrophoresis (PAGE 20%), followed by phosphorimaging and quantification analysis using Typhoon phosphorimager with the ImageQuantTL software (v.10.2) by CytivaTM.

Optimized reaction conditions for T3 DNA ligation on DNA template 4x-mix and 4x-mixS (large-scale analytical, 115 µL). DNA Primer **P1/P2** 100 µM (5.0 µL), DNA template **4x-mix/4x-mixS** 100 µM (7.5 µL), *DNA ligase buffer^{OPTI}* 10X (11.5 µL), PEG8000-50% (40.0 µL), DMSO (10.0 µL), T3 DNA ligase (11.0 µL), mix of DNA shortmers **1, 24, 27, 30** 100 µM (7.5 µL each), or shortmers **11, 25, 28, 31** 100 µM (7.5 µL each), or shortmers **17, 26, 29, 32** 100 µM (7.5 µL each), or shortmers **1, 33, 34** 100 µM (7.5 µL each), or shortmers **35, 36** 100 µM (7.5 µL each), or shortmers (**9** or **10, 17, 18, 20, 22, 23**), **24, 27, 30** 100 µM (7.5 µL each). *DNA ligase buffer^{OPTI} IX composition:* 66 mM Tris-HCl, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM DTT, 50 µM ATP, 7.5% polyethylene glycol (PEG8000), pH 7.6 at 25. Reaction mixtures were incubated at 4 °C for 12 h. The large-scale analytical reaction mixture was diluted with water to 500 µL final volume. Then washed and concentrated using an Amicon[®] Ultra centrifugal filter (3 kDa). Resulted DNA concentrate was additionally precipitated with ethanol, dried (typically 80–90% overall recovery), and used in LCMS analysis.

Synthesis of long and modified oligonucleotides

T3 DNA ligation of multiple shortmers for ultramer PU synthesis

Reaction conditions for T3 DNA ligation on DNA template TU (large-scale analytical, 200 µL). DNA Primer **P1** 100 µM (5.0 µL), DNA template **TU** 100 µM (6.0 µL), Mix of DNA shortmers: **4** 100 µM (15.0 µL), **25** 100 µM (15.0 µL), **28** 100 µM (7.5 µL), **31** 100 µM (15.0 µL), **37** 100 µM (15.0 µL), **38** 100 µM (7.5 µL), **39** 100 µM (7.5 µL), **40** 100 µM (7.5 µL), **41** 100 µM (15.0 µL), **42** 100 µM (7.5 µL), **43** 100 µM (7.5 µL), **44** 100 µM (7.5 µL), **45** 100 µM (7.5 µL). *StickTogetherTM DNA ligase buffer* 10X (20.0 µL), water (19.0 µL), T3 DNA ligase (15.0 µL). *StickTogetherTM DNA ligase buffer IX composition:* 66 mM Tris-HCl, 10 mM MgCl₂, 1 mM DTT, 1 mM ATP, 7.5% polyethylene glycol (PEG8000), pH 7.6 at 25 °C. Reaction mixture was incubated at 4 °C for 12 h.

Product PU isolation by magnetic separation. The reaction mixture obtained with biotinylated template **TU** was diluted with water to

500 μL final volume. Then washed and concentrated using an Amicon® Ultra centrifugal filter (10 kDa) (> 90% overall recovery). The reaction mixture was then subjected to magnetic separation using the general protocol (*vide infra*).

Protocol for magnetic separation. The T3 DNA ligation reaction mixture (~ 500 pmol of product) was subjected to magnetic separation from 5'-biotinylated template **TU** (107 nt) using streptavidin-coated magnetic beads (SMB).

