Transient Cyclic Structured Oligonucleotide Designs for Therapeutic Applications

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Oligonucleotide-based therapeutics are now widely used in clinical settings. From the late 1980s to the mid-1990s, efforts to improve therapeutic efficacy focused on imparting drug-like properties to oligonucleotides, emphasizing nuclease stability and target sequence affinity. These efforts resulted in the standard gapmer design for RNase H–mediated antisense and the prevalent use of chemical modification such as phosphorothioate and 2′-substituted oligoribonucleotides in oligonucleotide therapeutics. Progress made in the antisense field also enabled the development of splice-modulating oligonucleotide therapeutics and later siRNA therapies. All three modes of action are now widely employed in >25 approved drugs. Since then, we have learned that oligonucleotides and their chemical modifications can interact with pattern recognition receptors as well as various other proteins. This can have both positive and negative effects, such as aiding in oligonucleotide delivery or activating the intracellular innate immune system. My current work aims to optimize the drug-like properties of oligonucleotides by combining the early chemical advances with the more recent insights into off-target protein binding. The present article describes how this resulted in several different cyclic structured oligonucleotide designs, in which 3′ and 5′ ends are transiently held together via Watson-Crick base pairing. The transient nature of these cyclic structures protects the functional parts of the structure against nucleases during delivery and cell entry while allowing effective release of the oligonucleotide drug into the intracellular environment. These cyclic designs demonstrate significant improvements in potency and specificity over gapmer antisense and are broadly applicable to potentially all types of RNA therapeutics, irrespective of their mechanism of action.

Keywords: circular structure • cyclic structure • delivery • nuclease resistance • nucleic acids • oligonucleotide therapeutics

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INTRODUCTION

Early problems with oligonucleotide drugs were largely overcome by medicinal chemistry, optimizing chemical modifications to enhance nuclease stability and affinity. However, some solutions came with additional problems, such as unspecific protein binding and innate immune activation. New cyclic structured oligonucleotide (CSO) designs presented here overcome these problems and show increased efficacy compared to gapmer antisense. Three distinct designs that differ in

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how transient circularization is achieved are discussed in more detail, along with their *in vitro* activity relative to conventional antisense oligonucleotides (ASOs).

Nucleic Acid Medicinal Chemistry as the Main Driver of Recent Advances

As of November 2025, >25 oligonucleotide-based treatments, including antisense, siRNAs, and splice-switching therapeutics, have been approved by the FDA. The number of clinical successes in the field is rapidly increasing: the majority of these approvals have occurred in the last 7 years, with nearly half of those in the last 3 years alone. How has this rapid progress come about? Clearly, major challenges in the field have been overcome to achieve this.

Following the demonstration of the DNA double helix structure, many chemists became interested in the chemical composition of nucleic acids, synthesis and biochemical studies, and which nucleic acid modifications were naturally present or could be made. A summary of the evolution of oligonucleotide synthesis by Daniel M. Brown covers these developments exhaustively (Brown, 1993). During this time, modifications such as 2′-fluoro (2′-F) and 2′-O-methyl (2′-OMe), phosphorothioate (PS) internucleotide linkage (Eckstein, 1966) and their synthesis were described in the literature, mostly without any therapeutic application in mind (Fig. 1). Early efforts, by necessity, focused on solving fundamental problems with the synthesis of nucleic acids to have enough material for biochemical studies.

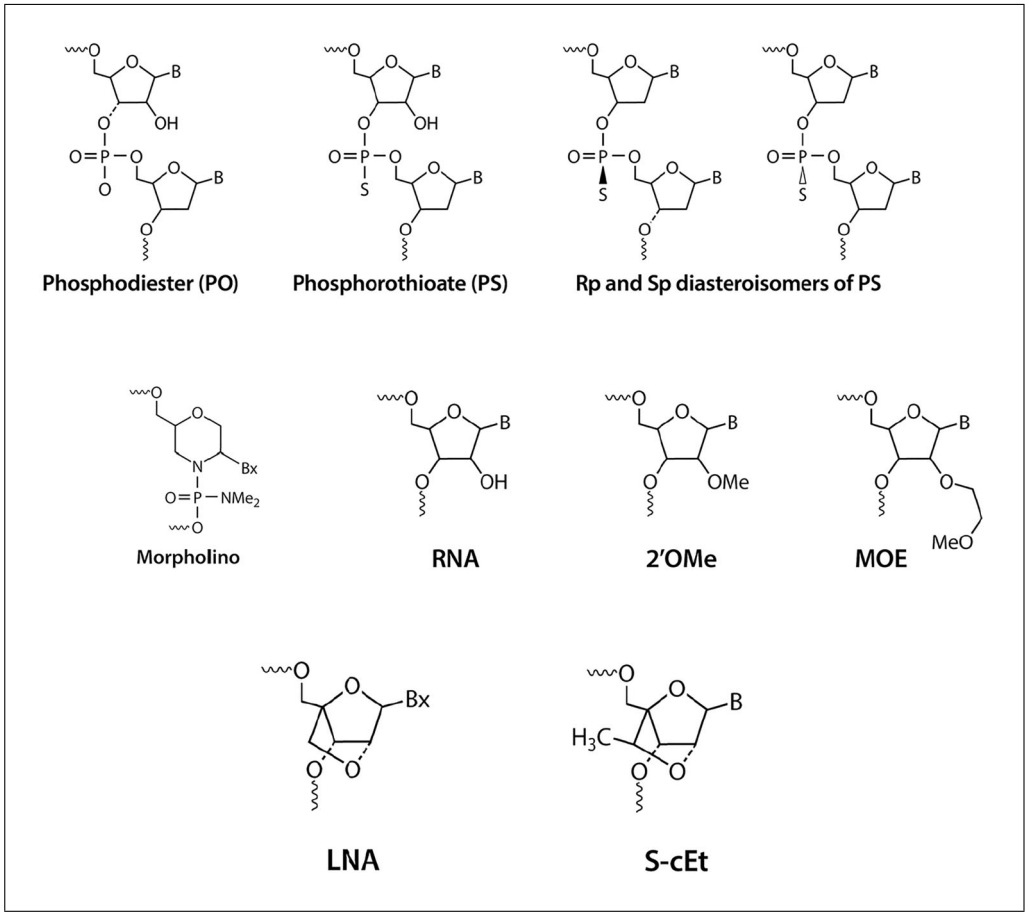

Figure 1 Chemical modifications of oligonucleotides. A phosphodiester (PO) nucleic acid backbone may be modified to phosphorothioate (PS) by replacing a non-bridging oxygen in the phosphate with sulfur. This creates a chiral center that may be in either the Rp or Sp orientation. In phosphorodiamidate morpholino oligonucleotides (PMOs), the sugar rings are replaced by methylenemorpholine rings linked via phosphorodiamidate groups instead of phosphates. In ribose sugar modifications, various groups are added to the 2′ hydroxyl of the ribose sugar, for example, a methyl for 2′-O-methylated RNA (2′-OMe) or a methoxyethyl group for 2′MOE. For locked nucleic acids (LNAs), a methylene bridge is added between the 2′ oxygen and 4′ carbon of the ribose sugar, "locking" it into a rigid 3′-endo conformation that is similar to A-form RNA. Constrained ethyl (S-cEt) is a structural analog of LNA that uses an ethylene bridge instead of methylene for the same reason.

