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Current Opinion in Chemical Biology

How custom polymerases are driving innovation in synthetic biology

Mohammad Hajjar[1], Alex R. Horton[1], Victoria A. Maola[1], Nicholas Chim[1] and John C. Chaput[1,2,3,4]

Polymerases are the molecular machines of information transfer, yet their natural catalytic repertoire is largely restricted to DNA, RNA, and a limited set of chemically modified analogs. In response to this constraint, custom polymerases have emerged as powerful tools in the synthetic biology toolbox. Advances in polymerase engineering are unlocking access to RNA polymers generated by primer-extension rather than a promoter driven process as well as synthetic genetic polymers with unnatural backbone structures, collectively termed xeno-nucleic acids (XNAs). In this review, we highlight examples of how custom polymerases are redefining the chemical boundaries of genetic function and driving innovation across the fields of synthetic biology, biotechnology, and molecular medicine.

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Introduction

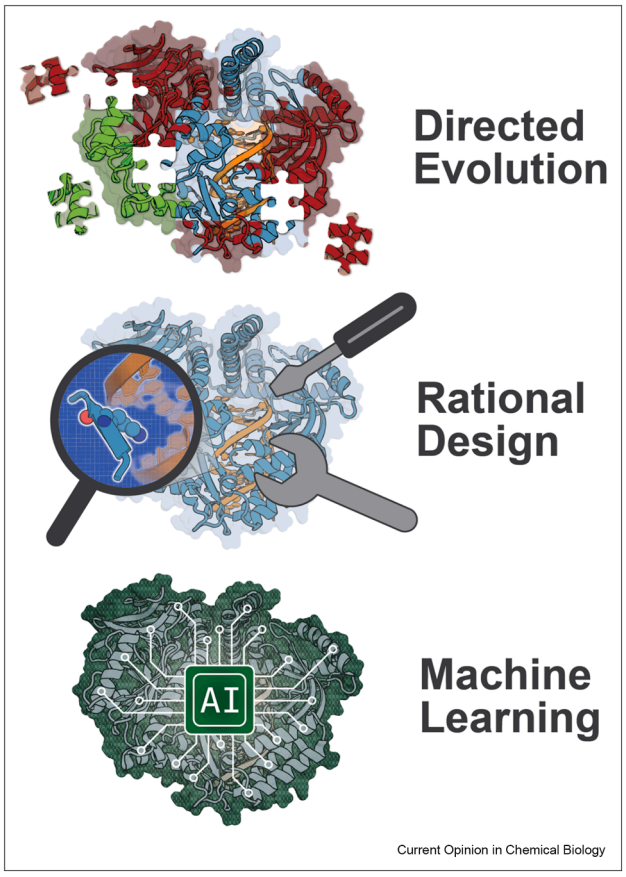
Polymerases lie at the heart of life's information processing system, catalyzing the synthesis of DNA and RNA as cells grow and divide, and respond to internal and external stimuli [1]. In synthetic biology, these enzymes have become indispensable tools for evolving new types of genetic molecules with specific ligand binding and catalytic activity [2–5]. This process has been accelerated by advances in enzyme engineering that enable polymerases to synthesize artificial genetic polymers [6], including unnatural base pairs that expand the genetic alphabet [7] and xeno-nucleic acids (XNAs) with backbone architectures distinct from DNA and RNA [8]. By extending the chemistries they can accept and functions they can perform, custom polymerases are transforming nature's information processing machinery into versatile engines of production and discovery. This review examines how polymerase engineering is reshaping synthetic biology and enabling new applications in biotechnology and molecular medicine. Since a thorough discussion of XNA is beyond the scope of this article, we direct readers interested in learning more about the diversity of artificial genetic polymers to a recent comprehensive review on the XNA alphabet [9]. We also guide readers interested in learning specific polymerase engineering techniques to several excellent reviews on this topic [10–12].

