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MEDICINAL CHEMISTRY RESEARCH

REVIEW ARTICLE

Oligonucleotides: evolution and innovation

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Abstract

Oligonucleotides, comprising single or double strands of RNA or DNA, are vital chemical compounds used in various laboratory and clinical applications. They represent a significant class of therapeutics with a rapidly expanding range of uses. Between 1998 and 2023, 19 oligonucleotides have received approval from the U.S. Food and Drug Administration (FDA). Their synthesis methods have undergone significant evolution over time. This review examines several oligonucleotide synthesis techniques, including phosphodiester, phosphotriester, and phosphoramidite approaches. It begins with a discussion of an early synthesis method involving a phosphoryl chloride intermediate, which proved unstable and prone to hydrolysis. The review then transitions to the solid-phase synthesis method, which uses polymer resins as a solid support, emphasizing its advantages over both phosphotriester and phosphoramidite techniques. This is followed by an exploration of recent advancements in oligonucleotide enzymatic synthesis, concluding with a discussion on modifications to bases, sugars, and backbones designed to improve their properties and therapeutic potential.

Keywords Oligonucleotides · Enzymatic synthesis · Modifications · Solid-phase synthesis · FDA approvals

Introduction

Oligonucleotides are oligomers composed of repeating nucleotide monomers, comprising deoxyribose or ribose sugar, nitrogenous bases, and a phosphate backbone [1]. Currently, oligonucleotides have garnered significant interest among chemists due to their extensive utilization in the pharmaceutical industry [2–4]. Oligonucleotides, short DNA or RNA molecules, serve as versatile tools with a wide range of applications in genetic testing, research, and forensics [5]. They are typically produced in the laboratory through solid-phase synthesis (SPS) and can be tailored as single- and double-stranded molecules with precise sequences [6–8]. Consequently, they play pivotal roles in numerous processes such as artificial gene construction [9], polymerase chain reaction (PCR) [10], DNA sequencing [11], molecular cloning [12], and molecular probing [13]. In nature, oligonucleotides are often found microRNAs molecules involved in gene expression regulation, including mRNA, or as byproducts resulting from the degradation of larger nucleic acid molecules [14].

Oligonucleotides possess a unique capability to bind specifically to their complements, such as DNA or RNA, leading to the formation of duplexes or, less frequently, higher-order hybrids. This characteristic enables the use of oligonucleotides as probes for identifying specific DNA or RNA sequences [14, 15]. Modern techniques for utilizing oligonucleotides include DNA microarrays, Southern blots, allele-specific oligonucleotide analysis, fluorescent in situ hybridization (FISH), PCR, and the synthesis of artificial genes [16].

Oligonucleotides have been utilized to treat rare diseases, including cardiovascular and metabolic disorders, oncology, infections, neurological and muscular disorders, as well as ophthalmological conditions [17–20]. They are also undergoing clinical trials for the treatment of dermatological, gastrointestinal, and hormonal disorders [21].

FDA-approved oligonucleotide therapeutics (Table 1) utilize various mechanisms: Antisense oligonucleotides

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Table 1 FDA approved oligonucleotides from 1998 to 2023

#	Generic name (Trade name)	Indication	Target	Mode of action	Administration route	Ref
1	Fomivirsen (Vitravene™)	Cytomegalovirus retinitis	CMV UL123	ASO	Intravitreal	[23]
2	Pegaptanib (Macugen™)	Neovascular age-related macular degeneration	VEGF-165	Aptamer	Intravitreal injection	[23]
3	Mipomersen (Kynamro™)	Homozygous familial hypercholesterolemia	APOB	ASO	Subcutaneous injection	[23]
4	Defibrotide (Defitelio™)	Hepatic veno-occlusive disease	NA b	Not elucidated	Intravenous injection	[23]
5	Eteplirsen (Exondys 51™)	Duchenne muscular dystrophy	DMD exon 51	ASO	Intravenous infusion	[22]
6	Nusinersen (Spinraza™)	Spinal muscular atrophy	SMN2 exon 7	ASO	Intrathecal injection	[23]
7	Patisiran (Onpattro™)	Hereditary transthyretin amyloidosis polyneuropathy	TTR	siRNA	Intravenous infusion	[24]
8	Inotersen (Tegsedi™)	Hereditary transthyretin; Amyloidosis polyneuropathy	TTR	ASO	Subcutaneous injection	[24]
9	Givosiran (Givlaari™)	Acute hepaticporphyria	ALAS1 mRNA	siRNA	Subcutaneous injection	[22]
10	Golodirsen (Vyondys 53™)	Duchenne muscular dystrophy	DMD exon 53	ASO	Intravenous infusion	[22]
11	Viltolarsen (Viltepso™)	Duchenne muscular dystrophy	DMD exon 53	ASO	Intravenous infusion using a peripheral or central venous catheter	[23]
12	Lumasiran (Oxlumo™)	Primary hyperoxaluria type 1 (PH1)	HOA1 mRNA	siRNA	Subcutaneous injection	[23]
13	Inclisiran (Leqvio™)	hypercholesterolemia	PCSK mRNA	siRNA	Subcutaneous	[26]
14	Casimersen (Amondys 45™)	Duchenne muscular dystrophy (DMD)	Exon 45	ASO	Intravenous infusion	[26]
15	Vutrisiran (Amvuttra™)	To treat polyneuropathy of hereditary transthyretin-mediated amyloidosis	TTR mRNA	siRNA	Subcutaneous injection	[27]
16	Tofersen (Qalsody™)	To treat amyotrophic lateral sclerosis in adults who have an SOD1 gene mutation	SOD1 mRNA	ASO	Lumbar puncture	[18]
17	Avacincaptad pegol (Izervay™)	To treat geographic atrophy secondary to age-related macular degeneration	C5 complement protein	Aptamer	Intravitreal injection	[18]
18	Nedosiran (Rivfloza™)	To lower urinary oxalate levels in patients 9 years and older with primary hyperoxaluria type 1 and relatively preserved kidney function	RISC	siRNA	Subcutaneous injection	[18]
19	Eplontersen (Wainua™)	To treat polyneuropathy of hereditary transthyretin-mediated amyloidosis	Transthyretin (TTR) mRNA	ASO	Subcutaneous injection	[18]

Refer to Table 2 for chemical structures

ASO antisense oligonucleotide, *N/A* not applicable, *siRNA* small interfering RNA, *ALSA1* aminolevulinate synthase 1, *SOD1* superoxide dismutase 1, *RISC* RNA-induced silencing complex

(ASOs) such as eteplirsen and viltolarsen modify splicing or promote mRNA degradation by masking exons and inducing their skipping, which reduces disease-causing proteins [22, 23]. Small interfering RNAs (siRNAs) like patisiran target and degrade specific mRNA to suppress gene expression [24]. Aptamers, such as pegaptanib, and avacincaptad pegol, are designed to selectively bind to particular proteins or molecules, thereby modulating their activity [25].

Overall, 19 oligonucleotides have been approved by the FDA from 1998 to 2023, 12 from 1998–2020, two in 2021, one in 2022, and four in 2023 (Table 1), indicating a growing focus on their synthesis in the coming years [18, 23, 26, 27]. The synthesis of oligonucleotides is currently facing significant challenges, primarily due to high synthesis costs and relatively inferior pharmaceutical properties. These properties include low chemical stability, limited oral absorption, and a short lifespan [28, 29].

The structure of oligonucleotides is primarily based on 2'-deoxyribonucleotides (oligodeoxyribonucleotides) or ribonucleotides, which can be modified in the backbone or at the 2' sugar position. These modifications result in various pharmacological effects. They bring about novel properties to oligonucleotides, making them an essential component of antisense therapy (Fig. 1).

This review aims to explore the significance of therapeutic oligonucleotides, their synthesis techniques, and the various modification methods used to enhance their therapeutic efficacy.