Then, in 1978, Stephenson and Zamecnik (1978a, 1978b) demonstrated that a synthetic oligonucleotide could bind to a complementary viral sequence and consequently inhibit viral replication. Synthesizing such oligonucleotides was an incredibly time-consuming manual process at the time, so research progressed slowly until automated oligonucleotide synthesizers became available in the late 1980s.

Overcoming nuclease susceptibility

After joining Paul Zamecnik in 1987, my first task was to enhance the nuclease stability of oligonucleotides. The first modification we studied was PS (Agrawal et al., 1988), as it was known that this increased nuclease resistance compared to phosphodiester (PO) backbones (Spitzer & Eckstein, 1988). Together, these publications launched the field of medicinal chemistry to improve antisense efficacy, demonstrating that nuclease resistance was directly correlated with higher antisense activity. We showed that duplexes between PS DNA oligonucleotides and cellular RNAs activated RNase H, further providing insights into increased potency (Agrawal et al., 1990).

Numerous reviews discuss PS and other modifications in detail (Agrawal, 2021b, 2024a; Gait & Agrawal, 2022), so these will not be discussed further. However, in the case of PS, it is necessary to point out that this backbone modification randomly presents as one of two stereoisomers, either Rp or Sp (Fig. 1). Therefore, PS containing oligonucleotides is a complex mix of 2^N possible diastereomers, where N equals the number of PS linkages. Cellular nucleases, including RNase H, have a specific recognition profile for each isomer, and thus, many attempts have been made to elucidate specific combinations of isomers that would maximize oligonucleotide efficacy (Yu, Kandimalla, et al., 2000).

It is also important to note that some backbone modifications, such as PS, do not change the inherent negative charge of nucleic acid backbones, whereas others neutralize it. Negative charges on nucleic acids can result in binding to a variety of extracellular and intracellular proteins, which is useful in some respects and detrimental in others, as discussed below. Charge-neutral backbones such methyl phosphonate (Agrawal et al., 1988) and phosphorodiamidate morpholino oligomer (PMO) do not facilitate such protein binding, though they are highly resistant to nuclease attack (Fig. 1).

Increasing target sequence affinity

Chemical modifications have also addressed another key issue for nucleic acid therapeutics: target sequence affinity. Once the idea took root that chemical modifications could help overcome limitations of nucleic acid therapeutics, the hunt was on to find additional useful ones. Attention soon turned to modifications of the 2′-hydroxyl group (OH) on the ribose, as 2′-OMe-modified oligonucleotides hybridized to native target sequences showed improved thermodynamic stability compared to completely native duplexes. However, the antisense potency of 2′-OMe-modified oligonucleotides was significantly reduced compared to PS DNA oligonucleotides, as the RNA/RNA duplex was not recognized by RNase H. Other widely used 2′ modifications were 2′-F and 2′-O-methoxyethyl (2′-O-MOE) (Fig. 1).

Thus, 2′ modifications cannot be used within the RNase H protein footprint when RNase H–based RNA degradation is the intended mechanism of action. Indeed, 2′-OH modifications, especially when combined with PS linkages, improve resistance to other nucleases. This led us to the design of mixed-backbone ASOs that combine PS DNA regions, enabling RNase H recognition, with 2′-OH-modified sections for increased affinity and nuclease stability (Agrawal, 1992; Metelev & Agrawal, 2002). The most common format for mixed-backbone oligonucleotides, also referred to as gapmer, consists of a central 10-nucleotide-long PS DNA "gap" with 3-5 2′-OH-modified PS nucleotide "wings" on both the 5′ and 3′ ends. Other formats are possible, however.

Obviously, such placement restrictions do not apply to oligonucleotides used in occupancy-mediated antisense mechanisms, for example, splice-switching oligonucleotides (Sierakowska et al., 1996; Wilton et al., 1999), or those used to inhibit or enhance translation. For many years, 2′-OH modifications were thought of as all-around winners, with increased affinity, nuclease protection, and reduced immune activation all in one package for most of them. Consequently, research focused on identifying 2′-OH modifications that further enhanced these characteristics, ultimately leading to the development of locked nucleic acid (LNA) and 2′-S-constrained ethyl (cEt) modifications (Fig. 1). Unfortunately, both modifications were associated with increased cytotoxicity and hepatotoxicity, with the latter being

significantly more severe for LNAs (Burel et al., 2016). Some toxicity was suggested for 2′-F, though contradictory reports have been published (Janas et al., 2019; Shen et al., 2018). There appears to be a connection between increased affinity (2′F < cEt < LNA) and severity of the toxicity (2′F < cEt < LNA), though of course the number of particular modifications within the oligonucleotide therapeutic and the administered dose will influence this. However, use of all three modifications is now much more limited and deliberate.

Improving cell-specific delivery

Another significant hurdle for nucleic acid therapeutics was (and to a large degree still is) not just tissue-specific but also cell type–specific delivery within target tissues. Although ASOs can readily enter mammalian cells due to their small size, systemically administered oligonucleotides are rapidly cleared from circulation by glomerular filtration. Very little partitions into tissues during that limited timeframe. Minimal, noneffective amounts enter the liver, kidney, and spleen, largely ending up in sinusoidal endothelial and Kupffer cells in the liver. Distribution to other tissues is ineffective and generally only occurs when the liver, kidney, and spleen have reached a saturation point.

Here again, PS backbone modifications showed an unexpected benefit: replacement of the smaller oxygen atom with the larger sulfur results in a wider spread of the negative charge. This facilitates much higher levels of protein binding than seen with PO oligonucleotides, provided there are a minimum of 10 to 12 PS modifications. Such binding, particularly to plasma proteins, enhances plasma half-life from mere minutes to an hour, therefore increasing delivery to tissues (Agrawal et al., 1991). Charge-neutral backbones such as PMOs do not exhibit such protein binding and are therefore rapidly filtered out, so considerably higher therapeutic doses are required for effective treatment.

Localized delivery of PS/2′-OH-modified oligonucleotides to confined tissue compartments such as the intrathecal space is clinically effective and leads to broad distribution in the brain (albeit with a focus on regions closer to the cerebrospinal fluid), as demonstrated by the success of nusinersen (Spinraza) and other gapmers targeting neurological diseases (Bennett et al., 2021). However, the intrathecal injection procedure can only be performed by specially trained medical professionals.

Targeted delivery remained a big challenge for quite some time, but as with the stability and affinity issues, medicinal chemistry provided a solution. In 2014, two reports described that conjugation of the hepatocyte-specific asialoglycoprotein receptor (ASGPR) ligand tri-antennary *N*-acetyl galactosamine (GalNAc) to antisense gapmers and siRNAs significantly improved selective delivery to hepatocytes (Nair et al., 2014; Prakash et al., 2014). This improved potency to such a degree that it allows delivery via subcutaneous injection that can be self-administered by patients.

The impact of GalNAc conjugation on the success of oligonucleotide therapeutics cannot be overstated: nearly all recently approved treatments employ this delivery modality. The only exceptions are those that are delivered intrathecally and splice-switching oligonucleotides for the treatment of Duchenne muscular dystrophy, which require delivery to muscle. The hunt for similarly effective targeted delivery ligands for other cell types has been a very active area of research over the last 10 years. Some potential applications, such as antibodies targeting Tfr for the delivery of oligonucleotides to muscle tissues, are in clinical trials and showing promise (Malecova et al., 2023).

