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Towards the enzymatic synthesis of oligonucleotides

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By early 2020, ten oligonucleotide drugs had received US FDA regulatory approval; with many more in the pipeline, it's only fair to say that this drug class is becoming big business

Acting at the interface between genes and proteins, therapeutic oligonucleotides are a class of drug based on the structure of DNA and RNA. By selectively binding genetic material such as RNA, they influence the expression of targeted proteins to elicit a beneficial therapeutic effect.

Oligonucleotides based on natural building blocks, whether based on DNA or RNA, are rapidly broken down inside cells by enzymes.[1] This has driven the need for chemical innovation to modify and stabilise these structures, which are typically oligomers comprising 20–30 nucleotide residues.

Some of these products are single stranded whereas others are double stranded. The synthesis of such molecules, combining natural and unnatural residues, has therefore presented many challenges during the discovery and development process, particularly when scaling-up oligonucleotide synthesis.

In addition to metabolic stability, the delivery of large and unwieldy oligonucleotides to their required site of action is also challenging, necessitating further chemical modifications and tagging strategies.[2] This article discusses how enzymatic approaches can contribute to better and cheaper methods for oligonucleotide synthesis.

The chemistry of oligonucleotide synthesis

DNA or RNA consists of deoxyribose or ribose sugar units — with their characteristic bases (A, C, G, T or U) — linked together by phosphodiester bonds.

Traditional oligonucleotide synthesis uses phosphoramidite chemistry and comprises a repeating sequence of four reactions (deprotection, coupling, capping and oxidation) with single

nucleotides added sequentially (Figure 1).

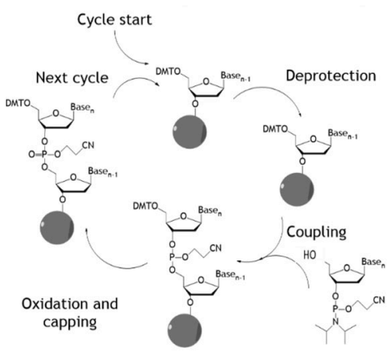

Figure 1: Traditional oligonucleotide synthesis

This is often performed in a solid phase mode wherein the oligonucleotide is extended via this cycle from a solid support. This chemistry has been adapted in numerous ways to allow unnatural monomer units to be incorporated into the oligonucleotide chain.

Various chemical approaches have been adopted to improve both stability and biological performance … and three exemplars (although there are many more) are shown in Figure 2 for derivatives of RNA.

One of these approaches uses a modified phosphodiester backbone during which sulphur is introduced to replace oxygen in the phosphodiester bonds.

A second approach introduces different chemical functionality to the ribose ring, usually in the 2′ position, with methoxy, methoxyethyl and fluoro being the groups most frequently used. A third approach "locks" the sugar ring conformation through the formation of bicyclic structures.

All of these can slow down the rate of metabolic breakdown of oligonucleotides to an acceptable level for effective therapeutic action.

As an oligonucleotide approaches market launch, there is a requirement to scale-up and synthesise kilo to tonne quantities of these building blocks and link them together in the correct sequence.

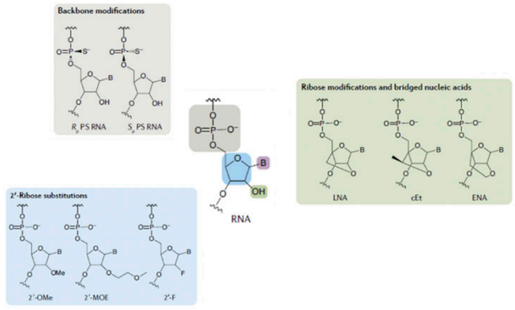

Figure 2: Examples of modification to facilitate oligo stability

However, large-scale oligonucleotide chemistry can be problematic. For example, the requirement for complex chromatography and its automated hardware can add significant cost to this active ingredient of the drug product.

Perhaps worse, the chemistry is linear and sequential, resulting in a geometric decrease in yield as the chain gets longer.

