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Chemical modifications for a next generation of nucleic acid aptamers

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Abstract: In the past three decades, *in vitro* systematic evolution of ligands by exponential enrichment (SELEX) has yielded many aptamers for translational applications in both research and clinical settings. Despite their promise as an alternative to antibodies, the low success rate of SELEX (~ 30%) has been a major bottleneck that hampers the further development of aptamers. One hurdle is the lack of chemical diversity in nucleic acids. To address this, the aptamer chemical repertoire has been extended by introducing exotic chemical groups, which provide novel binding functionalities. This review will focus on how modified aptamers can be selected and evolved, with illustration of some successful examples. In particular, unique chemistries are exemplified. Various strategies of incorporating modified building blocks into the standard SELEX protocol are highlighted, with a comparison of the differences between pre-SELEX and post-SELEX modifications. Nucleic acid aptamers with extended functionality evolved from non-natural chemistries will open up new vistas for function and application of nucleic acids.

1. Introduction

Aptamers (from the Latin word *aptus* which mean “fitting”) are oligonucleotide molecules that bind to a target with high affinity and specificity owing to their unique three-dimensional structures.^[1] They are short RNAs, ssDNAs or nucleic acids with other chemistries, typically comprising 20-100 nucleotides.^[2] Similar to a protein, the sequence of an aptamer determines its secondary and tertiary structures. An aptamer can adopt a multitude of structural motifs, such as helical duplex, stem-loop, hairpins, G-quadruplex, etc.^[3-4]

The emergence of aptamers has revolutionised our understanding of nucleic acids. It is now clear that nucleic acids function well beyond their pivotal role in the storage of genetic information and instructions for protein synthesis. During the 1980s, virologists discovered some non-coding viral RNAs could function as ligands and bind to viral proteins to facilitate their replications and infection,^[5] indicating that oligonucleotides had the potential to target specific biomolecules, resembling antibodies. At that time, there was no approach to systematically select an oligonucleotide against a particular target. The first major breakthrough happened in 1990 when two groups of scientists independently devised an *in vitro* strategy to direct the evolution of nucleic acids, known as the systematic evolution of ligands by exponential enrichment (SELEX), which has now become the universal method for aptamer selections.^[1, 6] Interestingly, this method has also been hijacked to isolate DNAzymes and ribozymes which are nucleic acids capable of catalysing chemical reactions^[7-10].

Three decades of research has investigated the possibilities of aptamers to act in therapeutics, diagnostics and biosensing. Aptamers have important merits, namely higher stability, lower immunogenicity, etc. making them excellent alternatives for antibodies.^[11] One drawback of aptamers is their lack of chemical diversity.^[12] Antibody side chains can be hydrophobic, hydrophilic or charged in nature, as they are made up from 20 chemically diverse amino acid species.^[13] They can utilize an

array of interactions, for instance, H-bonding, van der Waals' forces, hydrophobic interactions and stacking interactions to bind to their targets. On the contrary, nucleic acids are less chemically diverse. Composing of only four canonical nucleobases and the sugar phosphate backbone, aptamers are highly hydrophilic and negatively charged.^[14] They mainly form ionic interactions, H-bonding and stacking and polar interactions with their targets. The intrinsic shortcoming of aptamers limits the scope of their applications. It is estimated the success rate of SELEX using canonical oligonucleotide libraries is less than a third.^[15] This greatly hampers the potential of the development of aptamers. In light of overcoming this challenge and expanding the versatility of aptamers, alternative chemical moieties in aptamers are a promising option.^[12]

2. Molecular designs of novel aptamers

Natural chemistry aptamers mainly rely on shape complementarity and molecular forces that are polar or charged in nature while they bind to their targets. Consequently, SELEX does not always yield aptamers with good binding affinity and specificity. Moreover, nucleic acids are sensitive to certain conditions and subject to rapid nucleolytic degradation. This must be overcome if aptamers are to be applied as drugs or biosensors in cells. Thanks to modern synthetic chemistry, new methods have emerged to refine the chemistry of canonical nucleotides, including ribose modifications,^[16-17] introduction of new chemical moieties to nucleobases,^[12, 18-20] and genetic alphabet expansion.^[21-23] Recent, chemically modified nucleotides compatible with polymerase-mediated synthesis and useful in the SELEX method for the identification of modified aptamers are depicted in Figure 1.^[10, 24] This also reflects a merit of aptamers over antibodies – structural refinements are permitted in aptamers, whereas such modifications usually result in a loss of activity in antibodies.

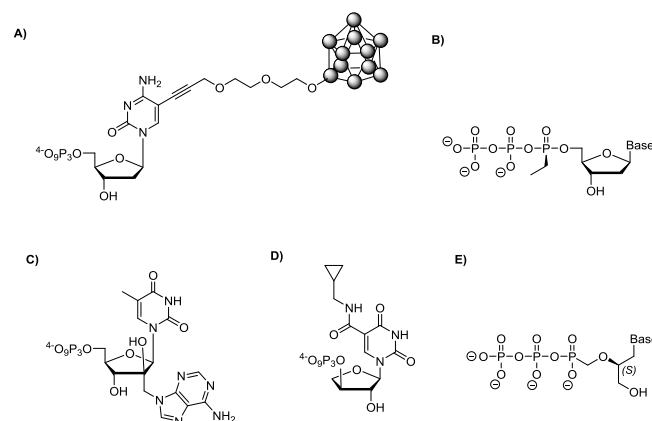


Figure 1. Recent examples of modified nucleoside triphosphates for the design of novel aptamers. A) dCTP analogue bearing a carborane modification at position C5 of the nucleobase which acts as an excellent substrate for the KOD XL DNA polymerase,^[25] B) Nucleotides bearing an (S)_P-ethyl-backbone modification and substrates for the RT521L mutant of the Tgo (*Thermococcus gorgonarius*) DNA polymerase,^[26] C) sugar modified dTTP analogue bearing a second (adenine) nucleobase and compatible with Terminator-mediated enzymatic DNA synthesis,^[27] D) Modified dUTP analogue bearing