1. Prior to the magnetic separation, the ligation mixture was washed and concentrated using an Amicon® (10 kDa) centrifugal filter, then distributed in three Eppendorf tubes ($3 \times 50 \mu\text{L}$).
2. Streptavidin magnetic beads (SMB) ($3 \times 150 \mu\text{L}$) were pre-washed $3 \times 200 \mu\text{L}$ with binding buffer TEN100 (10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5).
3. Next, the ligation mixture ($3 \times 50 \mu\text{L}$) was combined with pre-washed (SMB) ($3 \times 200 \mu\text{L}$) and mixed on a rotating wheel (speed 20) at room temperature for one hour.
4. Then, (SMB) was collected on the magnet using a magnetic separation rack (S1506S, NEB), then washed with wash buffer TEN1000 ($2 \times 300 \mu\text{L}$) (10 mM Tris, 1 mM EDTA, 1 M NaCl, pH 7.5) and water ($2 \times 300 \mu\text{L}$).
5. Next, (SMB) were re-suspended with water ($3 \times 100 \mu\text{L}$), and NaOH 1 M ($3 \times 10 \mu\text{L}$) was added for denaturation.
6. Then (SMB) were rapidly collected on the magnet, and the aqueous solutions with ssDNA product were combined and neutralized by dropwise addition of HCl 100 mM.
7. Finally, the aqueous solution with ssDNA product was washed and concentrated using an Amicon® (10 kDa) centrifugal filter for further analysis.

The resulting ultramer product **PU** (ssDNA, 112 nt) was verified by gel electrophoresis (Agarose 4%) (Supplementary Fig. 4A). The concentration was measured by NanoDrop to estimate the yield (230 pmol, 46%) and the purity was verified by LCMS analysis (Supplementary Table 6).

T3 DNA ligation of long sequences for ultramer PL synthesis

Reaction conditions for T3 DNA ligation on DNA Template TL (large-scale analytical, 100 μL). Fragment **VL** 100 μM (5.0 μL), DNA template **TL** 100 μM (5.0 μL), Fragment **IL** 100 μM (7.5 μL), *StickTogether*TM DNA ligase buffer 2X (50.0 μL), water (22.5 μL), T3 DNA ligase (10.0 μL). The reaction mixture was incubated at 16 °C over 6 h. *StickTogether*TM DNA ligase buffer 1X composition: 66 mM Tris-HCl, 10 mM MgCl₂, 1 mM DTT, 1 mM ATP, 7.5% polyethylene glycol (PEG6000), pH 7.6 at 25 °C. The reaction mixture was incubated at 16 °C for 6 h. The large-scale analytical reaction mixture was diluted with water to 500 μL final volume. Then washed and concentrated using an Amicon® Ultra centrifugal filter (10 kDa). The resulting DNA concentrate was additionally precipitated with ethanol and dried (90% overall recovery). The concentration was measured by UV spectroscopy on a NanoDrop, and the purity was verified by LCMS analysis (Supplementary Table 7).

Synthesis of Fomivirsen antisense drug, its analogs and conjugates

Synthesis on biotinylated template TF-Bio followed by magnetic separation

A) Synthesis of Fomivirsen (large-scale analytical, 100 μL). Shortmer **47** 100 μM (5.0 μL), DNA template **TF-Bio** 100 μM (5.0 μL), Shortmers **48, 49, 50** 100 μM (7.0 μL each), DNA ligase buffer^{OPTI} 10X (10.0 μL), PEG8000-50% (40.0 μL), DMSO (10.0 μL), T3 DNA ligase (10.0 μL). The reaction mixture was incubated at 4 °C for 24 h.

B) Synthesis of Fomivirsen FAM conjugate (large scale analytical, 100 μL). Shortmer **46** 100 μM (5.0 μL), DNA template **TF-Bio** 100 μM (5.0 μL), Shortmers **48, 49, 50** 100 μM (7.0 μL each), DNA ligase buffer^{OPTI} 10X (10.0 μL), PEG8000-50% (40.0 μL), DMSO (10.0 μL), T3 DNA ligase (10.0 μL). Reaction mixtures were incubated at 4 °C for 24 h.

