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Perspective

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Engineering considerations for next-generation oligonucleotide therapeutics

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Oligonucleotide therapeutics are revolutionizing disease treatment by regulating molecules at the genetic level, offering the possibility of treating conditions that were once considered 'undruggable'. However, delivering oligonucleotides to tissues beyond the liver remains a key challenge, limiting their clinical applications thus far to niche indications. To achieve broader applicability, extensive biomolecular engineering is necessary to enhance the stability, tissue targetability, pharmacokinetics and pharmacodynamics of these structures. The intricate design of these molecules also demands sophisticated process-engineering techniques. Here we provide a collaborative Perspective from academia and industry on the pivotal role of chemical engineering in expanding the use of therapeutic oligonucleotides to treat a wider range of diseases. We discuss how the interplay between biomolecular and process engineering impacts the developability of next-generation oligonucleotide therapeutics as well as their translation from bench to bedside.

Oligonucleotide therapeutics (ONTs) have emerged as a transformative approach to disease treatment. Unlike conventional drugs, which selectively bind to disease-related proteins to modulate their function, ONTs can regulate the production of target proteins by binding to their corresponding messenger RNA or non-coding RNA through Watson–Crick–Franklin base-pairing[1–3]. As such, ONTs address a substantial challenge in medicine—the 'undruggability' of up to 85% of the human proteome with traditional small-molecule- and protein-based therapeutics[4,5]. Consequently, ONTs have seen rapid market growth, approaching US$60 billion, with 2023 marking a notable milestone as the 20th ONT achieved regulatory approval[6,7].

ONTs are typically fewer than 30 nucleotides in length. Examples include antisense oligonucleotides (ASOs), small interfering RNAs (siRNAs) and microRNA modulators, among others[1,8]. These ONTs can be single-stranded or double-stranded, and their forms and structures can have a major impact on their functions, as discussed in recent reviews[1,3,9]. Although substantial progress has been made in developing mRNA-based therapies, such as those that produced vaccines for SARS-CoV-2, these mRNAs, being much longer than 30 nucleotides, are not included in this Perspective. Instead, we direct readers to excellent reviews on mRNA-based therapies[10,11].

Most approved ONTs are designed for liver-related disorders or require local administration to target other organs due to their propensity for liver sequestration upon systemic injection[9]. This bottleneck restricts ONTs to niche therapeutic areas. The Food and Drug Administration (FDA) approval of inclisiran in 2021 marked a defining moment, demonstrating that ONTs can treat common diseases like atherosclerotic cardiovascular disease, a leading cause of death globally[6]. However, inclisiran is still liver-targeted, highlighting the need for ONTs that can act on extrahepatic disease pathways while also being developable at large scales. To overcome these obstacles, innovative engineering solutions are required.

This Perspective explores how chemical engineering can expand the therapeutic reach of oligonucleotides. We delve into biomolecular engineering strategies, which include modifications to the chemical structure, the attachment of targeting ligands, and formulation

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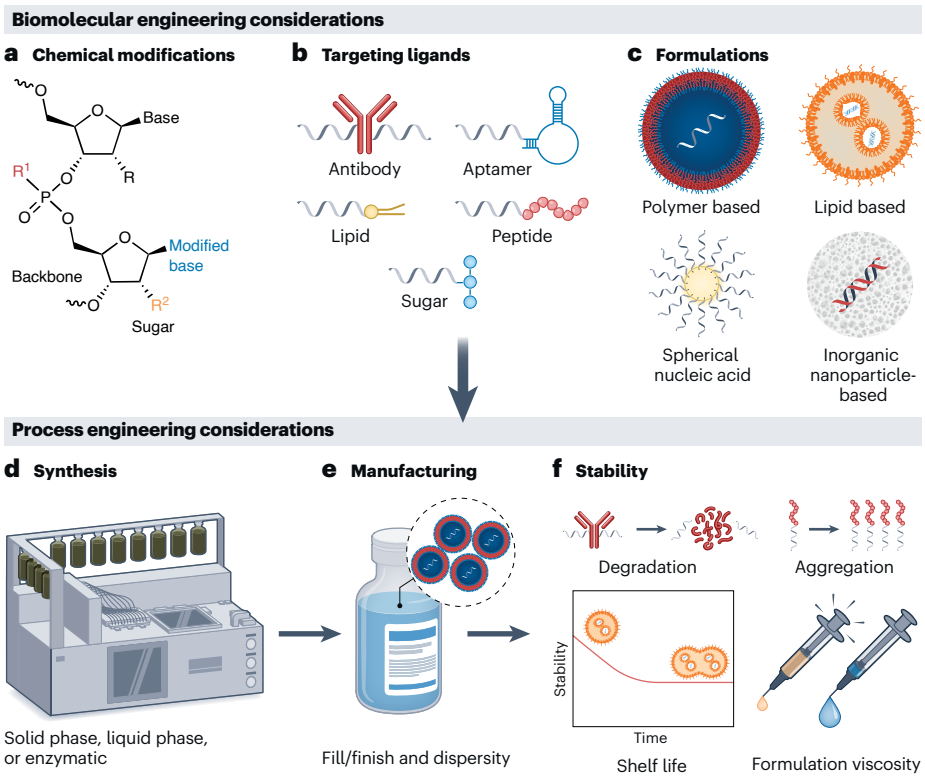

Fig. 1 | Broad categories of biomolecular engineering strategies and considerations for the process design and API/drug product attributes of the resultant structures. a, Chemical modifications can be made to the base, sugar or backbone. **b**, Targeting moieties like antibodies, aptamers, peptides, lipids and sugars can be conjugated to oligonucleotides. **c**, Oligonucleotides can also be compounded as nanoformulations. **d**, Synthesis of an oligonucleotide API is done via chemical and/or enzymatic methods. **e**, DP manufacturing consists of compounding followed by fill/finish. The dispersity/heterogeneity of the resultant structures is a key consideration. **f**, The final structures should be resistant to degradation/aggregation, exhibit high stability, and possess viscosities that do not inhibit injectability. Created with BioRender.com.

approaches. We highlight how advances in these areas can lead to the development of structures with new, and in some cases, unprecedented biological properties.