For example, if the yield for each nucleotide-linking phosphoramidite cycle was 95%, which would be very respectable for a four-step cycle, then the overall yield for a 20-mer product is only 36%. Impurity formation and removal is also often a confounding issue for the process. There is a need, therefore, for new approaches to this chemistry.

Biocatalytic synthesis is around the corner

Pharmaceutical synthesis has been transformed by biocatalysis in recent years. When enzymes have been introduced, routes have been shortened, costs lowered and processes generally "greened up" with spectacular results in many cases.

Almac Sciences has been at the forefront of this revolution in sustainable synthesis, tapping successfully into the transformative changes occurring in molecular biology and resulting in low-cost enzymes that can be confidently engineered for maximum performance.[3–5]

A strong oligonucleotide synthesis technology base is now available that includes the ability to produce panels of enzymes for screening, a deep understanding of biotransformation development, process optimisation and biocatalyst engineering at the molecular level.

From these foundations, biocatalysis is now starting to make in-roads into oligonucleotide synthesis with developments and concepts highlighted herein.

Addition of single nucleotides to form blockmers

Blockmers are loosely defined as very short single-stranded oligonucleotides of 4–8 residues that form the basis for the enzymatic synthesis of larger oligonucleotides. In the first example illustrated here, RNA ligase was used to form a series of RNA-based blockmers of four residues by adding a single nucleotide to a trimer (designated ABC).

Almac has applied state-of-the-art synthetic biology design to this process by creating panels of diverse RNA selectAZyme ligases for reaction screening.

The reactions in Figure 3 were achieved by screening the selectAZyme RNA ligase panel for residue X coupling (phosphate protected at the 3′ and 5′ positins), with some enzymes showing good activity for a given substrate. As will become apparent, access to enzyme screening panels is critical to identify good activity and is a recurring theme.

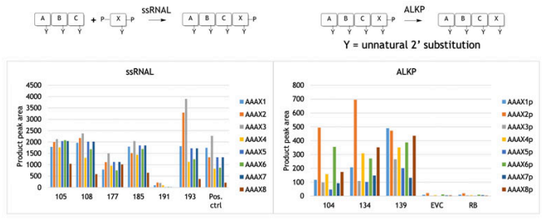

Figure 3: Nucleoside addition to blockmers using selectAZyme ligases

Figure 3 also shows the use of selectAZyme alkaline phosphatase, an enzyme that cleaves the phosphate group off the reaction product at the 3′ position, readying it for a further round of synthesis and chain extension.

This too showed that screening a panel of selectAZyme phosphatases allowed good activity to be identified for the substrates. Once the required blockmers have been assembled, larger strand construction can begin.

Building single stranded RNA oligonucleotides from blockmers

The use of enzymes to construct single strands of RNA by joining blockmers of RNA together was first described in the 1970s.[6,7]

This approach, which does not rely on a template to guide the addition of incoming substrates, is catalysed by the RNA ligase family of enzymes (as above) and linking together fragments based on combinations of natural and unnatural ribose-based nucleotides. This concept is shown in Figure 4, for example, whereby two unnatural fragments (blockmers) were joined together.

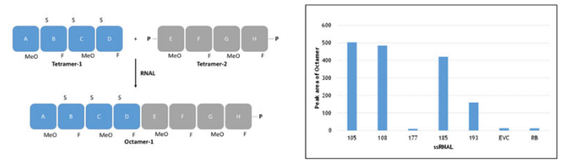

Figure 4: Blockmer to blockmer assembly (non-templated method) using selectAZyme ligases

In this example, the ribose residues both contained a substitution at the 2′ position (unnatural equivalent groups) and were linked together by phosphorothioate esters; the two strategies combined to stabilise the oligonucleotides against degradation.

To develop this successfully, it was critical to have access to a panel of RNA ligases for screening; some enzymes were not active whereas others showed good activity, as can be seen in the insert in Figure 4 (EVC is the empty vector control; RB is reaction buffer only).

Enzyme engineering for ligases

Almac's approach to enzyme engineering involves using its in-house INSIGHT platform, which allows rational enzyme design from metagenomes at a fraction of the cost associated with traditional directed evolution.

