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Mirror-Image Oligonucleotides: History and Emerging Applications

Brian E. Young^{a,†}, Nandini Kundu^{a,†}, and Jonathan T. Sczepanski^{a,*}

^aDepartment of Chemistry, Texas A&M University, College Station, Texas 77843, United States

Abstract

As chiral molecules, naturally occurring D-oligonucleotides have enantiomers, L-DNA and L-RNA, which are comprised of L-(deoxy)ribose sugars. These mirror-image oligonucleotides have the same physical and chemical properties as their native D-counterparts, yet are highly orthogonal to the stereospecific environment of biology. Consequently, L-oligonucleotides are resistant to nuclease degradation and many of the off-target interactions that plague traditional D-oligonucleotide-based technologies, making them ideal for biomedical applications. Despite a flurry of interest during the early 1990s, the inability of D- and L-oligonucleotides to form contiguous Watson–Crick base pairs with each other ultimately led to the perception that L-oligonucleotides have only limited utility. Recently, however, scientists have begun to uncover novel strategies to harness the bio-orthogonality of L-oligonucleotides, while overcoming (and even exploiting) their inability to Watson–Crick base pair with the natural polymer. In this review, we present a brief history of L-oligonucleotide research and discuss emerging L-oligonucleotide-based technologies, as well as their applications in research and therapy.

Graphical Abstract

*Corresponding Author: jon.sczepanski@chem.tamu.edu.

[†]B.E.Y and N.K. contributed equally

L-oligonucleotides (L-DNA and L-RNA) are enantiomers of native D-oligonucleotides. As such, L-oligonucleotides are orthogonal to the stereospecific environment of biology, making them ideal for use in biomedical applications. In this review, we discuss emerging applications of L-oligonucleotides, focusing on examples that illustrate how the unique properties of L-oligonucleotides can be exploited in order to obtain novel functionality, especially in biological contexts.

Graphical Abstract

Since the early 1990s, mirror image L-oligonucleotides have been pursued as potential tools for the development of bio-inert nucleic acid-based technologies. This review covers a brief historical perspective and emerging applications of L-oligonucleotides in research and therapy.

Keywords

Mirror-image oligonucleotides; L-DNA; L-RNA; spiegelmer; SELEX; heterochiral; modified oligonucleotide; L-(deoxy)ribose

Introduction

Oligonucleotides (ONs) are incredibly versatile biopolymers that have emerged as powerful materials for basic research and clinical applications. ONs can be easily programmed to interact with endogenous nucleic acids through simple Watson–Crick (WC) base pairing, which serves as the basis for a variety of exciting biotechnologies, including molecular beacons, fluorescent *in situ* hybridization (FISH) probes, and antisense therapeutic agents. In addition, the well characterized properties and predictable behaviors of ONs have allowed for the rational design of complex nanoscale structures and devices, giving rise to the burgeoning field of DNA nanotechnology.^[1] Not surprisingly, many of the most promising applications of ONs require that they operate within complex biological environments, for example, within living cells. However, exogenously delivered ONs have a half-life on the order of minutes and are susceptible to unintended interactions with endogenous macromolecules, making them poorly suited for such environments.^[2] In order to overcome these limitations, researchers have developed a variety of chemically modified nucleotide analogs, including 2'-*O*-methyl ribonucleotides, locked nucleic acids (LNAs), and phosphorothioate (PS) linkages.^[3] Although these modified nucleotides can dramatically increase the intracellular stability of ONs, and in some cases reduce off-target interactions, they often have a profound impact on the hybridization properties, thus undermining the perceived simplicity of nucleic acid programmability.^[4] Furthermore, some chemical modifications can result in increased toxicity and immunogenicity *in vivo*, making their introduction into existing ON systems unfavorable.^[5]