Tools of the trade

Natural polymerases follow stringent structural and kinetic checkpoints to ensure accurate DNA and RNA synthesis, enforcing proper substrate recognition, catalysis, and extension. Consequently, these enzymes have a limited tolerance for substrates bearing altered sugar, base, or backbone chemistries, either in the incoming nucleotide or encoding template. Overcoming this constraint often requires remodeling the active site to accommodate noncanonical substrates.

Custom polymerases are typically obtained by rational design or directed evolution (Figure 1). Rational design leverages high-resolution structural data to identify residues that influence activity or substrate specificity, which are then interrogated by mutagenesis [13]. Directed evolution, by contrast, explores sequence space through iterative cycles of diversification, selection, and amplification [14], often using large libraries generated by error-prone PCR (epPCR) [15] or homologous recombination [16]. More recently, machine-learning approaches have emerged as a promising complement to classical engineering strategies, with probabilistic models beginning to aid the prediction of beneficial polymerase mutations (Figure 1) [17].

Figure 1



Strategies for engineering custom polymerases. Expanding polymerase catalysis beyond natural substrates requires approaches that span empirical, mechanistic, and data-driven design. Directed evolution probes functional sequence space through iterative cycles of diversification and selection; rational design leverages structural and mechanistic understanding to introduce targeted mutations; and machine learning methods inform the computer-guided discovery of new variants. These complementary strategies enable the development of custom polymerases as adaptable biocatalysts for the synthesis of DNA, RNA, and XNA across diverse applications. Representative polymerase structure (PDB ID: 9MYE).

Collectively, these approaches have produced polymerases with altered substrate recognition, enhanced thermal stability, and improved performance under difficult reaction conditions [10].

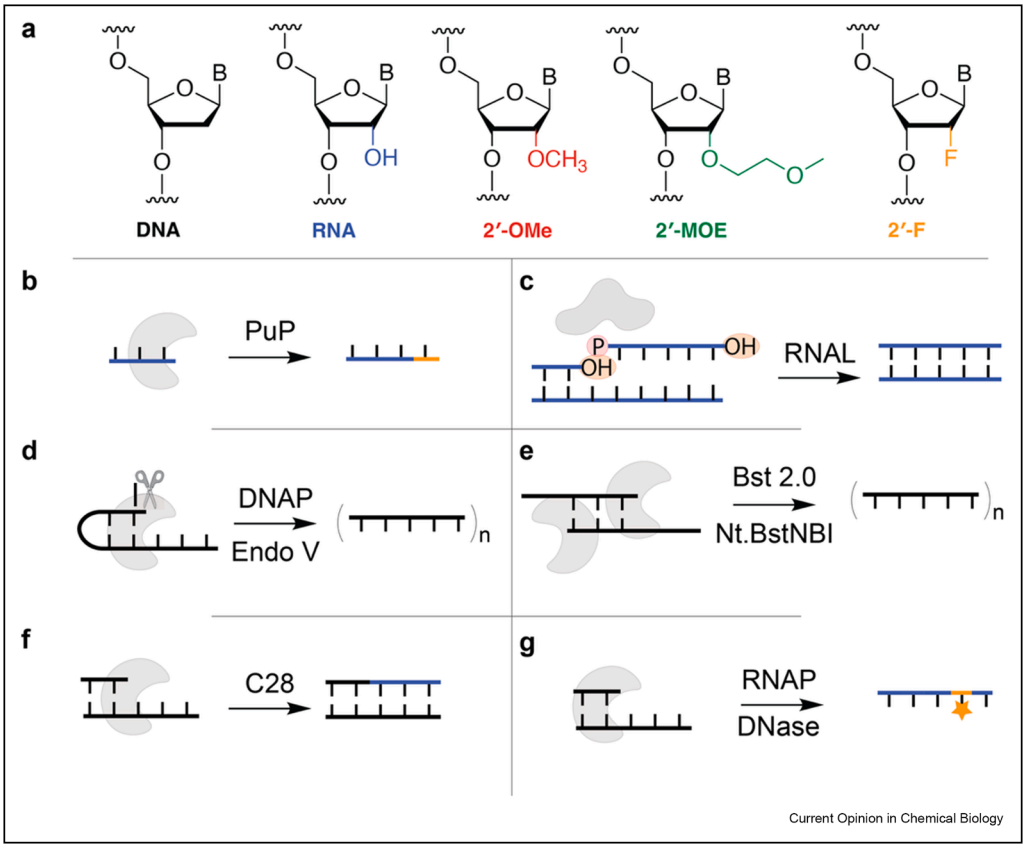
Candidate polymerases are evaluated using biochemical assays that assess such properties as catalytic efficiency, fidelity, substrate specificity, and thermal stability. While many properties can be measured by denaturing polyacrylamide gel electrophoresis, others, such as catalytic efficiency and substrate specificity, require careful quantitation. Polymerase kinetic profiling, for example, provides an integrated measure of the average nucleotide incorporation rate across a defined template, enabling direct comparison of polymerase variants and substrate classes [18]. Fidelity is typically assessed by sequencing the product of a replication cycle, which involves controls to show that the sequence is a true replication product rather than an artifact of the polymerase chain reaction (PCR) [19]. Once a high-performing polymerase is discovered, reaction conditions are then optimized by varying the temperature, pH, and magnesium concentration, and if desired, structural studies can be initiated to elucidate the molecular features responsible for achieving the altered activity.