Beyond stability and hybridization

Although these three main problems have largely been solved by medicinal chemistry—with the caveat that delivery to non-hepatic and non-neuronal tissues remains challenging—other less obvious issues have come to light. Indeed, in some cases, the solution to one of the main problems turned out to have some detrimental side effects.

As already discussed, the ubiquitously used PS modification increases binding to a broad array of extra- and intracellular proteins. Although such binding increases plasma half-life and therefore delivery, it can also cause significant thrombocytopenia, transient or sporadic acute reductions of platelet counts, and activation of the complement cascade, resulting in hemodynamic changes in non-human primates (Agrawal et al., 1995; Galbraith et al., 1994). These effects are dependent on dose and infusion rate.

Upon entry into the cells, oligonucleotides, depending on the sequence and modifications, can be recognized as pathogen-associated molecular patterns (PAMPs) and modulate immune responses by binding to pattern recognition receptors (PRRs). Most PRRs for nucleic acids are located intracellularly, in

endosomes and the cytoplasm. The PS modification on ASOs shows increased activation of the innate immune response compared to PO backbones. Detailed structure-activity relationship studies have illuminated the roles of sequence, secondary structures, modifications, and nucleotide motifs; their position within sequences; and flanking sequence context (Agrawal & Kandimalla, 2019).

During these studies, we made a surprising observation: accessibility of the 5′ end of the oligonucleotide is required for immune activation. Oligonucleotides containing two available 5′ ends (3′-3′ linked) showed significantly increased immune-stimulatory activity, whereas 5′-5′ linked oligonucleotides had minimal immune-stimulatory activity (Bhagat et al., 2011; Yu, Zhao, et al., 2000; Yu et al., 2002). ASOs lacking accessible 3′ ends (3′-3′ linked) also showed reduced potency, as opposed to increased potency with 5′-5′ linked antisense (Jiang et al., 1999). Thus, immune activation is due to not solely PS protein binding but also the accessibility of the oligonucleotide ends; for antisense, 3′-end accessibility is optimal (Bhagat et al., 2011).

Medicinal chemistry has been the major driver for overcoming these issues

Clearly, nucleic acid medicinal chemistry has been the main driver of the rapid advances in the field. We now know how to mix and match different chemical modifications within the same sequence to optimize desired oligonucleotide characteristics and accommodate specific modes of action. We understand the relationship between oligonucleotide sequence, particular chemical modifications, and their exact location within the sequence and can determine specific target sequences with the highest probability of resulting in effective ASOs. However, most of the commonly used chemical modifications in the current crop of therapeutic oligonucleotides were introduced into the antisense field in the mid-1990s. These include gapmer antisense for RNA knockdown (Agrawal et al., 1990; Agrawal, 1992) and 2′-substituted PS RNA for splice modulation (Sierakowska et al., 1996).

Have we reached the limits of the explorable chemical space for viable chemical modifications that can be used in antisense? How can we further optimize the oligonucleotide drug profile when so many chemical changes have already been explored and discarded? Does the massively improved delivery achieved with GalNAc, and hopefully other specific ligands in the near future, now allow use of chemistries that were previously unsuccessful due to their lack of systemic delivery? Do we need to move beyond medicinal chemistry and reconsider the basic structure of ASOs to better integrate all the knowledge we now have?

Beyond Medicinal Chemistry: Spatial Structures

As discussed, during previous studies, two ASOs linked via their 5′ ends showed minimal immune stimulation combined with increased antisense efficacy. However, when we originally investigated these constructs, systemic delivery was problematic because the linked constructs were twice as long, in linear format, as a standard antisense oligonucleotide and resulted in increased protein binding. The two accessible 3′ ends on these 5′-5′ linked constructs, however, were a problem because exonucleases largely attack the exposed 3′ end of ASOs.

The standard approach for blocking exonucleases is, of course, the use of 2′ modifications, but a feasible alternative to transiently hide the nuclease-susceptible 3′ ends was desirable. This was achieved by designing CSOs (Agrawal, 2023) (Fig. 2A). CSOs are composed of two domains, a functional domain and a circularizing domain, linked via their 5′ ends. The circularizing domain is complementary to the 3′ end of the functional domain and, upon intramolecular hybridization, will form a cyclic structure, thereby protecting the 3′ end. Such a circularizing domain need not be chemically modified, but the required transient nature of the hybridization necessitates careful selection of the length, nucleotide composition, and chemical modifications of the hybridizing sequences.

The length of the circularizing domain depends on the nucleotide sequence of the 3′ end of the functional domain and whether it is composed of PO DNA or PO RNA, given that DNA has a lower melting temperature.

For optimal formation and stability of the circularizing/functional domain duplex, the duplex melting temperature should exceed physiological temperature but not approach the melting temperature of the duplex between the functional domain and its intended target sequence. Thus, the melting temperature of the circularizing/functional domain duplex should be a minimum of 42°C but not exceed 55°C because the melting temperature of the duplex between the functional domain and target sequence will be about 60° to 75°C. If the composition of the circularizing/functional

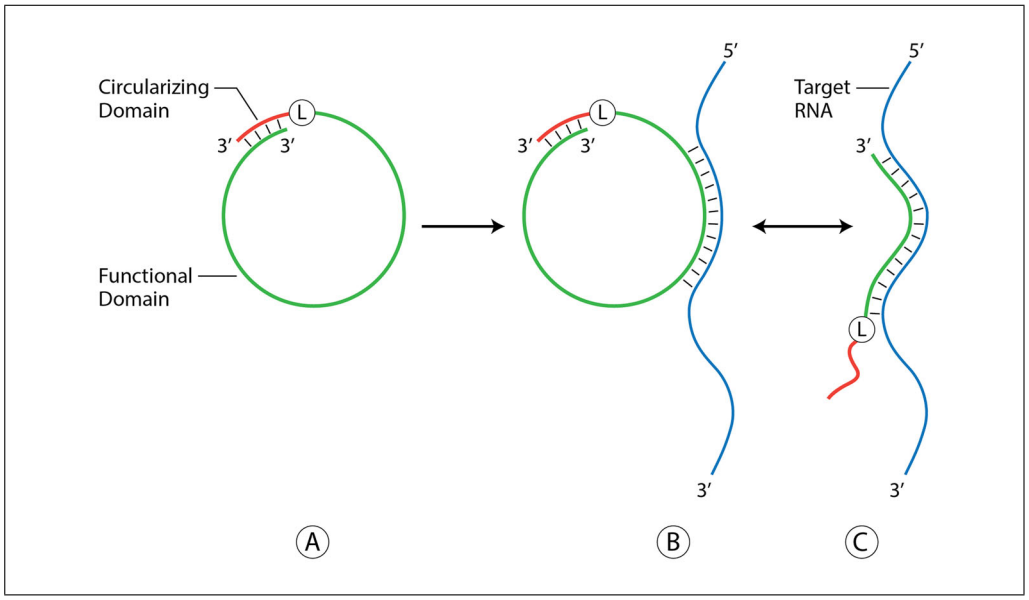

Figure 2 Design of a cyclic structured oligonucleotide (CSO). This design features two main segments: the functional domain (green) and a circularizing domain (red). The two domains are connected via a 5′-5′ linkage (L), resulting in two accessible 3′ ends. The circularizing domain is complementary to the 3′ end of the functional domain and, upon hybridization, forms a cyclic oligonucleotide structure (**A**). When a single-stranded region of the functional domain hybridizes with the target RNA (**B**), the circular structure destabilizes and opens (**C**).

domain duplex is GC rich and an RNA/DNA hybrid, then the length of the circularizing domain may be 5 to 6 base pairs. However, if it is AT rich and a DNA/DNA hybrid, the circularizing domain may require eight or more base pairs. It should be self-evident that the sequences of both the circularizing and functional domains must be carefully selected to avoid formation of hairpins as well as self-dimers and heterodimers other than the intended duplex formation between them. Hybridization of the circularizing domain with the functional domain must result in a more stable structure than any other conceivable intra- or intermolecular structures that may arise from any partial sequence complementarities.