Chemical synthesis

Phosphodiester and phosphotriester approaches

In 1955, Michelson and Todd reported the synthesis of dithymidinyl [30]. This groundbreaking achievement marked the inception of oligonucleotide synthesis and revolutionized the ongoing research in the field [30]. They discovered chemical transformation by creating the phosphate linkage between two thymidine nucleosides [30]. The process began with the initial synthesis of the 3′ phosphoryl chloride of a 5′ benzyl-protected thymidine using phenylphosphoryl dichloride. Subsequently, this compound was reacted with the 5′ hydroxyl group of a 3′ protected thymidine. Although the chemical reactions were found to be compatible and optimal, they proceeded at a relatively slower speed. Additionally, it was observed that the phosphoryl chloride intermediate was unstable and susceptible to hydrolysis under the reaction conditions (Scheme 1) [30].

The contribution of Khorana and coworkers for the synthesis of oligonucleotides by introducing the ON-Off protection, and the phosphodiester approach, which was considered as a noteworthy development. The ON-Off approach entailed the temporary protection of 5′-hydroxyl groups with acid-sensitive protecting groups such as 4-monomethoxytrityl or 4,4′-DMT. Conversely, the phosphodiester approach involved the reactivity of a 5′-activated phosphoester nucleotide with the nucleophilic 3′-hydroxyl group of another nucleoside using a coupling reagent or chloride as an activating agent (Scheme 2).

This methodology mimics the natural biosynthesis of nucleotides, in which the secondary alcohol on position 3′ acts as a nucleophile [31]. Khorana's discovery found a pivotal role in pushing forward the synthesis of oligonucleotides, which was a noteworthy development in the understanding of the genetic code and its significance in the protein synthesis process [32–34]. Khorana's remarkable discoveries were acknowledged with the Nobel Prize in Physiology and Medicine in 1968 [32–34].

The DMT protecting group approach was warmly welcomed due to its stability and the ability to be removed under mild acidic conditions, making it particularly advantageous [35]. However, this methodology still encountered issues with the quantitative formation of side products, particularly during the protection and deprotection stages. In the phosphotriester method, the nucleophilic 5′ alcohol position reacted with the activated alcohol in the 3′-position. The significant advancement was the introduction of the 2-cyanoethyl group for the first time as the protecting group into P–OH, which was then applied to SPS [35–38].

Phosphonate and phosphoramidite

Later in 1970, a different approach was put forward which was known as phosphite-triester approach. In this approach, reactive P(III) derivatives of nucleosides, such as 3'-*O*-chlorophosphites were utilized to react for the bond formation between nucleosides (Scheme 3). Caruthers' research group capitalized on the benefits of milder and more selective 1H-tetrazolidophosphites, implementing this method on solid phase.

In spite of remarkable progress on phosphotriester chemistry, this area of research is still facing serious challenges [39]. The average efficiency at each step for reproducibility has remained below 97%, specifically falling short of reaching the 95% threshold [39]. This drawback has particularly hampered the routine synthesis of oligonucleotides with chain lengths of less than twenty bases [39]. Another prominent problem in phosphotriester-based oligonucleotide synthesis is the longer reaction time for each step, exceeding one and a half hours [39, 40].

A large number of subsequent advancements in oligonucleotides synthesis technologies have revolutionized the phosphoramidite chemistry as an efficient oligonucleotides' synthesis method [38, 41–44].

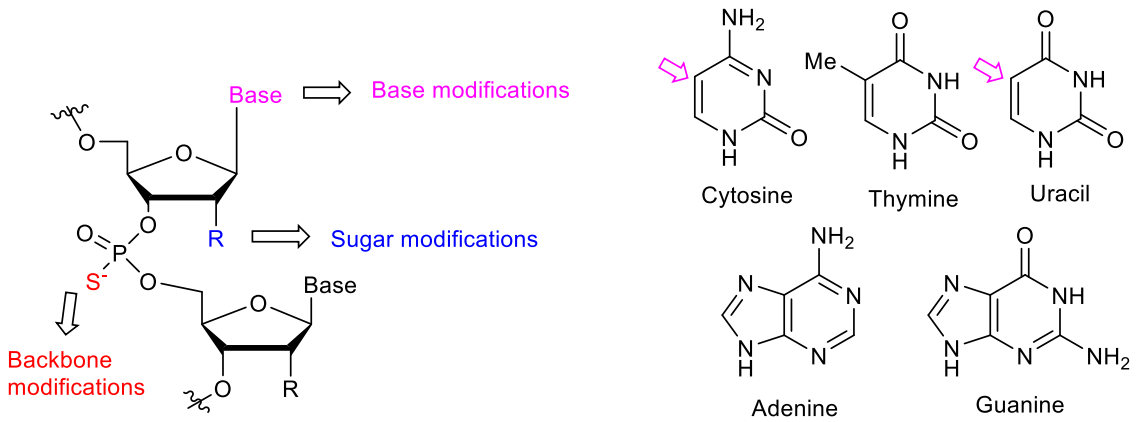
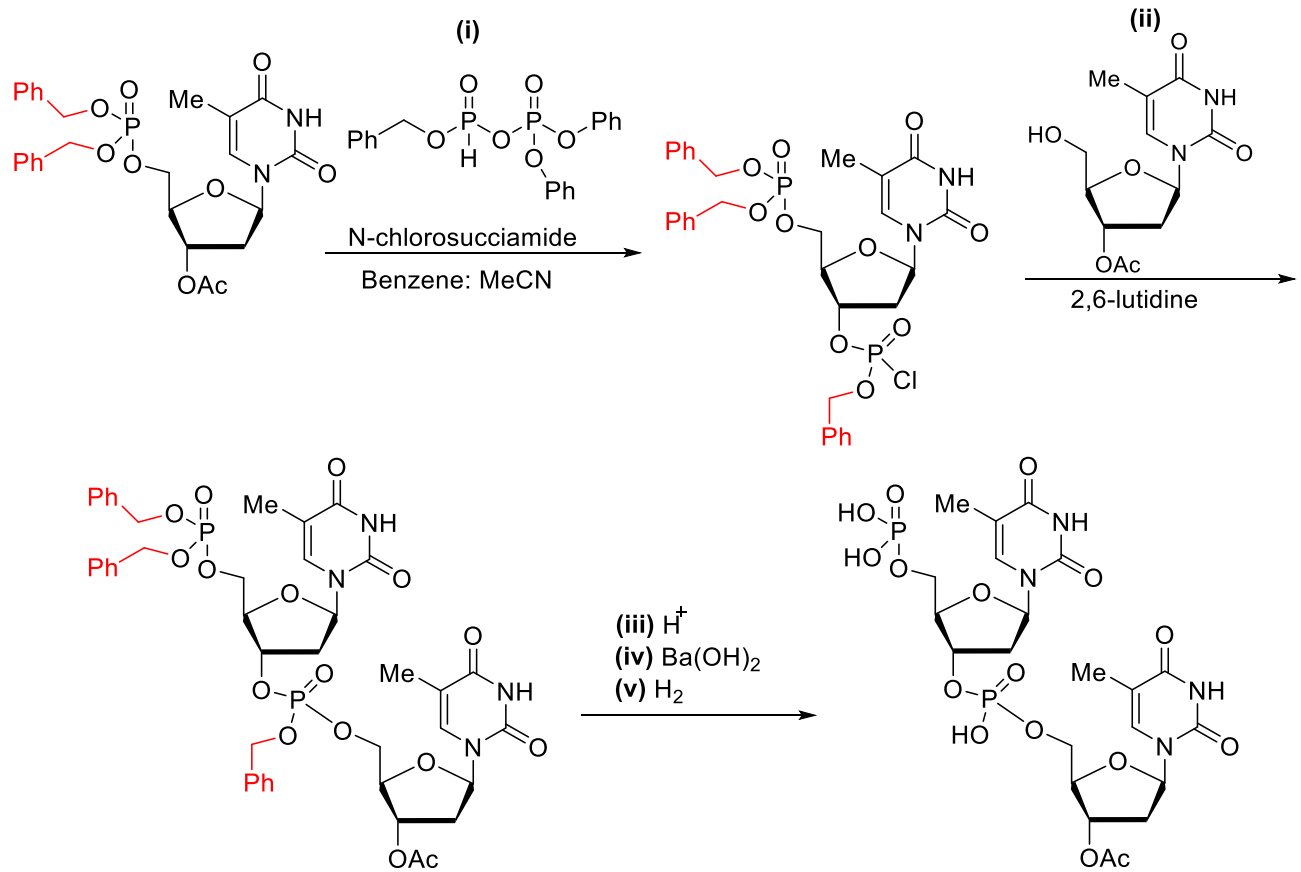