This was applied to RNA ligase to improve the addition of an unnatural nucleotide phosphate — protected at the 3′and 5′ positions (pX2Yp) — to the trimer ABC in the reaction shown in Figure 5.

INSIGHT design was used to create a small-but-smart panel of engineered RNA ligases based on an Almac selectAZyme enzyme, RNAL 105, which was redesigned around its active site to allow better interaction with pX2Yp and improved ATP binding; this was needed to drive the reaction.

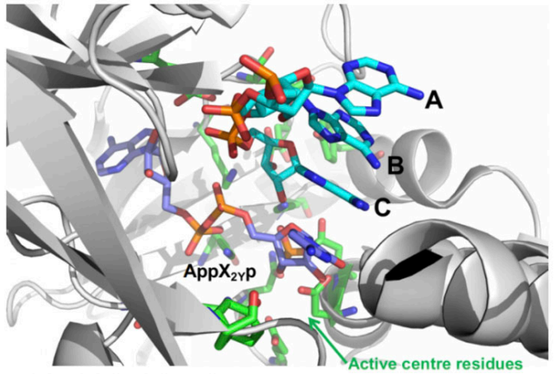

Figure 5: Ligase mediated coupling of ABC + pX2Yp involving mutation of the enzyme RNAL 105

When the resulting enzyme panel was screened, a near 10-fold increase in activity was achieved compared with the initial enzyme, showing the utility of INSIGHT technology in RNA ligase reactions.

Some oligonucleotide therapeutics are doubled stranded as opposed to single stranded. Ans whereas RNA ligase can be used to construct single-stranded products, it also has utility in double-stranded synthesis (innovators should be aware, however, of existing intellectual property.[8]

To create a double-stranded oligonucleotide, a range of short complementary RNA sequences (natural, unnatural or a mixture) can be assembled. Complementary base-pairing allows the fragments to assemble into a duplex structure, then RNA ligase (or DNA ligase) can be used to join the adjacent fragments together.

Figure 6 illustrates a typical approach for this self-assembly (annealing) and subsequent ligation chemistry. Panels of both RNA and DNA ligases can be screened from bacterial and viral sources, yielding several enzymes with good activity.

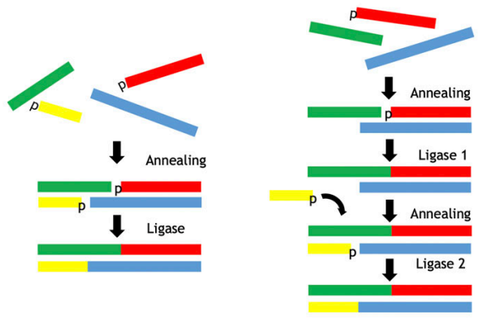

Figure 6: The ligase approach to double-stranded oligos via self-assembly

Almac has shown the importance of enzyme formulation in these types of reactions to minimise background reactions and oligo breakdown. The judicious selection of bacterial hosts and expression systems can prevent unwanted side-reactions by background host enzymes such as nucleases.

To enable low-cost manufacture, enzymes for oligonucleotide synthesis have been formulated as cell-free extracts to avoid expensive purification steps. This methodology should be considered when targeting these reaction classes.

Immobilisation strategies are evolving

It is apparent that oligonucleotide synthesis is a complex and challenging subject. So far, this article has shown how blockmers may be synthesised by single nucleotide addition, how they may be linked to form single strands and how they can also be linked to form double strands.

Numerous additional strategies are evolving, in which the immobilisation of substrates or enzymes is used to increase process performance.[9]

Immobilised templates have been used to make single-stranded oligos (as shown in Figure 7), whereby Almac has demonstrated proof of principle for oligo assembly using selectAZyme ligases.

These approaches use immobilisation technology in which immobilised substrate templates (blockmers) in various formats guide blockmer assembly prior to subsequent coupling.

