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The clinical potential of L-oligonucleotides: challenges and opportunities

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Chemically modified nucleotides are central to the development of biostable research tools and oligonucleotide therapeutics. In this context, L-oligonucleotides, the synthetic enantiomer of native D-nucleic acids, hold great promise. As enantiomers, L-oligonucleotides share the same physical and chemical properties as their native counterparts, yet their inverted L-(deoxy)ribose sugars afford them orthogonality towards the stereospecific environment of biology. Notably, L-oligonucleotides are highly resistant to degradation by cellular nucleases, providing them with superior biostability. As a result, L-oligonucleotides are being increasingly utilized for the development of diverse biomedical technologies, including molecular imaging tools, diagnostic biosensors, and aptamer-based therapeutics. Herein, we present recent such examples that highlight the clinical potential of L-oligonucleotides. Additionally, we provide our perspective on the remaining challenges and practical considerations currently associated with the use of L-oligonucleotides and explore potential solutions that will lead to the broader adoption of L-oligonucleotides in clinical applications.

1 Introduction

Oligonucleotides (ONs) have become vital tools in biomedical research and in the clinic. Their capacity to be rationally engineered through Watson–Crick (WC) base pairing makes ON highly adaptable, whereas robust synthetic methods and commercial availability make them accessible to most laboratories. In research, oligonucleotides are ubiquitous, with uses ranging from PCR amplification to gene editing. In the clinic, ON-based therapeutics have emerged as a powerful approach to treat a range of diseases by targeting undruggable proteins,[1,2] as well as the ever-growing list of disease associated non-coding RNAs.[3–6] Indeed, the number of ON-based therapeutics being approved by the U.S. Food and Drug Administration (FDA) is on the rise, including antisense oligonucleotides (ASOs) and aptamers.[7–9] ASOs and aptamers are both short, synthetic ONs that alter protein function either through sequence-specific hybridization with their mRNA or by directly binding to the protein target, respectively. Recent examples include the antisense oligonucleotide (ASO) Tofersen targeting SOD1 mRNA to treat amyotrophic lateral sclerosis (ALS)[10] and the aptamer-based drug Avacincaptad pegol targeting complement protein C5 for the treatment of geographic atrophy,[11] both of which were approved in 2023. Clinical applications of ONs are not without challenges, however. In particular, ONs are susceptible to degradation by cellular nucleases, which hampers their stability and overall effectiveness in biological environments. This vulnerability necessitates the use of chemical modifications that can resist enzymatic digestion, while also improving pharmacokinetic properties.[9] Another concern is potential off-target interactions. While oligonucleotides reagents such as ASOs and aptamers are designed *in vitro* to be highly specific, they often have unintended off-target interactions within living organisms, leading to undesired consequences, including immune-stimulatory effects and cytotoxicity.[12–15] For example, Mipomersen, an FDA approved ASO drug for familial hypercholesterolemia was withdrawn from the market in 2019 due to hepatotoxicity risks.[16,17] These issues are compounded by the fact that ONs can have limited cellular uptake and endosomal escape, further restricting their therapeutic potential.[18,19]

Significant progress has been made in addressing the limitations of ON-based therapeutics using chemical modifications and, thus, has been reviewed extensively.[9,20–24] Indeed, all FDA approved ON therapeutics are at least partially modified, often containing phosphorothioate (PS) backbones and 2′-OH modifications, such as 2′-*O*-methyl (2′-*O*Me) and 2′-*O*-methoxyethyl (2′-MOE).[9] Together, these modifications have been shown to increase nuclease resistance and cellular uptake, while reducing off-target interactions and toxicity.[25–28] More recently, xeno nucleic acids (XNAs), which contain non-(deoxy)ribose sugar backbones, have emerged as attractive alternatives to traditional chemical modifications.[29,30] Common XNAs include fluoroarabino nucleic acid (FANA), locked nucleic acid (LNA), threose nucleic acid (TNA), hexitol nucleic acid (HNA), and peptide nucleic acid (PNA). Like other modified ONs, many XNAs are capable of WC base pairing to native nucleic acids

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and, thus, retain the same programmability as their native counterparts.[31–35] However, XNAs offer several additional benefits, including superior biostability, improved bioorthogonality, and increased functionality due to their expanded chemical and structural diversity.[29,36,37] In particular, the utilization of XNA in DNAzymes has shown to improve their activity and stability.[38–40] Given the advantages offered by XNAs, coupled with an expanding biochemical toolbox to support XNA research (*e.g.*, XNA polymerases),[41,42] XNAs are expected to play a major role in the development of future ON-based therapeutics.

One promising class of XNAs are L-DNA and L-RNA (*i.e.*, L-ONs), which contain L-(deoxy)ribose sugar units (Fig. 1). L-ONs are mirror images (or enantiomers) of native D-DNA and D-RNA and, as such, are intrinsically orthogonal to the stereospecific environment of native biology. In particular, L-ONs are highly resistant to degradation by cellular nucleases, providing them with superior biostability.[43,44] Unlike other chemically modified ONs and XNAs, L-ONs have the same physical and chemical properties as naturally occurring D-nucleic acids, providing an important benefit from a rational design perspective.[45–47] Moreover, studies have shown that L-ONs are less susceptible to off-target interactions with endogenous biomacromolecules due, in part, to their inability to form contiguous WC base pairs with native D-nucleic acids.[43,45,47] Clinical studies have also shown that L-ONs have very low, possibly negligible immunogenic potential.[48] Given these properties, L-ONs have emerged as a promising platform for the development of biomedical technologies, including molecular imaging tools, diagnostic biosensors, and aptamer-based therapeutics (Fig. 1).[48,49] Herein, we briefly highlight these and other recent examples that demonstrate the clinical potential of L-ONs. We then provide our perspective on remaining challenges and practical considerations currently associated with the use of L-ONs and explore potential solutions that may lead to the broader adoption of L-ONs in clinical applications.

2 Recent advances of L-oligonucleotides in clinical applications

2.1 Spiegelmers: L-aptamer therapeutics

L-Aptamers (referred to as Spiegelmers) remain the most successful clinical application of L-ONs to date. As this topic has been reviewed previously,[48–53] we provide a brief summary here for context. Aptamers are ON-based affinity reagents that are isolated through the process of SELEX (Systematic Evolution of Ligands by Exponential Enrichment)[54–56] to bind molecular targets with high affinity and selectivity. Compared to protein antibodies, aptamers have many unique advantages, including ready availability through SELEX, high chemical stability and shelf life, easy chemical modification, small size, and inexpensive cost of production.[57] These desirable properties make aptamers well-suited for a variety of biomedical applications, including diagnostic assays, drug delivery vehicles, and therapeutics.[58–61] Indeed, the FDA has recognized the therapeutic potential of aptamers through the approval of pegaptanib sodium (Macugen) for macular degeneration[62] and Avacincaptad pegol (Izervay) for the treatment of geographic atrophy secondary to age-related macular degeneration

