

REVIEW

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Modified nucleic acids: replication, evolution, and next-generation therapeutics

Karen Duffy[†], Sebastian Arangundy-Franklin[†] and Philipp Holliger^{*}

Abstract

Modified nucleic acids, also called xeno nucleic acids (XNAs), offer a variety of advantages for biotechnological applications and address some of the limitations of first-generation nucleic acid therapeutics. Indeed, several therapeutics based on modified nucleic acids have recently been approved and many more are under clinical evaluation. XNAs can provide increased biostability and furthermore are now increasingly amenable to in vitro evolution, accelerating lead discovery. Here, we review the most recent discoveries in this dynamic field with a focus on progress in the enzymatic replication and functional exploration of XNAs.

Nucleic acids: natural and expanded functions

A heritable genetic system is a defining requirement for life. The natural nucleic acids, DNA and RNA, are exquisitely suited to store and propagate genetic information with sufficient stability and fidelity to support large genomes but also the flexibility to enable evolution. Nucleic acids are unique among biopolymers in that both accessible information and functional capacity coexist in a single molecule. Thus, function can be evolved at the molecular level yielding nucleic acid-based ligands and enzymes. In addition, nucleic acids fold through predictable and (to a large extent) programmable interactions, are highly water-soluble, and can be easily denatured and refolded. These advantages underpin their manifold uses in biotechnology, diagnostics, therapeutics, nanotechnology, material science, synthetic biology, and data storage.

The last four decades have seen the rise of nucleic acids as therapeutics [1], primarily in the form of antisense oligonucleotides (ASOs) [2], small interfering RNAs (siRNAs) [3], aptamers [4–6], microRNAs [7], mRNAs [8], and gene-editing guides [9]. Nucleic acids

afford completely new modalities for therapeutic intervention (e.g., direct protein expression or specific and programmable modulation of gene expression) that cannot be easily accessed by other biologics or small molecule drugs. However, several challenges have thus far prevented nucleic acids from reaching their full potential as medicines including poor chemical and biological stability and narrow chemical diversity.

To address these limitations, DNA and RNA analogues have been developed and evaluated for their ability to serve as useful biomaterials or functional molecules, encode genetic information, and support evolution. These synthetic genetic polymers, broadly termed xeno nucleic acids (XNAs), exhibit modified backbones, sugars, or nucleobases, and even novel bases or base pairs [10, 11]. While natural nucleic acids may also be modified to modulate their function in vivo (e.g., post-synthetic epigenetic modifications) [12–17], we constrain ourselves here to a discussion of nucleic acid congeners not found in nature. We do, however, consider certain modifications that occur sporadically in natural oligonucleotides, such as 2'OMe or C5 pyrimidine modifications, to be XNAs in the case of fully (or heavily) substituted oligonucleotides, the likes of which are not found in biology. Several new and prominent XNA chemistries are shown in Fig. 1. One attractive feature of XNAs is their generally improved chemical and biological stability [18–20].

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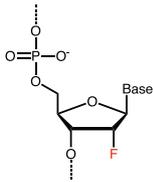
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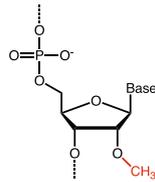
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Sugar Modifications

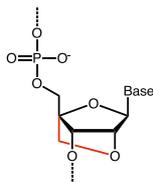
2'F RNA



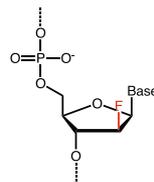
2'OMe RNA



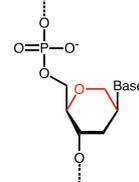
LNA



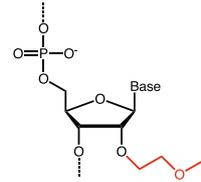
FANA



HNA

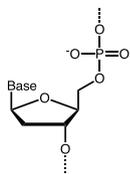


2' MOE

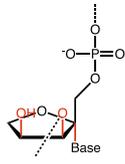


Sugar/Backbone Modifications

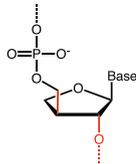
mirror DNA



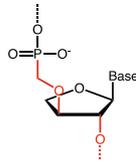
ribuloNA



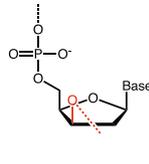
TNA



tPhoNA

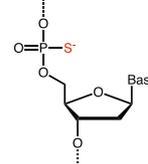


dXNA

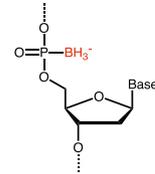


Backbone Modifications

PS

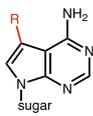


borano-phosphate



Base Modifications R=H, Cl, F, ...

