The Expanding World of DNA and RNA

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Abstract

DNA and RNA are remarkable because they can both encode information and possess desired properties, including the ability to bind specific targets or catalyze specific reactions. Nucleotide modifications that do not interfere with enzymatic synthesis are now being used to bestow DNA or RNA with properties that further increase their utility, including phosphate and sugar modifications that increase nuclease resistance, nucleobase modifications that increase the range of activities possible, and even whole nucleobase replacement that results in selective pairing and the creation of unnatural base pairs that increase the information content. These modifications are increasingly being applied both *in vitro* and *in vivo*, including in efforts to create semi-synthetic organisms with altered or expanded genetic alphabets.

Introduction

The template-directed enzymatic synthesis of DNA and RNA makes them unique among all materials and allows them to mediate the heritable storage and retrieval of biological information. The *in vitro* reconstitution of these processes has revolutionized biotechnology, enabling applications ranging from sequencing and cloning to a myriad of emerging techniques based on the genome-wide analysis of DNA and RNA. When combined with the range of structures available to single-stranded DNA and RNA, which allows them to recognize specific targets (aptamers) and even catalyze reactions, these processes allow for the laboratory evolution of functional oligonucleotides (or SELEX: systematic evolution of ligands by exponential enrichment) for applications ranging from affinity reagents and diagnostics to therapeutics. Finally, the emerging field of synthetic biology takes advantage of enzymatic synthesis *in vitro* and *in vivo* to create “parts” that may be used to impart living cells with new attributes or functions.

Despite these revolutionary applications, the limited composition (natural nucleotides), stability to nucleases, and range of physicochemical properties of natural DNA and RNA limit their potential. This has spurred the search for modified, and even wholly unnatural nucleotides, that bestow the corresponding oligonucleotides with desired properties but that remain compatible with enzymatic synthesis. For example, while the canonical nucleobases are capable of stacking, the types of motifs accessible are limited by the associated hydrophilic hydrogen-bond donors and acceptors, and thus DNA and RNA are largely unable to access many of the types of hydrophobic cores and surfaces that play such an essential role in protein folding and function.

In this review we focus on two types of nucleotides, natural nucleotides that are modified for improved properties (stability or function) and nucleotides that have wholly unnatural

nucleobases that mediate their selective pairing and that thus constitute unnatural base pairs (UBPs) with the potential to expand the compositional and thereby the informational content of DNA. We first summarize the progress made toward the identification and use of such nucleotides *in vitro*, with an emphasis on SELEX applications and the development of UBPs. We then provide a survey of recent progress toward the exciting goal of using modified nucleotides and UBPs *in vivo* as the basis of semi-synthetic organisms.

SELEX with modified RNA and DNA

Initially, SELEX was developed with RNA, which requires transcription of a DNA library prior to the selection step as well as reverse transcription back to DNA afterward to enable amplification of the selected sequences (Figure 1a). The enzymes commonly employed are the RNA polymerase from T7 bacteriophage (T7 RNAP) and Superscript™ or Superscript II™ reverse transcriptases. In principle, the use of DNA streamlines the SELEX protocol by requiring a single enzymatic step (Figure 1b). However, with modified RNA or DNA this requires the direct amplification of the modified oligonucleotides, which is generally challenging. Moreover, inefficient amplification is likely to introduce significant sequence biases that may result in the unwanted enrichment of more easily amplified sequences. Thus, it is common to use a protocol for DNA SELEX that is analogous to that of RNA SELEX: the DNA is first “transcribed” by a DNA polymerase using modified dNTPs, and then after selection, “reverse transcribed” by a DNA polymerase back into natural DNA for the more challenging amplification step. The DNA polymerases that appear most able to tolerate modifications include KOD Dash, Vent (exo⁻), Phusion, and Terminator.

Particularly with RNA, efforts have been directed toward the use of modified nucleotides that impart their corresponding oligonucleotides with resistance to nuclease-mediated degradation, including replacement of the non-bridging oxygen atom within the phosphate backbone with sulfur (α -thio) or BH_3 (α -borane), or modification of the sugar with 4'-thio or 2'-amino (NH_2), fluoro (F), or methoxy (OMe) substituents. Modifications at both sugar positions are best tolerated with pyrimidines, and as a compromise between stability and the efficiency of synthesis, mixed libraries of modified pyrimidines and natural purines are commonly employed. 2'-F substituents have been used the most (reviewed in [1]), and 2'-F-pyrimidine-modified libraries have recently been subjected to *in vivo* selection for stable aptamers targeting tumor cells in living animals [2] or that penetrate the blood-brain barrier [3], and the Soh group has recently demonstrated the utility of including selection pressure for specificity to identify 2'-F-pyrimidine-modified RNA aptamers that recognize different subunits of integrin $\alpha\text{V}\beta 3$ [4]. Optimized conditions and the use of the Y639F/H784A double mutant of T7 RNAP has also enabled the transcription of oligonucleotides with increased levels of 2'-OMe substitutions, which have been used for the identification of stabilized aptamers against vascular endothelial growth factor (VEGF) [5] and staphylococcal protein A [6]. The recent discovery of additional T7 RNAP mutants that better recognize 2'-OMe substrates [7,8] and a mutant of the Syn5 RNA polymerase from cyanophage Syn5 that better recognizes 2'-F substrates [9] should facilitate the identification of stabilized aptamers in the future.