In contrast, an ideal nucleic acid modification is expected to have absolute biostability, low toxicity and low immunogenicity, while maintaining compatibility with our existing understanding of nucleic acid thermodynamics. Although designing an ON analogue that meets these criteria may seem very challenging, the solution may only require looking into the mirror. As chiral molecules, native D-DNA and D-RNA have enantiomers, referred to as L-DNA and L-RNA. As enantiomers, L-ONs have identical physical properties in terms of solubility, hybridization kinetics, and duplex thermal stability as D-ONs, yet are completely orthogonal to the stereospecific environment of native biology. Consequently, L-ONs have been shown to be resistant to nuclease degradation, are nontoxic, and nonimmunogenic.^[6] Moreover, many design principles originally established for D-ON-based technologies can be directly applied to L-ONs without further optimization. Together, these properties seemingly make L-ONs an ideal polymer for applications in biological environments. However, D- and L-ONs are incapable of forming contiguous WC base pairs with each other.^[7] Because this property precluded the rational design of hybridization-based tools and reagents, L-ONs were initially perceived to have limited utility, especially in biomedical fields. Unfortunately, this perception briefly abated research interest into these unnatural polymers. However, the last two decades have seen a resurgence in L-ON research (Figure 1), due in part to the commercial availability of the L-phosphoramidite building blocks, resulting in the development of many promising L-ON-based technologies. In this review, we summarize the history of L-ONs and discuss emerging L-ON-based technologies and their applications in research and therapy. In particular, we highlight notable examples that illustrate how the

unique properties of L-ONs can be exploited in order to obtain novel functionality, especially in biological contexts.

L-Oligonucleotides: A Historical Perspective

The origins of L-ON research can be traced back to the 1930s, when advancements in synthetic sugar chemistry made it possible to access L-ribose and L-deoxyribose, which are otherwise unavailable in nature.^[8] Synthetically, L-ribose is most easily obtained by either chemical or enzymatic transformations of a variety of naturally abundant sugars (Figure 2).^[9] Most chemical methods employ complicated, multi-step synthetic strategies that are plagued by poor yields and unwanted by-products.^[10] Recent improvements involving the use of metal complexes, such as molybdate-based catalysts, avoid extensive protection/deprotection steps and have been shown to be suitable for the large-scale production of L-ribose.^[9i, 11] However, for commercial purposes, the biotransformation of either D-glucose or L-arabinose into L-ribose using either natural or engineered strains of microbial enzymes has proven to be the most viable route.^[9d, 9h] Following similar synthetic routes, L-deoxyribose can be obtained from naturally abundant sugars, with or without the use of L-ribose as an intermediate.^[9c, 9f, 12] Keeping pace with the growing interest in L-ribose from both the academic and clinical communities since the 1990s, the demand for L-ribose has increased to several metric tons per year in 2008.^[13]

L-nucleosides can be easily obtained from L-ribose using traditional chemical methods.^[14] In 1964, two independent research groups reported the first synthesis of an L-nucleoside, L-adenosine.^[9a, 15] Both groups utilized a common chloromercuri route,^[16] whereby the mercury(II) salt of adenine was used to catalyze the nucleophilic displacement of a halogen substituent from the anomeric carbon of the protected L-ribose sugar. In one example, contamination resulting from the presence of D-ribose in the starting material was selectively removed from the product mixture using a bacterial cell suspension, allowing for the isolation of enantiomerically pure L-adenosine.^[15] Importantly, this was one of the earliest demonstrations of enzymatic stereoselectivity towards nucleic acid substrates. Since these early studies, the growing availability of L-ribose, along with the development of better synthetic methodologies, has greatly expanded the repertoire of L-nucleosides (and their derivatives), many of which have found clinical utility as antiviral agents.^[9b, 17]

Early studies revealed that L-nucleosides interact with achiral environments in an identical manner as their natural counterparts, demonstrating identical spectroscopic properties.^[18] However, in the chiral environment of circularly polarized light (i.e. by circular dichroism spectroscopy), D- and L-nucleosides could be easily distinguished. Additional experiments also revealed the incredible nuclease resistance of L-nucleosides.^[15, 19] For example, 2',3'-cyclic phosphates of L-ribonucleosides were found to be completely resistant towards degradation by a variety of ribonucleases under conditions which were sufficient for degradation of the corresponding D-ribonucleosides.^[19] Motivated by the possibility of developing biocompatible L-ON-based antisense therapeutics, significant efforts were subsequently devoted towards the synthesis and characterization of L-ONs and their complexes. As expected, it was shown that homochiral helices comprised of two complementary L-ONs were left-handed and retained similar dynamic transitions and