Formation and integrity of the CSO structure can easily be determined using well-established techniques such as melting temperature or non-denaturing polyacrylamide gel electrophoresis. On such gels, migration of hybridized cyclic structures is expected to be faster than that of the unhybridized, and therefore linear; circularizing; and functional domains due to the duplex region and reduced overall charges.

If these guidelines are followed, the affinity between the circularizing and functional domains will easily be overcome when the functional domain binds to the target sequence, as the affinity will be higher (Fig. 2B and 2C). A functional consequence is that CSOs exhibit very high target sequence specificity, enabling them to distinguish target sequences with a single-nucleotide polymorphism from the wild-type more readily than gapmers.

CSOs also have many other advantages: the shape changes the spread of the negative charges on PS modifications and thus reduces PS-associated protein binding. Under physiological conditions, the cyclic structure remains intact during delivery and cell entry (Fig. 3A). Hiding the 5′ ends avoids their exposure to cellular PRRs and thus prevents activation of the innate immune response. Therefore, the fate of CSOs in the endosomal compartment and subsequent escape from endosomes will differ from that of linear oligonucleotides.

Once the CSOs have entered the cytoplasm (Fig. 3B) or nucleus (Fig. 3C) and hybridize to their target sequence (Fig. 3D and 3E), the circularizing domain is released from the self-hybridization with the functional domain. As the circularizing domain does not contain any chemical modifications, it is then susceptible to removal by cellular exonucleases. Other factors that may contribute to the significantly improved efficacy of this design over gapmers (Fig. 4) include enhanced cellular uptake and optimized RNase H kinetics and specificity. In the CSO sequence studied in Figure 4, the length of the circularizing domain is six base pairs. As discussed above, cyclic structure

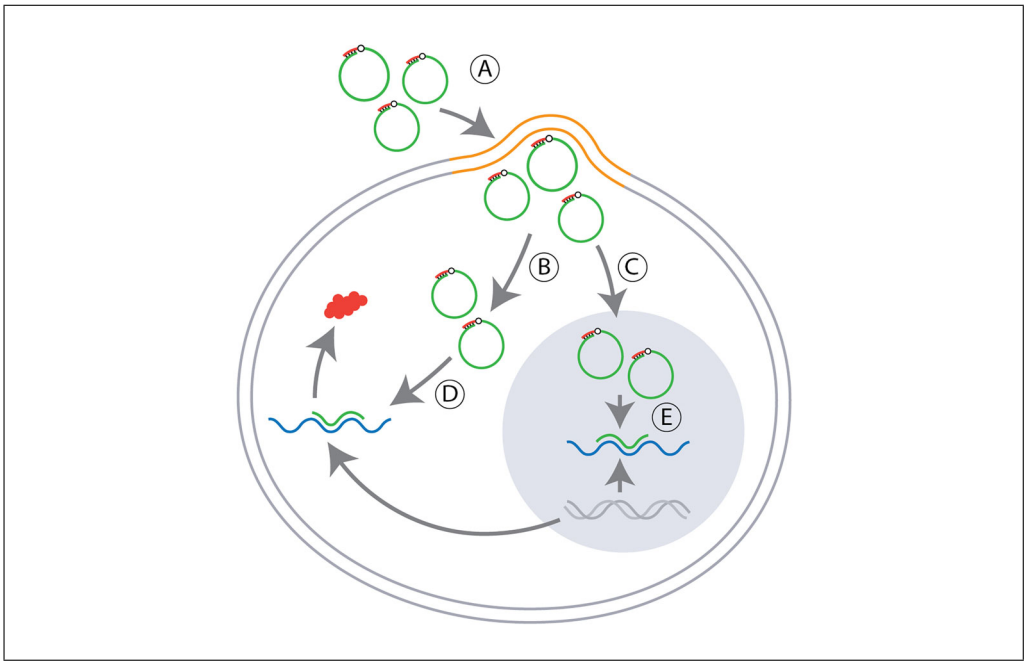

Figure 3 Delivery and intracellular fate of cyclic structured oligonucleotides (CSOs). This structure remains stable under physiological conditions, depending on the length of the circularizing domain and the nucleotide base composition (**A**). Once inside the cell, due to the circular structure and absence of accessible 5′ ends, its fate in the endosome will differ from that of linear oligonucleotides. After entering the cytoplasm (**B**) or nucleus (**C**), the cyclic structure will open upon hybridization with the targeted RNA (**D** and **E**, respectively). Following linearization and hybridization to the target sequence, the circularization domain may gradually be degraded by cellular exonucleases.

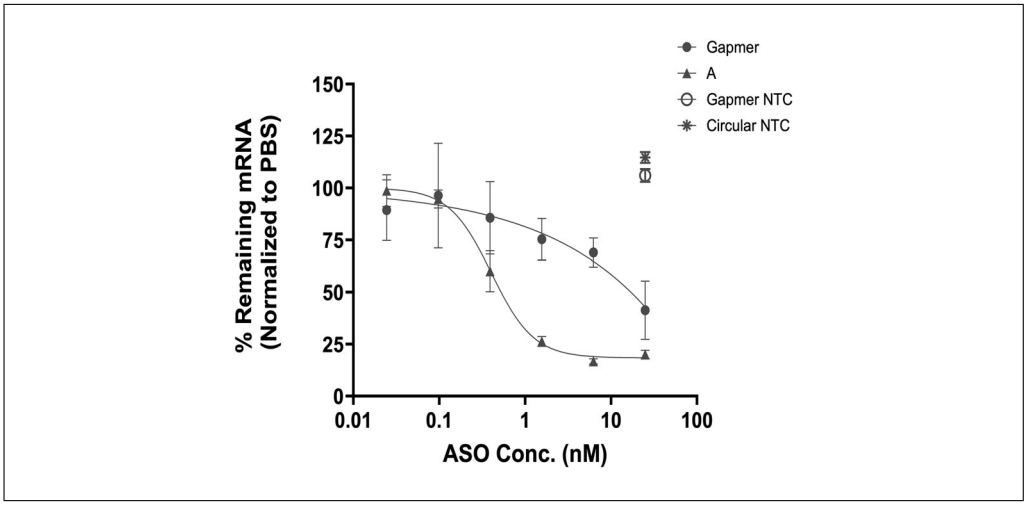

Figure 4 Efficacy of cyclic structured oligonucleotides (CSOs). A DGAT2-targeted gapmer (5′-TGCCATTTAATGAGCTTCAC), as previously published (Prakash et al., 2021), and a CSO containing the same functional sequence (gaagtg-5′-5′-TGCCATTTAATGAGCTTCAC; A) were transfected at various concentrations into Hep3B cells to obtain dose-response curves. Note the differences in chemical modification pattern. Gapmer and circular controls are non-targeted sequences consisting of equivalent structures and chemical modifications. Regular uppercase letters denote phosphorothioate oligodeoxynucleotides (PS DNA); underlined uppercase letters, 2′-methoxyethyl ribonucleoside phosphorothioates (PS MOE); lowercase letters, PO DNA; and the 5′-5′ linkage, phosphodiester. The data are from a prior publication (Agrawal & Vathipadiekal, 2024). The CSO sequence contains the Splitmer antisense design, as discussed previously (Agrawal, 2020, 2021a).

formation is dependent on the complementarity of the nucleotide sequences of the circularizing and functional domains. Competing intra- or intermolecular structures due to partial sequence similarities would compromise the cyclic structure and its characteristics, including potency, and must therefore be avoided.