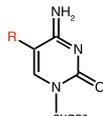
C7-modified deaza-adenine



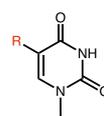
C7-modified deaza-guanosine



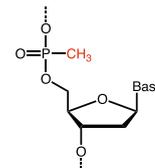
C5-modified cytosine



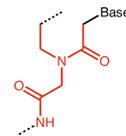
C5-modified uridine



phNA

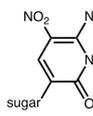


PNA

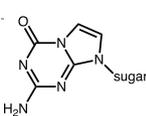


Unnatural Base Pairs (UBPs)

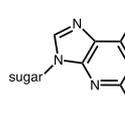
dZ



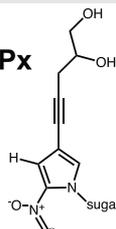
dP



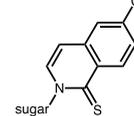
Ds



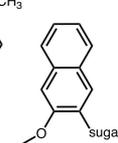
Px



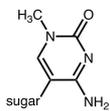
5SICSN



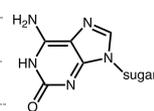
aM



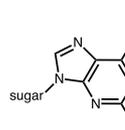
dS



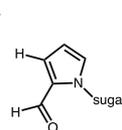
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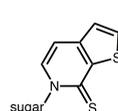
Ds



Pa



TPT3



NaM

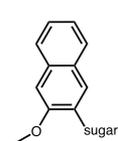


Fig. 1 (See legend on next page.)

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Fig. 1 The chemical diversity of XNAs. XNAs are often categorized by the component of the nucleotide (sugar, backbone, or base) carrying a modification. Shown here are XNAs discussed in this review, including both those of medical and historical relevance as well as several newly described chemistries. 2'F, 2'-fluoro; 2'OMe, 2'-O-methyl; LNA, locked nucleic acid; FANA, 2'-fluoro arabinose nucleic acid; HNA, hexitol nucleic acid; 2'MOE, 2'-O-methoxyethyl; ribuloNA, (1'-3')- β -L-ribose nucleic acid; TNA, α -L-threose nucleic acid; tPhoNA, 3'-2' phosphonomethyl-threosyl nucleic acid; dxNA, 2'-deoxyxylonucleic acid; PS, phosphorothioate; phNA, alkyl phosphonate nucleic acid; PNA, peptide nucleic acid

Decoration with diverse chemical substituents (e.g., hydrophobic groups) can also yield improved properties and functionalities such as new structural motifs and enhanced target binding [21–24]. While a wide range of XNAs have been synthesized at the chemical level, representing a rich palette to draw from, their functional potential and usage is only just beginning to be probed. Here, we explore recent discoveries in the enzymatic synthesis and functional exploration of XNAs with a view towards therapeutic applications.

Expanding the chemistry of nucleic acids

Advances in nucleic acid chemistry have enabled the development of XNAs with improved base-pairing stability over natural nucleic acids as well as enhanced activity in the context of living tissues, leading to several FDA approved nucleic acid therapeutics [25]. Research continues apace to identify novel chemistries with increased potency, bioavailability, stability, decreased toxicity, and minimal off-target effects.

An interesting development in this context is the discovery that mixed backbone chemistries can display novel emergent properties. For example, Krishnamurthy and colleagues probed the ability of XNA-RNA chimeric oligonucleotides to hybridize with their natural nucleic acid counterparts and found that the otherwise non-pairing ribuloNA can be interspersed with RNA or DNA building blocks to yield oligonucleotides with comparable or higher duplex thermal stability than natural systems and tuneable base-pairing properties [26]. The authors suggest that such a system could be used to mimic natural base-pairing rules with a reduced number of bases, storing information in the sugar in place of the GC base pair.

Similarly, the recent discovery that smaller “skinny” (pyrimidine-like) and larger “fat” (purine-like) base pairs can form stable helices challenges conventional assumptions of nucleic acid structure [27]. While the novel base pairs did not lend stability when interspersed in a natural oligonucleotide duplex, fully skinny or fat duplexes showed increased thermal stability over the corresponding natural duplexes.