strength of hydrogen bonding interactions as identical right-handed helices comprised of D-ONs.^[6a, 20] However, efforts to characterize heterochiral interactions between two ONs of opposite stereochemistry (D versus L) were less clear, and even controversial. Early modeling studies by Ashley suggested that the loosely wound A-form helix of RNA might facilitate formation of heterochiral complexes.^[6b] Indeed, several groups reported the formation of specific interactions between homopolymers of L-DNA/RNA (e.g. L-poly[rA] and L-poly[dA]) and their complementary natural nucleic acids, although the resulting heterochiral complexes were consistently less stable than the natural ones.^[6b, 21] These initial observations led some researchers to speculate that L-ONs were indeed capable of recognizing complementary natural DNA and RNA. However, this hypothesis was later refuted by Garbesi and co-workers, who showed that mixed-sequence L-DNAs containing all four bases were unable to hybridize to complementary D-DNA or D-RNA molecules.^[7]

Today, it is generally accepted that WC base pairing of complementary strands is strictly stereospecific.^[22] Because this precludes the use of L-ONs in hybridization-dependent technologies, such as antisense or RNA interference, L-ONs were once perceived to have little practical utility. However, researchers quickly realized that this property, coupled with the superior bio-stability of L-ONs, could be exploited in order to develop a variety of promising, bio-orthogonal L-ON-based technologies for applications in research and medicine. Recent notable examples of these technologies are discussed in the following sections.

Spiegelmers

Perhaps the most successful application of L-ONs to date has been the discovery and development of aptamers comprised entirely of L-DNA and/or L-RNA, referred to as *spiegelmers*).^[23] Aptamers are nucleic acid polymers that are capable of recognizing, with high specificity and affinity, a broad range of ligands,^[24] and are readily obtained through the iterative process of *in vitro* selection or SELEX (systematic evolution of ligands by exponential enrichment).^[25] As illustrated in Figure 1, *spiegelmers* and their related publications comprise nearly two thirds of all citations regarding L-ONs. Consequently, numerous reviews have addressed this class of L-ONs, and they will be discussed here only briefly.^[26]

Spiegelmers are initially selected as traditional D-aptamers against the enantiomer of the target ligand, which enables enzymatic amplification of the D-ONs during the process of *in vitro* selection. This is referred to as “mirror-image” *in vitro* selection (or “selection–reflection”, Figure 3).^[27] Once a suitable D-aptamer is identified, the corresponding L-aptamer (i.e. *spiegelmer*) is chemically synthesized and used to bind the desired, natural target. Following the rules of chiral symmetry originally put forward by Pasteur and Fischer in the 1900s,^[28] the *spiegelmer* must bind to the native target with the same affinity with which the D-aptamer binds to the mirror-image selection target.

Spiegelmers enjoy all the benefits of a bio-inert L-ribose backbone modification, while maintaining a high degree of specificity for their natural target. To date, *spiegelmers* have been evolved to bind a variety of targets, including small molecules, peptides, and proteins.^[29] In fact, Olea *et al* successfully identified an L-RNA *spiegelmer* capable of inhibiting the

ribonuclease barnase, highlighting the incredible resistance of L-RNA to nuclease degradation.^[30] Spiegelmers have been shown to retain high affinity for their targets *in vivo*, while being nontoxic and nonimmunogenic,^[6c, 26a, 26b, 26e] all highly desirable properties for therapeutic applications. Indeed, several spiegelmer therapeutics are currently undergoing clinical trials, with several drugs having completed phase I or II clinical trials in the US.^[26e]

Beyond their clinical utility as therapeutics, Spiegelmers have also been used to develop bioassays for the detection of clinically relevant analytes within complex biological mixtures, opening the door for future diagnostic applications. Early examples of such assays used immobilized Spiegelmers for the electrochemical and colorimetric detection of the growth hormone ghrelin.^[31] More recently, Yi *et al* employed Spiegelmers as affinity probes in order to develop a noncompetitive solution-phase assay for the detection of glucagon and amylin peptides.^[32] Capillary electrophoresis was employed to resolve spiegelmer-peptide complexes from excess spiegelmer in solution, allowing for the detection of hormone secretion from islets. Chovelon *et al* developed an alternative solution-phase detection method based on the loop-loop interaction of two RNA hairpins, referred to as a aptamer kissing complex (AKC).^[33] They engineered a previously identified spiegelmer against the arginine-vasopressin (AVP) hormone such that binding of AVP by the spiegelmer led to the formation of an AKC that could be detected by fluorescence anisotropy.