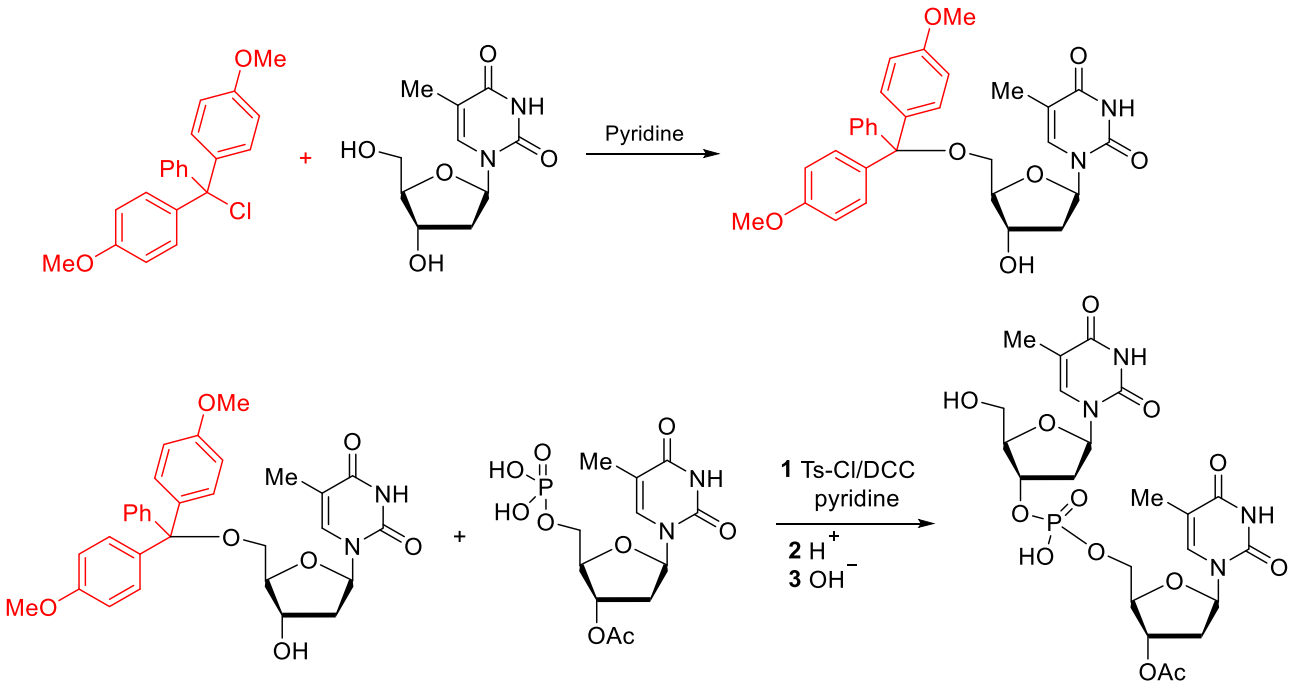
Fig. 1 Chemical structure of a modifiable oligonucleotide (left) and natural nitrogenous bases with predominant positions of modifications (right)

Scheme 1 Dithymidinyl nucleotide: synthesized by Michelson and Todd. Red color refers to the protecting groups

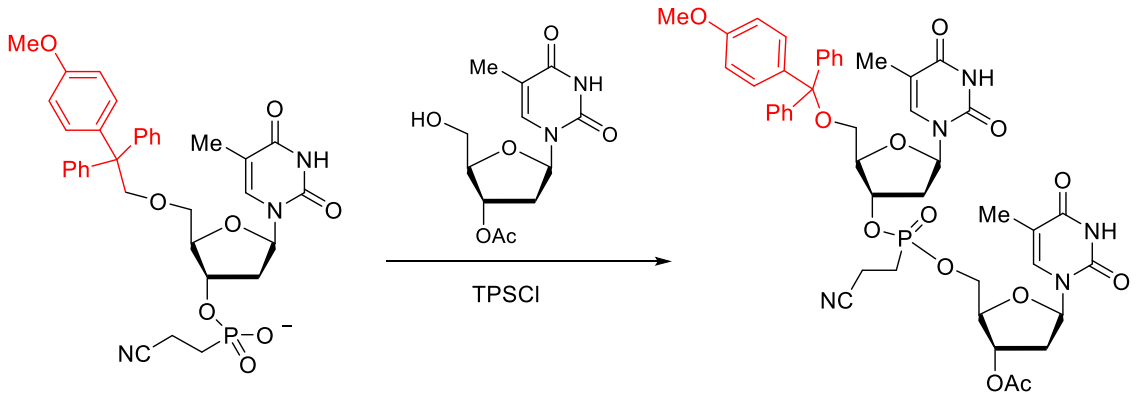


Baucage and Caruthers introduced the concept of phosphoramidite methodology for oligonucleotides synthesis [45]. At first glance, this approach seems to be a minor modification of the phosphotriester method developed by Letsinger, replacing chlorine leaving groups with an amine (Fig. 2). However, this simple modification proved very helpful for the routine synthesis of the oligonucleotides as it revolutionized the properties of the oligonucleotide molecules [45]. This tremendous discovery enabled chemists to pre-synthesize the intermediate compounds for oligonucleotides synthesis such as phosphitylated or phosphoramidite intermediates, which could be stored for extended periods [46]. Phosphoramidite intermediates can be activated on demand using a weak base when required for synthesis. This enhanced stability has made phosphoramidite intermediates commercially viable as reagents for nucleotide synthesis [47].

Scheme 2 Synthesis of dithymidinyl nucleotide through Khorana's method. Protecting groups are shown in red



Scheme 3 Phosphotriester approach for the synthesis of oligonucleotides. Red color refers to the protecting groups



The phosphoramidite intermediate, representing a nucleotide, typically consists of di-isopropylamine and 2-cyanoethoxy groups attached to the phosphorus atom at the 3'-position of a cyclopentanose sugar (Fig. 2).

Solution vs solid vs liquid phase approaches

The previous discussion centered on solution-phase oligonucleotide synthesis. In 1965, Letsinger utilized a styrene-divinylbenzene polymer, also known as a popcorn polymer, as a solid support for the synthesis of oligonucleotides through the phosphotriester approach [35].

As of today, SPS of oligonucleotides via phosphoramidite remains a widely utilized approach, with numerous modifications and advancements made over time. These

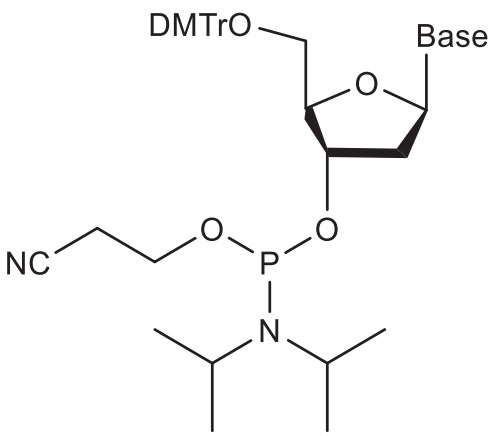


Fig. 2 Chemical structure of a phosphoramidite nucleotide intermediate

include alterations in solid support materials, variations in protecting groups, modifications to sugar structures, and innovations in deprotecting reagents and oxidizing agents [48]. The pathway for synthesizing oligonucleotides via the phosphoramidite intermediate approach typically involves four main steps: deprotection, coupling, oxidation (sulfurization), and capping [49].