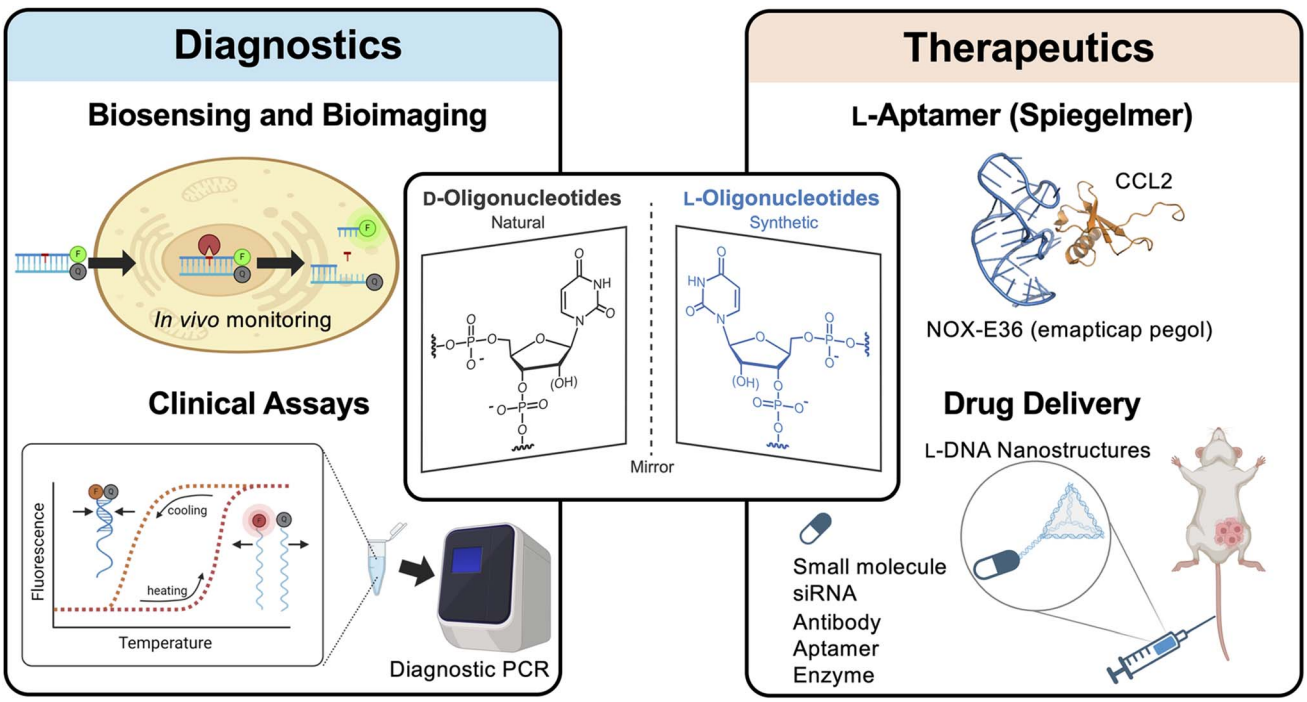

Fig. 1 L-Oligonucleotides are the enantiomer of native D-oligonucleotides. Due to their beneficial properties and general bioorthogonality, L-oligonucleotides have been employed in a variety of biomedical technologies. Some examples are shown here.

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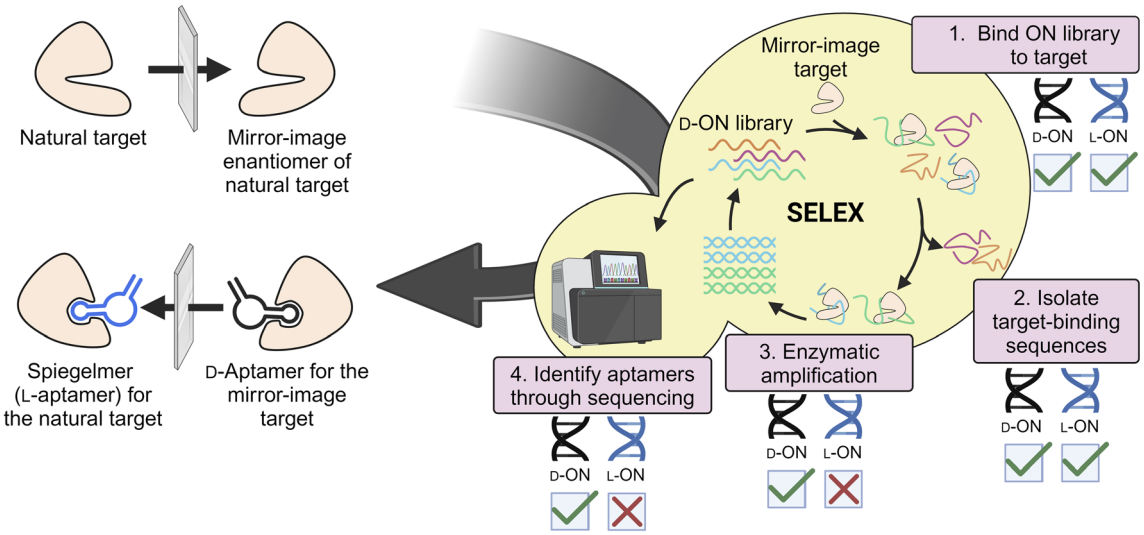

Fig. 2 Schematic depicting the "selection-reflection" process. The enantiomer of the intended target is used to select for D-ON aptamers. D-Aptamers with suitable binding properties are then synthesized in the L-form and used to bind the native target. Key steps of the SELEX process are highlighted, indicating their compatibility with L-ONs.

(AMD).[11] These approvals underscore the growing significance of aptamers as therapeutics.

Given the potential of aptamers in clinical applications, significant efforts have been made towards increasing their biostability and improving other pharmacological properties. Towards this end, L-ONs have proven to be a highly effective approach. L-ON aptamers can be generated using "mirror-image" SELEX (or "selection-reflection").[63–65] Here a D-ON library is evolved against the enantiomeric variant of the desired target enabling enzymatic amplification and sequencing of the ON library (Fig. 2). Once a suitable D-aptamer is identified, the corresponding L-aptamer is chemically synthesized and used to bind the desired, natural target. To date, Spiegelmers have been generated for a variety of targets, including small molecules, peptides, and proteins.[64–67] Like D-aptamers, Spiegelmers fold into distinct structures that bind their targets with high affinity and selectivity.[68,69] However, their inverted stereochemistry affords Spiegelmers high plasma stability and immunological passivity.[70–74] Not surprisingly, Spiegelmers have shown promise as drugs, especially for targeting small secreted proteins, such as hormones and cytokines.[48] A notable example is Olaptesed pegol (NOX-A12),[73] which targets the C-X-C motif chemokine ligand 12 (CXCL12/SDF-1) that is involved in several aspects of tumor progression, including metastasis, angiogenesis, and survival.[75] NOX-A12 is currently in phase 1/2 clinical trials as a combination therapy for hard-to-treat glioblastomas.[76] When combined with radiotherapy and the anit-VEGF antibody Bevacizumab, NOX-A12 significantly improved survival rates and reduced tumor sizes in glioblastoma patients without causing dose-limiting toxicities, indicating a strong safety and efficacy profile.[76–78] The success of NOX-A12 has shown that Spiegelmers have a promising future as therapeutics in clinical applications.

In addition to proteins and peptides, Spiegelmers have recently shown promise in targeting pharmacologically relevant nucleic acids, such as RNA. Because WC base pairing is stereospecific, Spiegelmers must instead interact with native D-ONs through tertiary interactions (or shape), much like how proteins and antibodies recognize their targets. Despite the lack of complementarity, these so-called "cross-chiral" interactions between Spiegelmers and D-ONs occur with high affinity and excellent selectivity.[79–82] This was recently exemplified by a Spiegelmer that was able to distinguish between the stem-loop II-like motif (s2m) RNA from SARS-CoV-2 and SARS-CoV-1, which differ by a single nucleotide in the target region.[79] Importantly, Spiegelmers targeting structured RNAs have been shown to modulate RNA function through several modes of action, including by blocking functional RNA–protein interactions.[81–85] For example, the Kwok lab has developed an L-RNA Spiegelmer targeting the human telomerase RNA G-quadruplex (hTERC) and demonstrated inhibition of telomerase activity in cell lysates.[84] Given current challenges associated with the discovery of molecules capable of binding disease-relevant RNA structures with high affinity and selectivity, Spiegelmers, which can be readily obtained for most RNA targets, represent a promising approach to address this technological gap.

2.2 L-Oligonucleotides as drug delivery vehicles

ONs have emerged as a promising class of drug delivery vehicles. In particular, the programmability of WC base pairs allows for the self-assembly of DNA nanostructures having precise sizes, shapes, surface chemistries, and functions. As drug delivery platforms, these properties can be easily modulated to improve the therapeutic effectiveness of drugs by enhancing their solubility, improving distribution, and promoting cellular internalization.[86,87] Towards the development of DNA nanostructure delivery platforms with improved biostability and drug efficacy, several groups have turned to L-DNA. Because D-DNA and L-DNA have identical physical properties, such as

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duplex thermostability, well-established principles for engineering self-assembling D-DNA nanostructures can be directly applied to L-DNA without further optimization, making L-DNA an ideal nucleic acid analogue from a design perspective.[45–47] The use of L-DNA to engineer self-assembled nanostructures was first demonstrated by the Yan group, who also showed that L-DNA-based nanotubes are highly resistant to nuclease degradation.[88] More recent studies have confirmed that L-DNA nanostructures resist exonuclease-mediated degradation for up to 24 hours in serum[89] and are considerably more stable in live cells compared to those constructed using D-DNA.[90,91] Subsequently, L-DNA nanostructures have been harnessed as versatile delivery platforms for small molecule drugs,[92–95] anti-proliferating aptamers,[90] enzymes,[95,96] and siRNAs.[91] Importantly, L-DNA nanostructures have been shown to be more effective in tumor-specific localization compared to nanostructures constructed from native D-DNA, 2′-*O*Me modified RNA, and 2′-fluoro modified RNA, which can be attributed to their superior serum stability, high rate of cancer cell uptake, and minimal macrophage uptake.[95] For example, when intravenously injected into HeLa tumor-bearing mice, pyramid shaped L-DNA nanostructures showed a threefold higher accumulation in tumors compared to the liver, showcasing superior relative tumor specificity compared to other reported nanoparticle delivery vehicles.[95] Taken together, L-DNA nanostructures represent a promising approach for drug delivery, especially for targeting the tumor environment, and we encourage future growth in this area.