It is important to note that the 3′-3 and 5′-5′ linked CSOs will form a similar structure, but surprisingly, improvement in efficacy was only observed with 5′-5′ linked CSOs, whereas 3′-3′ linked CSOs showed marginal potency. In our early work, discussed above, the focus was on increasing the nuclease stability of ASOs. Cyclic structures with 3′-3′ links are significantly more resistant to nucleases than 5′-5 ones (Jiang et al., 1999). However, the potency of 5′-5′ linked cyclic structures is greater than that of 3′-3′ linked cyclic structures, suggesting that nuclease stability is not the only factor affecting potency. This confirms our previous observations and shows that the current CSO design is feasible, with the insight that the 5′ ends are critical (Jiang et al., 1999). Interestingly, 3′-3′ linked CSOs, where the functional domain is a PAMP, show a significant increase in immunostimulatory activity and permit the creation of a library of novel immune modulators for immunotherapy (Agrawal, 2023).

This CSO structure has broad applicability: although the example in Figure 4 uses an antisense sequence, a variety of sequences, varying in length and modifications, can be accommodated. In addition, the functional domain could be an antisense sequence targeting an miRNA, a pre-mRNA for splicing, or a long non-coding RNA (lncRNA).

Circular prodrug oligonucleotides

In the cyclic structure discussed above, the 3′ end of the functional domain is involved in intramolecular hybridization, which must be complementary to the sequence of the circularizing domain, and the opening of the cyclic structure is dependent upon hybridization with the target RNA. This requirement may limit the applicability to potential sequences and applications. I therefore developed the cyclic design concept further. In this new circular prodrug design (CPO), the sequence needed for the intramolecular hybridization required for circularization is not part of the functional domain (Fig. 5), and the structure opens in response to intracellular factors rather than hybridization to the target sequence (Agrawal, 2024b).

As with the CSO design, the circular prodrug structure consists of two domains: the functional domain and the circularizing domain (Fig. 5). The circularizing domain comprises two sequences; one is part of the 3′ end of the functional domain, and the second sequence is a reverse complement to the first that is linked via a 5′-5′ or 3′-3′ linkage. The structure shown in Figure 5 is an example of a prodrug design with a 5′-5′ linkage, resulting in two 3′ ends. The composition of the selected circularizing domain sequences and resulting

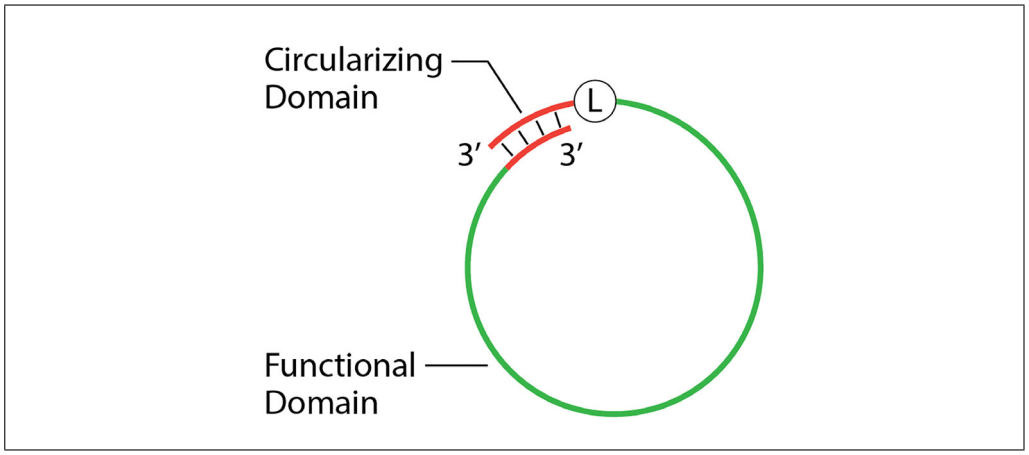

Figure 5 Design of a cyclic prodrug oligonucleotide (CPO). As with cyclic structured oligonucleotides (CSOs), this design comprises two segments: the functional domain (green) and the circularizing domain (red). However, in this design, the circularizing domain comprises sequences that are present at both ends of the functional domain, not just at one end. Thus, the circularizing domain on the 3′ end of the functional domain is selected to be complementary to the circularizing domain on the 5′ end of the functional domain, with the latter connected via a 5′-5′ linkage. The two circularizing domains can be composed of PO DNA, PO RNA, or a combination thereof to enable recognition of and release from the duplex by intracellular factors, such as RNase H.

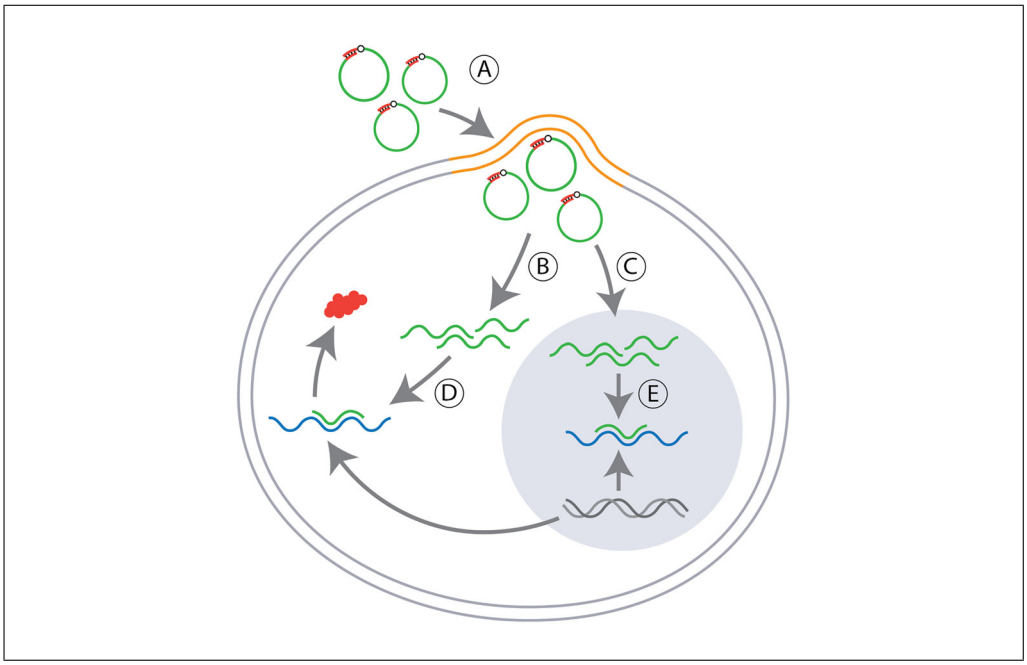

Figure 6 Delivery and intracellular fate of cyclic prodrug oligonucleotides (CPOs). This structure remains stable under physiological conditions depending on the nature of the circularizing domain (**A**). Once inside the cell, due to the circular structure and absence of accessible 5′ ends, its fate in the endosome will differ from that of linear oligonucleotides. After entering the cytoplasm (**B**) or nucleus (**C**), CPOs designed to form a duplex of PO DNA and PO RNA within the circularizing domains will be cleaved by RNase H, resulting in the opening of the circular structure and release of the functional domain to hybridize with its target sequence (**D** and **E**, respectively).