Deprotection of the 5'-position involves treating the compound with di- or trichloroacetic acids in chlorinated solvents like dichloromethane (DCM) or 1,2-dichloroethane (1,2-DCE). This step liberates the 5' alcohol group, making it available for reaction with an activated nucleotide molecule [50]. While the phosphoramidite pathway represents an advancement in oligonucleotide synthesis methods, it still encounters challenges related to the use of non-green halogenated solvents. Stronger acids or prolonged reaction times can lead to undesirable side reactions, such as depurination, limiting the length of oligonucleotides synthesized through chemical processes [50].

After deprotection of the solid-supported nucleotide, it is prepared to react with an already activated phosphoramidite intermediate nucleotide. The free 5'OH group on the nucleotide can react with the incoming phosphoramidite nucleotide. The phosphoramidite, which contains a 2-cyanoethyloxy group and a di-isopropylamino phosphate at the 3' position, is activated using an acid, typically a tetrazole or another nitrogen heterocycle. The phosphoramidite and acid activator are combined in a reactor-type tube, where the acid protonates the di-isopropylamino group at the 3' position, making it ready for substitution by the incoming OH group. To ensure maximum yield, it is crucial that the activated nucleotide is present in higher equivalents compared to the supported chain with the free OH group [51].

During the coupling step, efforts are focused on ensuring that all free 5'OH groups on the growing oligonucleotide chain react with the activated phosphoramidite nucleotide. To address any remaining free OH groups that might not have reacted, capping is employed. Capping protects these residual free OH groups to prevent unwanted reactions and ensure the integrity of the growing oligonucleotide chain [52]. Capping is crucial to prevent the free 5'OH groups from reacting with phosphoramidite nucleotides in subsequent steps. If left uncapped, these OH groups would necessitate n-1 deletions, which would be challenging to isolate from the synthesized oligonucleotides. Additionally, in the case of guanosine nucleotides, the O6-position may sometimes react with the activated phosphoramidate intermediate nucleotides. Moreover, depurination of the growing chain can occur when the chain is oxidized in the presence of I_2 and water. Therefore, capping is an essential step to prevent these reactions and is typically carried out in the presence of *N*-methyl imidazole (NMI) and acetic anhydride [53]. These specialized reagents facilitate the acylation of any free unreacted OH groups present at the 5' position [53]. In the final step of the cycle, oxidation at the P-atom of the growing oligonucleotide chain is performed to convert it from a phosphite triester to a phosphate triester. This oxidation is achieved with the aid of molecular iodine (I_2) in water (Scheme 4).

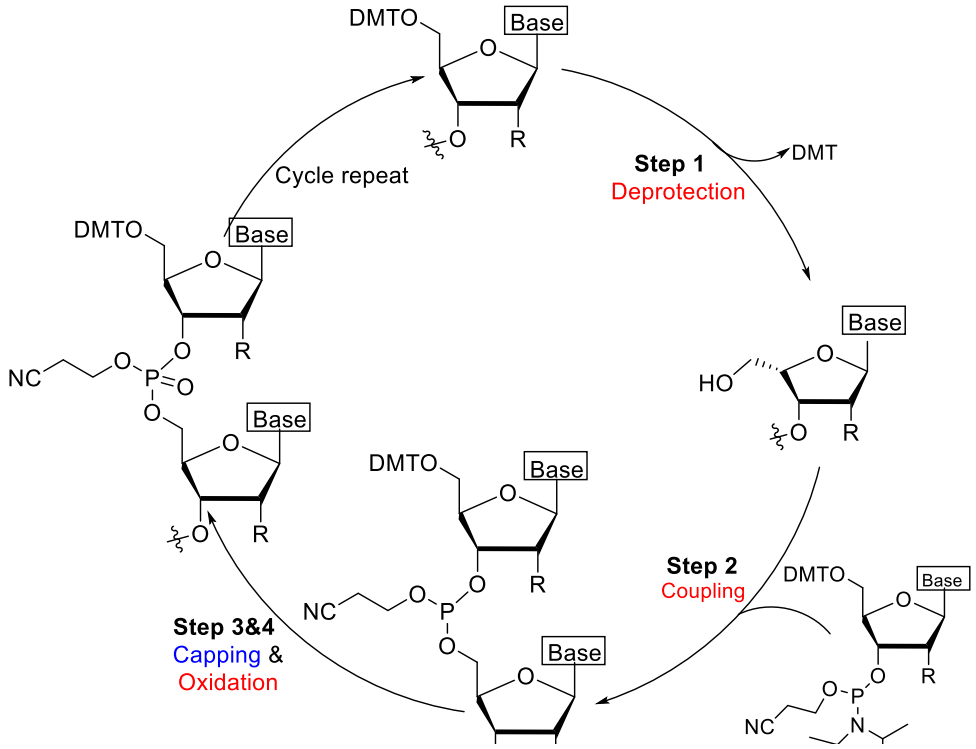
Selecting an appropriate base is crucial to prevent depurination during the reaction. Researchers have explored alternative oxidizing agents to avoid the presence of water. For instance, tertiary butyl hydrogen peroxide (TBHP) can be utilized in organic solvents under anhydrous conditions [54, 55]. Repeating these four steps enables the synthesis of oligonucleotides with the desired characteristics and length. The terminal nucleotide of the synthesized oligonucleotide is typically blocked with a dimethoxytrityl (DMT) group, which can either be retained or capped with a non-nucleoside moiety at the 5' position [56]. This final protective group is instrumental in modifying the properties of the oligonucleotide, rendering it hydrophilic, hydrophobic, or UV-active, depending on the intended application [56]. Finally, the acyl-protected bases and phophotriesters are deprotected during the isolation of the synthesized oligonucleotides chain from the solid support. These bases include methyl amine ($MeNH_2$), ethyl amine ($EtNH_2$), among others. Subsequently, the oligonucleotides are purified and characterized [57].

Recently, there has been significant progress in advancing phosphoramidite chemistry for the 3'-dephosphorylation of oligonucleotide chains. Yamamoto and colleagues reported the use of mild and efficient O-alkylphosphoryl groups as a replacement for conventional O-cyanoethylphosphoramidites (Scheme 5). These alkylated phosphotriesters were found to be more stable in the presence of alkali compared to their cyanoethyl counterparts. Another exciting achievement was the synthesis of nucleoside phosphoramidites bearing 1,2-diols and their incorporation into oligonucleotides [58].

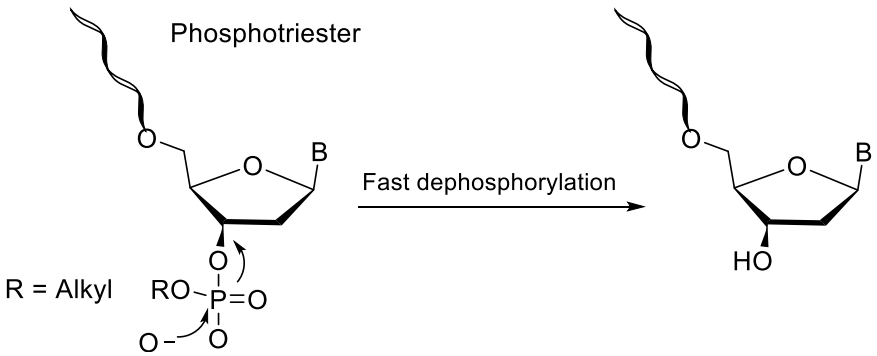
In 2023, a more facile tandem synthesis of the oligonucleotide approach has been adapted for the development of chemically modified DNA and RNA with easily accessible and cleavable linkers [59]. The tandem synthesis was performed with 2,2′-sulfonyldiethylene cleavable linkers (X-linker and X-amidite) that was incorporated in a single sequence of many-fragments oligonucleotides [59]. The 2,2′-sulfonyldiethylene linkers were investigated for their ability to self-immolate during the deprotection of DNA and RNA. This approach was presented as a generalized methodology for oligonucleotide tandem synthesis (Fig. 3) [59].