Moving beyond phosphoramidite chemistry

The growing impact of RNA therapeutics has intensified interest in alternative approaches to solid phase phosphoramidite chemistry [20], which faces challenges in scalability and sustainability [21]. Enzymatic approaches offer an attractive solution by enabling oligonucleotide production in aqueous environments, bypassing the need for hazardous reagents (Figure 2). Convergent strategies involving DNA and RNA ligases, for example, have already proven effective for assembling antisense oligonucleotides and small interfering RNAs from their chemically synthesized fragments [22,23]. More transformative are enzymatic strategies for programmable oligonucleotide synthesis. Terminal deoxynucleotide transferase (TdT) and polyU polymerase (PUP) variants have enabled template-free synthesis using tethered or 3′ reversibly protected nucleotides that are sequentially added to the 3′ end of a growing primer [24–26]. Literature reports indicate that these reactions can achieve coupling efficiencies of >95%, with some enzymes, like PUP, showing increased tolerance for 2′ modified RNA substrates common to many RNA therapeutics [27].

A uniquely different approach involves establishing a biocatalytic cycle in which a polymerase and nuclease work together to generate highly pure, single-stranded oligonucleotide products. In this strategy, iterative cycles of oligonucleotide synthesis are performed using a polymerase to copy a template into DNA and an endonuclease to selectively nick the primer-product junction. At elevated temperatures (∼55 °C), the product strand dissociates into the aqueous solution, regenerating the template for another round of catalysis (Figure 2). For example, Lovelock and colleagues showed that a 123 mg of an 18 nt DNA strand could be generated in 89% purity using Kod DNA polymerase and endonuclease V [28]. Iwahara and colleagues developed a similar strategy, called palindrome-nicking-dependent amplification (PaNDA), which uses commercial Bst 2.0 DNA polymerase and Nt.BstNBI nicking enzyme to produce isotopically labeled DNA for structure elucidation studies by nuclear magnetic resonance (NMR)

Figure 2



Strategies for synthesizing natural and modified DNA and RNA. a) Chemical structures of DNA, RNA, and their common analogs. **b**) PUP-mediated non-templated extension of an RNA primer. **c**) Stepwise assembly of double-stranded RNA by sequential ligation of short oligonucleotide fragments using a double stranded-RNA ligase (RNAL). **d**) DNA polymerase (DNAP) mediated extension of a self-priming hairpin template, followed by endonuclease (EndoV, scissors) cleavage adjacent to an inosine (I) residue, releasing the product strand. **e**) Enzymatic production of single-stranded DNA following a similar format as (d) but using a strand-displacing DNA polymerase (Bst 2.0) and a nicking enzyme (Nt.BstNBI) to synthesize and release the product strand. **f**) RNA synthesis by an engineered RNAP (C28) following a primer extension mechanism. **g**) Staggered RNA extension using a specialized DNA polymerase to build long RNA molecules with site-specific chemical modifications (star).