Delivering ONTs beyond the liver involves more complex molecular designs, making the process engineering required to manufacture these drugs inherently more demanding. Additionally, treating more common diseases will necessitate manufacturing these drugs at much larger scales than currently done. Therefore, this Perspective also focuses on the synthesis, scale-up and drug product (DP) stability of these intricate molecules, delineating how each class of biomolecular engineering strategy impacts various considerations in these key areas. We believe that this holistic view (Fig. 1) is essential to enhance the developability of emerging oligonucleotide candidates and accelerate their transition from research to therapeutic reality.

Biomolecular engineering considerations

The design of ONTs is driven by the biological attributes desired, such as cell permeability, endosomal escape, high affinity for complementary sequences, circulation time, stability and reduced side effects[1]. Rather than describing how each attribute can be controlled, we will concentrate on a select few, particularly those pertinent to extrahepatic delivery.

Chemical modifications

Unmodified oligonucleotides exhibit rapid degradation, unfavorable pharmacokinetics and an inability to enter cells in high amounts in vivo[1]. Biomolecular engineering (Fig. 2) of oligonucleotides through chemical modifications represents a simple and effective approach for tuning their biological properties, and can be accomplished through modification of the backbone (for example, phosphorothioate, PS), sugar (for example, 2′-*O*-methyl) or nucleobase (for example, 5-methyl-C). For instance, the first ONT to be approved by the FDA, fomivirsen, contains a PS backbone. Since then, PS modifications have been employed in several more ASO- and siRNA-based ONTs, including inotersen, nusinersen and givosiran[6]. This modification can confer the oligonucleotide with enhanced resistance to nuclease degradation, increased serum protein binding that yields structures that are not rapidly cleared by the kidneys, and the ability to enter cells in amounts sufficient to impart a therapeutic effect. So far, all FDA-approved oligonucleotides contain one or more chemical modifications, exhibiting the utility and importance of these approaches[6].

Generally, these modifications increase the delivery to target tissues through a ‘passive’ mechanism. For example, enhanced stability (for example, resistance to nuclease degradation) and longer circulation times allow ONTs to evade clearance from the body long enough to access tissues of interest. However, these strategies do not usually unlock broad biodistribution. Modified oligonucleotides incorporating PS, morpholino and 2′-*O*-methyl groups still primarily accumulate in the kidney and liver, with limited access to organs such as the brain or heart[12].

It is important to recognize that enhancing one property through chemical modification may compromise another. For example, although morpholino modifications improve cell permeability and nuclease stability, they decrease hybridization melting temperatures to complementary sequences, potentially undermining therapeutic effectiveness[13]. This issue can be alleviated by concurrently incorporating other groups into these sequences, such as locked nucleic acids (LNAs) or 2′-*O*-methyl, that increase binding strength[14]. In some cases, modifications might make the structures

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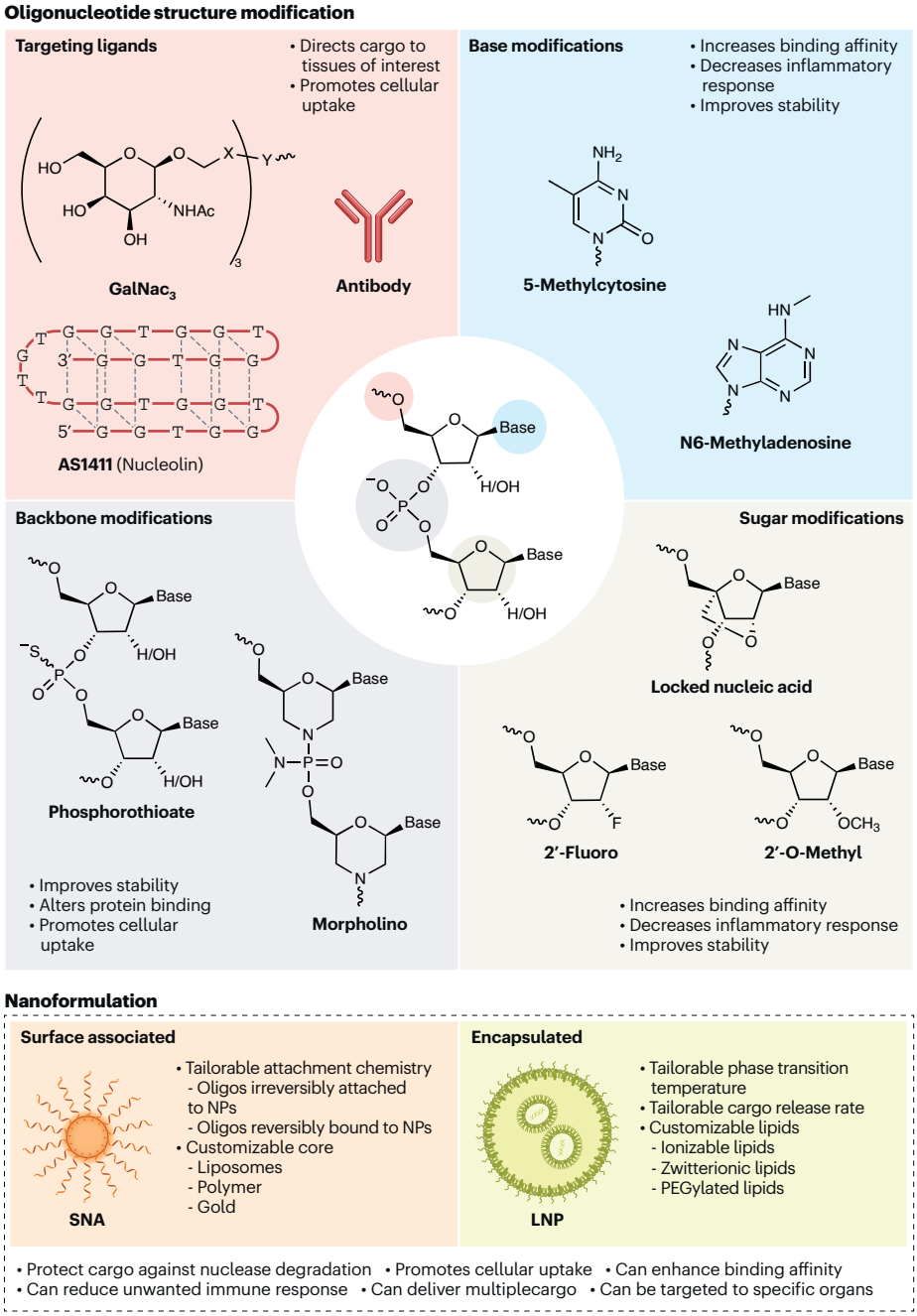