Oligonucleotides are currently synthesized using automated SPS with phosphoramidite chemistry. While this method produces high-quality oligonucleotides on a kilogram scale, the challenge of scaling up for widespread pharmaceutical use remains. SPS is limited by the high cost of resins and the need for excess expensive and hazardous

Scheme 4 A generalized synthesis cycle of an oligonucleotides through phosphoramidite approach



Scheme 5 Dephosphorylation of oligonucleotide through phosphotriester intermediate



reagents, resulting in a high carbon footprint [60]. The simple concept of atom economy (EA) cannot be applied to the SPOS synthesis due to the repetition of synthetic steps and the use of on–off protections considerably affecting the concept of EA, as a huge amount of the waste is produced at each synthetic cycle [39, 61]. Additionally, purification is crucial, as pharmaceutical-grade oligonucleotides require extremely high purity.

A solution-phase approach to oligonucleotide synthesis can overcome many limitations, offering scalability similar to conventional organic synthesis and reducing reagent use by utilizing homogeneous solutions [62]. While solution-phase methods like phosphotriester and H-phosphonate chemistries have been widely studied [63, 64], they require laborious column chromatography after each step and can face solubility issues with long oligomers.

The liquid-phase method, which uses soluble polymers instead of solid-phase resins, addresses these issues by combining the benefits of both synthesis methods. Various approaches have been developed, such as the use of cellulose acetate, β-cyclodextrin, and PEG [65, 66]. Early PEG-based liquid-phase approaches used phosphotriester chemistry, with phosphoramidite and H-phosphonate chemistries also being applied [7]. Non-polymeric methods like ionic liquid tag-assisted synthesis [67], fluorous tag-assisted synthesis [68], and AJIPHASE® have further expanded oligonucleotide synthesis strategies.

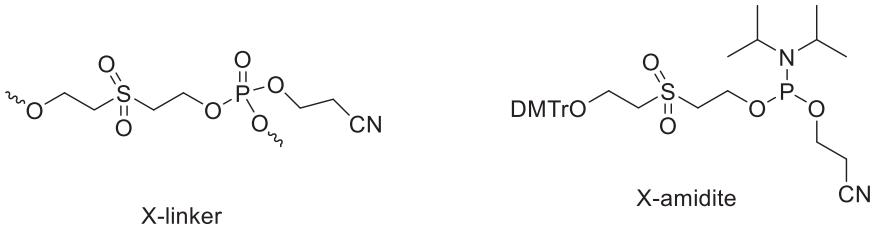


Fig. 3 Chemical structure of self-immolate 2,2,sulfonyldiethylene linkers

Creusen and colleagues developed a one-pot liquid-phase DNA synthesis method, allowing nucleotide addition through coupling, oxidation, deprotection, and a single precipitation step [69]. They highlighted the importance of the right oxidizing agent, effective DMT cation scavenging, and using 2-propanol over diethyl ether for precipitation to prevent depurination during adenine addition [69].

The Livingston group developed an iterative synthesis platform using organic solvent nanofiltration (OSN) for scalable purification in liquid-phase oligonucleotide synthesis (LPOS). They synthesized and characterized 5-mer and 9-mer 2′-O-methyl phosphorothioate oligoribonucleotides through eight cycles of chain extension, with OSN membranes enabling purification by separating the product from smaller reagent debris on a soluble PEG support [70].

Protecting group scheme and the concept of orthogonality

Protecting groups are essential in the synthesis and manipulation of oligonucleotides, providing the tools necessary for creating complex and highly specific sequences. These include: NPPOC (2-(2-Nitrophenyl)propoxycarbonyl), which is a photolabile group used for orthogonal protection in oligonucleotide synthesis, allowing for precise microarray production [71]. Wang and co-workers utilized this approach to assemble oligosaccharides through stereoselective glycosylation [72]. Levulinyl (Lev), an orthogonal protecting group that integrates well with other protection strategies in oligonucleotide synthesis [73]. Fmoc (9-Fluorenylmethyloxycarbonyl), a commonly used protecting group that can be removed under mild basic conditions and is orthogonal to many other groups [74]. Gaytán and colleagues employed a combination of DMT-mononucleotide and Fmoc-trinucleotide phosphoramidites in oligonucleotide synthesis to create codon-level degenerate oligodeoxyribonucleotides [75]. Dma (Dimethylacetamidine), it prevents unwanted hybridization at undesired sites while facilitating intended hybridization in oligonucleotides [76].

Enzymatic synthesis

To overcome limitations such as suboptimal yields and purities, sequence length restrictions, environmental impact, and lengthy purification steps inherent in traditional chemical synthesis methods, enzymatic oligonucleotide synthesis is being developed as an innovative approach. These advancements aim to improve the efficiency, scalability, and sustainability of oligonucleotide production. The approach holds great promise for advancing DNA and RNA synthesis, particularly in applications requiring high precision and sustainability. Different approaches have been developed so far [77–80].

Schmitz and Reetz developed a SPOS method using T4 RNA ligase with tentagel or kieselguhr/polydimethylacrylamide supports [81]. Their results show that, although T4 RNA ligase operates slowly, it effectively catalyzes the attachment of various 3',5'-diphosphates and pre-adenylated 3'-nucleoside phosphates to primers on solid supports under mild conditions. The researchers highlighted that the slow rate is inherent to the enzyme, not due to immobilization effects [81].

Flamme and colleagues synthesized a nucleoside triphosphate analog with dual modifications at the sugar (LNA) and backbone (thiophosphate) levels and tested its compatibility with enzymatic DNA synthesis, potentially addressing some synthetic limitations [82]. While this novel analog is less well tolerated by polymerases compared to α-thio-dTTP or LNA-TTP, α-thio-LNA-TTP can still be successfully used in enzymatic synthesis on universal templates to introduce phosphorothioated LNA nucleotides [82].

Lu and coworkers engineered TdT orthologs, identified from terminal deoxynucleotidyl transferase from *Zonotrichia albicollis*, creating (ZaTdT) with over 1000-fold greater catalytic activity for 3′-ONH2-dNTPs than MmTdT [83]. They used this enzyme to synthesize a decamer oligonucleotide via iterative extension and deblocking, achieving de novo DNA synthesis with coupling efficiencies similar to the phosphoramidite process [83].

Later, Sabat and colleagues developed an enzymatic method to protect 3′-O-modified LNA and DNA nucleotides from phosphatase and esterase activity [84]. They found that the Poly(U) polymerase (PUP) is a suitable alternative to TdT and explored engineered DNA polymerases for better tolerance of modified nucleotides [84].

Wiegand and coworkers developed a novel enzymatic platform for RNA oligonucleotide synthesis that operates in an aqueous, template-independent manner [85]. The enzyme adds a reversible terminator NTP to the 3'-end of

the initiator oligonucleotide, followed by deblocking with mild hydrolysis [85]. This iterative process continues until the full-length RNA is synthesized and released enzymatically. Unlike chemical methods, no final global deprotection is required [85].

The Lovelock group reported a biocatalytic method for efficiently producing oligonucleotides in a single operation, bypassing the iterative chain extension, oxidation, capping, and deprotection steps used in traditional synthesis [86]. Utilizing unprotected building blocks and aqueous conditions, their process employs polymerases and endonucleases to amplify complementary sequences within catalytic self-priming templates [86]. To demonstrate the effectiveness of this methodology, they synthesized clinically significant oligonucleotide sequences with various modifications, such as prexigebersen, fomivirsen, alicaforsen, trabedersen, and aganirsen [86].