spectroscopy [29]. Together, these examples offer an exciting paradigm for manufacturing oligonucleotide products in aqueous reactions that are scalable and environmentally sustainable.

RNA synthesis by primer extension

Engineering DNA polymerases to synthesize natural and modified forms of RNA is an important goal with significant implications for biotechnology and medicine. DNA polymerases discriminate against ribonucleotides (NTPs) through a steric-gate residue, yet mutating this position alone yields polymerases with limited RNA synthesis activity, indicating that substrate selectivity is distributed across the active site [30]. Early rational design efforts produced Tgo-TGK [31], a B-family DNA polymerase isolated from the archaeal species *Thermococcus gorgonarius*, which was used in several studies, including a synthetic biology effort to engineer a bacterium with an RNA genome [32]. Tgo-TGK has since been optimized by directed evolution using droplet microfluidics to isolate C28, a variant that synthesizes RNA with a rate of $\sim$3 nt s^{-1} and >99% fidelity [33]. C28 is capable of long-range RNA synthesis, reverse transcription, and chimeric DNA-RNA amplification. Notably, it accepts sugar- and base-modified ribonucleotides found in FDA-approved RNA therapeutics [27].

The widespread occurrence of 2′ modified ribonucleotides in RNA therapeutics has prompted a search for engineered polymerases that can support the evolution of functional oligonucleotides from 2′ modified RNA libraries. Early efforts by Romesberg and colleagues yielded SFM19, a variant of the Stoffel fragment of Taq DNA polymerase, isolated from a phage display library [34]. Subsequent optimization

by directed evolution resulted in SFM4-6 and SFM4-9, which enable the transcription and reverse transcription, respectively, of fully 2′-*O*-methyl RNA oligonucleotides [35]. More recently, Holliger and colleagues used protein design to identify Tgo variants that facilitate 2′-*O*-methyl and 2′-*O*-methoxyethyl RNA synthesis, enabling the discovery of 2′ modified aptamers and ribozymes [36]. Other protein design efforts include work by Obika and co-workers on a Kod variant that can synthesize 2′-*O*-methyl RNA, and mixed 2′-*O*-methyl RNA and locked nucleic acid (LNA) sequences in the presence of Mn^{2+} ions [37] as well as Liu and colleagues who reported a Taq DNA polymerase variant that can accept a range of analogs [38]. These examples represent promising tools for synthesizing functional RNA molecules with enhanced biostability and base pairing properties.

Position-specific labeling of RNA molecules by staggered extension

The ability to install chemical modifications at defined positions within RNA molecules is critical for RNA biotechnology. While solid phase synthesis can introduce modifications with high precision, chemical synthesis is expensive and generally limited to shorter (≤60 nt) RNA strands. This problem is partially addressed by *in vitro* transcription using T7 RNA polymerase, which enables the synthesis of long RNAs. However, T7-mediated RNA synthesis is restricted to uniformly labeled products with one modification per nucleotide type. Staggered extension strategies overcome this constraint by assembling RNA in segments using engineered polymerases capable of RNA synthesis [38–41]. Using this strategy, RNA molecules are generated by extending a DNA duplex with RNA, removing the DNA portion with DNase, annealing a new DNA template to the RNA product, and repeating the process until each modification has been sequentially installed at its desired position. Relative to earlier ligation strategies, staggered extension benefits from the higher yield, fidelity, and selectivity offered by a polymerase.