Traditionally, efforts to employ SELEX with sugar-modified DNA have been less common, however, a DNA aptamer containing 2'-F dG that binds thrombin has recently been disclosed [10]. In addition, we recently reported the directed evolution of a variant

of Stoffel fragment (the catalytic subunit of Taq DNA polymerase), SFM4-3, that efficiently PCR amplifies DNA partially modified with 2'-F or 2'-OMe substituents, including purines and/or pyrimidines [11]. An increasingly diverse range of sugar modified nucleotides that are recognized by wild type or mutant DNA polymerases has recently attracted attention, including threose nucleic acids (TNA), hexitol nucleic acids (HNA), cyclohexenyl nucleic acids (CeNA), locked nucleic acids (LNA), arabinonucleic acids (ANA), and 2'-fluoro-arabinonucleic acids (FANA) (Figure 2a). For example, Chaput and co-workers used a variant of SELEX known as SELMA (SElection with Modified Aptamers [12,13]) to select TNA aptamers that bind thrombin [14]. Terminator DNA polymerase was used to extend a DNA hairpin primer/template with the modified nucleotides, permitting amplification of the DNA primer-template after selection and re-synthesis of the selected TNA aptamer. Kuwahara and co-workers used a mixture of mutant and/or wild type KOD DNA polymerases to “transcribe” and “reverse transcribe” mixed LNA/DNA oligonucleotides during selection of an anti-thrombin aptamer [15,16]. Holliger and co-workers have dramatically expanded the potential of these analogs by evolving a series of TgoT DNA polymerase mutants to better “transcribe” and “reverse transcribe” these modified nucleotides, and they have used them to select HNA aptamers targeting HIV trans-activating response RNA and hen egg lysozyme [17•], a FANA aptamer targeting HIV-1 RT [18], and catalytic nucleic acids containing ANA, FANA, HNA, and CeNA moieties [19]. In addition, we have evolved several mutants of the Stoffel fragment that better “transcribe” and “reverse transcribe” fully modified 2'-F and 2'-OMe oligonucleotides [11].

To increase the physicochemical potential of oligonucleotides, efforts have been directed toward the use of nucleotides with modified nucleobases, and while modification at the 5-position of pyrimidines is best tolerated, modification at the 7-position of deazapurines

is also possible. It is useful to consider the modifications as being composed of “linkers,” to which the polymerase is very sensitive, and attached functionalities, to which the polymerase is generally less sensitive. This allows for a significant simplification of the literature as most modifications are based on one of five linker groups, the most common of which consist of several subgroups (Figure 2b). Far from being inert, these linkers mediate interactions with the polymerase during incorporation [20] and within the oligonucleotide once incorporated [21•]. To expand the range of modifications that are compatible with enzymatic synthesis, Mayer and coworkers recently demonstrated that after PCR-mediated incorporation of C5-ethynyl-dU, the ethynyl groups may be modified via Click chemistry, allowing access to a wide range of triazole-linked functionalities [22•].

While it is clear that the backbone and sugar modifications achieve the goal of increasing stability, whether nucleobase modifications actually increase the range of potential functions is less clear. This question has been elegantly addressed by SomaLogic Inc., who has structurally characterized three “SOMAmers” (Slow Off-rate Modified Aptamers) which were selected against PDGF [23], IL-6 [24] or NGF [25] and where each dT is replaced by dU analogs with simple aromatic rings (typically benzyl rings) attached to the 5 position via carboxamide linkers. Mutational analysis revealed that the 8 to 10 appended aromatic rings in each SOMAmer are generally required for binding, and structural analysis revealed that they nucleate the formation of clusters that present optimal surfaces for target recognition or that act as hydrophobic cores that support unique structure formation (Figure 3). Two particularly remarkable motifs observed were a four-ring stack in the IL-6 SOMAmer (with one ring provided by a tyrosine side chain from the protein; Figure 3e) and two “benzyl-dU zippers” in the NGF SOMAmer, where the benzyl group of one modified nucleotide packs with the uridine

ring of the other (Figure 3f). These motifs demonstrate the potential of augmenting the repertoire of forces naturally available to nucleic acids with hydrophobic and packing forces, and are reminiscent of interactions common to proteins, as well as to the mode of pairing observed with predominantly hydrophobic unnatural base pairs (see below).