duplex is crucial, depending on which intracellular mechanism is intended to be appropriated for cleavage of the duplex. For example, if cleavage is supposed to utilize RNase H, then the circularizing duplex must consist of PO DNA, or PS DNA and PO RNA, with the DNA segment of a length sufficient to activate RNase H. In general, this is six or more bases (Agrawal et al., 1990). Alternatively, the circularizing duplex could be composed of two PO DNA sequences and thus recognized by a restriction enzyme for cleavage. As discussed in the preceding section, the circularizing domain could be longer, namely 6 to 20 bases, depending on the sequence and composition of the functional domain.

These circular prodrug oligonucleotides share many characteristics with the CSOs described in the previous section, such as shape, polyanionic nature, and lack of protein binding and accessible 5′ ends. However, after cell entry (Fig. 6A), the functional domain is released into linear form by the action of RNase H in the cytoplasm (Fig. 6B) or nucleus (Fig. 6C), before hybridization to the target sequence takes place (Fig. 6D and 6E).

Therefore, this design offers a platform for delivering any RNA therapeutic molecule, regardless of mechanism of action, into the intracellular space. The functional domain can vary in length, sequence composition, and modifications and support any application, including antisense, splice modulation, and guide RNA for CRISPR-based genome, base, or prime editing. The functional domain can also act as the passenger strand of an siRNA, where the guide strand is simply hybridized to the functional domain before the CPO is used (Agrawal, 2024b).

Results from a proof-of-concept study of a CPO that utilizes RNase H to release the circular structure and contains an antisense oligonucleotide in the functional domain are shown in Figure 7. Two CPO chemistries were compared: the circularizing domain of CPO A only contains PO DNA, whereas in CPO B, both PO DNA and PO RNA are used. In this example, the length of the circularizing domain is 10 base pairs. All compounds showed dose-dependent knockdown of the targeted RNA (Fig. 7), with both CPOs superior to the standard gapmer control. As expected, CPO B with a circularizing domain forming a clearly defined RNA/DNA duplex can engage RNase H and thus shows superior efficacy, whereas the DNA/DNA circularizing domain of CPO A is not able to activate RNase H. However, the increased potency of CPO A

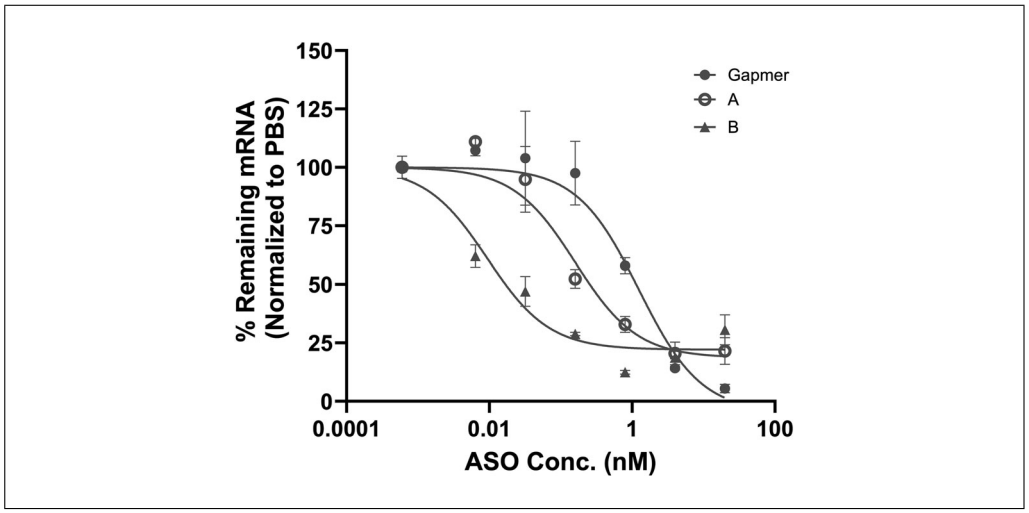

Figure 7 Efficacy of cyclic prodrug oligonucleotides (CPOs). In this study, a TAU-targeted gapmer (5′-CCGTTTTCTTACCACCCT) described by DeVos et al. (2017) and two different corresponding CPOs (tgtgatccct-5′-5′-CCGTTTTCTTACCACCCTacactaggga, A, and tgtgatccct-5′-5′-CCGTTTTCTTACCACCCTacactaggga, B) were transfected at various concentrations into 251 MG cells to obtain dose-response curves. Note the differences in chemical modification pattern: in CPO A, both parts of the circularizing domain consist of PO DNA, whereas CPO B's circularizing domains will form a 10-mer PO DNA/PO RNA duplex that is recognized and cleaved by RNase H. Regular uppercase letters denote phosphorothioate oligodeoxynucleotides (PS DNA); underlined uppercase letters, 2′-methoxyethyl ribonucleoside phosphorothioates (PS MOE); lowercase letters, PO DNA; and lowercase underlined letters, PO RNA. The 5′-5′- linkage is phosphodiester. The data are from a prior publication (Agrawal, 2024b).

compared to the standard gapmer design may be due to advantages of cyclic structures already discussed above. It is also possible that the CPO may be linearized upon hybridization of the antisense functional domain with the target RNA. Clearly, the CPO enhances the potency of the therapeutic moiety contained in the functional domain.

As discussed above, the functional domain can be antisense for RNase H–mediated knockdown, splice-modulating oligonucleotides, antisense sequences for ADAR-mediated RNA editing, guide RNA for CRISPR-mediated genome editing, or an mRNA construct for protein expression as used in mRNA vaccines. Moreover, the functional domain can be double stranded, with the prodrug strand acting as a hybridization partner for a complementary sequence as needed for the siRNA or microRNA mimic mechanisms of action. After cell entry, RNase H then releases the circularized structure and thus the double-stranded molecule. For each application, the sequence composition and length of the circularizing domain need to be optimized (Agrawal, 2024b).

Ring-shaped oligonucleotides

Studies with CSOs and CPOs show that the shape, protein binding, and accessibility of the 3′ or 5′ ends affect the potency and specificity of the functional domains. In both designs, the circularizing domain is covalently attached to the functional domain. This may limit the nucleotide sequences at the ends of the functional domain, thereby affecting the nature and length of the sequence. To address this, I created a ring-shaped oligonucleotide (RSO) where the functional domain is not covalently attached to the circularizing domain, and therefore, there are no limits on its sequence and modifications.