2.3 L-Oligonucleotide-based biosensors

L-ONs also have clinical applications in the fields of sensors and diagnostics. Many L-ON-based biosensors have been created by simply inverting the stereochemistry of existing D-DNA and D-RNA designs. One prominent example of this is the molecular beacon (MB).[97] MBs are hairpin-shaped ONs with an internally quenched fluorophore that can be activated upon denaturation of the MB structure, for example, upon heating or binding to a complementary sequence. Due to their predictable design, ease of synthesis, high sensitivity, and rapid response, MBs have found broad utility in a variety of bioanalytical and biomedical applications.[98–102] Construction of MB using L-ONs offers a straightforward strategy to generate biostable and bio-orthogonal molecular sensors. For example, L-ON-based MB probes have been used to image stimuli-dependent temperature changes in live cells[103,104] and to accurately monitor melting and annealing during PCR in real-time, facilitating the development of highly sensitive and selective PCR-based diagnostic assays.[105,106] Ligand-dependent DNAzymes are also amenable to this "inversion" approach, assuming the analyte is achiral. Indeed, mirrored versions of several metal ion-dependent DNAzyme sensors have been reported,[107–109] which show dramatically improved stability and functionality compared to their D-ON counterparts when employed in live-cell imaging assays.[108]

While simply inverting the stereochemistry of known ON-based sensors allows for straightforward construction of more robust probes, this approach is limited to achiral analytes. This is due to the principle of reciprocal chiral substrate specificity,[110] which dictates that stereochemical inversion of a chiral receptor (*i.e.*, the sensor) also requires inversion of the chiral ligand. Thus, D-ON-based sensors that rely on interactions with chiral analytes, such as proteins and nucleic acids, are unable to engage these ligands upon stereochemical inversion of their backbones. To overcome this limitation, several groups have turned to "chimeric" ONs constructed from both D- and L-DNA. Here, a region of natural D-DNA is embedded within (or linked to) the L-DNA-based probe to enable its engagement with native ligands. For example, chimeric MBs have been constructed with L-DNA stems and D-DNA loops (Fig. 3a).[111] Use of L-DNA in the stem provides improved stability and reduced off-target

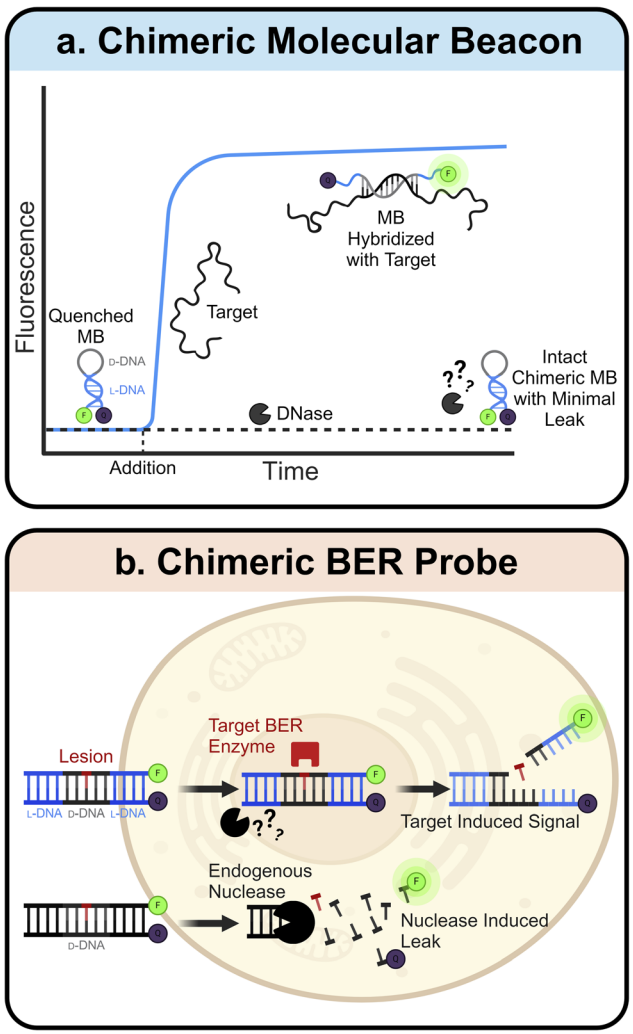

Fig. 3 Chimeric D/L-DNA probes. (a) Schematic depiction of the chimeric molecular beacon. D-DNA binding domain (grey) is protected by an L-DNA stem (blue), adding superior biostability to the existing design. Blue curve represents target induced signal increase, while the black dotted line indicates minimal leak due to resistance to nuclease degradation. (b) Schematic depiction of the chimeric BER probe. The D-DNA domain (black) containing a lesion is protected by flanking domains of L-DNA (blue), allowing for detection of target BER enzyme activity in live cells with minimal background.

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interactions, whereas use of D-DNA in the loop enables hybridization of the probe with a target nucleic acid sequence. A similar chimeric approach has been used to interface L-DNA-based fluorescence *in situ* hybridization (FISH) probes with target RNA sequences in fixed cells, enabling the sensitive imaging of endogenous mRNA and microRNA biomarkers within single cells.[112] Recently, we developed chimeric D/L-DNA probes for imaging specific DNA repair activities in living cells, demonstrating that the chimeric approach can also be used to interface L-ON-based probes with native proteins (Fig. 3b).[113] These probes consist of an L-DNA duplex containing a centrally positioned region of D-DNA with the target lesion, therefore enabling recognition by native repair enzymes. Upon recognition and repair of the lesion by the corresponding repair enzyme(s), the probe disassembles giving rise to a fluorescent signal. We showed that these chimeric probes can be used to monitor relative DNA repair activity, evaluate the efficiency of inhibitors, and study enzyme mutants in live cells. Given the involvement of DNA damage and repair pathways in human diseases and aging,[114–116] these probes should enable a broad spectrum of clinical applications.

The field of electrochemical aptamer-based (E-AB) sensors is another area where L-ONs are starting to have an impact. E-AB sensors rely on ON-based aptamers to translate the presence of a specific ligand into an electrochemical signal.[117] Although E-AB sensors have been used successfully for continuous, real-time measurement of specific molecular targets within living animals, their *in vivo* operation is limited to a few hours due, in part, to degradation of the aptamer component.[118–120] Use of L-ON-based aptamers presents a straightforward solution to this problem. Indeed, the Arroyo-Currás lab recently showed that conversion of a D-DNA aptamer into its enantiomeric form does not affect performance of E-AB sensor signalling, at least where achiral ligands are concerned.[121] Furthermore, they showed that L-DNA-based E-AB sensors are significantly more resistant to nuclease-dependent signal loss than those employing D-DNA, indicating that the use of L-ONs is a promising approach for prolonging the lifespan of invasive E-AB sensors of future clinical applications.

3 Perspectives and outlook

3.1 Overcoming the bioorthogonality of L-oligonucleotides

While the bioorthogonality of L-oligonucleotides (*e.g.*, nuclease resistance) is often touted as a key advantage of this polymer, it also represents the key weakness, which limits the types of applications that can be currently accessed and hinders the broader adoption of L-oligonucleotides into the clinic. This section will discuss recent efforts to overcome the practical challenges associated with using this bio-orthogonal polymer and our viewpoint on where future initiatives should be focused, providing a blueprint for bringing L-oligonucleotides to the forefront of biomedical technologies.

3.1.1 Expanding the L-oligonucleotide toolbox. Due to their bioorthogonality, L-oligonucleotides cannot be amplified by PCR, sequenced, labeled, or manipulated in many of the ways that native D-ONs can, representing the major hurdle impacting research and development of L-oligonucleotide therapeutics and other clinical tools. In this section, we will discuss recent efforts to expand the L-oligonucleotide toolbox, and where we believe future efforts should be focused. In particular, we will discuss what we believe are the most critical tools still needed to advance the field and how the successful development of each will benefit future clinical applications.

3.1.1.1 Polymerases. Perhaps the most glaring challenge of working with L-ONs is their incompatibility with native DNA/RNA polymerases, precluding straightforward enzymatic synthesis and amplification of L-ONs in the laboratory. In particular, the polymerase chain reaction (PCR), the cornerstone of biomedical research and medical diagnostics, is mostly unavailable to L-ON researchers, severely limiting the types of applications that can be accessed using this powerful nucleic acid analogue. Furthermore, in the absence of enzymatic methods, L-ONs are almost exclusively prepared using solid-phase phosphoramidite chemistry, which imposes a limit on the length and quality of L-ONs that can be obtained. While there is not yet an ideal solution to this problem, significant progress has been made towards the enzymatic synthesis of L-ONs.