Although numerous Spiegelmers against peptides and proteins have been discovered, the ability to isolate Spiegelmers against nucleic acid targets remains largely unexplored. This is despite the unique opportunity for Spiegelmers to bind nucleic acid through novel, WC-independent interactions. Previous work by the groups of Toulmé and Famulok showed that *in vitro* selection techniques could be used to isolate D-RNA aptamers against native D-RNA targets.^[34] Importantly, however, these studies showed that selection experiments employing ON libraries and targets having the same chirality ultimately succumb to the tyranny of WC base pairing, resulting in the isolation of aptamers that bind the target preferentially through WC base-pairing interactions. In 2013, Szczepanski *et al* reported isolation of the first spiegelmer capable of binding to a native D-RNA target, the HIV-1 trans-activation response (TAR) element.^[35] Unlike all previous D-aptamers, this spiegelmer (6-4t; Figure 4) had no discernible complementarity to the nucleic acid target, suggesting that Spiegelmers are potentially capable of binding structured D-RNAs through tertiary interactions (or shape) rather than sequence complementarity. This was only possible due to the inability of ONs of opposite chirality to form contiguous WC base pairs with each other, which encouraged the evolution of sequence-independent binding motifs during *in vitro* selection. Consequently, spiegelmer 6-4t was shown to be highly sensitive to subtle differences between RNA sequences, and less susceptible to off-target interactions due to its inability to WC base pair with native nucleic acids. Importantly, binding of 6-4t to TAR RNA inhibited formation of the Tat-TAR ribonucleoprotein complex that is required for TAR function, demonstrating the ability of Spiegelmers to modulate RNA function. The above properties, coupled with the intrinsic nuclease resistance of L-oligonucleotides, suggest that Spiegelmers hold great promise as novel RNA-targeted therapeutics. Since this initial study, several additional RNA-binding Spiegelmers have been reported, including the first spiegelmer to be evolved using chemically modified L-nucleotides.^[36]

Applications of L-ONs in Molecular Biology

Outside of spiegelmers, many of the first reported applications of L-ONs sought to exploit their strict bio-orthogonality in order to expand the scope and versatility of traditional molecular biology assays. In one example, Hauser *et al* developed a novel universal microarray platform comprised of L-DNA “ZIP-codes” spotted on a glass slide (Figure 5a).^[6d] The array was used in conjunction with a chimeric probe consisting of a D-DNA region complementary to a target gene, for example, and an L-DNA region complementary to the ZIP-code array. In this way, specific genomic sequences were immobilized on the array for subsequent analysis using various techniques. For example, this approach was used to detect single-nucleotide polymorphisms and protein-DNA interactions (Figure 5a), both independently and simultaneously on the same microarray slide. The use of L-DNA ZIP-codes ensured that there was no risk of cross-hybridization between the microarray positioning ONs and the D-DNA sample, significantly reducing the background compared to traditional D-DNA-based microarrays. Furthermore, L-DNA ZIP-code arrays were shown to be completely resistant to nuclease degradation. Therefore, in addition to handling advantages, L-DNA ZIP-code arrays allow for prolonged incubation and capture with direct sample analysis within complex biological environments.

In a second example, Adams *et al* reported the use of an L-DNA-based hybridization sensor for real-time optimization of temperature settings during a PCR reaction, referred to as “adaptive PCR” (Figure 5b).^[37] Here, L-DNA analogs of both the target duplex and the template-primer complex were added directly into the PCR reaction. Each of the L-DNA analogs were designed to produce a unique fluorescent response during temperature cycling. As the PCR reaction was heated (melting step), the L-DNA target analog would denature, producing a fluorescent signal that indicated the corresponding D-DNA target duplex was fully denatured. Likewise, as the sample was cooled (annealing step), the L-DNA primer analog would anneal to the L-DNA template resulting in fluorescence quenching, indicating that the corresponding D-DNA primer had also annealed to the D-DNA template. Because the fluorescent analogs are comprised of bio-inert L-DNA, they can be added directly to the PCR sample in order to monitor, in real-time, when optimal primer annealing and target melting have been achieved without interfering with the amplification reaction.