In another example of XNAs reshaping classical assumptions [28], enzymatic synthesis of phosphonate nucleic acid (phNA), in which the negatively charged non-bridging oxygen of the phosphodiester linkage is replaced by an uncharged alkyl group, has enabled the

demonstration of genetic function in an uncharged backbone chemistry for the first time as well as in vitro evolution of phNA aptamers [29].

As certain individual modifications become easier to synthesize and their unique properties better understood, combining two or more of these known modifications to sugars, backbones, and bases will further push the boundaries of chemical diversity in XNAs [30–35].

The development of ever more diverse chemistries raises the question: how much of what we know about natural nucleic acids translates to XNA? To what extent can we borrow design strategies from natural nucleic acids in order to rapidly develop useful new XNA chemistries? Some design rules, such as the ability of certain sequence motifs to form G-quadruplexes, have been shown to translate to TNA [36] and FANA [37]. However, the generality of such parallels remains to be explored.

Templated synthesis of non-natural nucleic acids

The phosphoramidite approach to solid-phase DNA synthesis [38, 39] developed in the early 1980s led to the mainstream use of synthetic DNA in a myriad of applications. Similarly, improvements in the synthesis of XNAs can be expected to increase their usage and unlock new applications in the coming decades. XNA synthesis can be broadly divided into enzymatic and non-enzymatic approaches. Some widely used XNAs (e.g., 2'OMe, LNA, PS, 2'MOE) can be chemically synthesized, although yields of even the most synthetically accessible XNAs are limited to around 150 bp [40]. For other XNA chemistries, no reliable solid-phase synthetic route is known [41]. Furthermore, solid-phase synthesis depends on explicit knowledge of the sequence being synthesized. In contrast, templated synthesis enables general information transfer and is essential for the evolution of functional oligonucleotides.

Non-enzymatic templated synthesis

Non-enzymatic templated synthesis traditionally uses activated nucleotides or short oligonucleotide building blocks that self-organize on a template via base-pairing interactions and react to polymerize. Such template copying, pioneered in the 1970s [42–44], has been extended to several XNA chemistries [45–53], often in the context of prebiotic nucleic acid replication. For example, systems for templated replication of PNA pentamers [54, 55] using reductive amination are efficient enough to permit model selection experiments [56]. Similar strategies have also

been exploited to assemble nucleic acids via copper-catalyzed click ligation of oligonucleotides [57, 58] and by phosphoramidate ligation [59], and the resulting backbones have shown some biocompatibility [60]. In an interesting extension of this work, nucleic acid templates have also been used to organize chemical entities by proximity for programmed reactions [61–63]. In particular, the Liu group has extended such templated synthesis approaches to chemically ligate diverse non-nucleic acid entities in precise sequences defined by a DNA template [64]. Such templated chemical syntheses allow increasingly divergent chemistries to be synthesized and replicated without constraining polymer diversity by the ability of its constituent monomers to serve as substrates for polymerases or other nucleic acid-modifying enzymes.

Enzymatic ligation and modification of XNAs

Several DNA ligases have been shown to accept non-natural substrates and have been used alone or in conjunction with XNA polymerases to synthesize XNA oligonucleotides. Much like the aforementioned chemical ligation strategies, enzymatic ligation of pre-organized oligomers on a template allows for positioning of modified nucleotides, including multiple different modifications, at defined loci. It has recently been shown that several commercially available ligases can catalyze ligation of XNA substrates including 2'OMe, HNA, LNA, TNA, and FANA [41, 65]. Moreover, rational design and molecular modeling approaches have now resulted in the first XNA-templated XNA ligase [66]. The Liu and Hili groups have also worked extensively with ligase-mediated synthesis of modified DNA from diversely functionalized DNA 3- or 5-mers [67, 68], allowing a great variety of modifications, including hydrophobic, aliphatic, aromatic, acid, and basic moieties, to be incorporated using these short “quasicodons.” In agreement with results obtained with SOMAmer (Slow Off-rate Modified Aptamer) selections [22], they find that functionalization with nonpolar moieties appears to promote faster selection convergence and stronger aptamer binding [21]. Incorporating chemical diversity beyond that found in proteins (e.g., halogenated residues), the group isolated high affinity aptamers against PCSK9 and interleukin-6 [69]. The above strategies encapsulate the paradigm of leveraging the unique properties of nucleic acids (i.e., encoded synthesis, evolvability) coupled with an expanded set of chemical substituents to create functional molecules with therapeutic potential.