Fig. 2 | Summary of biomolecular engineering strategies for enhancing oligonucleotide properties. These properties can be improved by either modifying the oligonucleotide structure—such as using modified bases, backbones or sugars—or by attaching targeting ligands. Alternatively, nanoformulations can be utilized in which the oligonucleotide is either bound to the surface of a nanoparticle (NP) (for example, emerging structures such as spherical nucleic acids (SNAs)) or encapsulated within (for example, LNPs). Created with BioRender.com.

less functional. For example, siRNAs with PS backbones are less active than their phosphodiester (PO) counterparts[1]. As such, for this application, PS modifications are often limited to the termini only, or alternate strategies such as nanoformulations (vide infra) are pursued to enhance biological properties. Furthermore, although certain chemical modifications like 2′-*O*Me can help reduce unwanted innate immune responses, others such as PS might exacerbate them[15,16]. Taken together, approaches based on chemical modifications are the most clinically advanced strategy to enhance biological properties of oligonucleotides, but often must still be interfaced with more intricate targeting approaches (for example, active targeting through ligands) to broaden their distribution in vivo.

Targeting ligands

Typically, >50% of systemically administered ONTs accumulate in the liver, kidney or spleen[17]. By conjugating targeting ligands, delivery to desired cell types or organs can be increased significantly, often by more than tenfold[18,19]. Ideally, receptors that are chosen should be highly specific to the organ of interest, expressed in sufficiently high quantities, and internalized from the cell surface to facilitate the entry of bound molecules. Currently, six of the approved ONTs incorporate targeting ligands[20]. An example is givosiran, an approved siRNA that uses GalNac to target the asialoglycoprotein receptor on liver hepatocytes. Following its approval, four more siRNA–GalNac conjugates have entered the market, as well as one ASO–GalNac conjugate[2]. This success

has prompted the exploration of various targeting ligands—such as sugars, lipids, antibodies, aptamers and peptides—to expand delivery to extrahepatic tissues[21,22]. Receptors that have been targeted include transferrin for the brain or muscle[23,24], human epidermal growth factor for tumors[25], and mannose receptor 1 for the lung[26].

In some cases, ligands can lead to accumulation in extrahepatic tissues through less specific interactions. For example, emerging work has shown that cholesterol-conjugated DNA/RNA heteroduplexes can cross the blood–brain barrier (BBB), although the specific mechanisms behind this are not yet fully understood[22]. Additionally, a recent study demonstrated that C_{16}-lipid-conjugated siRNA can achieve sustained gene silencing in the brain for up to three months following intrathecal injection[27].

Each class of targeting ligands has advantages and drawbacks. Ligands that perform well in vitro might lack sufficient affinity or stability in vivo, especially aptamers and sugars[28,29]. Multivalent presentation of targeting moieties can enhance binding but may also result in strong agonist or antagonist activity[28]. Therefore, as higher affinity structures are developed, their impact on downstream signaling must be studied.

In situations where targeting is limited by low receptor expression, targeting ligands that support high drug loading are advantageous. This allows the delivery of multiple ONTs from a single receptor-targeting ligand interaction. Examples include antibodies modified at cysteine or lysine residues with ONTs. However, larger structures have more difficulty diffusing within organs to reach target cells. Consequently, smaller targeting moieties like short aptamers or peptides may be preferable to larger ones like antibodies.

The interaction between the targeting ligand and the oligonucleotide must be carefully considered. Oligonucleotides should not reduce the ligand's binding affinity due to steric blocking or negative charge. Similarly, the targeting ligand should not impair the oligonucleotide's activity. For example, in siRNA, it is generally preferable to conjugate targeting ligands to the passenger strand. This avoids perturbing the interactions of the guide strand with the AGO2 complex, which is essential for silencing activity[1]. In some cases, these concerns are alleviated by using linkers that are cleaved in the cell, thereby releasing the oligonucleotide strand from the ligand[30].