L-ON-Based Biosensors

One of the most common biosensors is the molecular beacon (MB). In general, MBs are hairpin shaped ONs with an internally quenched fluorophore whose fluorescence is restored upon denaturation, for example, upon binding to a complementary target ON. Given their relatively simple design, MBs can be easily prepared using L-nucleotides in order to generate bio-stable molecular sensors. Ke *et al* used this approach to prepare an L-DNA MB capable of detecting temperature changes in real-time based on thermal denaturation (Figure 6a). This so called “molecular thermometer” was shown to be compatible with live-cell imaging, enabling the real-time detection of temperature changes based on various external stimuli.^[38] Chimeric MBs comprised of both D- and L-DNA have also been reported.^[39] Here, only the stem domain of the MB hairpin was comprised of L-DNA, whereas the loop domain of the hairpin (which is required for target recognition) was retained in the D-configuration.

This MB design takes advantage of the superior bio-stability of L-DNA, while still maintaining the ability to target native D-ONs.

The use of ligand-dependent deoxyribozymes (or DNAzymes) represents another common biosensing approach that can benefit from the intrinsic resistance of L-ONs to nuclease degradation. RNA-cleaving DNAzymes are of particular interest for biosensor development due to their fast reaction rate and because RNA cleavage, which can be made dependent on a specific analyte, can be easily converted into a detectable signal via the routine installation of fluorophore-quencher pairs (Figure 6b). Importantly, many DNAzyme-based sensors have been engineered to detect achiral analytes, which are compatible with both enantiomers of the sensor. Therefore, by simply preparing the sensor using L-ONs rather than D-ONs, a bio-stable version can be produced without further optimization. This approach was recently used to develop bio-stable DNAzyme-based sensors for the detection of both Cu²⁺ and Pb²⁺.^[40] Not surprisingly, both L-ON-based sensors had dramatically improved stability and functionality in complex biological matrixes compared to their D-counterparts, enabling reliable monitoring of metal ion concentrations within living cells.

L-ONs for the Detection and Separation of Chiral Analytes

Researchers have also begun to explore the use of L-ONs for the detection and separation of chiral analytes. Taking advantage of the chiral substrate specificity of ON aptamers, Feagin *et al* developed a rapid platform for measuring the enantiopurity of the small molecule tyrosinamide (Tym, Figure 7a).^[41] They prepared both D- and L-DNA versions of an aptamer-based biosensor specific for Tym. These sensors function through a “structure-switching” mechanism, whereby a ligand-induced conformational change of the aptamer domain triggers the dissociation of a quencher ON, and thus, fluorescent activation. Because Tym is a chiral molecule, each enantiomer of the sensor is only able to detect a specific enantiomer of the target (i.e. the D-DNA sensor detects L-Tym and L-DNA sensor detects D-Tym). By labeling the D- and L-DNA sensors with orthogonal fluorophores, the authors showed that the sensors could be added together to a mixture of the target, enabling the concentration of each enantiomer of Tym to be determined simultaneously. This approach was successfully used to determine the enantiomeric distribution of the Tym following a variety of synthetic conditions. Expanding this methodology to the detection of other chiral small molecules may be useful for the rapid screening of enantiomeric purity, which is an important consideration in many industrial applications.