3.1.1.1.1 Mirror-image polymerase constructed of D-amino acids. The most obvious method to resolve compatibility issues between L-ONs and natural polymerases is to also invert the chirality of enzymes. Indeed, early studies on mirror-image proteins consisting of D-amino acids demonstrated their capability to bind and process mirror-image substrates, such as the D-HIV-1 protease.[110,122,123] Thus, it is only natural that researchers considered inverting the stereochemistry of natural polymerase enzymes to facilitate L-ON synthesis. These efforts were pioneered by Zhu and coworkers, who performed the total chemical synthesis of the enantiomer of the smallest known DNA polymerase, African swine fever virus polymerase X (ASFV pol X, 20 kDa).[124] This 174-residue protein was assembled *via* native chemical ligation (NCL) of three D-peptide fragments obtained *via* solid-phase synthesis. Using this D-amino acid polymerase, the authors successfully demonstrated the template-directed polymerization of L-DNA and transcription of L-RNA for the first time.[124] Building on these results and employing more refined synthetic methods, researchers have pursued the synthesis of larger mirror-image polymerases having greater processivity, enhanced fidelity, and thermal stability. Indeed, mirror-image versions of *Sulfolobus solfataricus* P2 DNA polymerase IV (Dpo4) analogue (40.8 kDa),[125–127] *Pyrococcus furiosus* (Pfu) DNA polymerase (90 kDa),[128] and T7 RNA polymerase (100 kDa)[129] have now been reported. This progress has enabled the assembly and amplification of gene-sized L-DNA fragments and transcription of full-length ribosomal L-RNAs,[129] bringing the field one step closer to a mirror-image ribosome-based translation system. While this progress is both promising and inspiring, the chemical approaches used to synthesize large mirror-image polymerases remain highly specialized, labor intensive, costly, and have proven difficult to scale. Thus, they are not practical solutions for the average researcher, at least not yet.

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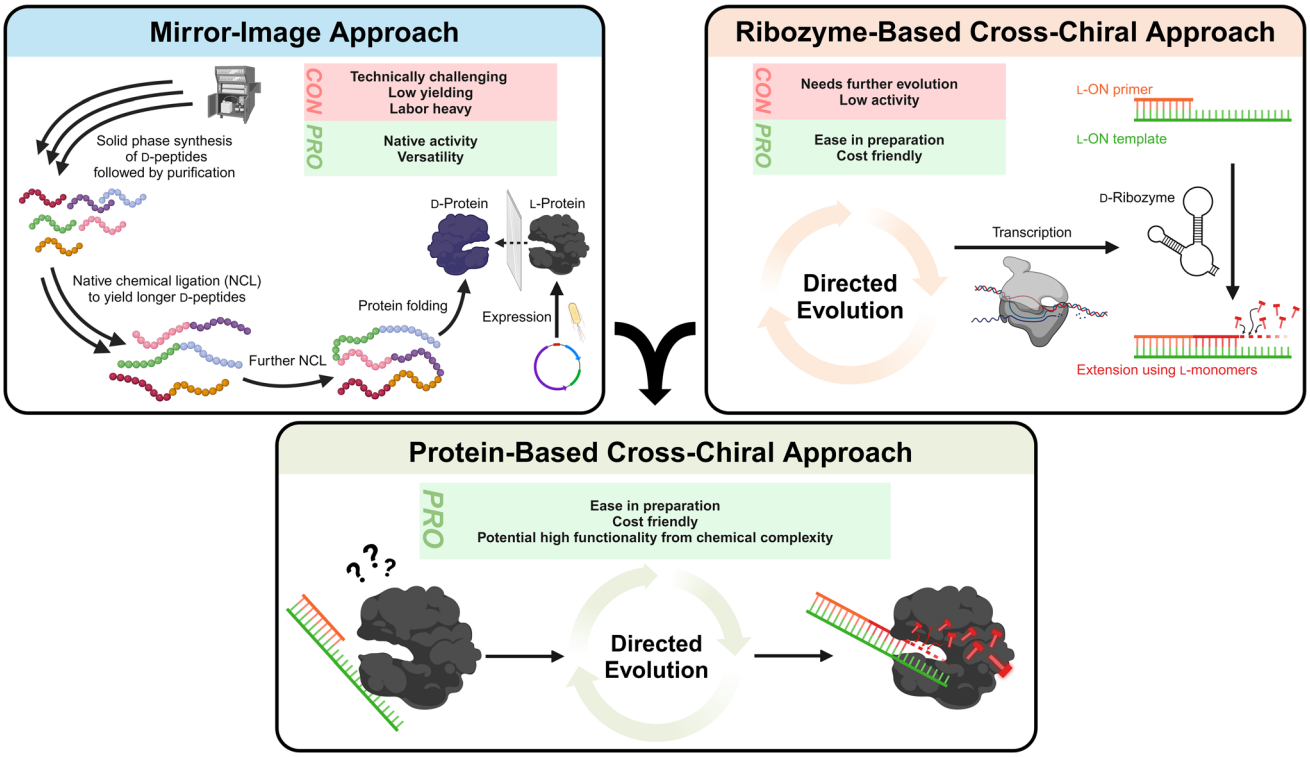

Fig. 4 Current strategies to expand the L-ON toolkit include inverting the chirality of native protein enzymes (*i.e.*, the mirror-image approach) and the development of native D-RNA ribozymes that can act directly on L-ONs (*i.e.*, the ribozyme-based cross-chiral approach). Each approach has its pros and cons. We encourage integration of these two approaches, whereby directed evolution is used to generate native protein enzymes that can act directly on L-ONs, as an alternative strategy with several practical advantages. The example shown here is for polymerases, but this idea can be applied to other enzymatic functions as well (ligases, nucleases, *etc.*).

3.1.1.1.2 Cross-chiral polymerases: a more practical approach? An alternative path for polymerization of L-ONs that warrants more attention is the use of cross-chiral enzymes, *i.e.*, enzymes composed of native polymers (D-ONs and L-amino acids) that can act directly on L-ONs (Fig. 4). Such enzymes could be readily prepared using standard biochemical and molecular biology techniques, making them much more accessible to a broader range of researchers. Indeed, some progress has been made towards this goal. In 2014, *in vitro* evolution was used to discover a cross-chiral ligase ribozyme.[130] The D-RNA enzyme was capable of joining two L-RNA stands on a complementary L-RNA template, demonstrating for the first time that cross-chiral synthesis of L-ONs is possible. Further evolution of this D-RNA enzyme aimed at generating a general and processive cross-chiral polymerase yielded variants capable of assembling long L-RNAs from a mixture of L-trinucleotide building blocks.[131,132] Furthermore, the cross-chiral polymerase ribozyme was used to perform exponential amplification of L-RNA, providing a proof-of-principle for cross-chiral ribo-PCR amplification of L-RNA. Despite its promise, however, this enzyme is still quite early in its evolutionary trajectory and therefore stills suffers from slow catalytic activity and processivity of polymerization, limiting the size of L-RNAs that it can produce. Nevertheless, given the progress that has already been made, we expect that further *in vitro* evolution could ultimately yield cross-chiral polymerase ribozymes with activity comparable to native protein enzymes. The success of the cross-chiral polymerase ribozyme also begs the question: Is cross-chiral polymerization possible for proteins of native chirality (Fig. 4)? There have been examples of some proteins that have ambidextrous operating capabilities. For example, the bacterial GroEL/ES chaperone protein can surprisingly fold a D-protein.[133] Furthermore, many polymerases have been engineered to tolerate non-native sugar backbones, including 2′-fluoroarabino nucleic acid (FANA),[134] arabino nucleic acid (ANA),[41] hexitol nucleic acid (HNA),[135] threose nucleic acid (TNA),[136–138] and phosphonomethylthreosyl nucleic acid (PMT).[139] Therefore, it is not unreasonable to expect that native polymerase could be evolved to accommodate L-ONs. The primary obstacle, however, may be the opposite (left-handed) helicity of L-ONs. This is evident in the crystal structure of Dpo4 polymerase bound to L-DNA, which shows that accommodation of L-DNA would require a major reconfiguration of the active site and DNA-binding surfaces.[140] However, as rational and high-throughput protein engineering and screening methods continue to advance,[141] it may become possible to completely invert the substrate specificity of native polymerase enzymes.

It is clear that the future of L-ONs rests, in part, on the ability to routinely synthesize and amplify L-DNA and transcribe L-RNA through enzymatic means, which will provide greater accessibility of these polymers to researchers and open the door to exciting new technologies, including those unforeseen. Routine synthesis of long L-ONs would, for example, enable construction of bioorthogonal versions of many promising riboswitch-based

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biosensors in support of bioimaging and diagnostic applications[142–144] and allow for the construction of larger, more complex L-DNA nanotechnologies with potential applications in drug delivery.[94] Interestingly, the hypothetical cross-chiral acting polymerases discussed above could be expressed in living organisms and used to genetically encode robust L-ON-based technologies (*e.g.*, biosensors) or even establish an orthogonal L-DNA/RNA replication system in the host, allowing for accelerated and continuous evolution of functional L-ONs.[145] Perhaps the area that would benefit the most (and most immediately) from routine polymerization and amplification of L-ONs is *in vitro* selection of functional L-ONs, such as Spiegelmers and L-ON-based enzymes. This is discussed in detail below (Section 3.1.2).