For recent advancements in the biocatalytic manufacturing of nucleosides and cyclic dinucleotides, as well as progress in enzymatic strategies for producing oligonucleotide therapies, readers are encouraged to consult Giesen et al.'s review [80].

Oligonucleotides modifications

Oligonucleotides can be challenging to deliver and are often rapidly cleared by the kidneys. To overcome these limitations and improve their pharmacological properties, a range of chemical modification strategies have been extensively studied [87–89]. These modifications are designed to increase stability, enhance target affinity and bioavailability, and improve cellular uptake [90]. The effectiveness of these improvements depends on the specific chemical modifications employed. The modifications in the oligonucleotides structure can be generalized into several categories, each aiming to enhance the stability, specificity, and efficacy of the oligonucleotides. These categories include: sugar modifications, phosphate modification, backbone modification and finally the end 5' group modifications [91]. These modifications of oligonucleotides can be associated with diverse properties and various classes of therapeutic molecules, significantly enhancing their utility in clinical applications. Here, we will explore various chemical modifications, including alterations to the backbone, base, sugar, and other intriguing modifications (Table 2).

Base modifications

Base modifications significantly enhance the interaction of oligonucleotides with the target nucleotide, resulting in a thermodynamically stable oligonucleotide-target RNA complex. This heightened interaction makes the oligonucleotide molecule more conducive for sensing applications of the target RNA molecules [92]. Furthermore, this increased affinity of the complex is utilized for selectively blocking mRNA molecules through binding, which can conceal the mRNA splice site. Consequently, mRNA translation can be halted [93].

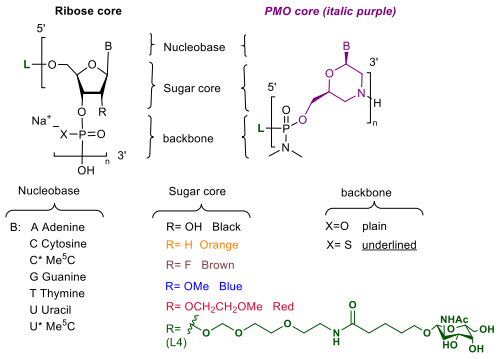
Among the two types of bases, purines and pyrimidines, the 5-position of the latter is predominantly utilized for base modifications (Fig. 1) [92]. There is a plethora of studies have focused on modifying this base position with a C-methyl group, rendering it highly stable, particularly when the methyl group is stacked within the main groove of the RNA duplex [94]. The critical aspect of base modification lies in targeting positions more exposed to the solvent at the major groove. Hence, this position is predominantly selected for modifications. Examples of these modifications can be seen in mipomersen, eteplirsen, nusinersen, inotersen, tofersen, nedosiran, and eplontersen (Table 2). On the other hand, purine bases are typically modified at the 6- and 7-positions, while pyrimidines are functionalized at the 4- and 5-positions [92], However, there are no FDA-approved oligotherapeutics yet that exhibit these specific modifications.

Besides naturally occurring pyrimidine and purine scaffolds, other bases termed universal bases have been utilized. These bases are derivatives of pyrrole, imidazole, and indoles, relying on aromatic ring π-stacking instead of the hydrogen bonding characteristic of natural purine and pyrimidine bases (Fig. 4) [91, 95]. The π-stacking interaction serves as the primary driving force for the stability of the DNA-RNA duplex. Chemical modification of universal bases is a strategy to enhance this π-stacking interaction, as demonstrated by the synthesis of oligonucleotides incorporating tricyclic phenoxazine and G-clamp cytosine derivatives [96, 97].

Sugar modifications

The ribose sugar moiety in RNA, including oligonucleotides, can be modified at the 2' position to adopt a favorable puckered conformation, thereby increasing the likelihood of duplex formation and enhancing the stability of the oligonucleotides against nucleases. As a result of this enhanced stability, oligonucleotides can prolong their pharmacokinetic lifespan for weeks. Studies have shown that certain specific groups at the 2' position of the sugar can exert a beneficial effect on stabilizing the conformation of oligonucleotides [98]. The 2'-fluoro (2'-F) modification on the ribose moiety boosts oligonucleotide activity by enhancing nuclease resistance and improving binding affinity to target RNA or DNA. This modification also preserves the RNA-like structure, increasing stability and overall effectiveness in therapeutic applications. Drugs featuring this modification

Table 2 Chemical structures of FDA approved oligonucleotides



#	Generic Name	Sequence	Modification			Ligand 5'-L
			Base	Sugar	Back-bone	
1	Fomivirsen	5' GCG TTT GCT CTT CTT CTT GCG 3'	---	--	X=S	H
2	Pegaptanib	5' L_1 CGG AAU CAG UGA AUG CUU AUA CAU CCG dt 3'	---	2'-position	---	L_1
3	Mipomersen	5' GC*C* U*C*A GTC* TGC* TTC* GC*A C*C* 3'	C*, U*	2'-position	X=S	H
4	Defibrotide	Not specified-mixture of oligonucleotides	N/A	N/A	N/A	N/A
5	Eteplirsen	5' L_2 *CTC CAA CAT CAA GGA AGA TGG CAT TTC TAG* 3'	T(U*)	*PMO*	phosphorodiamidate	L_2
6	Nusinersen	5' U*C*A C*U*U* U*C*A U*AA U*GC* U*GG 3'	C*, U*	2'-position	X=S	H
7	Patisiran	sense strand 5' GUA ACC AAG AGU AUU CCA Udtdt 3' antisense strand 3' dtdt CAU UGG UUC UCA UAA GGU A 5'	---	2'-position	---	H
8	Inotersen	5' U*C*U* U*GG TTA C*AT GAA AU*C* C*C* 3'	C*, U*	2'-position	X=S	H
9	Givosiran	sense strand 5' CAG AAA GAG UGU CUC AUC UUA L96 3' antisense strand 3' UG GUC UUU CUC ACA GAG UAG AAU 5'	---	2'-position	X=S	5', L=H 3', L96
10	Golodirsen	5' L_2 *GTT GCC TCC GGT TCT GAA GGT GTT C* 3'	---	*PMO*	phosphorodiamidate	L_2
11	Viltolarsen	5' *C"CT CCG GTT CTG AAG GTG TT C* 3'	---	*PMO*	phosphorodiamidate	*C"*
12	Lumasiran	sense strand 5' GAC UUU CAU CCU GGA AAU AUA-L96 3' antisense strand 3' AC CUG AAA GUA GGA CCU UUA UAU 5'	---	2'-position	X=S	5', L=H 3', L96
13	Inclisiran	sense strand 5' CUA GAC CUG UdTU UGC UUU UGU-L96 3' anti-sense strand 3' AA GAU CUG GAC AAA ACG AAA ACA 5'	---	2'-position	X=S	5', L=H 3', L96
14	Casimersen	5' L_2 *C AAT GCC ATC CTG GAG TTC CTG* 3'	---	*PMO*	phosphorodiamidate	L_2
15	Vutrisiran	sense strand 5' UGG GAU UUC AUG UAA CCA AGA **L96** 3' anti-sense strand 3' CU ACC CUA AAG UAC AUU GGU UCU 5'	---	2'-position	X=S	5', L=H 3', L96
16	Tofersen	5' C*AG GAT AC*A TTT C*C*A C*AG C*U* 3'	C*, U*	2'-position	X=S	H
17	Avacincaptad pegol	5' L_3 CGC CGC GGU CUC AGG CGC UGA GUC UGA GUU UAC CUG CG dT 3'	---	2'-position	---	H
18	Nedosiran	sense strand 5' AUG UUG UCC UUU UUA UCU GA G CAG CC G A A anti-sense strand 3' GG UAC AAC AGG AAA AAG AGA CU* C GUC GG A 5' 3'	U*	2'-position, L4	X=S	5', L=H
19	Eplontersen	5' L_5 U*C*U* U*GG TTA C*AT GAA AU*C* C*C* 3'	C*, U*	2'-position	X=S	L_5