In this design, there are two oligonucleotides: the functional oligonucleotide and an anchor oligonucleotide, which serves as a circularizing domain. The anchor sequence is selected to be complementary to the 3′ and 5′ ends of the functional oligonucleotide, and upon hybridization, the two form a ring-shaped structure (Fig. 8), with the anchor sequence presenting like a precious stone in a solitaire setting on a traditional engagement ring. The anchor sequence can be designed so that the duplex region is cleaved by an intracellular enzyme. For instance, if the duplex contains PO DNA hybridized to PO RNA, RNase H cleaves it, thereby releasing the functional domain inside the cell.

In an example experiment, an RSO consisting of a modified 18-mer antisense sequence

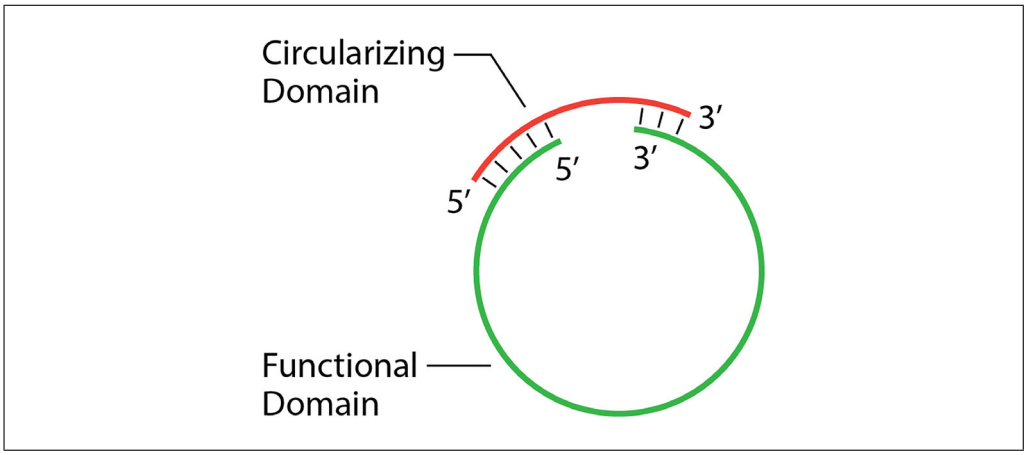

Figure 8 Design of a ring-shaped oligonucleotide (RSO). This design involves two oligonucleotides: a functional oligonucleotide (green) and a circularizing oligonucleotide (red). The circularizing oligonucleotide is complementary to both the 3′ and 5′ ends of the functional oligonucleotide, enabling hybridization between the two and thus bringing the 3′ and 5′ ends of the functional oligonucleotide together. The two oligonucleotides can be composed of PO DNA, PO RNA, or a combination thereof to enable recognition of the functional oligonucleotide and release from the duplex by intracellular factors, such as RNase H.

was extended at both ends with an 11-mer PO DNA sequence (A). A 24-mer anchor sequence complementary to these 11-mer extensions was then hybridized to the antisense sequence to form the ring-like structure. The anchor sequence consisted of PO RNA (B), thus forming a DNA/RNA duplex recognizable by RNase H, or PO DNA (C) for control purposes. The nature and length of the anchor domain will depend on the nucleotide base composition, that is, whether it is composed of PO DNA, PO RNA, or a mixture of both. The overall goal of the anchor domain is to form a ring-shaped structure and to engage an appropriate intracellular factor to cleave the duplex and thus release the functional domain.

All three compounds (A, A+B, and A+C) demonstrated dose-dependent reductions of the target RNA. As expected, the (A+B) RSO showed greater potency than (A) alone, whereas the (A+C) RSO exhibited a stronger effect than (A) alone, but not to the level of the (A+B) RSO (Fig. 9). This is likely due to the advantages associated with the circular nature of the RSO and replicates the results seen with CSOs and CPOs. Slower release of the anchor domain by other cellular nucleases that can cleave DNA/DNA duplexes may also play a role here.

Future studies should focus on optimizing the sequence, length, and modifications of the anchor oligonucleotide for each functional domain.

Chemical synthesis of all three types of cyclic structures described above is straightforward and can be performed on automated synthesizers using standard nucleoside amidites and reverse amidites (Bhagat et al., 2011; Yu, Zhao, et al., 2000; Yu et al., 2002). Reverse amidites can facilitate the incorporation of the non-standard 3′-3′ or 5′-5′ linkage via a PO bond but can also accommodate PS or other backbone modifications and may include a spacer in the linker region.

CONCLUDING REMARKS

With all the progress made in the antisense field, it is easy to forget how antisense was once viewed with derision and doubt. When I first joined Paul Zamecnik's laboratory at the Worcester Foundation for Experimental Biology in Shrewsbury, MA, I noticed two stacks of yellowed reprints. These were reprints of his 1978 papers (Stephenson & Zamecnik, 1978a, 1978b). Paul used to joke that no one requested them because there was skepticism that oligonucleotides could even enter cells, let alone do what the papers showed. However, these two papers were the beginning of antisense. Now, several antisense drugs are approved and in fact helping patients.

So far, medicinal chemistry has been the primary driver of the main advancements necessary to achieve clinical success, improving nuclease resistance and target sequence affinity as well as targeted delivery. However, further progress requires alternative approaches. Here, I have shown how rethinking the structure instead of exclusively focusing on medicinal chemistry can significantly enhance the therapeutic efficacy of ASOs.

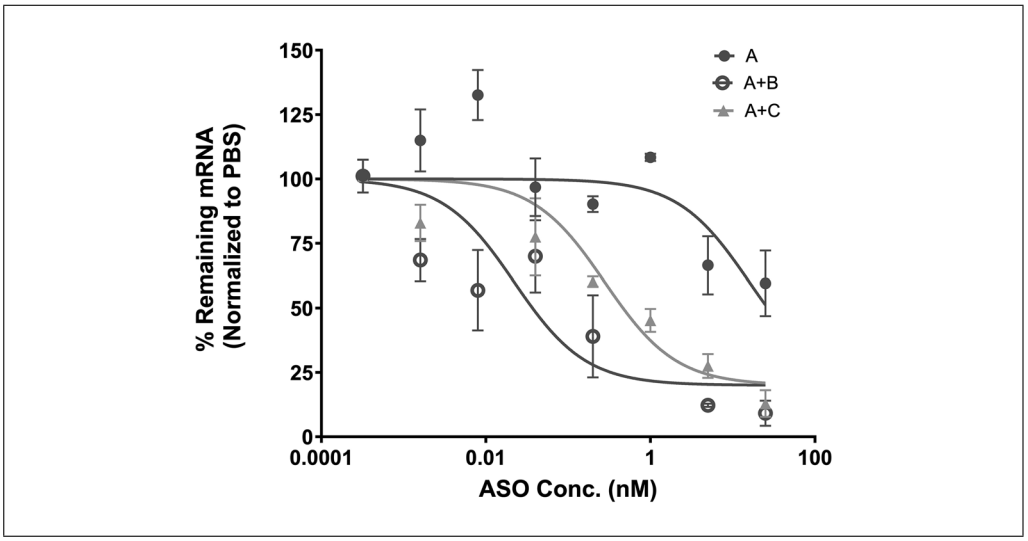

Figure 9 Efficacy of ring-shaped oligonucleotides (RSOs). A functional oligonucleotide in Splitmer format (5′-gagagtctggcCCGTTTTCTTACCACCCTgagagtcctga), corresponding to the TAU-targeted gapmer used in Figure 7, was transfected into 251 MG cells either alone (A) or hybridized to the circularizing oligonucleotides 5′-gccagactctcaatcaggactctc (A+B) or 5′-gccagactctcaatcaggactctc (A+C) at various concentrations to obtain dose-response curves. Note the differences in chemical modifications: for (A+B), the duplex formed between circularizing and functional oligonucleotide will consist of PO DNA/PO RNA, and for (A+C), the duplex will be PO DNA/PO DNA. The (A+B) RSO duplex is easily recognized and cleaved by RNase H. However, there is an increase in potency of (A+C) over A alone due to hybridization with the target RNA and partial destabilization of the ring-shaped functional domain. Further details are available in a prior publication (Agrawal, 2024c). Regular uppercase letters denote phosphorothioate oligodeoxynucleotides (PS DNA); underlined uppercase letters, 2′-methoxyethyl ribonucleoside phosphorothioates (PS MOE); lowercase letters, PO DNA; and lowercase underlined letters, PO RNA. The data are from the prior publication (Agrawal, 2024c).