Nanoformulations

After synthesis, oligonucleotides are generally formulated as liquid injectables, either in water or with more complex excipients (for example, spermine and lysine) to promote their stability[31]. Compounding oligonucleotides with nanocarriers via encapsulation[32], surface adsorption[29] or surface functionalization[33] is another strategy for improving their biological properties. Nanomaterials offer several advantages: they protect the cargo from nuclease degradation, enhance circulation time, and promote endosomal escape[32]. In some cases, nanoformulations can lead to unprecedented biological properties. For example, when oligonucleotides are presented in a spherical nucleic acid architecture, they can enter cells in high quantities and bind to complementary nucleic acids with up to 100-fold greater affinity[33]. These formulations can also be designed to display a broad tissue distribution and facilitate high cellular uptake[34]. Commonly used carriers include those that are lipid-, polymer-, inorganic nanoparticle-, DNA nanostructure- or extracellular vesicle-based[21]. The most clinically advanced example is Onpattro, which uses lipid nanoparticles (LNPs) for the delivery of siRNA[6].

Nanoparticles primarily accumulate in the liver and kidneys, but their modularity allows for extensive engineering to target other tissues. Examples include SORT-LNPs, where a single lipid group's identity is changed to drive distribution to the spleen or lungs[35], and polyplex nanoparticles identified through high-throughput screening to target lung cancer cells for siRNA delivery[36]. Additionally, tuning nanoparticle size has shown that ultrasmall gold nanoparticles (<10 nm) penetrate tumors more effectively than larger ones[37]. Beyond modifying the physiochemical properties of the nanoparticles, incorporating targeting ligands on their surface can further enhance tissue-specific delivery (vide supra).

The modularity of nanoformulations allows for tuning structural parameters such as size, shape, valency, surface functionality and composition, providing a vast design space for programming delivery outside the liver. One nanoparticle can be encapsulated or functionalized with multiple oligonucleotide sequences. As a result, uptake of a single nanoparticle facilitates entry of a large ratio of oligonucleotides. However, compared to ligand conjugation or linear sequences, it is more difficult to control stoichiometry in nanoformulations (for example, drug loading in a nanoparticle). Certain nanoparticles exhibit high polydispersity (vide infra), leading to varying drug loading and structural heterogeneity, which can result in different biological activities. Some nanoparticles may be harder to translate clinically due to long-term toxicity concerns (for example, inorganic nanoparticles that are not cleared from the body) or the fact that they elicit immune responses at therapeutic doses (for example, certain LNPs)[32,38].

To overcome these issues and enhance extrahepatic delivery, nanoformulation strategies are often combined with chemical modifications and/or targeting ligands. These combinations improve tissue/organ targetability, increase potency and reduce the required dose. Moreover, in some situations, 'first-generation' nanoformulations have been enhanced by introducing a non-nanoformulation DP based on improved chemical modifications and a targeting-ligand approach. For instance, Onpattro uses an LNP-based vehicle to deliver siRNA in the treatment of polyneuropathy associated with hereditary transthyretin-mediated amyloidosis, but a different DP (Amvuttra) was approved four years later that relies on enhanced stabilization chemistry and GalNac conjugation to promote delivery to target cells[39]. In contrast to Onpattro, which is delivered intravenously once every three weeks, Amvuttra enables subcutaneous dosing once every three months with comparable clinical efficacy.

Process engineering considerations

The complex biomolecular engineering strategies outlined above impact the process engineering aspects of development (Fig. 3 and Box 1). With continued strides in unlocking access of oligonucleotides to hard-to-reach tissues, larger scales of production will also be needed.

The scale required for producing ONTs per year varies widely as a result of multiple factors, including the prevalence of the disease, whether the drug targets the entire patient population or only a specific subgroup with particular mutations, the existence of competing medications, and the drug's potency, which affects the frequency and strength of dosages.

For example, viltolarsen, an FDA-approved drug, is used to treat a subset of patients with Duchenne muscular dystrophy (DMD) who carry a specific genetic mutation. DMD is considered a rare disease, affecting <0.02% of people in the United States, and just 10% of this population (5,000 patients) exhibit the mutation targeted by viltolarsen. The drug is administered intravenously on a weekly basis at a dosage of 80 mg kg^{-1}. For a patient weighing ~50 kg (110 lbs), this equates to ~200 g of ONT per year. If every eligible patient were treated, the total annual production required would be ~10^3 kg. However, because viltolarsen competes with another drug, golodirsen, the actual production volume is less than this maximum potential. Nevertheless, current manufacturing facilities are well-equipped to handle these production volumes on a routine basis in a single synthesis line.

For diseases affecting a larger percentage of the population (for example, over 10%), production scales would need to scale up significantly, potentially exceeding 10^6 kg annually. As the market for ONTs expands, enhancing global manufacturing capacity and reducing production costs become crucial. In the following we discuss the interplay between the desired structure and scale and how this influences process engineering considerations.

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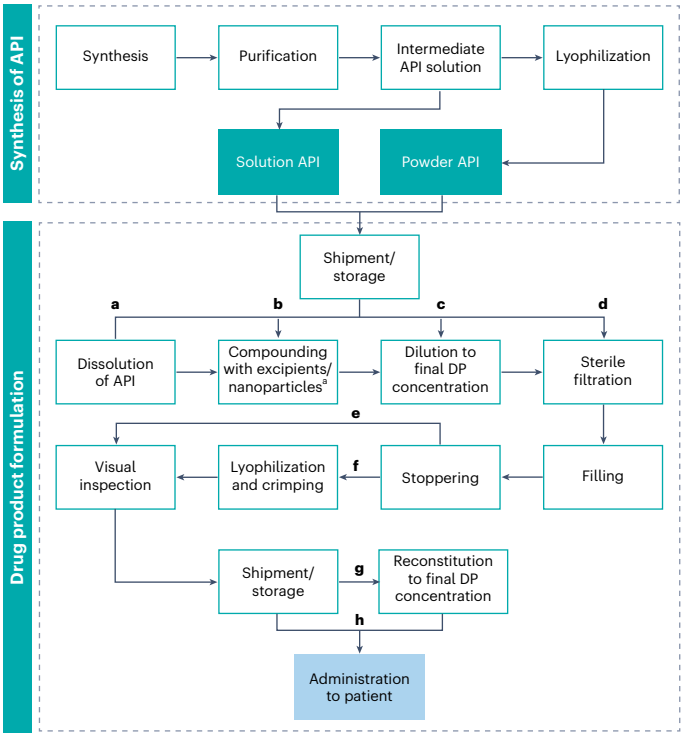