3.1.1.2 Ligases. Efficient ligation is also an important roadblock towards the clinical advancement of L-ONs. As with polymerases, ligases are ubiquitous in biomedical research and have important applications in medical diagnosis.[146] For example, DNA amplification by the ligase chain reaction (LCR) has emerged as powerful platform for genotyping applications, such as the detection of gene mutations and viral/bacterial pathogens.[147] In the absence of tools capable of ligating L-ONs, development of more robust versions of these technologies using L-DNA is out of reach. Furthermore, ligation represents another potential solution to the L-ON synthesis problem by allowing long L-ONs to be assembled from shorter fragments and amplified *via* mirror-image LCR.[130] Ligation also opens the door to diverse bioconjugation techniques and other ON manipulations not currently feasible using L-ONs. 3′ terminal labelling of L-RNA is one example.[148,149] In the long run, L-ON ligation could facilitate the isothermal assembly of gene-size L-DNA fragments in support of a D-amino acid protein production using mirror-image ribosomes,[150] and may prove to be essential for next-generation sequencing (NGS) of L-ONs, either for sample preparation or for the sequencing reaction itself (*e.g.*, sequencing by ligation).[151]

Realizing the need for and implications of tools that are capable of ligating L-ONs, researchers have pursued several strategies to overcome the current deficit. While purely chemical approaches, such as phosphorothioate (PS) and "click" chemistry-based ligation,[152–154] may prove useful for ligating L-ONs, we focus our attention on enzymatic approaches as the most promising pathway forward. Similar to polymerases, the Hoheisel group synthesized the D-amino acid version of the DNA-ligase of H. influenzae (D-LigA; 252 amino acids) and demonstrated efficient ligation activity on L-DNA substrates using an L-ATP cofactor.[155] They also demonstrated ligation-mediated assembly of a 300-mer double-stranded L-DNA from 18 shorter L-DNA oligonucleotides, providing a proof-of-principle for ligase-mediated assembly of gene-size L-DNAs. Although promising, this approach still relies on the synthesis of D-amino acid proteins and, as discussed above with polymerases, is not yet practical for the average researcher. With this in mind, our group developed a strategy to ligate L-ON using native protein ligases. Here, two strands of L-RNA were joined by native T4 RNA ligase by incorporating D-ribonucleotides at the ligation junction.[156] By carefully positioning these D-RNA ligation junctions at non-critical positions within the otherwise all L-RNA structure, we demonstrated that this approach could be used to assemble functional L-RNAs of considerable length, including an L-RNA version of a 124 nt theophylline biosensor that retained activity in serum. The ability to prepare nuclease resistant versions of RNA-based sensors using this straightforward approach greatly expands the utility of such technologies for applications in molecular sensing and imaging. It is important to note that L-ON ligation needs not be limited to the use of protein enzymes. As we discussed above, *in vitro* evolution was used to generate a cross-chiral D-RNA ribozyme that is capable of joining two or more strands of L-RNA. With further *in vitro* evolution to improve activity, such ribozymes should provide an attractive approach for routine L-ON ligation, as large quantities of the D-RNA enzyme can be easily generated using *in vitro* transcription. *In vitro* directed evolution could also be used to obtain cross-chiral protein ligases, either by engineering known ligases to accept L-ON substrates or by being created *de novo* using mRNA display or similar strategies.[157] Interestingly, these cross-chiral L-ON protein ligases could serve as a starting point for the generation of cross-chiral L-ON polymerase activity through further evolution, as has been demonstrated previously with ribozyme ligases.[132,158]

3.1.1.3 Nucleases. Many important biomedical applications rely on the ability to readily break phosphodiester linkages between nucleotides. Nucleases have found widespread use in biomedical applications that require precise manipulation of ONs, including restriction digestion, molecular interaction probing, and sequencing.[159–161] Programmable nucleases, such as CRISPR/Cas9, are powerful gene editing tools with broad applications for the prevention or treatment of numerous diseases.[162] As the field of L-ONs matures and the ability to amplify and replicate L-ONs becomes routine, inevitably too will the need for nucleases to support a growing list of applications. There are several purely chemical approaches that can be used to cleave L-ONs directly. For example, the group of reactions used during Maxam Gilbert sequencing allow for nucleotide-specific cleavage of DNA irrespective of stereochemistry.[163] This was recently demonstrated through the sequencing of a 55 nt long L-DNA.[164] However, the future surely lies in the development of programmable nucleases capable of cleaving any L-ON sequence with precision. One potential solution is the use of RNA- and DNA-cleaving DNAzymes.[165,166] These DNAzymes typically consist of a defined catalytic core flanked by two binding arms that can be exchanged to recognize most substrates through complementary interactions. Thus, they can be readily synthesized in their mirror-image form and programmed to cleave most L-RNA and L-DNA in a sequence-specific manner. Similarly, one can synthesize protein nucleases in their mirror-image form. In 2015, several groups reported the synthesis of mirror-image *B. amyloliquefaciens* ribonuclease (barnase) and demonstrated its nuclease activity toward L-RNA.[67,167] Moving forward, it will be important to demonstrate this approach using programable nucleases, such as Cas9 and TALENs (transcription activator-like effector nucleases). This will not be without its challenges, however, as chemical synthesis of the enantiomeric forms of these systems,

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which are at least 600 amino acids, will require a Herculean amount of effort. Instead, we again encourage the community to explore the development of cross-chiral enzymes capable of cleaving L-ONs. In particular, the successful *in vitro* evolution of a cross-chiral D-RNA ribozyme that can join L-RNA bodes well for evolving the reverse activity using similar methods.[130] Beyond expanding the L-ON toolbox, we expect that cross-chiral nucleases will have immediate clinical utility. For example, L-DNA versions of cross-chiral RNA-cleaving DNAzymes could be used in gene silencing applications by targeting endogenous mRNAs (*e.g.*, oncogenes) for degradation. This approach represents a promising strategy to improve the biostability and other pharmacological properties of DNAzyme therapeutics and warrants further attention in the future.

3.1.2 *In vitro* selection/evolution of L-oligonucleotides. The inability to directly evolve L-ONs in the laboratory represents another key bioorthogonality "problem" and, as discussed above, is a major driving force behind the development of tools to enzymatically synthesize and manipulate L-DNA/RNA. Some of the most promising clinical applications of L-ONs, including Spiegelmer therapeutics, DNAzymes, and ribozymes, rely on the use of *in vitro* evolution techniques. However, the sparce availability of L-ON polymerases precludes direct *in vitro* evolution of functional L-ONs. Instead, research must rely on indirect approaches. For example, the current repertoire of Spiegelmer therapeutics were all obtained using the "selection-reflection" approach discuss above (Fig. 2), wherein D-ON libraries are selected against the enantiomer of the target ligand.[48,63–65] While this approach has clearly been successful (See Section 2.1), it has several important limitations. In particular, this indirect method renders many targets inaccessible, as their enantiomers may be very difficult or impossible to obtain using current methods. For example, cell-SELEX, which aims to generate aptamers that can bind selectively to a cell-type of interest,[168] is not possible using selection-reflection approaches. In addition, "counter-selection" steps, which are often employed during SELEX to increase target specificity and/or minimize off-target interactions, are challenging using selection-reflection techniques.[169] For instance, cell lysates are often used in the counter selection step during SELEX for protein-binding aptamers in order to remove nonselective binders from the aptamer pool. Again, this approach is simply not practical when enantiomeric targets must be employed. These challenges further highlight the need for widely available L-ON polymerases and other biochemical tools that can facilitate the direct *in vitro* selection or evolution of functional L-ONs.