UBPs and their *in vitro* applications

While modification of the natural nucleotides promises to stabilize and expand the potential functions available to oligonucleotides, the development of a UBP that functions along side the natural base pairs promises to expand their information content. Three families of UBPs have been extensively validated and are exemplified by **dZ-dP**, developed by the Benner laboratory, **dDs-dPx**, developed by the Hirao laboratory, and **dNaM-d5SICS**, developed by our laboratory [26-28] (Figure 4a). The first of these UBPs is based on a hydrogen bonding pattern that is orthogonal to those employed by the natural base pairs, while the latter two are based on hydrophobic and packing forces. When incorporated into DNA, each may be efficiently replicated and transcribed into RNA.

The structures of **dNaM-d5SICS** and several derivatives have been determined within duplex DNA, and the unnatural nucleobases are found to pair via cross-strand intercalation [29] (Figure 4b). This accounts for their stability, but is inconsistent with their efficient replication by polymerases, which is based on the recognition of Watson-Crick-like structures. However, the structure of the UBP formed by pairing an unnatural triphosphate with its cognate nucleotide in a template within the active site of a DNA polymerase revealed a Watson-Crick-like, planar edge-to-edge structure (Figure 4c) [29]. Thus, the **dNaM-d5SICS** family of UBPs (and perhaps **dDs-dPx** as well) is stable and efficiently replicated because the hydrophobic and packing forces that mediate

pairing are strong but sufficiently plastic to adopt to their duplex or polymerase environment. Moreover, this mode of interaction has been observed with other hydrophobic nucleobase analogs [30-33] and with the hydrophobic aromatic rings of SOMAmers (see above), which suggests that it is robust and easily accessed with modified nucleotides.

DNA containing the **dDs-dPx** or **dZ-dP** UBPs has recently been used to evolve aptamers with novel properties. The Hirao group used a modification of the **dDs-dPx** UBP to evolve aptamers that use the unique functionality provided by **dDs** to recognize VEGF-165 or IFN- γ [34•], and the Benner group used libraries containing the unnatural nucleotides **dP** and **dZ** to evolve aptamers that bind breast or liver cancer cells [35,36]. In each case the unnatural nucleotides are required for binding.

Along with linkers akin to those used to modify natural nucleotides, the UBPs allow for the *site-specific* attachment of functionality within an amplifiable context, and the use of **dDs-dPx** and **dNaM-d5SICS** to array small molecules or proteins with unprecedented spatial resolution has been demonstrated [37,38]. In addition, we also recently reported the use of a derivative of the **dNaM-d5SICS** UBP to direct the transcription of the central domain of *Thermus thermophilus* 16S ribosomal RNA site-specifically labeled with fluorophores at two positions, which with single molecule FRET characterization revealed the existence of previously unknown assembly complexity [39].

Reading and writing modified oligonucleotides *in vivo*

An increasingly central goal in the field is to use modified oligonucleotides or UBPs within cells to control, alter, or augment their physiology. Towards this goal, several groups have reported the successful conversion of modified DNA into natural DNA *in*

vivo. Kool and co-workers demonstrated that a short stretch of DNA with size-expanded analogs of natural nucleobases can be copied into their natural counterparts in living *E. coli* cells [40], and Marlière and co-workers showed that a short stretch of CeNA, ANA, or HNA can be converted into DNA [41]. Most recently, the Matsuda group demonstrated that 4'-thio-DNA can be transcribed into RNA in mammalian cells [42].

The *production* of modified nucleic acids *in vivo* is more challenging, as the modified triphosphates must be made available and must be selectively utilized by polymerases. The replication of modified DNA *in vivo* was first demonstrated by Marlière and co-workers in 2011 when they showed that the 5'-chlorouracil analog of dT could replace the majority of dT nucleotides in the genome of *E. coli* cells [43••]. The triphosphate was made available by providing 5'-chlorouracil to the growth medium and expressing a deoxyribosyltransferase from *Lactobacillus leichmannii*. Competition with dTTP was reduced by disabling its major route of biosynthesis, and the efficiency of unnatural triphosphate use was optimized by adaptive evolution.