Fig. 3 | Process flow diagram for the synthesis of oligonucleotide API and DP formulation. a, Lyophilized API requires dissolution at the beginning of DP manufacturing. **b**, Oligonucleotides in solution can be compounded with excipients or nanoparticles. **c**, If necessary, oligonucleotides in solution are diluted to the final DP concentration. This gives the benefit of being able to store APIs in more concentrated form, therefore requiring less storage space for the API. This step is also useful if different dosage strengths are required, which can be achieved by varying the dilution buffer volume. **d**, Solution API already compounded with excipients to the target DP concentration can be moved directly to sterile filtration. **e**,**f**, Vial- and syringe-based presentations are the two predominant options for oligonucleotides. For vials (**f**), the final DP may be lyophilized, or if in solution moved directly from stoppering to crimping and then to visual inspection. For conventional syringes (**e**), lyophilization is generally not an option, and the process moves directly from stoppering to visual inspection. It should be noted that other presentations exist, such as dual chamber syringes that enable lyophilized presentation, but that is out of the scope of this diagram. **g**,**h**, DP presented as a liquid (**h**) is administered directly to patients, whereas DP presented as a lyophilized powder (**g**) must be reconstituted before use. [a]If oligonucleotides are compounded with nanoparticles, an additional purification step (for example, size-exclusion chromatography) may be necessary to remove unencapsulated or unassociated oligonucleotides.

Synthesis

The predominant method for manufacturing ONTs is solid-phase oligonucleotide synthesis (SPOS) using an automated synthesizer[40]. SPOS is preferred for its speed, consistency and high coupling efficiency. In this method, sequences are extended from the 3′ to 5′ direction on a solid support through repeated steps of deprotection, coupling, capping and oxidation. SPOS enables the synthesis of highly complex ONTs with multiple chemical modifications (for example, gapmers) or targeting ligands (for example, GalNac) in one pot, as long as the corresponding phosphoramidite building blocks are available. Unreacted reagents can be removed by simple washing. After synthesis, oligonucleotides are deprotected and cleaved from the support for purification and further downstream processing.

However, a key challenge with SPOS is scale. Solid supports limit production to <10 kg per batch due to limitations associated with the bed height and pumps used to flow the reagents through the resin. Scaling up would necessitate larger resin beds, where reagents cannot flow evenly, thereby compromising the efficiency of the coupling reactions needed for synthesis. A larger bed height would also increase the pressure required to maintain optimal flow rates, shortening the lifespan of the manufacturing equipment. Moreover, excess monomer building blocks are needed to drive the reactions to completion. Assuming a typical 99% coupling efficiency for each base addition, overall yields drop below 75% (0.99^{30}) even for a 30-mer. The synthetic process also requires large volumes of solvents like acetonitrile, leading to a substantial generation of waste and high process *E* factors (defined as the mass of waste generated per unit product)[41].

To address these issues, alternate methods such as liquid-phase oligonucleotide synthesis (LPOS) and enzymatic synthesis are starting to be explored, but so far they have not been developed to the same level of commercial maturity as SPOS. When attached to a lipophilic group to increase organic solvent solubility, LPOS can be run as a batch process without the same scale limitations as SPOS[42]. Batches of up to ~10 kg have been produced using this method[8]. However, LPOS involves additional extraction, wash and isolation unit operations compared to SPOS, which require optimization to maximize the yield and final product quality, and also contribute to higher synthesis costs.

Enzymatic synthesis, although still in its early stages, is advancing rapidly and has the potential to greatly increase both the accessibility of ONT synthesis and its range of applications. Enzymatic synthesis can offer several advantages over chemical synthesis, including unparalleled specificity, mild aqueous reaction conditions with elimination of organic solvents, significant reduction of hazardous waste, and the ability to initiate synthesis from simpler building blocks that reduce or eliminate the use of protecting groups (for example, nucleotide triphosphates instead of protected phosphoramidites)[43]. Template-independent enzymes such as terminal deoxynucleotidyl transferase and CID1 poly(U) polymerase have proven effective for making short strands of DNA and RNA de novo by single-base extension, but require a new synthetic step for each base addition[40,44]. These methods have also not yet reached the scale and yields achievable with conventional SPOS. In contrast, conventional template-dependent polymerases have the potential to make therapeutic polynucleic acids in a single operation, but suffer from the inability to control the site-selective incorporation of the same base with two different modifications. Moreover, both classes of enzyme can be less active when synthesizing ONTs with modified base, ribose and backbone linkage combinations.

Given the unique strengths and weaknesses of both chemical and enzymatic methods, more research is needed to fully leverage their complementary properties to enhance ONT synthesis strategies. The success of such hybrid approaches has already been demonstrated. For example, 'shortmers' (5–10 nucleotides) can be synthesized efficiently via SPOS, then these fragments can be enzymatically ligated to form longer sequences. This convergent synthesis approach substantially increases the final yield, with the potential to also increase final product purity[45].