Very recently, the direct *in vitro* selection of L-aptamers using mirror-image polymerases has been reported.[170] This selection process utilized an engineered version of D-Dpo4 DNA polymerase (D-Dpo4-5m) for PCR amplification of an L-DNA library following affinity enrichment steps. The target for this selection was native human thrombin, which would be challenging to synthesize in the opposite handedness due to its large size (294 amino acids) and intensive glycosylation.[171] After nine rounds of selection, two L-DNA aptamers were identified and shown to bind native human thrombin with K_d values in the low nanomolar range. This groundbreaking study proved that, with the appropriate toolbox, the direct *in vitro* selection of L-ONs is possible. Nevertheless, this report also highlighted some of the remaining challenges. Producing useable quantities of high-fidelity mirror-image DNA polymerases, which tend to be quite large, still presents a significant synthetic challenge. Consequently, this work utilized the shorter, more synthetically tractable D-Dpo4-5m, which suffers from low fidelity and suboptimal amplification efficiency, especially for long DNA sequences, that together could hinder the amplification of rare sequences in the pool. Sequencing of enriched L-DNA pools is another technical hurdle highlighted by this work. In the absence of bacterial cloning or high-throughput L-DNA sequencing, individual L-DNA sequences from the enriched pool were isolated by denaturing gradient gel electrophoresis (DGGE), which separates different DNA sequences of similar lengths based on their different melting temperatures.[172] Individual bands were then isolated, amplified by mirror-image PCR using D-Dpo4-5m, and sequenced by the phosphorothioate approach with L-deoxynucleoside α-thiotriphosphates (L-dNTPαSs) and cleavage by 2-iodoethanol.[128] This approach is extremely labor intensive and does not guarantee single-sequence resolution, especially for sequences with similar melting temperatures. Moreover, it dictates that many rounds of selection be carried out, as sequence isolation by DGGE requires a high level of convergence within the L-DNA pools. Nevertheless, the progress already made provides confidence that these technical hurdles can be overcome. For example, the accuracy and throughput of L-DNA sequencing could be improved by adopting mirror-image versions of nanopore sequencing or massively parallel sequencing-by-synthesis techniques using the appropriate mirror-image toolbox,[173,174] thereby increasing the overall efficiency and practicality of the L-ON selection process.

Direct *in vitro* selection/evolution of L-ONs using straightforward and practical methods would open the door to a number of exciting opportunities for researchers and clinicians. For example, without the need to generate the enantiomer of the desired target, as is currently required for mirror-image SELEX, the pool of clinically relevant molecules that could be targeted by Spiegelmers is virtually limitless. Even whole cells could be targeted with relative ease to discover binders, inhibitors, and biomarkers without pre-existing knowledge of the target itself. Cell-based SELEX approaches would also benefit from the biostability of L-ON pools. Given the ability of Spiegelmers to evade biodegradation during storage and use, coupled with their convenient production, we expect that as the repertoire of Spiegelmers grows, so too will their utility in applications currently dominated by traditional aptamers and antibodies. Potential applications of Spiegelmers in nuclease-rich environments could include enzyme-linked immunosorbent assays (ELISAs), immunohistochemistry, and drug delivery (using cell-targeting Spiegelmers). Furthermore, the ability to directly isolate L-aptamer-based molecular switches that undergo a binding-induced conformational change (*e.g.*, *via* "Capture SELEX")[175] should fuel advances in the areas of biosensing and diagnostics, such as the development of more robust E-AB sensors for invasive molecular

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monitoring.[176] Beyond Spiegelmers, direct L-ON selection schemes are expected to find applications in the discovery of L-ribozymes and L-DNAzymes that are capable of catalyzing the manipulation (*e.g.*, cleaving, tagging, *etc.*) of clinically relevant proteins and nucleic acids.

3.1.3 Sequence-specific interfacing of oligonucleotide enantiomers. Since the first L-ONs were synthesized, efforts have been made to characterize their interactions with the native polymer. Although early studies reported the formation of stable complexes between homopolymers of L-DNA/RNA (*e.g.*, L-poly[A]) and their native complements, leading researchers to speculate that heterochiral base pairing was possible, later studies employing mixed sequence demonstrated that L-ONs are unable to form stable hybrids with complementary D-ONs.[43,45,47] While non-canonical interactions are possible, such cross-chiral interactions between Spiegelmers and their D-ON targets,[79–81,85] the vast majority of experimental evidence and computational modeling confirms that WC base pairing of complementary strands is stereospecific.[45,177,178] The inability to rationally design sequence-specific interactions between D- and L-ONs precludes the use of L-ONs in common hybridization-based technologies, such as antisense oligonucleotides (ASOs), molecular beacons, and DNAzymes, which would otherwise benefit from the unique properties of L-ONs. To circumvent the incompatibility of ON enantiomers, several indirect approaches for sequence-specific interfacing of D- and L-ONs have been reported. For example, our group showed that peptide nucleic acid (PNA) can be used as an intermediary between the two.[179] Unlike native DNA and RNA, PNA has no inherent chirality and hybridizes to DNA and RNA irrespective of chirality.[47] On this basis, we developed a series of toehold-mediated strand-displacement (TMSD) reactions that exploit DNA/PNA heteroduplexes in order to interface the two enantiomers of DNA in a sequence specific manner. By directly comparing D- *versus* L-ON-based TMSD reactions in cells, we showed that L-DNA-based reactions have reduced background, faster kinetics, and greater reliability inside live cells compared to their conventional D-DNA counterparts.[180] Importantly, we showed that this technology could be used to interface L-DNA-based molecular sensors with endogenous microRNA biomarkers in live human cells, laying a foundation for future clinical applications aimed at microRNA profiling.[181] An alternative strategy to interface D- and L-ONs involves the use of chimeric D/L-ONs, wherein a functional L-ON domain is directly linked to a D-ON domain for sequence specific targeting.[182,183] This approach has also been used in TMSD reaction systems to translate DNA inputs of one chirality into DNA outputs of the opposite chirality in a sequence-specific manner, thus further bridging the chirality gap.

While indirect approaches for interfacing D- and L-ON are promising, future efforts should be directed towards the establishment of a "heterochiral code" that would enable the rational design of stable heterochiral complexes in a sequence-specific manner. *In vitro* selection methods already allow for direct D- and L-ON interactions to be discovered from random sequence space.[80,81,85] With more examples, as well as structural information, patterns may emerge that point to an alternative set of "heterochiral" base pairing rules. Indeed, molecular dynamics simulations reveal numerous recurring geometric patterns that suggest potential structural motifs between heterochiral RNA strands,[177] indicating that computational modeling and machine learning could be employed for discovering novel sequence-specific interactions between the enantiomers. It is important to note that the sequence-specific interface between D- and L-ONs does not necessarily have to adhere to the traditional anti-parallel duplex model. Alternative structural arrangements, such as parallel-stranded duplexes, triplexes, and patterned mismatches, as well as the use of chemically modified nucleotides with alternative base pairing properties, should all be considered when exploring the potential for sequence-specific cross-chiral interfacing. Overall, the ability to rationally design stable heterochiral complexes in a sequence-specific manner would represent a major breakthrough in the field of L-ONs, enabling the integration of this polymer into a number of clinically relevant applications.

3.2 Biological interactions of L-oligonucleotides

If L-ONs are to be routinely employed in a clinical setting, then it is imperative that we understand how they interact with biology and their potential consequences. Indeed, extensive *in vivo* investigations into the mechanisms of action and off-target interactions of D-ONs, such as ASOs[26,184–187] and aptamers,[15,61] have greatly benefited the clinical translation of ON-based therapeutics. In contrast, despite the enormous promise of Spiegelmer therapeutics, we are still just beginning to understand how L-ONs behave in living systems, how they interact with these environments, and the potential consequences. Although careful *in vivo* pharmacological studies have been carried out on Spiegelmer therapeutics (and have been reviewed elsewhere),[48] the vast majority of these Spiegelmers have been developed against extracellular targets and, consequently, little attention has been paid to the behavior of L-ON at the intracellular level. As the field of L-ON-based therapeutics matures and shifts towards intracellular targets, it will become increasingly important to understand how L-ONs behave within the complex environment of the cell. Therefore, we focus our discussion below on the current understanding of how L-oligonucleotides behave inside cells, the known and potential off-target effects, and where we believe future efforts should be focused with an eye toward broader clinical adoption.