N/A not applicable

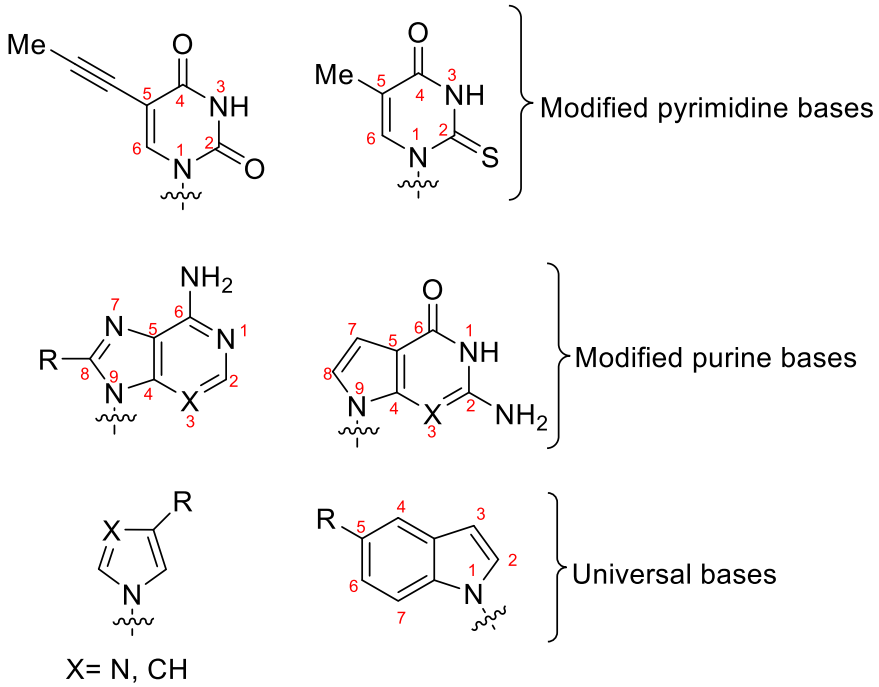


Fig. 4 Chemical structure of other modified nitrogenous bases used for the synthesis of modified oligonucleotides

include pegaptanib, patisiran, givosiran, lumasiran, inclisiran, vutrisiran, avacincaptad pegol, and nedosiran. Additionally, the OMe group at the 2' position, achieved by replacing OH with Me, has been widely studied for its ability to improve duplex stability, with patisiran being one of nine FDA-approved oligonucleotide drugs utilizing this modification.

Martin first reported 2'-O-methoxyethyl (MOE)-modified building blocks and oligonucleotides in 1995 [99]. Additionally, MOE and 2' F groups have been explored and shown some efficacy when present at the 2' position of the sugar [100]. Examples of 2'-MOE-modified therapeutic oligonucleotides include fomivirsen, mipomersen, nusinersen, inotersen, tofersen, and volanesorsen [100].

Another key modification replaces the ribose or deoxyribose sugar backbone with a morpholine ring, forming Phosphorodiamidate Morpholino Oligomers (PMOs) and connecting the bases through phosphorodiamidate bonds instead of phosphodiester bonds. This modification greatly enhances the stability of the oligonucleotide, making it resistant to nucleases and other degrading enzymes. PMOs are widely used in antisense therapies due to their improved stability, low toxicity, and effective ability to block specific RNA sequences, thereby inhibiting gene expression [101, 102]. FDA-approved PMOs include eteplirsen, viltolarsen, golodirsen, and casmersin, used to treat Duchenne muscular dystrophy by targeting specific exons in the dystrophin gene. These drugs exemplify modifications in both the sugar unit and the phosphorodiamidate backbone.

A more sophisticated approach for sugar modification involves locking, leading to the generation of Locked

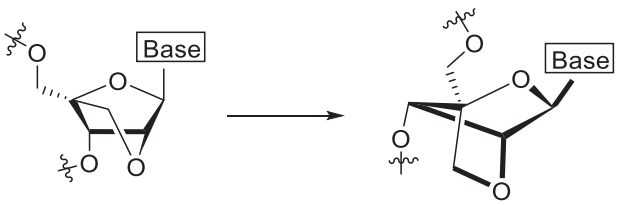


Fig. 5 A segment of a locked nucleic acid with normal confirmation (left) and its puckered confirmation (right)

Nucleic Acid (LNA), where the 2' and 4' positions are linked through an oxymethylene linkage (Fig. 5) [103].

This modification has revolutionized the properties of oligonucleotides, particularly in the therapeutic realm. The puckered conformation has shown enhanced binding affinity towards complementary DNAs and RNAs. In addition, the puckered LNA oligonucleotide molecule has demonstrated reduced length within the sequence and an improvement in mismatch discrimination or base-pairing selectivity in the Watson-Crick model [104–106]. This stability also improved the resistance of LNA to biological degradation, such as enzymatic degradation. The antisense properties of therapeutic oligonucleotides have also been enhanced, as exemplified by the mRNA inhibition with Ribonuclease (RNase) H. Furthermore, There are some reports where non-RNase has also been investigated for antisense properties [107]. Wahlestedt et al. were the first to discover the potent and safe antisense role of other LNA molecules as a modification in vivo [108]. Following this discovery, numerous reports have been published on the antisense role of LNA-modified oligonucleotide molecules,

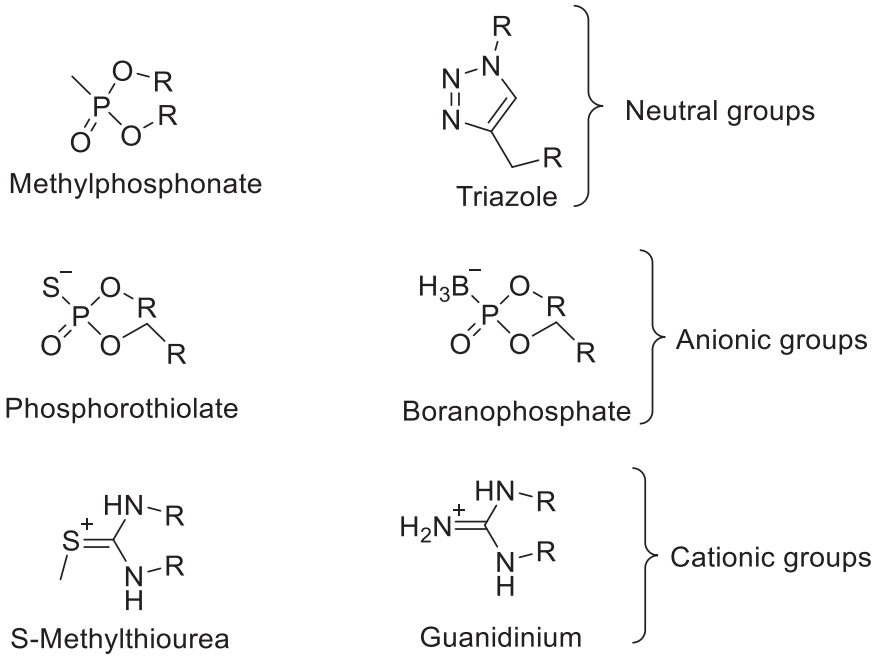


Fig. 6 Anionic, cationic and neutral groups used for backbone modification of oligonucleotides

encompassing gene silencing both in vivo and in vitro. Recent studies have also explored the inhibition of HIV-I expression using LNA-modified oligonucleotides [109–112].