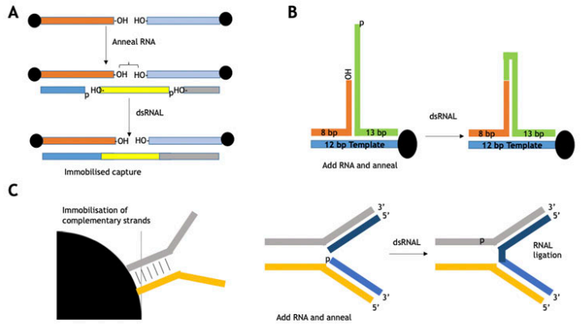

Figure 7: Immobilised blockmers used to assemble two complementary blockers for subsequent selectAZyme ligase mediated ligation

Alternatively, the enzymes themselves may be immobilised. Single nucleotide extension using selectAZyme RNA ligase to generate specific, short, single-strand fragments was outlined in Figure 3.

This same approach is illustrated in Figure 8 using immobilised RNA ligase and alkaline phosphatase for template-free single nucleotide addition. This raises the prospect of a flow-based biocatalytic process with the enzymes immobilised on solid supports and packed in columns as an evolution forward from batch processing.

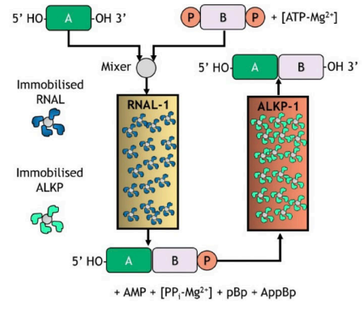

Figure 8: Immobilised selectAZyme ligase and phosphatase for blockmer synthesis

Building block biotransformations

Whatever strategy is taken for oligonucleotide synthesis, it is dependent on having access to the relevant building blocks. Biocatalysis also has an important role in this regard, to mediate regioselective introduction of phosphate groups, for example, or to address stereochemistry requirements.

When sulphur is introduced in place of oxygen in the oligonucleotide backbone, a chiral phosphorous centre is formed. If several sulphur residues are present in the oligonucleotide, then the possibility of multiple diastereomeric products exists.

It is possible to address such chirality issues by resolving mixtures of dimers bonded by a racemic phosphorothioate bond (illustrated in Figure 9).

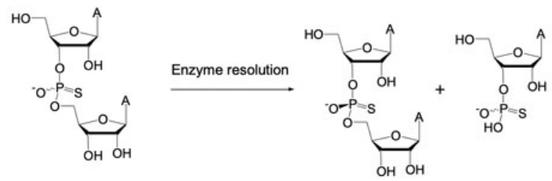

Figure 9: Enzyme-mediated resolution of dimers

Selective hydrolytic bioresolution may be catalysed by phosphodiesterases, phosphotriesterases and phosphoramidases.

In readiness for the next generation of oligonucleotides, wherein a single isomer is needed, Almac has been developing panels of these enzyme classes and taking inspiration from Nature regarding the microbial degradation of organophosphate herbicides to assist and guide enzyme panel design.

These three classes of enzymes have been cloned and screening collections are available to address the need for good stereochemistry control.

When single-stranded products are being elongated enzymatically, it may be necessary to remove a phosphate group to allow the next stage of enzymatic extension.

Alkaline phosphatases are enzymes that cleave an unprotected phosphate under selective and mild conditions (as shown in Figure 3). Almac has developed a panel of 96 diverse phosphatases for this type of reaction.

Modification of the phosphodiester backbone and the ribose

ring have been outlined as strategies to improve the therapeutic performance of oligonucleotides.

Another type of modification is in the base when, for example, methylation may be desired. Biocatalysis provides a synthesis route for such modified substrates.

Purine and pyrimidine nucleoside phosphorylases can exchange the natural base for an unnatural base as shown in Figure 10. Almac has assembled panels of diverse purine and pyrimidine nucleoside phosphorylases (PNPs) to assist the construction of nucleoside building blocks when an unnatural base is needed.

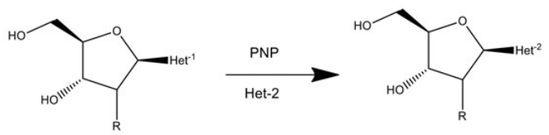

Figure 10: Application of PNPs to make nucleosides

As in oligonucleotide strand synthesis, building block synthesis is greatly assisted by access to various enzyme screening panels; this allows multiple routes and synthetic strategies to rapidly be assessed for a given target.