Although reports of circular oligonucleotides as antisense agents date back to 1999, these studies mostly focused on closed-loop structures in which the oligonucleotides' ends are covalently linked. This complicates the synthesis, post-synthesis processing, and purification of such constructs and limits the use of chemical modifications. The same is true of a number of studies published in recent years that describe various designs of circular oligonucleotides and their applications in antisense, siRNA, microRNA decoy, and mRNA therapies (Hagiwara et al., 2020; Jahns et al., 2021; Rowley et al., 1999). The circularized structures I have described here are transient, with the ends of the oligonucleotides non-covalently held together by Watson-Crick base pairing. They are thermodynamically more stable as intramolecular structures (Kandimalla & Agrawal, 2000).

These structures can be efficiently synthesized on automated synthesizers using commercially available reagents and include modifications. The proof-of-concept data shown in this article represent a sample of much larger previously published datasets (Agrawal, 2023; Agrawal, 2024b, 2024c; Agrawal & Vathipadiekal, 2024). These data show significant increases in potency with these cyclic structures compared to gapmer antisense, suggesting that only a fraction of the intracellular concentration of gapmers is available to hybridize with the targeted RNA, whereas the majority is bound to other factors. Further research to understand the cellular uptake and processing of these structures will shed more light on these topics. Furthermore, mitigating inflammatory responses using circularized structures is advantageous for developing therapeutics.

Each structure has unique characteristics and is composed of two key components: a functional domain and a circularizing domain, connected by a linker. Further structure-activity relationship studies varying the length and modification pattern of these components and employing different mechanisms of action for linearization can fine-tune the therapeutic profile of the functional domain, thereby enhancing therapeutic efficacy. Nucleotide sequences and the biological environment will affect the integrity of these structures; hence, careful consideration must be given to their design.

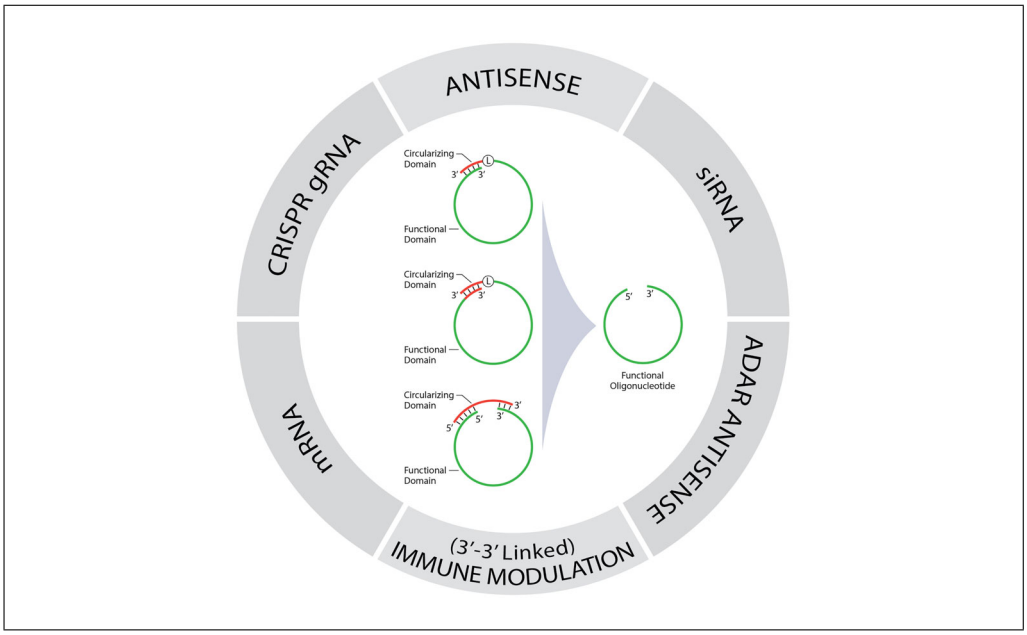

Figure 10 Designs of spatial structures of oligonucleotides and their uses. Three distinct types of oligonucleotide structures are described. All of these share some features, including shape and accessibility of the 3′ and 5′ ends. However, each structure is unique in how the functional domain is held within the structure and released inside the cell. These structures have a wide range of potential applications for any therapeutic modality requiring exogenous RNA or DNA sequences within cells. These include all antisense mechanisms, be it RNase H–based or occupancy-only mechanisms; siRNA; guide RNAs for genome, base, or prime editing mediated by CRISPR, ADAR, or other enzymes; or mRNA expression constructs. One application not discussed here is the use of corresponding 3′-3′ linked structures that have accessible 5′ ends to modulate immune responses through pattern recognition receptors.

Regarding delivery, these circularized designs can be administered alone, encapsulated in lipid nanoparticles, or conjugated to delivery moieties, such as antibodies, lipids, or peptides. However, formulations, encapsulations, or conjugations for *in vivo* delivery of these structures will require consideration to maintain the integrity of the cyclic structures.

One point not discussed here is the use of 3′-3′ linked cyclic structures as immune-stimulatory agents (Agrawal, 2023). Cyclic structures will likely provide various iterations that could act as PAMPs and thus be useful in immunotherapy.

In conclusion, these designs are applicable universally: in addition to oligonucleotide therapeutics, they can also be used to transfer guide RNAs for genome, base, and prime editing mediated by CRISPR, ADAR, or other enzymes and can accommodate mRNA transcription units as used in mRNA vaccines or for protein expression (Fig. 10).

Thus, these designs represent a significant advancement for all nucleic acid therapeutics, potentially surpassing what has been achieved through medicinal chemistry.

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My journey with antisense began with Paul Zamecnik in 1987. He was a wonderful teacher, mentor, and father figure in my research career for over 20 years. I am grateful to him for his guidance, advice, and support. After retiring in 2017, I felt that some missing links in previous observations needed clarification, so I founded ARNAY Sciences to conduct new research to connect the dots. The work described here is an example of connecting the dots. I am grateful to Petra Disterer for her expert writing and editorial assistance with this article and to Beth Mellor for the figures.

AUTHOR CONTRIBUTIONS

Sudhir Agrawal: Conceptualization; validation; writing—review and editing; writing—original draft; supervision; methodology.

CONFLICT OF INTEREST

S. A. is the founder of ARNAY Sciences LLC and the inventor on the cyclic antisense patent applications described and cited in this article.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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