Polymerase synthesis of XNAs

Enzymatic polymerization of nucleic acids is orders of magnitude faster and more accurate than chemical copying or synthesis and is now being pursued for next-generation DNA oligonucleotide synthesis [70, 71], with

a view to supersede phosphoramidite technology. Similarly, enzymatic XNA synthesis may facilitate production of XNA oligonucleotides. XNAs with chemically conservative modifications are accepted as substrates by natural polymerases, while engineering of polymerases to accept a broader range of XNA substrates has also met with success [72, 73]. Indeed, some polymerases have proven highly adaptable to new substrates. Thermophilic B-family polymerases, especially those from *Pyrococcus* and *Thermococcus* genera, have proven especially amenable to engineering for XNA substrates [33, 74–81]. This functional plasticity may be aided by high thermostability, which promotes greater tolerance for mutations that would otherwise be excessively destabilizing. However, A-family [82–90] and mesophilic polymerases such as Phi29 [91, 92] as well as RNA polymerases [93–97] have also been engineered to accept XNA substrates with success (Table 1). However, the efficiency, fidelity, and kinetics of engineered polymerases on XNA substrates are usually compromised relative to the native enzymes and natural substrates. Often, “forcing” conditions (e.g., superstoichiometric polymerase concentrations or the presence of Mn^{2+} , which in turn reduces fidelity) are needed to boost synthetic yields [106].

Capitalizing on the promiscuity of natural polymerases

It is perhaps remarkable that natural polymerases can incorporate modified nucleotides at all, given their need for stringent substrate specificity and accuracy. However, at some positions in the nucleotide, modifications are readily tolerated. For example, modifications at the C5 position of pyrimidines and C7 position of N7-deazapurines project into the duplex's major groove and do not interfere with Watson-Crick base pairing. Therefore, even bulky adducts at these positions are generally well tolerated by polymerases [22, 107–112]. Enzymatic synthesis of nucleotides with reactive groups (e.g., for copper-catalyzed click chemistry) at these positions can allow elaborate post-synthetic modifications [113–117].

The plasticity of polymerases has also allowed researchers to carry out wholesale replacement of natural bases with close chemical analogues [118, 119]. In some cases, synthesis is efficient enough to produce modified PCR products of up to 1.5 kb [119]. Moreover, entirely new unnatural base pairs (UBPs) have been developed based on novel hydrogen bonding patterns [105, 120], hydrophobicity [121, 122], and shape complementarity [123–125]. These UBPs can be replicated by engineered and natural polymerases, demonstrating successful expansion of the genetic code, and recently achieving information encoding in an unprecedented eight-letter genetic alphabet [120].

XNA synthesis in vivo

The synthesis and replication of XNAs in vivo represents an important frontier in XNA research towards aims

Table 1 Polymerase-mediated synthesis of XNAs

Pol Family	Polymerase	Novel Activity	Ref.
Pol A	Taq Tth Pol θ	2'F RNA 2'OME RNA 2'-azido RNA	[83, 84, 87, 88, 90, 98]
Pol B	Tgo KOD ϕ N Pfu phi29	CeNA LNA phNA HNA FANA CyDNA 2'F RNA ANA TNA 2'azido RNA tPhoNA	[29, 33, 75, 78, 81, 82, 92]
Pol Y	(D-aa) Dpo4	L-DNA	[99–101]
Pol X	(D-aa) ASFV pol	L-DNA L-RNA	[102]
RNAP	T7 RNAP Syn5	2'F RNA 2'OMe RNA Ds-Pa UBP	[95, 97, 103, 104]
RT	HIV-RT	pyDAD-puADA UBP	[105]

Natural and engineered polymerases across of wide range of families have been used to synthesize unnatural nucleic acids. Polymerases are shown as space-filling models with primer shown in blue and templates shown in red. PDB codes are 4BWJ (Taq), 5OMF (KOD), 4G3I (Dpo4), 5HRF (ASFV), 1H38 (T7 RNAP), and 6HAK (HIV RT)

such as stable encoding of unnatural amino acids and genetic orthogonality for “firewalling” synthetic biology. To this end, complete replacement of cytidine and/or thymidine with 5-substituted pyrimidine analogues in bacterial genomes has now been achieved by careful metabolic engineering and evolution [126–129].