L-ONs have also been applied to chiral separations. Efficient separation of enantiomers is often difficult to achieve, since the two molecules are chemically identical other than the inverted stereochemistry. The most common method to accomplish this is the use of chiral stationary phases (CSPs) during HPLC. CSPs are generally prepared by coupling a single enantiomer of a suitable chiral compound to the surface of a chromatography support. Because two enantiomers of a chiral analyte will interact differently with the single enantiomer of the CSP, they will elute from the column at different times. In particular, researchers have utilized existing D-RNA aptamers as CSP components for the chromatographic separation of chiral analytes.^[42] This approach takes advantage of the chiral substrate specificity of ON aptamers in order to enrich only a single enantiomer of the

target molecule. Aptamer-based CSPs can be easily prepared by immobilizing a biotin-labeled RNA aptamer to a streptavidin conjugated chromatography support. However, initial attempts to utilize aptamer-based CSPs were hampered by the rapid degradation of the aptamer, which were invariably comprised of natural D-ONs. For example, Brumby *et al* showed that an aptamer-based CSP designed to separate the enantiomers of arginine lost approximately 65% of its target retention capacity after only 8 days of storage.^[43] To overcome this limitation, several groups turned to the use of L-aptamers as CSPs.^[44] This approach dramatically improves the stability of aptamer-based CSPs, while maintaining specificity for the analyte. Only the order in which each enantiomer of the target molecule is eluted has changed (Figure 7b). This approach allows for the straightforward development of robust aptamer-based CSPs, enabling the uniform and repeatable separation of traditionally difficult to separate mixtures.

Applications of L-ONs in DNA Nanotechnology

The unparalleled precision and programmability of DNA hybridization has enabled the rational design and construction of discrete DNA nanostructures in a bottom-up manner. Despite the relative infancy of this field, DNA nanostructures have already demonstrated a myriad of uses in applications ranging from basic research to medicine.^[1a] Given the increasing appreciation of DNA nanostructures, especially in the development of biomedical technologies, it is not surprising that researchers are now beginning to explore the use of L-DNA in their construction. Obvious advantages include increased resistance to nuclease degradation and reduced non-specific interaction, which may aid to further broaden the utility of DNA nanostructures. Importantly, as enantiomers, well-established principles for designing D-DNA nanostructures can be directly applied to L-DNA without further optimization. However, the inability to prepare L-DNA by enzymatic polymerization restricts the size of building blocks that can be obtained, potentially limiting the types of structures that can be assembled using L-DNA. Nevertheless, Lin *et al* were able to demonstrate that a series of short L-DNA ONs could reliably self-assemble into a various structures, including canonical 4-way junctions, extended L-DNA nanotubes, and large two-dimensional arrays (Figure 8a).^[45] In all examples, the L-DNA structures were physically and chemically identical to their native D-DNA counterparts under achiral conditions. Similar observations were made by Simmons *et al*, who reported the self-assembly of D- and L-DNA porous crystals from a series of short ONs.^[46] L-DNA tetrahedrons have also been reported.^[47] This work was motivated by previous observations that D-DNA tetrahedrons can facilitate delivery of molecular cargo into various cell types without the aid of traditional transfection reagents.^[48] Therefore, DNA tetrahedrons comprised of bio-inert L-DNA were assumed to provide a superior delivery platform. Indeed, L-tetrahedrons were shown to be virtually indestructible in serum, which in turn resulted in improved cellular uptake and pharmacokinetics *in vivo* compared to natural D-tetrahedrons. Furthermore, doxorubicin (DOX)-loaded L-tetrahedrons were shown to promote tumor-selective delivery of DOX and improve survival rates in tumor-bearing mice, demonstrating a promising application of L-DNA nanostructures in cancer therapy. Building on this work, a number of additional mirror-DNA structures have been developed for the delivery of small molecules and proteins.^[49]

Very recently, our group reported a novel methodology for sequence-specifically interfacing orthogonal D- and L-ONs, enabling for the first time development of DNA-based nanotechnology having fully-interfaced D- and L-ON components.^[50] Our approach takes advantage of peptide nucleic acids (PNA), which unlike native DNA and RNA, has no inherent chirality. As a result, PNA hybridizes to DNA and RNA irrespective of chirality. Based on this property, we designed and tested two toehold-mediated strand-displacement schemes that exploit a DNA:PNA heteroduplex in order to sequence-specifically interface the two enantiomers of DNA (Figure 8b). These reactions were then used to construct a series of “heterochiral” DNA strand-displacement circuits, including an L-DNA-based circuit capable of detecting D-microRNAs. Importantly, this work provides the foundation for interfacing bio-inert L-DNA-based circuits and other devices with living cells and organisms for exciting applications in bioengineering, synthetic biology and clinical diagnostics.