Another crucial class of LNA-modified oligonucleotides includes aptamers, which are short DNA or RNA molecules [113], with a higher affinity to bind to the target due to the three-dimensional puckering of the sugar moiety [114]. Hence, they surpass their non-LNA counterparts due to their high affinity for the target and their increased resistance to nucleases [108]. Non-LNA aptamers, whether DNA or RNA, can be modified into their LNA counterparts through the common systematic evolution of ligands by exponential enrichment (SELEX) process, and by post-chemical modifications of the aptamers sequences LNA nucleotides [115, 116]. The second method involves the utilization of LNA-modified sequence libraries for the generation of LNA aptamers through standard SELEX methodology [117–119]. Other classes of LNA-modified oligonucleotides include LNA-modified siRNA and microRNA modified through LNA [120–124].

Sugar modifications have recently gained traction due to the growing application of oligonucleotides in the biomedical field. A wealth of literature is available on the synthesis of various sugar-modified oligonucleotides [125, 126].

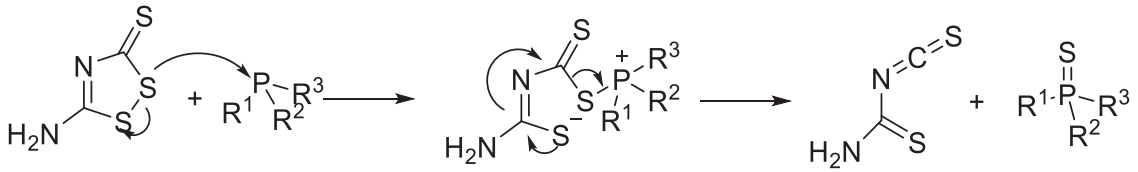
Backbone modifications

Backbone-modified oligonucleotides represent the first generation of antisense oligonucleotides, featuring modifications that include functionalities and groups such as guanidinium, amides, amines, thioethers, thioesters, triazole, boranophosphate, methylphosphonate, N-3′-phosphoramidate, and S-methylthiourea [59]. These modifications can exist as anions, cations, or neutral groups, enhancing the physical and biological properties of oligonucleotide molecules and ultimately improving their performance in target applications (Fig. 6) [59, 91].

Among these, phosphorothioate (PS) diesters and phosphorodiamidate in the morpholino-containing oligonucleotides (PMOs) have proven to be of pivotal importance in oligonucleotide modification. The remarkable effect of the PS di-ester modification was the reduction of the melting temperature (Tm) by 0.5–0.7 °C per each modification [127]. A moderate balance was sought to achieve optimal properties with a balanced possible modifications [128]. Thus, PS modification was observed to enhance the bioavailability and cellular uptake of PS oligonucleotides. This improvement is attributed to the increased hydrophobicity of sulfur atoms in the modified oligonucleotides compared to the hydrophilic oxygen in the unmodified molecules (Scheme 6).

Gene silencing is an important biological process achieved using the phosphorothiolate (PS) internucleoside linker. This PS linker provides firm resistance to nuclease enzymes, resulting in better bioavailability of the oligonucleotide molecules. Moreover, the anionic nature of the PS group enhances the base-pairing affinity of the oligonucleotide molecules [129]. Higher plasma-membrane affinity led to observed improvements in pharmacokinetic properties and cellular uptake. The first FDA-approved antisense drug, fomivirsen, containing the PS group, was approved

Scheme 6 Phosphorothioate backbone modification of a segment of oligonucleotides molecule



for treating AIDS-related cytomegalovirus (CMV) retinitis. However, it exhibited reduced specificity in binding to target oligonucleotide sequences [130].

Another significant modification is *N*-acetylgalactosamine (GalNAc), which acts as a ligand molecule binding to the asialoglycoprotein receptor (ASGPR). The RNA-GalNAc conjugate is crucial in the treatment of acute hepatic porphyria, approved by the FDA and commercially available as givosiran [131].

Backbone modifications are introduced by either on-resin coupling of a monomer or by coupling a dinucleoside with the artificial linkage. The monomer method faces challenges due to potential chemical incompatibilities, while the dimer approach is often preferred for constructing more complex backbones. The monomer method has been used for modifications such as PS [132], phosphorodithioates [133], boranophosphates (borano) [134], phosphoramidates (PA) [135], methylphosphonates [136], and amides [137]. The dimer approach is often favored for synthesizing more complex backbones on a solid support [138]. The lack of optimized, user-friendly protocols for on-resin artificial backbones and the difficulties of the dimer approach are key obstacles in therapeutic oligonucleotides research [138].

Other modifications

Studies have shown that sugar-modified oligonucleotides are less toxic than their base-modified counterparts (phosphorothioates); however, their potential to cleave target RNA is inferior. RNase cleavage is considered a crucial and vital process in the application of therapeutic oligonucleotides. Therefore, more advanced modifications have led to the development of 'gamer' type oligonucleotides, achieved by simultaneously incorporating 2'-O-alkyl groups on the sugar and phosphorothioate base modifications [139].

Peptide Nucleic Acids (PNAs) represent the third generation of oligonucleotide molecules, introduced by replacing the phosphodiester group with a synthetic, flexible pseudopeptide polymeric *N*-(2-aminoethyl)glycine molecule [140]. This unique structure has the base molecule directly attached to the backbone via a methylene carbonyl group. The PNA class of oligonucleotides revolutionized the interaction with the complementary RNA or DNA strand through hybridizing with greater affinity and outstanding location specificity compared with the natural counterparts (Fig. 7) [141].

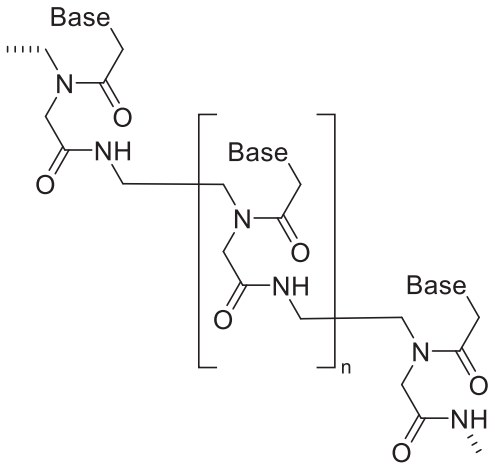


Fig. 7 Chemical structure of peptide nucleic acid (PNA)

Conclusion

Oligonucleotides have attracted significant attention from researchers due to their potential use as alternative medicines. These short DNA or RNA molecules can be designed to target specific sequences within the human genome, allowing for highly precise therapeutic interventions. The initial synthetic methodologies employed reagents and starting materials that created isolation problems, such as the water-sensitive phosphoryl chloride intermediate, which hindered the reaction by producing side products. A modified approach used a 4-monomethoxytrityl and 4,4-DMT protecting group, which was acid-sensitive. Besides, early solution-based methodologies required large quantities of organic solvents, which were not considered environmentally friendly. These methodologies were later replaced by SPOS. Recently, new and improved methods of oligonucleotide synthesis have been developed. One such approach is the use of O-alkylphosphoryl groups, which replaced the conventional O-cyanoethylphosphoramidites. These O-alkylphosphoryls were found to be more stable in the presence of alkali than their cyanoethyl counterparts. In 2023, easily accessible and easily cleavable 2,2′-sulfonyldiethylene linkers (X-linker and X-amidite) were introduced for the synthesis of modified oligonucleotides. Enzymatic synthesis has greatly advanced oligonucleotide production by enabling single-step biotransformation, removing the need for repetitive chain extension, oxidation, capping, and deprotection steps. This

method provides access to a broad range of oligonucleotide sequences and modifications, which can be further enhanced through the discovery or engineering of biocatalysts with a wider substrate scope.

Due to the increased use of oligonucleotides in biomedical applications, many modifications to the oligonucleotide nucleus have been made to synthesize antisense oligonucleotides. These modifications include changes to the sugar, backbone, and base modifications such as guanidinium, amides, amines, and thioethers. These modifications have revolutionized the properties of oligonucleotides, significantly enhancing their antisense capability and target specificity. As a result, third-generation peptide nucleic acids and phosphorodiamidate morpholino oligomers were developed, demonstrating remarkable locus-specificity and gene inhibition efficiency.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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