Remarkably, Romesberg and colleagues have been able to engineer *E. coli* strains with the capacity to replicate and maintain their unnatural NaM–TPT3 base pair in both plasmid [130] and genomic [131] contexts. To achieve this, they engineered a nucleoside triphosphate transporter [132], invoked Cas9 to degrade sequences having lost the UBP, and made several tweaks to the *E. coli* DNA synthesis and repair machinery. They further demonstrated the compatibility of the UBP with translation machinery to site-specifically encode unnatural amino acids [133, 134]. Further semi-synthetic organisms with substituted genomes or the ability to propagate UBPs will likely follow these early successes [135, 136].

Directed evolution for XNA polymerase engineering

While base modifications and even completely new base pairs are tolerated by natural polymerases to varying extents, modifications to the sugar and backbone generally prove more challenging, especially at full substitution. To improve activity with such unnatural substrates, polymerase engineering approaches, ranging from rational engineering driven by structural insights or computational analysis to screening and directed evolution, are often employed. Among these, emulsion-based methods and phage display have been especially useful [137, 138].

A range of directed evolution strategies have been employed in the field. Compartmentalized self-replication (CSR) [139–141] challenges polymerases to PCR amplify their own gene inside an emulsion compartment. The exponential enrichment of highly active clones, as opposed to simple partitioning of active variants, makes CSR a powerful method. However, it places stringent demands on polymerase performance, which are somewhat relieved in short-patch CSR (spCSR) [85] where amplification is confined to only a short section of the polymerase gene. Nested CSR allows selection of sequences or features not present in the polymerase gene itself (e.g., novel base pairs, reverse transcription of XNA templates) by using out-nesting primers with these features during the CSR amplification step [79, 86]. A further extension called compartmentalized partnered replication (CPR) can be used to select for enzymatic activities other than nucleic acid polymerization by constraining the selectable PCR reaction by the fitness of a partner gene [86, 142]. Recently, CSR has also been adapted for isothermal amplification reactions (iCSR), both at high temperatures [143] and at lower

temperatures for evolution of non-thermostable enzymes [91]. Compartmentalized self-tagging (CST) [144] allows selection for much more difficult substrates or conditions; selection is based on a primer extension templated by the encoding plasmid. The biotinylated primer along with any plasmids that have been “captured” by sufficient primer extension are partitioned on streptavidin beads. CST has yielded polymerases for a range of XNAs [78], most recently for dxNA [145]. In contrast to bulk emulsification methods, microfluidic devices have been used to generate highly homogeneous emulsion droplets, enhancing the ability to accurately distinguish small incremental improvements important in stepwise selections [146, 147]. Another directed evolution strategy is phage display, whereby polymerases displayed on phage particles are challenged to extend a primer with separable (e.g., biotinylated) nucleotides [80, 89, 148]. In a notable recent use of phage display for polymerase evolution, Chen et al. identified several Taq variants capable of synthesizing and reverse transcribing 2'OMe RNA [88], an XNA of particular interest for therapeutics due to its high biostability and nuclease resistance.

Rational engineering of XNA polymerases: structural insights

Polymerases make many key contacts with the incoming nucleotide triphosphate and primer strand during the catalytic cycle [149]. Structures of polymerases in complex with modified substrates may therefore aid in both rational engineering and retrospective understanding of XNA polymerases. Recently, Kropp et al. solved ternary structures of KlenTaq polymerase with a C5-modified cytidine at each of six successive positions [150] in the catalysis cycle, recapitulating the movement of the modified substrate through the polymerase's active site and revealing a surprising level of flexibility in both the modified nucleotide and the interacting polymerase residues to accommodate the modification.

Further structural insight into XNA polymerization has been reported by Singh et al. [151] with structures of an engineered KlenTaq in a binary pre-incorporation and a ternary post-incorporation complex with the unnatural base pair dZ:dP. Although none of the mutated amino acids are in direct contact with primer, template, or incoming dZTP, the structure suggests that increased flexibility, particularly in the thumb subdomain, and an increased angle of closure in the finger subdomain facilitate primer elongation with the unnatural base pair.