Synthesis of base- and backbone-modified DNA

Over the years, polymerases have been developed to synthesize a broad range of chemically modified forms of DNA. Prominent among these efforts is the use of thermostable enzymes such as Vent, Taq, and Phusion DNA polymerases to PCR-amplify six-letter genetic alphabets incorporating a third base pair designed around the principles of alternative hydrogen-bonding, shape complementarity, or hydrophobicity [7]. These foundational studies enabled key advances in genetic code expansion [42] and facilitated the discovery of highly sensitive protein-binding aptamers [43] and diagnostic agents [44].

In parallel, a substantial effort has been devoted to the enzymatic synthesis of functionally enhanced forms of DNA and RNA in which nucleobases are augmented with additional chemistry. Structural and biochemical studies have shown that archaeal B-family DNA polymerases, including Kod, vent, and deep vent, are generally more permissive toward nucleobases carrying substitutions at the C5 position of pyrimidines and the C7 position of 7-deaza-purines than their A-family counterparts [45]. Most of the work in this area has focused on the C5 position of 2′-deoxyuradine, which tolerates a wide range of functional groups, including hydrophobic moieties, charged side chains, reactive handles, and fluorescent dyes. These efforts have yielded aptamers with enhanced pharmacological properties, including several recent examples featuring cubane and aromatic side chains [46,47].

More recently, Kod and Vent DNA polymerases have enabled the synthesis of hypermodified DNA containing chemically diverse base substitutions across all four nucleotides. Through the enzymatic incorporation of modified analogs of adenine (A), guanine (G), cytosine (C), and uracil (U), nucleic acid polymers can now be generated with pronounced hydrophobic [48], anionic [49], or zwitterionic character [50]. In doing so, these systems approach the chemical complexity of proteins while retaining the solubility and folding properties of nucleic acids. Such advances increasingly blur the boundary between nucleic acids and proteins, providing new opportunities to mimic the complexity and function of biological systems.

By contrast, far fewer studies have examined the synthesis of DNA bearing modifications to the phosphodiester backbone. Notable recent examples include polymerase engineering efforts in which Tgo variants were used to synthesize nucleic acids containing 2′-5′ linkages [51] and uncharged alkyl phosphonate nucleic acids [52]. Together, these studies demonstrate the feasibility of expanding the chemical parameters of genetic polymers and provide a foundation for exploring how backbone chemistry influences information storage and function.

Reverse transcriptases for molecular biology

Reverse transcriptases (RTs) have become indispensable tools for molecular biology, forming the foundation of molecular transcriptomics. The conversion of RNA into complementary DNA allows researchers to profile RNA patterns in cells, detect pathogens, and diagnose disease. However, most viral RTs suffer from limited thermal stability, low fidelity, and poor sensitivity that hinder the detection of low abundance cellular transcripts. In addition, premature stalling on base modifications, highly structured motifs, and long RNA transcripts result in biases due to incomplete synthesis.

Polymerase engineering offers an appealing solution to this problem. Researchers at Qiagen, for example, have engineered a thermostable RT by shuffling the sequences of orthologs derived from metagenomic data [53]. The most active enzyme was fused to the 5' → 3′ exonuclease domain of Taq DNA polymerase to facilitate probe detection in quantitative assays [53].

Departing from viral RTs, Ellington and colleagues demonstrated that the native activity of Taq DNA polymerase can be implemented to achieve a one-enzyme qRT-PCR reaction [54]. By carefully optimizing the buffer conditions, a one-enzyme TaqMan assay was established that can detect as few as 2 copies/μL of viral RNA from SARS-CoV-2. A subsequent directed evolution study yielded an RT with proofreading activity from natural Kod DNA polymerase [55]. The enzyme, termed RTX, provides a single-enzyme strategy for high fidelity RT-PCR and direct RNA sequencing without cDNA isolation. A similar study by Marx and colleagues produced a Taq DNA polymerase variant capable of analyzing four different RNA targets in a multiplex assay [56]. Progress in this area demonstrates that the reverse transcription reaction can be adapted in ways that simplify the assay format and address outstanding challenges in sensitivity and coverage.