Post-synthesis processing of an active pharmaceutical ingredient

After synthesis, oligonucleotides are generally purified using chromatographic separation to remove failure sequences. For some ONTs, resolving these failure sequences may be challenging, such as in PS-modified strands, where the different diastereomers present in the mixture can contribute to wide peaks and insufficient resolution[46]. Following this, samples can be concentrated and buffer/salt-exchanged using ultrafiltration/diafiltration (UF/DF). However, achieving desirably high concentrations (>150 mg ml^{-1}) may not be possible because of membrane fouling. For these situations, distillation-based techniques such as thin-film evaporation or rotary evaporation are alternate options. It should be noted that these methods are not able to do buffer/salt exchange and are, therefore, a complementary technique to

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BOX 1

Impact of biomolecular engineering strategies on process design and API/DP attributes

			Process engineering						
			Synthesis	Purification	DP formulation	Need for lyophilization[a]	Dispersity	DP stability	Viscosity
Biomolecular engineering	Chemical modifications	Common modifications[b]	●	●	●	●	●	●	●
		Uncommon modifications	●	●	●	●	●	●	●
	Targeting ligands	Antibody	●	●	●	●	●	●	●
		Aptamer	●	●	●	●	●	●	●
		Peptide	●	●	●	●	●	●	●
		Lipid	●	●	●	●	●	●	●
		Sugar	●	●	●	●	●	●	●
	Nanoformulation[c]	Encapsulated	NA	●	●	●	●	●	●
		Surface associated	NA	●	●	●	●	●	●

[a]Lyophilization may be needed either for API or DP.
[b]Defined as backbone, sugar, or base modifications that are used in an FDA-approved therapeutic or have commercially available phosphoramidites.
[c]For nanoformulation, purification refers to the need to separate NP-associated oligonucleotides from free oligonucleotides.
NA, not applicable.
The questions that were considered in compiling the table in this Box are shown below. In general, the complexity the biomolecular engineering strategies bring to the context of these questions is indicated by circle color as follows: pink, substantial complexity; yellow, moderate complexity; green, little complexity.

Synthesis. Does the strategy make scalability challenging? Does the strategy require the use of harsh reaction conditions (for example, toxic compounds) or lead to large amounts of waste? Do additional components other than the oligonucleotide need to be made (for example, peptide, nanoparticle and so on)? Can synthesis be done using a conventional oligonucleotide synthesizer or is a post-synthesis reaction required?

Purification. Can the structure be purified with conventional techniques used for oligonucleotides (for example, chromatography)? Do the individual components add complexity in terms of purification compared to unmodified DNA (for example, antibody purification)? Does the synthesis strategy require extra purification steps compared to unmodified oligonucleotides (for example, separating out unconjugated DNA from nanoparticle-oligonucleotide conjugates)?

DP formulation. Does the structure add complexity in terms of formulation compared to unmodified oligonucleotides (for example, need for nanoparticle, need for novel excipients to promote stability and so on)? Are extensive formulation development studies required (for example, finding a formulation that co-optimizes the stability of both the antibody and oligonucleotide component in antibody-oligonucleotide conjugates)? Do small changes in formulation properties (pH, excipient concentration and so on) lead to large changes in product attributes (for example, stability)?

Need for lyophilization. Does the structure have sufficient stability to offer flexibility in terms of solution-based versus powder-based storage of API/DP?

Dispersity. Are final structures prone to large amounts of polydispersity or heterogeneity?

DP stability. Does the final structure have sufficient shelf-life to be developable as a commercial product? Is the structure resistant to the stresses that would routinely be encountered during manufacturing, shipping or administration?

Viscosity. Does the final structure/formulation exhibit viscosity that hinders injectability?

UF/DF[47]. For ONTs with heat-sensitive modifications (for example, antibody conjugates), distillation is not a viable choice due to the elevated temperature involved in the process.

An important next decision to make is whether the active pharmaceutical ingredient (API) is stored in solution or a lyophilized form before the start of DP manufacturing[47]. In this regard, each choice has its advantages and drawbacks.

Although oligonucleotides are generally stable at 2–8 °C in solution, certain structures necessitate lyophilized storage because of chemically labile groups. Structures that are stored in solution may have additional bioburden risks if excipients required for stabilization promote microbial growth. Storing samples frozen is an effective way to mitigate this risk. Leachables from the container closure are also a greater concern in solution than lyophilized form.

On the other hand, storing APIs in a lyophilized form adds complexity to the DP manufacturing process, as an additional step to reconstitute the oligonucleotide is needed before fill/finish. Moreover, lyophilization is a time- and energy-intensive process. Nevertheless, the current industry standard is to store ONT APIs as lyophilized powders.

DP manufacturing

The various paths of DP manufacturing based on the API presentation and desired final structure are shown in Fig. 3. In the following we cover key attributes of the DP that are influenced by the process design, formulation and product presentation, including structural heterogeneity, stability and viscosity.

Dispersity/heterogeneity. A key consideration for the final DP is the heterogeneity of the final structures. This is particularly important, because structural variants in a population can each have different biological activities.

Linear sequences are relatively monodisperse, as conventional synthesis and purification schemes achieve purities of full-length products greater than 90% (ref. 41). However, as biomolecular engineering of ONTs leads to more complex structures, it also introduces more sources of heterogeneity. For example, PS linkages result in racemic mixtures of oligonucleotides, which can have inferior biological activity compared to stereopure structures[48]. Heterogeneity can also arise in ONTs with targeting ligands if the ligand is prone to degradation or has multiple attachment sites that result in structures with varying oligonucleotide loadings (for example, antibodies).