Chim et al. recently reported ternary structures of a hyperthermophilic B-family polymerase, an engineered KOD mutant, polymerizing TNA [152]. Given the prevalent use of B-family hyperthermophiles in polymerase engineering, the structures of the apo, binary, and ternary polymerase complexes will prove useful in

developing hypotheses for further work in the field. Subsequently published ternary structures of KOD and 9^oN with DNA primer and template and an incoming dNTP [149, 153] suggest possible explanations for the predisposition of hyperthermophilic archaeal B-family polymerases to engineering: an unusually wide and positively charged channel between the finger and thumb subdomains may allow space for substrate modifications while maintaining strong binding to the template and high processivity. Additionally, whereas the primer template duplex adopts an A-form in the active site of A-family polymerases, it adopts a B-form in KOD, possibly contributing to accommodation of nucleotide modifications in the B-form duplex's wider major groove.

Rational engineering of XNA polymerases: translation of mutations across substrates and polymerases

There are numerous examples of transferable mutations across XNA substrates and polymerases. A recent report [154] details engineering of a previously described Taq mutant to better incorporate 2' modified XNAs. From kinetic data on incorporation of different 2' modified nucleotides, relevant mutations from the literature were chosen based on the hypothesis that the rate-limiting step may be recognition of the modified primer strand. Several mutants showed enhanced synthesis of 2'F and 2'OMe and/or reverse transcription of 2'OMe RNA. In another instance, diversification at eight "specificity determining residues" selected by computational analysis, evolutionary conservation, and literature precedent yielded improved polymerases for RNA and TNA [75]. Furthermore, testing the top mutation sets in the context of different homologous B-family polymerases evidenced their general utility as well as revealing the structural context in which they functioned best. Similarly, polymerase synthesis of increasingly diverse XNAs was achieved with a TNA polymerase by combining the TNA sugar with base modifications known to be polymerase-compatible [31, 32]. While it has been generally believed that mutation of conserved residues is highly deleterious, it is becoming increasingly apparent through this and other work that such mutations can be key in allowing activity with unnatural substrates [75, 155].

Liu et al. recently reported engineering of a polymerase to synthesize a newly described XNA, tPhoNA, with both sugar and backbone modifications [33]. Impressively, the polymerase engineering strategy consisted solely of successive introduction and evaluation of mutations at positions known to affect synthesis for other XNA substrates. Their success is a testament to the accumulating knowledge base in the field of polymerase engineering and the extraordinary ability of a small number of specific key mutations to confer an expanded substrate spectrum.

RNA polymerases have also been engineered to synthesize XNAs. It has been shown that T7 RNAP can transcribe content-expanded RNA from DNA containing the Ds-Pa UPB as well as further modified Pa bases [103]. Through a small screen of previously identified mutations, Kimoto et al. identified a T7 RNAP mutant that incorporates 2'-F uracil and cytidine triphosphates and shows improved incorporation of Pa nucleotides and their analogues in difficult sequence contexts [93]. Surprisingly, several bulky modifications of the Pa nucleotide designed to expand chemical diversity for aptamer and ribozyme selections appear to be even better substrates than the original Pa triphosphates. Similarly, a Tgo polymerase mutant previously evolved to synthesize HNA was recently shown to incorporate HNA nucleotides with various aromatic modifications on the uridine base [35]. Here too, some of the bulkier base modifications demonstrated superior incorporation.

Reverse transcription of XNAs

Identification of enzymes capable of reverse transcribing XNAs demonstrates the potential of these divergent chemistries as genetic materials and is crucial for in vitro selections [156]. Some XNAs can be reverse transcribed by natural polymerases (e.g., TNA and FANA by Bst polymerase [157–159]). Alternately, engineering approaches must be taken [79, 88]. A small number of mutations in Tgo polymerase yielded a reverse transcriptase with a generally expanded substrate spectrum that can reverse transcribe several XNAs to DNA [29, 78].

Analysis of XNAs and XNA polymerases

One difficulty in working with XNAs is that they are often incompatible with traditional methods of nucleic acid manipulation or analysis such as restriction enzymes and sequencing technologies. Thus, an important parallel effort to the development of XNAs and XNA polymerases is the development of tools to analyze them. For example, fidelity has not been evaluated in detail for many of the described XNA polymerases. A recent method for assaying fidelity reported finding that even relatively small and natural RNA modifications can significantly increase polymerase error rates [160]. Deep sequencing has also been used to investigate error profiles and polymerase read-through of templates with backbone modifications [161] and found that some commonly used isosteric modifications, such as phosphorothioates, caused significant copying errors. This fidelity data foreshadows an important limitation in enzymatic XNA synthesis that will likely need to be addressed as the field moves forward. On the other hand, the promiscuous behavior of polymerases has been leveraged to read and record the presence of epigenetic DNA and

RNA modifications via misincorporation “signatures” at the modifications [162–170]. In addition, the first instance of direct sequencing of an XNA was recently reported; FANA was sequenced using nanopore technology, albeit with relatively short read lengths [171].