mRNA therapeutics

The success of the COVID-19 mRNA vaccine motivated interest in efforts to refine the synthesis of mRNA therapeutics by improving their yield, immunogenicity, translational efficiency, and stability. Researchers at Moderna have pioneered new versions of T7 RNA polymerase by rational design that produce substantially less immunostimulatory dsRNA during *in vitro* transcription as compared to the wild-type enzyme [57]. More recently, EVOLVEpro, an in silico protein engineering method that combines protein language models with few-shot active learning, was used to generate a variant of T7 RNA polymerase, named epT7, that provides higher RNA yields, reduced immunogenicity, and improved translation efficiency [58]. In addition to these methods, other strategies are being explored to further increase the yield and stability of mRNA transcripts. These include the ligation of branched chemically modified poly(A) tails [59] and circular mRNA strategies that bypass the need for internal ribosomal entry sites for translation initiation [60]. Collectively, these developments may prove useful for optimizing the manufacture of mRNA therapeutics.

Genome editing tools

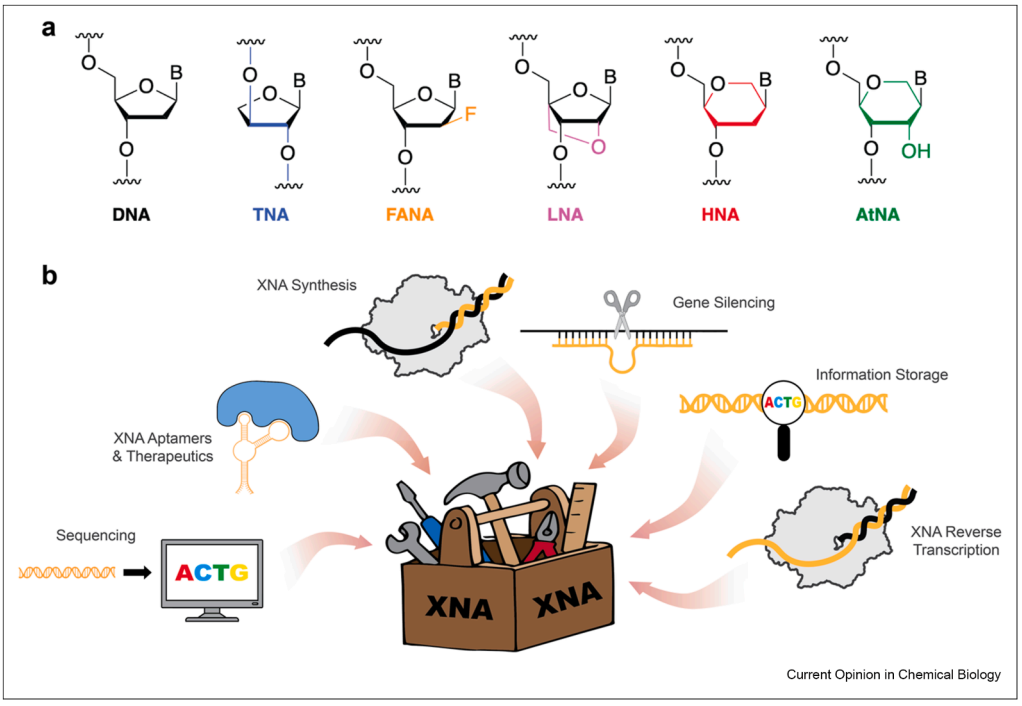
The ability to make targeted genetic changes in living cells has long been a central goal of the life sciences. Advances in CRISPR-Cas9 gene editing enabled programmable DNA cleavage through the generation of double-strand breaks (DSBs). Prime editing further expanded the field by converting Cas9 into a search-and-replace technology, where a Cas9 nickase is fused to an engineered RT, guided by a prime editing guide RNA (pegRNA) that encodes the desired edit [61]. Although prime editing avoids DSBs and enables precise sequence changes, its performance is limited by pegRNA optimization, instability, and reduced efficiency for long edits. To address these challenges, the RT enzyme was replaced with a DNA polymerase to yield a DNA polymerase editing (DPE) system. In DPE, a nicked 3′ DNA end is extended using a donor template that recruits the DNA polymerase through an RNA aptamer–protein interaction [62]. Because this approach relies on the activity of a replicative polymerase, DPE can support longer genome edits with higher fidelity. Building on this concept, click editors were developed by fusing a DNA-dependent polymerase to the Cas9 nickase and incorporating a HUH endonuclease that covalently attaches the single-stranded DNA donor [63]. The click linkage ensures efficient delivery of the editing template; thus, expanding the precision-writing capabilities of prime editors.