Summary and Outlook

Despite initial setbacks due to the inability of L-ONs to hybridize to native D-ONs, which precluded their use as antisense reagents, a series of technological advancements have now proven the utility of the L-ONs elsewhere: (1) Mirror-image *in vitro* selection has enabled the discovery of Spiegelmer therapeutics, (2) L-ON-based biosensors improve detection capabilities in biological matrixes, and (3) DNA nanotechnologies constructed using L-ONs pave the way for next-generation devices with a myriad of potential biomedical applications.

Currently, L-ONs can only be prepared using standard solid-phase phosphoramidite chemistry, potentially limiting the types of applications that can be accessed using this powerful nucleic acid analog. Even with improved synthetic methods, however, L-ONs still cannot be amplified by PCR, sequenced, labeled, or manipulated in many of the ways native D-ONs can. This represents a major hurdle impacting the adoption of L-ONs in every day research. For example, the inability to replicate L-DNA and L-RNA requires that L-aptamers be initially selected as D-aptamers against the enantiomer of the target molecule during SELEX. This severely limits the utility of this approach because it is difficult or even impossible to prepare the enantiomer of many of the most promising aptamer targets (e.g. proteins and complex natural products). To address these issues, several groups have begun to explore alternative strategies to manipulate L-ONs enzymatically. In 2014, Szczepanski *et al* reported an RNA enzyme (or ribozyme) that catalyzes the ligation and polymerization of RNA of the opposite chirality.^[51] The D-RNA version of this “cross-chiral” ribozyme was shown to catalyze the ligation of two or more L-RNAs, allowing for the assembly of long L-RNAs from a series of short, synthetically accessible fragments. Beyond ribozymes, there has been substantial progress made recently regarding the chemical synthesis of mirror-image D-peptides and D-proteins. Similar to L-ONs, D-proteins are comprised of mirror-image building blocks (i.e. D-amino acids), and thus, utilize the enantiomer of the native substrate. In a landmark study, Wang *et al* successfully synthesized the entire D-version of the African swine fever virus polymerase X (174 amino acids), which they used to enzymatically transcribe L-RNA for the first time.^[52] More recently, two research groups independently synthesized the D-amino acid version of DNA polymerase IV (D-Dpo4, 358 amino acids) using two different synthetic routes.^[53] The mirror-image polymerase was shown to transcribe and PCR amplify L-RNA and L-DNA, respectively, using synthetic L-

DNA templates. As researchers' ability to synthesize D-enzymes improves, we can expect a dramatic increase in the throughput and efficiency of L-ON assembly and manipulation. Importantly, these advances will bring us one step closer to direct *in vitro* selection of L-ONs, opening the door to the discovery of mirror-image aptamers and L-(deoxy)ribozymes that recognize a vast number of previously inaccessible chiral targets. Success in this regard would undoubtedly lead to a rapid expansion of L-ON-based technologies and increase the presence of L-ONs in the fields of clinical and biomedical chemistry.

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Biographical Sketches

Brian Young was born in Atlanta, Georgia (USA) in 1989. He received his B.Sc. in chemistry and biology in 2012 from the University of North Georgia. He is currently pursuing his Ph.D. at Texas A&M University under the supervision of Dr. Jonathan Sczepanski. His current project focuses on the design and characterization of heterochiral nucleic acid technologies.

Nandini Kundu was born in Kolkata, West Bengal, India. She received her B.Sc in Chemistry from Presidency College (University of Calcutta) in 2013. She then joined the Indian Institute of Technology, Kharagpur for her M.Sc. degree in Chemistry. She is currently enrolled in the Ph.D. program in Chemistry at Texas A&M University, where she is working with the Sczepanski group. Her current project involves the development of novel cross-chiral ribozymes.

Dr. Sczepanski was born in Stephen, MN, and received his B.Sc. in chemistry from the University of Minnesota in 2005. He received his Ph.D. from Johns Hopkins University in

2010, before joining The Scripps Research Institute as a postdoctoral research associate. In 2015, he began his independent career at Texas A&M University, where he is currently an Assistant Professor of Chemistry. Dr. Szczepanski's research interests lie in the design of L-oligonucleotide-based technologies and the development of chemical biology approaches to study chromatin-related processes.