An XNA particularly limited by a lack of tools is L-DNA. Such “mirror image” DNA provides both complete orthogonality in macromolecule-scale features and interactions, while maintaining identical properties to DNA at the chemical level. Thus, enzymatic synthesis of L-DNA requires mirror image polymerases made of D-amino acids. Zhu and colleagues first made the D-form of the smallest known polymerase, African Swine Fever Virus polymerase X, and used it to synthesize L-DNA, showing that the polymerase was strictly enantio-specific with no crossover inhibition from D-nucleotide triphosphates [102]. The group subsequently reported synthesis of a mutant of the larger thermostable Dpo4 polymerase with D-amino acids, which enabled PCR amplification of an L-DNA product [99, 100]. Klussmann and colleagues had also reported synthesis of a D-Dpo4 mutant [101], which was used to assemble gene-length L-DNA sequences. A mirror image ligase has also been reported [172]. Mirror image nucleic acids are of immense interest for therapeutics and are being actively pursued in research and clinical development [173]. However, their usage remains limited by the arduous process of synthesizing D-polymerases and the incompatibility of L-DNA with traditional nucleic acid manipulation tools.

Non-natural nucleic acids for therapeutic applications

Current FDA- and EMA-approved nucleic acid therapeutics (Table 2) can be broadly defined in three categories: antisense (ASO), aptamer, and most recently, siRNA. Given the inherent instability of natural nucleic acids in biological fluids, it is not surprising that every example from these categories is either fully or partly modified. Fomivirsen, a PS ASO to treat cytomegalovirus retinitis (CMV), was the first nucleic acid therapeutic to gain regulatory approval. It was subsequently withdrawn from the market due to a lack of demand as incidence of (CMV) infections decreased sharply [184]. Nevertheless, it proved an important step in demonstrating the safety and potential of nucleic acid medicines. Eteplirsen and Golodirsen are fully composed of a morpholino phosphoramidate (PMO) backbone, while Mipomersen, Nusinersen, Inotersen, and Volanesorsen feature both phosphorothioate (PS) and 2′-methoxyethyl (2′MOE) modifications. Patisiran contains 2′OMe pyrimidine residues in both siRNA strands. The siRNA Givosiran also carries PS linkages and 2′F and 2′OMe modifications.

Pegaptanib, while discovered as an RNA aptamer, underwent rounds of medicinal chemistry-like optimization and modification with 2′OMe and 2′F moieties to increase its *in vivo* stability and potency. Over 100 oligonucleotide drugs are currently in clinical trials [185], evidencing the belief and momentum behind these new therapeutic modalities able to address previously undruggable target classes.

Somalogic has pioneered aptamer discovery with their SOMAmer platform [186], which employs modifications at the C5 position of pyrimidine nucleotides to append protein-like side chains to aptamer libraries, generating aptamers of high affinity and specificity for diagnostic and clinical applications.

As mentioned above, aptamers modified with 2′F and 2′OMe moieties have been selected previously, but Liu and colleagues have recently made possible direct selections in the latter backbone at full substitution using two Taq polymerase variants [104]. Similarly, direct selections of TNA have recently yielded aptamers against proteins [187] and small molecule targets [188]. Pursuant to the hypothesis that UBPs increase the sequence space and thus functional diversity of nucleic acid sequences, aptamers containing UBPs have been selected against both protein targets [189–191] and whole cells [192, 193].

In many cases, aptamers are selected as DNA or RNA sequences and then modified post-selection. Commonly, DNA and RNA aptamers are converted to their 2′ modified counterparts, producing useful affinity reagents for applications that would be incompatible with natural nucleic acids (e.g., for stability in alkaline conditions) [194]. In many cases, such post-SELEX modifications decrease affinity of the original aptamer, arguing for direct selection in XNA systems where possible. However, this is not always the case. A single base modification introduced in a thrombin aptamer increased its binding affinity by up to 7-fold [195]. In another recent report, Sullenger and colleagues modified RNA aptamers against prostate cancer cells with 2′F pyrimidines. Upon conjugation with small molecule drugs or therapeutic peptides, these aptamers displayed significant specificity and toxicity against cancerous but not normal cells [196, 197]. We direct the reader to a recent review for a more detailed panorama of modified aptamer chemistry and related technologies [198].