C) Synthesis of Fomivirsen Amino/Azide FG conjugates (large-scale analytical, 100 μL). Shortmer **51/52** 100 μM (5.0 μL), DNA template **TF-Bio** 100 μM (5.0 μL), Shortmers **48, 49, 50** 100 μM (7.0 μL each), DNA ligase buffer^{OPTI} 10X (10.0 μL), PEG8000-50% (40.0 μL), DMSO (10.0 μL), T3 DNA ligase (10.0 μL). Reaction mixtures were incubated at 4 °C for 24 h.

D) Synthesis of Fomivirsen Fatty acid conjugate (large-scale analytical, 100 μL). Shortmer **53** 100 μM (5.0 μL), DNA template **TF-Bio** 100 μM (5.0 μL), Shortmers **48, 49, 50** 100 μM (7.0 μL each), DNA ligase buffer^{OPTI} 10X (10.0 μL), PEG8000-50% (40.0 μL), DMSO (10.0 μL), T3 DNA ligase (10.0 μL). Reaction mixtures were incubated at 4 °C for 24 h.

E) Synthesis of Fomivirsen Cholesterol-TEG conjugate (large-scale analytical, 100 μL). Shortmer **47** 100 μM (5.0 μL), DNA template **TF-Bio** 100 μM (5.0 μL), Shortmers **48, 49, 54** 100 μM (7.0 μL each), DNA ligase buffer^{OPTI} 10X (10.0 μL), PEG8000-50% (40.0 μL), DMSO (10.0 μL), T3 DNA ligase (10.0 μL). Reaction mixtures were incubated at 4 °C for 24 h.

F) Synthesis of Fomivirsen LNA gapmer (large-scale analytical, 100 μL). Shortmer **55** 100 μM (5.0 μL), DNA template **TF-Bio** 100 μM (5.0 μL), Shortmers **48, 49, 56** 100 μM (7.0 μL each), DNA ligase buffer^{OPTI} 10X (10.0 μL), PEG8000-50% (40.0 μL), DMSO (10.0 μL), T3 DNA ligase (10.0 μL). Reaction mixtures were incubated at 4 °C for 24 h.

Fomivirsen products isolation by magnetic separation. Large-scale analytical reaction mixture using a biotinylated template was diluted with water to 500 μL final volume. Then washed and concentrated using an Amicon® Ultra centrifugal filter (3 kDa) (> 90% overall recovery). The reaction mixture was then subjected to magnetic separation using the general protocol (see below).

General protocol for magnetic separation of Fomivirsen and analogs. The T3 DNA ligation reaction mixture of Fomivirsen or its analogs (~ 500 pmol of product) was subjected to magnetic separation from 5'-biotinylated template **TF-Bio** (21 nt) using streptavidin magnetic beads (SMB).

1. Prior to magnetic separation, the ligation mixture was washed and concentrated using an Amicon® (3 kDa) centrifugal filter, then distributed in three Eppendorf tubes ($3 \times 50 \mu\text{L}$).
2. Streptavidin magnetic beads (SMB) ($3 \times 150 \mu\text{L}$) were pre-washed with binding buffer TEN100 ($3 \times 200 \mu\text{L}$) (10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 7.5).
3. Next, the ligation mixture ($3 \times 50 \mu\text{L}$) was combined with pre-washed (SMB) ($3 \times 200 \mu\text{L}$) and mixed on a rotating wheel (speed 20) at room temperature for one hour.
4. Then (SMB) was collected on the magnet using a magnetic separation rack (S1506S, NEB), then washed with wash buffer TEN1000 ($2 \times 300 \mu\text{L}$) (10 mM Tris, 1 mM EDTA, 1 M NaCl, pH 7.5) and water ($2 \times 300 \mu\text{L}$).
5. Next, (SMB) were re-suspended with water ($3 \times 100 \mu\text{L}$), and NaOH 1 M ($3 \times 10 \mu\text{L}$) was added for denaturation.
6. Then, (SMB) was rapidly collected on the magnet, and the aqueous solutions with the ssDNA product were combined and neutralized by dropwise addition of 100 mM of HCl.