The deployment of a UBP in a living cell would allow for the creation of semi-synthetic organisms that store (and eventually retrieve) increased information. Following the validation of d**NaM**-d**5SICS** *in vitro*, we set out to test whether DNA containing this UBP can be replicated within *E. coli* cells [44••]. The first challenge of getting the unnatural triphosphates into the cells was solved by the expression of an algal nucleoside triphosphate transporter. Cells expressing the transporter were then transformed with a plasmid containing the UBP and then grown in media supplemented with d**NaM**TTP and d**5SICS**TTP. Analyses of the plasmid after various periods of growth revealed that not only was the UBP retained, the fidelity of replication (retention per doubling) was greater

than 99.4%. This modified *E. coli* is the first semi-synthetic organism able to store increased information in its DNA (Figure 5).

Conclusions

DNA and RNA are remarkable molecules and our ability to manipulate them has impacted virtually every area of the biological sciences. The increasing ability to efficiently synthesize backbone- or sugar-modified oligonucleotides has allowed for the discovery of aptamers against a variety of proteins and small molecules that are stable to nuclease-mediated degradation. Recent successes in the directed evolution of polymerases tailored for the modified substrates should now accelerate progress.

While too few studies have focused on if and how nucleobase modifications allow access to an increased range of function, comparative analysis of SELEX experiments suggest that they increase the likelihood of aptamer identification, and structural analyses suggest that they do so by allowing oligonucleotides to adopt a broader range of conformations and structural motifs [21•,45,46]. Moreover, the observation of similar structural motifs when hydrophobic aromatic moieties are introduced, either via modification (SOMAmers) or as the nucleobases of UBPs, suggests that common themes, and perhaps even rules that will facilitate design, are possible.

Two examples where modified or wholly unnatural DNA was propagated within living cells have been reported, and the further optimization and use of the resulting semi-synthetic organisms seems imminent. The next step towards retrieving information encoded by UBPs is the *in vivo* transcription of RNA containing unnatural nucleotides, and work towards this goal is in progress. UBPs have already been used for protein

translation *in vitro* [47,48] and their use *in vivo* represents the final step of creating a fully functional semi-synthetic organism capable of possessing and evolving new and useful attributes or functions, for example, the production of novel proteins for development as therapeutics. The substrate repertoires of the polymerases clearly extend beyond their natural substrates, and the synthesis of novel nucleotides, increasingly augmented with genetics and directed polymerase evolution, is enabling extension of the *in vivo* and *in vitro* applications of DNA and RNA and a dramatic increase in the potential of these already remarkable molecules.

Acknowledgements

FER acknowledges financial support from the National Institutes of General Medical Sciences (GM060005 and GM097489) and the Defense Advanced Research Projects Agency Folded Non-Natural Polymers with Biological Function Fold F(x) Program (Award No. N66001-14-2-4052). Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of DARPA.

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Figure captions

Figure 1. Evolution of nucleic acids via SELEX. (a) RNA SELEX. (b) DNA SELEX. In both RNA and DNA SELEX, larger libraries of oligonucleotides (up to $\sim 10^{15}$) with different sequences are subjected to selection for a desired function (binding or catalysis), and the recovered oligonucleotides are then amplified and subjected to additional rounds of selection.

Figure 2. Modified nucleotides used in SELEX. (a) Chemical structures of ANA, FANA, TNA, LNA, HNA, and CeNA. (b) Position and structure of common nucleobase modifications. Sugar and phosphates omitted for clarity. The five common classes of linkers are shown with additional and variable functionality indicated with R'.

Figure 3. Structures of SOMAmers bound to their respective target proteins, including an expanded view of each hydrophobic cluster. (a, d) PDGF SOMAmer (PDB ID 4HQU). (b, e) IL-6 SOMAmer (PDB ID 4NI9). (c, f) NGF SOMAmer (PDB ID 4ZBN).

Figure 4. UBPs that may be replicated and transcribed by native polymerases *in vitro*. (a) Chemical structures (sugar and phosphates omitted for clarity). (b) Structure of an early progenitor of the d**5SICS**-d**NaM** UBP [30]. (c) Structure of d**5SICS**-d**NaM** in duplex DNA [29]. (d) Structure of d**5SICS**-d**NaM** as formed in the active site of a DNA polymerase (PDB ID 3SV3).

Figure 5. *E. coli* based semi-synthetic organism. Triphosphates provided in the growth medium diffuse into the periplasm (likely via porins) and then are transported into the

cytoplasm by a nucleoside triphosphate transporter where they are used to replicate DNA containing the d**5SICS**-d**NaM** UBP.

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Graphical Abstract