XNA replication

Modern biotechnology applications increasingly require the use of genetic polymers with unique physicochemical properties, including enhanced stability under physiologically relevant conditions. XNAs address this problem by harnessing backbone architectures that are distinct from natural DNA and RNA [9]. Impressive advances in polymerase engineering have made it possible to copy genetic information back and forth between a limited set of XNA polymers and DNA [10]. Recent work has focused on expanding the list of engineered polymerases to include XNAs (Figure 3) that were previously only accessible by chemical synthesis, along with efforts to improve the rate, fidelity, and substrate specificity of existing XNA polymerases. In the former category, Obika used rational design to produce a Kod DNA polymerase variant that can synthesize locked nucleic acid (LNA) polymers up to 1 kilobase in length, and a slightly different variant that allows LNAs to be reverse transcribed back into DNA [37].

In the area of XNA polymerase optimization, we showed that homologous recombination could be used to isolate a threose nucleic acid (TNA) polymerase with superior activity [64]. In this case, droplet-based microfluidic selection yielded 10–92, an engineered TNA polymerase that functions with a catalytic rate that is within 5-200-fold the rate of the parent enzymes with their natural DNA substrates. Interestingly, subsequent structural and biochemical studies found that fidelity and catalytic activity evolved as partially decoupled activities guided by separate ground- and transition-state discrimination events [65]. Significant efforts have also been underway to develop polymerases that can copy XNA back into DNA.

Figure 3



Chemical diversity and applications of xeno-nucleic acids (XNAs). a) Chemical structures of DNA and common XNA analogs. **b**) Overview of key XNA-enabled technologies. Engineered polymerases enable the synthesis and reverse transcription of XNAs, permitting the storage and transmission of sequence information for further applications such as gene-silencing, information storage, aptamer-based therapeutic development, and sequencing. These advances position XNAs as programmable biomolecules capable of expanding the functional space of nucleic acid chemistry.

While natural Bst DNA polymerase has been shown to function with general XNA reverse transcriptase activity [66], Holliger developed a strategy for evolving XNA reverse transcriptases using a compartmentalized bead labeling strategy that enables the discovery of polymerases that recognize 2′-*O*-methyl RNA, hexitol nucleic acid (HNA), altritol nucleic acid (AtNA), and 2′-methoxyethyl RNA (MOE) templates [67]. Together, these innovations provide a powerful enzymatic platform for replicating XNA using DNA as an intermediate, which is conceptually similar to retroviral replication.

XNA evolution

For over three decades, *in vitro* evolution has produced nucleic acid aptamers and catalysts against clinically relevant targets [68]. Extending this methodology to unnatural genetic polymers requires polymerases that can copy genetic information back and forth between DNA and XNA. In 2012, Holliger and Chaput were the first to show that functional XNA molecules could be isolated by *in vitro* selection using engineered polymerases to propagate XNA information under conditions of increasing selective pressure [2,3]. This work heralded an era of synthetic genetics, in which XNA polymerases are used to explore the informational, structural, and functional properties of XNA [6].