DNA strands can also be produced enzymatically

Oligonucleotides may be based on DNA rather than RNA, warranting different synthetic approaches. Terminal deoxynucleotidyl transferase (TdT) is a specialised DNA polymerase that does not require a template and adds single nucleotide triphosphates (NTPs) to the 3′ end of an oligonucleotide or DNA molecule.

This is in contrast to the examples above that show how larger fragments may be joined together. If the donor NTP is blocked, for example with a 3′-O-allyl or 3′-aminoxy group, then it can be added to an oligonucleotide by TdT … but indiscriminate further addition of NTPs by the enzyme is prevented by the blocking group.

The product can be unblocked and the cycle repeated so that a DNA chain grows as shown in Figure 11. This pioneering approach may form the basis of future enzymatic oligonucleotide synthesisers and, in time, may also be applicable for unnatural DNA-based oligonucleotides.

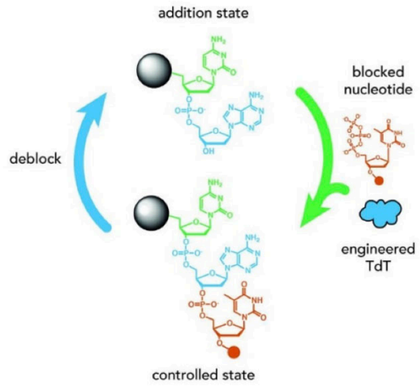

Figure 11: Application of TdT enzymes to assemble DNA

Summary

An increasing demand for oligonucleotides as therapeutics has focused attention on methods for their synthesis. Chemical routes using phosphoramidites have drawbacks relating to the yield and purity of the product and this has stimulated considerable interest in biocatalytic approaches.

Enzymes offer the benefits of sustainability, selectivity and cost reduction in pharmaceutical synthesis. There are multiple synthetic strategies for oligonucleotides (and their building blocks) and these products invariably have considerable structural complexity.

Enzymatic synthesis therefore requires a sophisticated suite of enzyme technologies to effectively tackle both building block and strand construction. Almac has developed a range of biocatalytic products and services to facilitate the challenges posed in biocatalytic oligonucleotide synthesis and we can expect spectacular success stories to emerge in the coming years.

Conclusion

The proven ability of biocatalytic technology to produce hard cost savings for pre-existing processes or to provide economical access to NCEs in the pharmaceutical sector ensures increased year-on-year investment in this area.[10]

There is a phalanx of oligonucleotides coming through the pipelines of innovators that all need materials for clinical trials. As these products move through the pipeline, larger quantities will be required, putting intense pressure on existing solid-phase capacities.

It is time to think of alternative methods and to reap the benefits of enzyme catalysis in your oligo synthesis. The work showcased here is not only alleviating pressure on capacity, it is unlocking new routes of synthesis and improving the profiles of products that are challenging to purify. Now is the time to unlock the power of enzymes for your oligo project.

References

1. F. Czauderna, et al., Nucleic Acids Res. 31, 2705–2716 (2003).
2. I.J. Huggins, et al., Molecules, 24(18), 3287–3300 (2019).
3. A.T.P. Carvalho, et al., Phys. Chem. Chem. Phys. 20, 2558–2570 (2018).
4. A.T.P. Carvalho, et al., Phys. Chem. Chem. Phys. 19, 26851–26861 (2017).
5. D.F.A.R. Dourado, et al., ACS Catalysis 6, 7749–7759 (2016).
6. Y. Kikuchi and K. Sakaguchi, Nucleic Acids Res. 5, 591–598 (1978).
7. O.C. Uhlenbeck and V. Cameron, Nucleic Acids Res. 4, 85–98 (1977).
8. www.chemicalsknowledgehub.com/article/14595/.
9. R. Mullin and T.S. Moody, Chemical & Engineering News 97, 34–35 (2019).
10. T.S. Moody and S. Mix, Specialty Chemicals Magazine, 22–25, (February 2019).

For more information

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Part 1 and Part 2 are available to read on:

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