3.2.1 Intracellular localization of L-oligonucleotides. Regarding the intracellular localization and dynamics of L-ONs, the various L-ON-based probes and other devices that have been implemented in living cells provide some insights into these behaviors, which we attempt to summarize here (Table 1). To begin, it is clear that L-ONs and D-ONs localize differently inside cells, likely due to unique interaction profiles. For example, when transfected into human cells, double-stranded L-DNA appears to localize within the nucleus, whereas double-stranded D-DNA localizes primarily to the cytosol.[113,180] Interestingly, time course experiments show that, although D-DNA duplexes are present in the nucleus shortly after transfection, they are rapidly exported to the cytosol. In contrast, chimeric D/

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Table 1 Intracellular localization of L-ONs

Nucleic acid	Features	Delivery method	Cell line	Localization	Reference
L-RNA	Single stranded G-rich	Transfection	HeLa	Nucleoplasm, nucleolus, nuclear foci	188
	Double stranded G-rich	Transfection	HeLa	Nucleoplasm, nucleolus	188
	G-quadruplex	Transfection	HeLa	Nucleoplasm, nucleolus, nuclear foci	188
	G-quadruplex	Cell penetrating peptide	HeLa	Cytosol	189
	A-rich	Transfection	HeLa	Nucleoplasm	188
	Double stranded; mixed-sequence aptamers	Cholesterol	HeLa	Cytosol	181
L-DNA	Duplex	Transfection	HeLa	Nucleus	180
	Duplex	Direct uptake	SCC7	Cytosol	93
	Duplex; D/L chimeric	Transfection	HeLa	Nucleoplasm	113
	5′-L-DNA capped D-DNA duplex	Transfection	HeLa	Cytosol, nucleus	190
	G-quadruplex	Transfection	HeLa	Nucleoplasm	188
	ATP aptamer	Graphene oxide complex	HeLa	Cytosol	191
L-DNA nano-structure	Tetrahedron	Direct uptake	HeLa	Cytosol	92
	Pyramid, triangular prism, cube and rugby ball-like construct	Direct uptake	HeLa, RAW264.7	Cytosol	95

L-DNA duplexes composed primarily of L-DNA are strongly retained in the nucleus.[113] This phenomenon likely reflects the inability of L-DNA to interact with nuclear export proteins, such as Exportin-5,[192] and may provide an advantage for applications requiring nuclear localization. Differences in the intracellular localization of D- and L-RNA have also been observed (Table 1). For example, while both D- and L-RNA versions of 5′-r$(GGAA)_8$ localized to the nucleus following transfection, only the L-RNA version accumulated in the nucleolus.[188] This behavior was proposed to be the result of unique interactions of the L-RNA strand with nuclear paraspeckle proteins, which have been shown to translocate to the nucleolus upon binding to exogenously delivered ON reagents, such as ASOs.[184] However, nucleolar localization of L-RNA appears to be dependent on sequence and, specifically, on guanine content, as L-rA_{32} did not accumulate in the nucleolus. Not surprisingly, the intracellular localization of L-ONs also appears to be dependent on the method of delivery. In the absence of transfection reagents, double-stranded L-DNA nanostructures are internalized by endocytosis and localized mainly in the cytosol.[90,95] Cytosolic localization was also observed for a cholesterol-conjugated L-RNA probe that is uptaken into cells by receptor-mediated endocytosis.[181] Little to no nuclear localization was observed for either L-DNA or L-RNA in the absence of transfection reagents.[92,93,95,191] Taken together, these examples demonstrate that the subcellular localization and dynamics of L-ONs are distinct from D-ONs and are dependent on various factors that we are just now beginning to understand.

3.2.2 Interactions of L-oligonucleotides with endogenous biomacromolecules. It is also important to understand the interactions of L-ONs with endogenous biomolecules. The stereospecific nature of many protein–ON interactions implies that L-ON are inherently less susceptible to off-target interactions with the diverse intracellular proteome compared to their native counterparts, representing a key advantage for future clinical applications. However, mounting evidence, including the intracellular localization studies discussed above, suggests that L-ONs are still susceptible to non-specific protein interactions inside cells, and that they may be distinct from their native counterparts. Early evidence of off-target protein interactions was observed in a study comparing the behavior of the Bcl-2 targeting ASO G3139 to its enantiomer.[193] The authors showed that the basic fibroblast growth factor (bFGF) bound both enantiomers of the ASO equally well. Furthermore, both enantiomers of G3139 inhibited the voltage-dependent anion-selective channel (VDAC), resulting in loss of channel conductance. More recently, we showed that polycomb repressive complex 2 (PRC2), a promiscuous RNA binding protein with important gene regulatory functions, can bind G-rich RNA sequences irrespective of chirality, mirroring the results above.[194] Given these results, it is not unreasonable to expect that many other nucleic acid-binding proteins have the capacity to interact with L-ONs, potentially resulting in undesirable effects. Indeed, we showed that exogenously delivered L-ONs have the potential to be highly cytotoxic to human cells, with single-stranded G-rich L-RNAs being the most potent.[188] Upon transfection into human cells, cytotoxic L-RNAs were shown to form nuclear foci and accumulate in the nucleolus, consistent with their binding to nuclear paraspeckle-associated proteins, a property that has been attributed to the cytotoxic effects of other ON reagents.[184] Moreover, RNA-seq data revealed that cytotoxic L-RNA sequences induce dramatic perturbations in gene expression levels. Given that L-ONs are incapable of binding endogenous DNA and RNA through WC base pairing, these effects are most likely the result of L-RNA–protein interactions.

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3.2.3 Immunogenicity of L-oligonucleotides. Preclinical animal studies and clinical trials have shown that Spiegelmers therapeutics have very low, possibly negligible immunogenic potential.[48,70–73] Thus, L-ONs are often cited as being non-immunogenic. However, when delivered into cells, immunological effects have been observed. Specifically, the aforementioned cytotoxic G-rich L-RNAs were found to stimulate an innate immune responses and induce the production of pro-inflammatory cytokines, including (tumor necrosis factor) TNF, interleukin (IL)-1, IL-6, IL-12, and CXCL8, following transfection into HeLa cells.[188] One possible explanation for these effects is that endosomal toll-like receptors (TLRs) such as TLR3 and TLR7, which recognize pathogenic D-RNAs, also recognize L-RNA.[195] Indeed, knockdown of TLR3 and TLR7 led to a dramatic reduction in TNF expression following G-rich L-RNA treatment. The lack of immunostimulatory effects in prior studies with L-ONs[48] may reflect the sequence and/or structure dependency of these interactions and warrants further investigation. Overall, these findings suggested that pattern recognition receptors, such as TLRs, may exhibit promiscuous recognition of nucleic acids regardless of their stereochemistry, and such interactions should be considered for future clinical applications of L-ONs in cells.

3.2.4 Towards the rational design of predictable intracellular behaviors. The examples above demonstrate that L-ONs, while nuclease resistant, should not be viewed as being completely bioorthogonal and, importantly, they highlight the knowledge gaps in our understanding of how L-ONs behave in cells. Below, we outline our priorities for overcoming this knowledge gap and achieving the overarching goal of establishing a set of design principles for engineering functional L-ON, such as Spiegelmer therapeutics, with predictable intracellular behaviors (Fig. 5).

First, it will be important to further characterize how common features such as the length, sequence, structure, and sugar chemistry influence the cellular uptake and distribution of L-ONs. It will also be important to establish detailed structure toxicity/immunogenicity relationships. Such information can be used to predict toxicity from sequence and will potentially allow for toxic motifs, such as single-stranded G-rich sequences, to be eliminated at the design stage. These studies may also reveal previously unrecognized therapeutic opportunities for L-ONs. For example, a better understanding of L-ON immunogenicity could lead to the development of L-ON-based vaccine adjuvants having superior biostability and reduced off-target hybridization compared to current immunomodulatory ONs.[196]