7. Finally, the aqueous solution with ssDNA product was washed and concentrated using an Amicon® (3 kDa) centrifugal filter for further analyses.

The nature of the resulting Fomivirsen products (ssDNA, 21 nt) was verified by gel electrophoresis (Agarose 4%) (Supplementary Fig. 7). The concentration was measured by NanoDrop to estimate the yield, and the purity was verified by LCMS analysis (Supplementary Table 8).

Statistics and reproducibility. All analytical scale experiments were independently repeated at least three times before scaling up. For experiments where representative gel electrophoresis images are shown (both in the manuscript and supplementary information files), the results were consistent across three independent replicates.

LCMS analysis of enzymatic reactions

LCMS settings and method. Chromatographic separations for LC-MS experiments were performed on a Thermo Scientific™ Vanquish™ Flex Binary UHPLC system (Thermo Fisher Scientific, Reinach, Switzerland).

The column used for all separations was a Waters Aquity Premier BEH C18 Peptide 2.1*50 mm 1.7 μm 300 Å (Waters (CH) AG, Baden-Dättwil, Switzerland). The solvents were A: 15 mM Amylamine (ALDRICH, W424201 (inhouse re-distilled), CAS 110-58-7, SIGMA-ALDRICH CHEMIE GMBH (CH), Buchs Switzerland) and 50 mM 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, ACROS ORGANICS, ACRI4754 (99.5+%, PURE), CAS 920-66-1, ThermoFisher Scientific, Reinach Switzerland) in water (Milli-Q® IQ 7000, Millipore, Merck & Cie, Schaffhausen, Switzerland) and B: methanol/acetonitrile (9/1;v/v) both gradient grade. Both solvents contained 1 μM Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), SIGMA, E5134-100G (99.0–101.0%, titration), CAS 6381-92-6, SIGMA-ALDRICH CHEMIE GMBH (CH), Buchs Switzerland, helping to suppress metal adducts forming in the mass spectrometer. A flow rate of 400 μL min⁻¹ was applied, and the column compartment was held at 80 °C. The gradient system was A vs B, starting at 5%B with a rise to 20%B within 3 min and reaching 35%B at 25.5 min, followed by a short step to 100% B before coming back to the initial 5% B for reinjection.

For mass spectrometric data acquisition, a Thermo Scientific™ Fusion Lumos™ Hybrid Quadrupole-Orbitrap mass spectrometer equipped with a heated electrospray ionization-II (HESI-II) probe in a standard Thermo Scientific™ Ion Max™ ion source (Thermo Fisher Scientific, San José, CA, USA) was used. Data acquisition was performed with Thermo Scientific™ Xcalibur 4.5. HR LC-MS measurements were performed under Thermo Scientific™ Xcalibur™ Orbitrap Fusion Lumos Tune Application 3.5. The mass range was 600–2000 Da at a resolution of 120 K. In addition, the full DAD but also a 260 nm UV trace were acquired.

MS raw files were exported to ThermoFisher Scientific BioPharma Finder (BPF) 5.1 software and analyzed using the ReSpect algorithm via the sliding window deconvolution feature either against the full structure of the compounds or just against the molecular mass. All data evaluation was done based on the Thermo Scientific BioPharma Finder User Guide Software Version 5.1, XCALI-98492 Revision A, July 2022.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The Supplementary Information provides experimental procedures and characterization data. Correspondence and requests for materials should be addressed to M.H.

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Author contributions

Conceptualization was done by M.H., K.P., F.S., S.H., S.B., and N.S. Methodology was done by M.H. and N.S. Validation was carried out by N.S. and M.H. Formal analysis was done by all authors. The 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 was 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.

Competing interests

The authors declare no competing interests.

Additional information

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