XNAs are also being explored for other new therapeutic modalities beyond siRNA, ASOs, and aptamers. Modified oligonucleotides capable of cleaving or ligating other oligonucleotides have been developed as fully modified “XNAzymes” [156]. In other cases, incorporation of modified nucleotides at specific positions can expand chemical functionality or otherwise enhance catalysis [199, 200]. Therapeutic XNAs are also being

Table 2 FDA-approved nucleic acid therapeutics as of February 2020

Drug name (trade name)	Target	Modifications	Mechanism	Indication	Approval	Ref.
Fomivirsen (Vitravene)	mRNA of the CMV immediate-early (IE)-2 protein	PS	ASO (translation blocking)	Cytomegalovirus retinitis (CMV)	FDA (1998) and EMA (1999) approved. FDA (2001) and EMA (2002) withdrawn	[174]
Pegaptanib (Macugen)	Vascular endothelial growth factor (VEGF165)	2'F, 2'OMe, PEG conjugate	Aptamer	Neovascular (wet) age-related macular degeneration	FDA approved (2004)	[175]
Mipomersen (Kynamro)	Apolipoprotein B-100 mRNA	2'MOE, PS, 5mC	ASO (RNase H)	Homozygous familial hypercholesterolemia	FDA approved (2013)	[176]
Eteplirsen (Exondys 51)	Exon 51 in dystrophin mRNA	PMO	ASO (splicing modulation)	Duchenne muscular dystrophy	FDA approved (2016)	[177]
Nusinersen (Spinraza)	Survival of motor neuron 2 (SMN2) pre-mRNA	2'MOE, PS, 5mC	ASO (splicing modulation)	Spinal muscular atrophy	FDA (2016) and EMA (2017) approved	[178]
Patisiran (Onpattro)	Transthyretin (TTR) mRNA	2'OMe	siRNA	Hereditary transthyretin-mediated amyloidosis	FDA and EMA approved (2018)	[179]
Inotersen (Tegsedi)	Transthyretin (TTR) mRNA	2'MOE, PS, 5mC	ASO (RNase H)	Hereditary transthyretin-mediated amyloidosis	FDA and EMA approved (2018)	[180]
Volanesorsen (Waylivra)	Apolipoprotein C ₃ (apo-CIII) mRNA	2'MOE, PS, 5mC	ASO (RNase H)	Familial chylomicronemia syndrome	EMA approved (2019)	[181]
Givosiran (Givlaari)	Aminolevulinatase synthase 1 (ALAS1) mRNA	PS, 2'F, 2'OMe, GalNAc conjugate	siRNA	Acute hepatic porphyria	FDA approved (2019)	[182]
Golodirsen (Vyondys 53)	Exon 53 in dystrophin mRNA	PMO	ASO (splicing modulation)	Duchenne muscular dystrophy	FDA approved (2019)	[2, 183]

explored in the context of mRNAs [201], microRNAs [202], anti-gene oligonucleotides [203], and CRISPR guides [204, 205].

Delivery of oligonucleotides into the cytosol of target cells remains one of the major challengers in the development of nucleic acid therapeutics [206, 207]. Conjugation or encapsulation in lipids has demonstrated promise in improving pharmacokinetics and biodistribution [208], and cell-penetrating peptides can increase transport of biomolecules across cellular membranes [209, 210]. Other conjugations can enable targeting to specific tissues with high efficiency; *N*-acetylgalactosamine (GalNAc) has proven highly efficient for targeting biomolecules to the liver [211]. However, on the whole, effective delivery is a major challenge that remains to be addressed by the field.

The future of unnatural nucleic acid therapeutics

As tools such as XNA polymerases become available for an increasing number and diversity of nucleic acid chemistries, it is becoming possible to explore in greater detail the molecular properties and therapeutic potential of these unnatural polymers. Several chemistries show promise in addressing the shortcomings that limit the utility of natural nucleic acids in therapeutic contexts. We anticipate that exploration in this field will lead to a

steep increase in clinical-stage nucleic acid medicines in the coming decades.

Authors' contributions

The manuscript was written and revised jointly by K.D. S.A.-F., and P.H. All authors reviewed and approved the manuscript.

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Competing interests

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