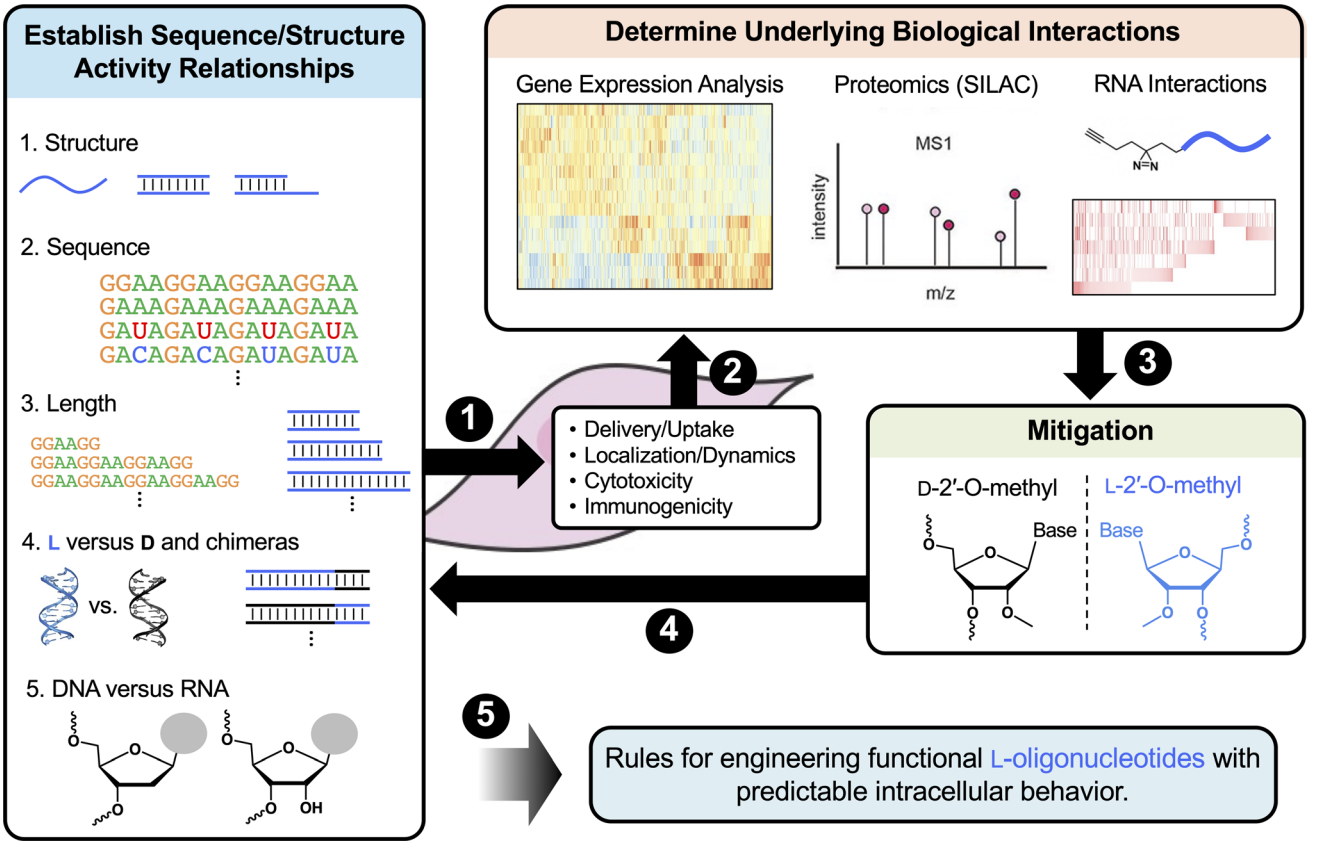

Fig. 5 Proposed workflow for characterizing the intracellular behaviors of L-ONs. (1) Determine the impact of length, sequence, structure, and sugar chemistry on cellular uptake, distribution, cytotoxicity, and immunogenicity. (2) Determine the biological interactions underlying key behaviors. (3) Use information gained from steps 1 and 2 to devise strategies to mitigate unwanted effects (*e.g.*, 2′-*O*Me to mitigate protein interactions) and verify through repeated analysis (4). (5) Collectively, these data will contribute to the establishment of rules for rationally designing L-ONs with predictable intracellular behaviors.

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Second, it is critical that we map the interactions of L-ONs with cellular proteins, especially for cytotoxic sequences. Mass spectrometry (MS)-based proteomics techniques, such as stable isotope labeling by amino acids in cell culture (SILAC),[197] have been shown to be an efficient assay for detecting interactions of D-ONs with proteins and could be readily adopted for the detection and quantitation of the L-ON–interacting proteome.[198,199] Such information will not only shed light on the mechanistic basis for observed intracellular behaviors of L-ONs, but may also inform strategies to mitigate off-target cytotoxicity. For example, studies have shown that the cytotoxicity resulting from interaction of D-ONs with nuclear paraspeckle proteins can be mitigated *via* the incorporation of 2′-*O*Me modifications.[184] If similar interactions are observed for cytotoxic L-RNAs, as proposed, it would be very interesting to test whether this mitigation strategy translates across the chiral mirror.

Third, although canonical WC base pairing is not possible between D- and L-ONs, potential interactions between them should not be overlooked. Indeed, previous studies suggest that homopolymers between D- and L-ONs can form stable complexes, particularly in the context of RNA, which has a greater propensity for binding the enantiomer of its complement than DNA.[200–202] The greater cytotoxicity found for L-RNA compared to L-DNA of the same sequence may be explained, in part, by these interactions.[188] However, further investigations are needed to determine the extent to which L-ONs interact with endogenous ONs and the consequences of these interactions, for which almost nothing is known. Cross-linking and immunoprecipitation (CLIP)-based approaches provide one potential strategy to map the interactions between L-ON and endogenous nucleic acids.[203] For example, *in situ* crosslinking of biotinylated L-ONs with endogenous nucleic acids would permit selective pulldown and identification of these interactions *via* high-throughput sequencing.

Finally, even with a comprehensive understanding of L-ON behavior in hand, intracellular applications of L-ONs, especially L-ON-based therapeutics, will require effective delivery strategies. For most research applications, ONs can be delivered into cells using common liposome-based transfection reagents, regardless of chirality. Indeed, work from our lab and others have shown that L-ONs are efficiently delivered into diverse cells lines (*e.g.*, HeLa, MCF7, A375, HEK293T) using Lipofectamine and related reagents.[113,180,188,193] While this approach is sufficient for basic research and preclinical studies, it is not ideal for most therapeutic applications. Instead, we should look to the many promising delivery systems currently being employed for D-ON-based therapeutics, such as direct conjugation to carriers (*e.g.*, peptides, lipids, receptor ligands, *etc.*) or incorporation into lipid-derived nanoparticles (LNPs), all of which function independent of the chirality of the ON.[21] Thus, we expect that they can also be applied directly to L-ONs with little optimization. For example, cholesterol conjugation, which has been used extensively to enhance siRNA delivery,[204] was shown to facilitate efficient cellular uptake of L-RNA-based molecular probes.[181] Similarly, conjugating a cell-penetrating peptide to the G-quadruplex-targeting Spiegelmer L-Apt.4-1c resulted in cellular uptake efficiencies similar to traditional transfection reagents.[189] Formulations of Spiegelmers with branched polyethyleneimine (PEI) have also been shown to permit efficient cellular delivery and targeting of intracellular proteins.[205] The ability to borrow proven delivery strategies from the D-ON therapeutics field is encouraging for the future development of therapeutic L-ONs.

4 Conclusions and outlook

Recent advancements have put a spotlight on ON-based therapeutics. The COVID-19 pandemic and the first approved mRNA vaccines have highlighted the power of nucleic acid-based therapeutic strategies.[206,207] This is further exemplified by the growing list of approved ASO drugs for diseases that have long lacked treatment options.[1,2,7] Nevertheless, native nucleic acids and their chemically modified counterparts have well-recognized limitations that L-ONs are poised to overcome. Indeed, the superior biostability and low immunogenicity of L-ONs have already been exploited to develop Spiegelmer therapeutics, with several examples advancing to clinical trials.[208] Even with the advantages offered by L-ONs, however, major challenges remain. Herein, we have provided our perspective on these challenges and explored potential solutions that we believe will pave the way toward the broader adoption of L-ONs in clinical applications:

(1) An expanded L-ON toolkit will allow for more practical synthesis and manipulation of L-ONs, while enabling powerful applications such as direct *in vitro* selection of Spiegelmer therapeutics and other functional L-ONs. Future focus: we encourage researchers to explore the development of cross-chiral enzymes, *i.e.*, enzymes composed of native polymers (D-ONs and L-amino acids) that can act directly on L-ONs (Fig. 4). Compared to mirror-image enzymes, which must be produced synthetically, cross-chiral enzymes could be readily prepared using standard biochemical and molecular biology techniques, making these tools much more accessible to a broader range of researchers.

(2) Establishment of a "heterochiral code" will enable the rational design of stable heterochiral complexes in a sequence-specific manner, facilitating the use of L-ONs in common hybridization-based technologies, such as ASOs. Future focus: *in vitro* selection methods already allow for direct D- and L-ON interactions to be discovered from random sequence space. Pairing these methods (and their datasets) with machine learning approaches may allow for prediction of heterochiral nucleic acid interactions. Such efforts will be aided by structural studies of heterochiral complexes.

(3) An improved understanding of the behavior of L-ONs inside cells will allow for the engineering of functional L-ONs, such as Spiegelmer therapeutics, with predictable intracellular behaviors, a critical step towards expanding the therapeutic potential of L-ONs. Future focus: efforts should be directed at characterizing how common features such as the length, sequence, structure, and chemical modifications impact the cellular uptake and distribution of L-ONs. Establishing protein and nucleic acid interactomes of L-ONs will provide the

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mechanistic basis for observed intracellular behaviors and inform strategies to mitigate off-target effects.

We hope that the challenges and opportunities discussed herein will inspire nucleic acid researchers and serve as a roadmap for future investigations. Indeed, given the unique advantages offered by L-ONs and the recent clinical success of Spiegelmers, we expect that interest in this area will continue to grow, bringing about unique and exciting solutions to each of the challenges discussed herein. Success in this regard will undoubtedly lead to a rapid expansion of L-ON-based technologies with tremendous potential to advance biomedical research and improve patient care.

Data availability

No primary research results, software or code have been included and no new data were generated or analysed as part of this review.

Author contributions

Victoria Shearer: writing – original draft, writing – review & editing. Chen-Hsu Yu: writing – original draft, writing – review & editing. Xuan Han: writing – original draft, writing – review & editing. Jonathan Sczepanski: writing – original draft, writing – review & editing, supervision, project administration, funding acquisition.

Conflicts of interest

The authors declare no competing financial interests.

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