The last few years have witnessed several exciting examples of XNA evolution. In the area of therapeutic aptamers (Figure 3), a parallelized method for isolating functionally enhanced TNA aptamers with low to sub-nanomolar binding affinity was established based on a high throughput screening strategy [69]. Yu and colleagues extended the reach of XNA aptamers to include PROTAC reagents that function as a combination therapy for triple-negative breast cancer [70] or block the PD-1/PD-L1 interaction for cancer immunotherapy [71]. Strong gains have also been made in the evolution of XNA catalysts, including examples of XNAzymes that mediate allele-specific RNA cleavage [72,73] or facilitate RNA ligation [74,75]. These examples illustrate how XNA polymerases are facilitating the discovery of functional XNA molecules by *in vitro* selection.

Information storage

Advances in information storage have expanded beyond DNA with engineered polymerases enabling digital data to be written, read, and preserved in chemically modified nucleic acids. While DNA offers unmatched information density and energy efficiency, its susceptibility to enzymatic degradation limits its durability as a medium for data archiving. Early demonstrations established the feasibility of large-scale DNA data

storage, encoding megabytes of digital content with high accuracy using next-generation synthesis and sequencing technologies [76]. However, achieving enhanced biological stability requires engineered polymerases that can copy genetic information between DNA and XNA. Examples of synthetic biology systems (Figure 3) established to store and recover digital information in genetic polymers that are stable against biological nucleases include TNA [2,77]'-*O*-methyl RNA [78], and L-DNA [79]. Together, these studies demonstrate how polymerase engineering is advancing molecular data storage, transforming modified nucleic acids from inert synthetic materials into a high-fidelity medium for long-term data archiving.

Closing the gap

Despite remarkable progress, several fundamental challenges continue to limit the broader adoption of custom polymerases as foundational tools across synthetic biology. Chief among these is the absence of an XNA replication system that functions independently of DNA, as opposed to existing systems that rely on DNA as an informational intermediate through PCR-based amplification [6]. While some progress has been made in copying 2′ modified RNA templates with 2′ modified RNA products [80], achieving a fully autonomous XNA→XNA replication system with more diverse sugar chemistries is a key technical milestone that would greatly advance our understanding of life's genetic system and produce an important tool for recovering XNA molecules from biological samples. Realizing this goal will require engineered polymerases that accommodate noncanonical backbone geometries while preserving efficient rates of catalysis and fidelity.

A second critical bottleneck is the lack of robust, scalable, and low-cost methods for reading sequence information encoded in XNA oligonucleotides. Although continued advances in nanopore sequencing offer a promising solution, current technologies remain limited in accuracy, throughput, and generality across XNA chemistries. They also fail to detect modified bases used to augment the functional properties of the molecule. This challenge is not merely dependent on improved motor proteins, but also on the development of new algorithms trained on chemically diverse datasets that recognize the sequence signatures of individual XNA systems, which is a labor- and resource-intensive process.

Future progress in protein engineering techniques will play a decisive role in addressing these limitations. Machine learning strategies that integrate sequence, structural, and kinetic data offer a powerful complement to directed evolution, enabling a more efficient navigation of sequence space and the fitness landscape of engineered polymerases. When coupled with high throughput screening and selection platforms, these approaches may accelerate the discovery of polymerases with novel substrate preferences, enhanced robustness, and fidelity across chemically diverse genetic systems. In combination with newfound knowledge surrounding the mechanisms of polymerase evolution [65], such applications may also enable the rational co-evolution of properties that have traditionally been hidden to advanced computational programs.

In summary, the examples provided in this review offer evidence that polymerases have become enabling tools for applications in synthetic biology and healthcare. Continued effort in this area is expected to advance the field by providing the tools needed to replicate and modify a growing body of alternative genetic polymers, collectively viewed as the XNA alphabet.

Author contributions

JC and NC conceived the review. All authors wrote and edited the manuscript.

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Declaration of competing interest

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Data availability

No data was used for the research described in the article.

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- • of special interest
- •• of outstanding interest

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