Heterogeneity is an especially important consideration when pursuing nanoformulations. A polydispersity index (PDI) < 0.3 is generally desirable for organic nanoparticles, and certain classes of nanoparticles produced at commercial scale, such as LNPs, can routinely attain PDI values of <0.2 (ref. 49). However, achieving intra-batch or inter-batch consistency may be prohibitively challenging with nanoparticles derived from natural sources, such as exosomes[50]. Aside from the dispersity of the nanoparticle itself, the relative loading of oligonucleotides, either encapsulated or associated with the surface, can vary from particle to particle within a batch.

To address these issues, further research is needed on biomolecular engineering strategies that reduce dispersity. For example, controlling stereopurity in sequences with PS linkages and enhancing homogeneity in chirality is an ongoing area of research[48,51]. Conjugating targeting moieties such as aptamers, peptides and sugars to linear sequences can generally be done with stoichiometric precision, minimizing additional heterogeneity.

For nanoformulations, developing 'molecularly pure' particles is a key research area[52]. Advancements in analytical characterization techniques are needed, particularly those providing single-particle resolution instead of bulk information[53]. These tools can also aid in high-throughput screening of nanoparticle/oligonucleotide compounding conditions to achieve maximum monodispersity.

Moreover, API/DP processes can introduce stresses that lead to the degradation of ONTs, thereby adding to their heterogeneity. Therefore, manufacturing processes should be designed to minimize these pathways by controlling factors such as solvent exposure time during synthesis, temperature and light exposure.

Stability. The stability of the final DP is crucial for ensuring a shelf-life that is clinically translatable. Among the currently approved products, all are offered as solutions with shelf lives varying from 18 to 60 months. These products have recommended storage temperatures ranging between 2 °C and 30 °C (ref. 20). Several factors can determine DP stability, including ONT degradation pathways, intra- and intermolecular interactions, the presence of targeting ligands and the formulation of the ONTs. As the complexity of the ONT structure increases, the number of variables that influence its stability grows.

Oligonucleotides can degrade through various pathways, such as hydrolysis, deamination, depurination and desulfurization, driven by temperature, pH and light exposure. For instance, the deamination of 5-methylcytosine to form thymine accelerates at high temperatures and extreme pH levels; it is estimated that at 80 °C and pH 8, the half-life of this reaction is ~3 weeks, compared to over 1,000 years at 5 °C (ref. 54). Therefore, formulations maintained around physiological pH and processes avoiding extreme temperatures, such as terminal sterilization (typically performed at 121 °C), are advantageous[31,55]. Additionally, some sequences are sensitive to metal-catalyzed degradation. Monitoring trace metal levels in raw materials and preventing metal leaching from manufacturing equipment are crucial. Adding a metal chelator such as ethylenediaminetetraacetic acid (EDTA) to the formulation can help mitigate this degradation pathway[56].

Instability can also arise from intra- and intermolecular interactions of oligonucleotides. Certain G-rich sequences tend to self-associate and aggregate, forming supramolecular assemblies that increase viscosity and hinder developability (vide infra)[57]. Formulation design can modulate these interactions by adjusting buffering agents, salt concentration and pH.

The conjugation of targeting ligands introduces additional stability considerations. Antibodies and peptides, for example, have their own degradation pathways[58]. Therefore, formulations must strike a balance between stabilizing the oligonucleotide and ligand module. Hydrophobic ligands, such as lipids, can promote self-interaction and aggregation when conjugated to oligonucleotides[32]. While oligonucleotides generally withstand freeze/thaw cycles, conjugated ligands such as antibodies may be sensitive, affecting processes involving frozen storage and thawing.

Nanoformulations present unique instability challenges. Nanoparticles such as liposomes or inorganic nanoparticles can undergo colloidal instability, leading to aggregation[32]. Surface modifications with polymers such as polyethylene glycol can slow such aggregation[59]. Other nanoparticles, like poly(lactic-*co*-glycolic acid), are prone to hydrolysis in solution and thus require lyophilization for long-term storage[60]. Similarly, lipid components containing ester bonds in LNPs can also be susceptible to hydrolysis[61]. The rates of nonspecific leakage or leaching of cargo from the nanoparticles must also be understood. For instance, for encapsulated payloads, the encapsulation efficiency—typically expressed as a percentage—is measured over time as a proxy for nonspecific release[62–64]. During process development, identifying critical manufacturing parameters such as scale, shear forces and temperature is necessary, as these factors may affect nanoparticle stability differently from oligonucleotide stability. For example, LNPs are sensitive to shaking stresses associated with shipping, which can increase particle size and increase ONT leakage[65].

Finally, the interaction of components with the final DP container (for example, vial or syringe) must be considered. Protein-based nanoparticles can adsorb to glass surfaces, reducing the effective concentration and potentially leading to protein denaturation in the adsorbed state[58]. Ensuring stability and efficacy thus requires a comprehensive understanding and control of these various factors throughout the development and manufacturing processes.

Viscosity. Viscosity is a crucial parameter in the formulation of ONTs, as most ONTs are designed for delivery through injections. The viscosity is dependent on a number of factors, including concentration, temperature, sequence, excipients and pH. From a practical standpoint, formulation viscosity should not exceed ~20–25 cP, with 50 cP representing a commonly accepted absolute limit that can be used with 25/27-G needles[66,67]. High viscosity can substantially impact the injectability of these formulations. Elevated viscosities require greater injection forces, which can be difficult to achieve and can cause a great deal of pain at the injection site (>~40 N)[68]. This makes high-viscosity formulations undesirable for routine use, particularly for subcutaneous injections, which are preferred over intravenous methods due to their convenience and improved patient compliance.

While managing viscosity is important, formulating ONTs at high concentrations is also essential (>150 mg ml^{-1}). Such formulations are necessary to deliver therapeutic doses to hard-to-reach organs like the

brain. High-concentration doses can also enable less frequent dosing, enhancing patient adherence to the treatment regimen.