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Figure 1. Timeline showing significant milestones leading to the modern era of L-ON-based technologies. Number of citations per year referencing L-ON (blue) or spiegelmers only (orange) is depicted between the years 2001 and 2018. Data queried from Web of Science™.

Figure 2.

L-Ribose can be obtained from a variety of naturally abundant sugars. References for each transformation are given in the figure.

Figure 3.

Mirror-image *in vitro* selection is used to generate spiegelmers against chiral targets. Initially, the enantiomer of the intended target is used to identify a D-aptamer from a random library of D-ONs. Once a suitable D-aptamer is identified, the L-version is chemically synthesized and used to bind the native form of the target molecule.

Figure 4.

Sequences and secondary structures of D-TAR RNA and 6-4t (spiegelmer). Red shading is used to indicate protection against hydrolytic cleavage under mild alkaline conditions (pH 8.5) in the aptamer-TAR complex compared to either molecule in isolation, suggesting a potential involvement of those residues in binding interactions. Increasing red intensity corresponds to increasing levels of protection. Boxes indicate residues having increased susceptibility to hydrolysis in the complex. Reprinted with permission from reference [35]. Copyright 2013 American Chemical Society.

Figure 5.

Applications of L-ONs in microbiology assays. (a) Representative ZIP-code array assay for detecting DNA-protein interactions. Target genes are amplified using a chimeric primer to give double-stranded DNA products containing a single-stranded L-DNA tag that is complementary to ZIP-codes on the array. The amplified DNA products are then immobilized onto the array via the bio-orthogonal ZIP-codes and protein-DNA interactions are assayed using fluorescently labeled proteins. (b) An orthogonal L-DNA probe set for real-time optimization of PCR cycling temperature (i.e. “adaptive PCR”). L-DNA analogs of

both the primer-template complex and product allow real-time monitoring of the denaturation and annealing steps via orthogonal fluorescent responses. Reprinted with permission from reference [37]. Copyright 2017 American Chemical Society.

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Figure 6.

L-ON-based biosensors. (a) Principle of the L-MB thermometer. Reprinted with permission from reference [38]. Copyright 2012 American Chemical Society (b) Sequence and secondary structure of the Pb^{2+} -dependent RNA-cleaving (rA) DNAzyme biosensor. Because the analyte (Pb^{2+}) is achiral, both D- and L-DNA versions of the biosensor function identically. Reproduced from reference [40b] with permission from The Royal Society of Chemistry.

Figure 7.

Use of L-ONs for chiral analysis and separations (a) Mirror-image structure-switching aptamer sensors allow for simultaneous monitoring of D- and L-Tym in solution. Binding of the ligand results in folding of the corresponding aptamer (dotted line), resulting in release of the quencher oligonucleotide and a fluorescent signal. (b) Comparison of chromatograms demonstrating the separation of a racemic mixture of arginine using a CSP comprised of either D-RNA (left) or L-RNA (right) arginine-specific aptamers. Reprinted with permission from reference [43]. Copyright 2005 American Chemical Society.

Figure 8.

L-ONs in DNA nanotechnology. (a) Self-assembly and characterization of DNA nanotubes comprised of D-DNA and L-DNA. Lower panel shows atomic force microscopy (AFM) images of L- (left column) and D- (right column) DNA nanotubes. Defects on the tubes (arrows) observed by AFM reveal that D-DNA and L-DNA nanotubes exhibit opposite supramolecular helicity. Reprinted with permission from reference [45]. Copyright 2009 American Chemical Society. (b) Example of a heterochiral strand-displacement reaction. DNA is depicted as lines with half arrows denoting the 3' end and an asterisk indicating complementarity between sequence domains. The toehold domain (t^*) resides on the achiral PNA strand in the L-DNA:PNA heteroduplex (Complex A). Therefore, the D-Input can still

bind to Complex A (via t and t^*) and displace the L-Output. In this way, a D-ON input can generate an L-ON output in a sequence-specific manner.