However, increasing the concentration of ONTs leads to several problems. The siRNA-based drug inclisiran has a viscosity of 20 cP when formulated at 200 mg ml^{-1}, which approaches the limit of desirable viscosity for subcutaneous injection (20–25 cP)[20]. In some cases, high concentrations result in increased interactions between oligonucleotide molecules, forming supramolecular structures that substantially raise the solution's viscosity[69]. These solutions often become non-Newtonian, wherein their viscosity changes based on the applied stress and does not scale linearly with ONT concentration. This not only complicates the handling and administration of the formulations, but also poses challenges in the manufacturing process, such as UF/DF, where high viscosities can cause gelling on the membrane and high back-pressures. In one study, Maksudov and colleagues observed an up to sixfold increase in viscosity as an oligonucleotide's concentration was increased from 25 mg ml^{-1} to 100 mg ml^{-1} (1.1 cP to 6.7 cP). Moreover, based on molecular dynamics simulations, the group proposed that increased concentrations lead to increased levels of intermolecular interactions between oligonucleotide molecules in solution[70].

Additionally, the inclusion of targeting ligands to enhance the specificity and efficacy of ONTs can further increase viscosity. For example, GalNac-conjugated sequences show a 50% increase in viscosity compared to their unmodified counterparts[31]. Addressing these challenges may involve using viscosity-reducing excipients (for example, lysine and spermine) or innovative formulation strategies, such as incorporating enzymes that degrade hyaluronan locally to allow higher-volume subcutaneous injections (>10 ml versus the traditionally used <2-ml injection volumes)[31,71].

Outlook

Despite the groundbreaking potential of ONTs, important challenges in their delivery, stability and specificity have restricted their clinical success predominantly to liver-targeted applications[9]. Chemical engineering, in particular, plays a crucial role in overcoming these barriers and bridging the gap between molecular design and clinical implementation[6,8].

Biomolecular engineering of oligonucleotides has led to impressive advances and unlocked structures that can better target tissues beyond the liver, including in tumors, the lungs and the brain. However, in many current examples, extrahepatic delivery is still relatively low compared to the dose administered. Ongoing study of fundamental biology to help identify new receptors to target outside the liver will be crucial to overcoming this challenge.

The use of synthetic strategies that enable the rapid creation of large libraries of materials (for example, nanoformulations) can help realize new extrahepatic targeting 'hits' while also helping to elucidate structure–biofunction relationships. In this regard, the use of emerging methods such as DNA barcoding to probe the activity of these materials in high throughput in vivo will be important to make this successful. Combinatorial chemistry techniques and emerging machine-learning methods have proven promising for identifying new structures with desirable in vivo properties, such as greater than tenfold transfection potency compared to commercial benchmarks[72]. The ultimate goal is to gain sufficient knowledge to enable rational design in tuning material properties to enhance extrahepatic targeting.

The continued development of new administration techniques that facilitate exposure of oligonucleotides to target organs will also be key. When subcutaneous or intramuscular administration is used, one important factor to consider is the risk of injection-site reactions (ISRs) caused by a local immune response to the drug[73]. Therefore, continued study of how different chemical modification strategies (for example, LNA and 5-methyl-cytosine) drive ISRs will be key to minimizing their occurrence.

From a process-engineering perspective, acknowledging the hurdles associated with the large-scale synthesis and DP development of complex structures resulting from biomolecular engineering optimization is essential. As ONTs evolve to treat a broader range of therapeutic areas, scaling up the manufacturing of both APIs and DPs is a major challenge. On the other hand, ONTs offer unique opportunities for personalized medicine by enabling the rational design of drugs for patient-specific mutations. This approach has led to 'N-of-1' clinical trials, where drugs are tailored to individual patients[74]. Such personalized approaches are crucial, particularly in addressing urgent medical conditions like neurodegenerative diseases in young children, where treatments must be developed rapidly, ideally within 12–15 months after mutation detection. However, the costs associated with producing a drug for a single patient can exceed US$3 million[75]. Adapting existing process engineering methods to these specific needs could help mitigate these challenges[76]. Since the inception of 'N-of-1' studies in 2017, the best practices for modifying process engineering in this evolving field are still being developed. Close collaboration between academia and industry will be required to address these considerations.

Early in the development process, it is crucial to choose the synthesis strategy and manufacturing workflow that best aligns with a specific drug's scale demands and structural sensitivities. Further developments in increasing scale and purity while reducing costs will rely on the invention of novel reagents and processes. A large source of the costs is in the purification of oligonucleotides using chromatographic separation. In this regard, the development of techniques such as hybrid enzymatic synthesis (that is, convergent synthesis), which results in purities of >90% without the need for purification, will be important. The unsustainable amounts of waste generated (~4,300 kg of waste per 1 kg of API) through SPOS and the shortage of acetonitrile as larger scales are unlocked will lead to further motivation to find alternate synthesis methods[77]. The large diversity of structures in the pipeline—from early research stages to clinical candidates—means that a one-size-fits-all approach to process or formulation development is not feasible. The expanded use of process analytical technology to measure the critical quality attributes of complex oligonucleotides in real time will be a vital tool for optimizing process parameters and manufacturing workflows. By addressing these considerations proactively and expanding the process engineering toolbox, the field will be well-positioned to translate the exciting and often complex generation of new ONTs from the bench to the bedside.

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Author contributions

S.B.E. and D.S. conceived the Perspective. S.B.E. and D.S. wrote the paper with input from H.B., S.S., D.F., P.S.D. and Y.L. S.B.E. and D.S. created the figures and tables. All authors reviewed and edited each section of the paper.

Competing interests

The authors declare no competing interests.

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