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modifications at the level of the sugar moiety (TNA) and nucleobase (at position C5) which can be used together with the engineered polymerase Kod-RSGA to synthesize modified oligonucleotides;^[28] E) Nucleoside triphosphate displaying simultaneous alterations to the backbone and sugar moieties and compatible with Klenow (*exo*) and Vent (*exo*) DNA polymerases mediated polymerization reactions.^[29]

2.1. Ribose modifications

Modifications on ribose are common techniques to confer stability to aptamers. This is especially significant to aptamers that are applied *in vivo*. Susceptible to nucleolytic degradation, unmodified aptamers have poor pharmacokinetic parameters. They are readily truncated into pieces by nucleases, which can be found in both intracellular and extracellular spaces.^[2] An early attempt to enhance the stability of aptamers in a nucleolytic environment was the development of 2'-modifications. Examples of such modifications include halogenation,^[30] amination^[31] and alkoxyl group substitution.^[32] These chemical modifications are common features of aptamers. Pegaptanib, an FDA-approved aptamer for the treatment of age-related macular degeneration (AMD), is a mixture of canonical and non-canonical nucleotides. All 13 pyrimidine nucleotides are fluorinated at the 2'-position of their ribose rings, while 12 out of 14 of the purine nucleotides are methoxylated at their 2'-positions.^[33] The elimination half-life of this drug is estimated to be approximately 10 days,^[34] whereas an unmodified aptamer has a half-life of only 1.4 hours.^[33] Computational simulation of methoxy-dinucleotide on the Klenow fragment polymerase suggests the introduction of a methoxy group at the 2'-position of the ribose induces steric hindrance between the oligonucleotide and amino acid residues at the active site (Figure 2). As a result, the affinity between the polymerase and the methoxy-dinucleotide is reduced, thereby explaining nuclease-resistance of ribose modified aptamers. Progress in enzyme evolution has allowed the identification of polymerases capable of accepting nucleotides with bulky substituents at the 2'-position. This feature was recently exploited for the identification of aptamers binding to the human neutrophil elastase and the blood coagulation protein factor IXa with high affinity^[35].

With improvements in synthetic methodology, locked nucleic acid (LNA) was introduced as a novel variant of nucleotides in 1998.^[36-38] LNA is a bicyclic nucleotide, in which a methylene group links the 2'-oxygen to 4' position of the ribose sugar (Figure 3a). This methylene linkage constrains LNA nucleotides into an RNA-like C3'-endo conformation, instead of a C2'-endo conformation in ordinary B-DNA (Figure 3b).^[39] Aptamers containing LNA nucleotides have strong *in vivo* stability. Take TTA1, a 39-mer Tenascin-C binding aptamer, as an example.^[17] When ten of its nucleotides are changed to LNA-modified nucleotides, its half-life increases from 42 to 53 hours in plasma. This approach increases stability more significantly than 2'-methoxy modifications. The introduction of this extra bridge not only increases the nuclease resistance of an oligonucleotide, but also its thermal stability.^[39-40] LNA-DNA/RNA duplexes have substantially higher melting temperature than DNA or RNA duplex. A 9-mer oligonucleotide, with 3 LNA nucleotides (all G-C base pairs), has a melting temperature of 49°C, whereas in the case of DNA duplex, the melting temperature is only 33°C. Thus, the incorporation of some LNA nucleotides can enhance the thermal stability of the duplex region of an aptamer.

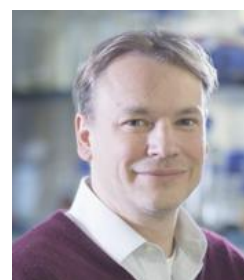
Kwing Yeung Chan is a bachelors graduate in biomedical sciences and chemistry from HKU. He has a strong interest in the field of chemical biology and medicinal chemistry. He completed a project on aptamer-mediated detection of RNA-protein interactions as his final year project in Tanner's group.



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Julian Tanner is a professor in biomedical sciences and also the assistant dean of the LKS Faculty of Medicine, HKU.



Marcel Hollenstein received his Ph.D. degree in 2004 working in the group of Prof. Christian Leumann (University of Bern, Switzerland). He was a postdoctoral fellow in the group of Prof. David Perrin (UBC, Vancouver, Canada) and since 2016 he has been an independent junior research group leader at Institut Pasteur (Paris, France). His research interests focus on the identification of modified aptamers for therapeutic, biosensing, and medical imaging purposes.



2.2. Nucleobase modifications

The addition of abiotic chemical moieties on nucleobases is another major direction to develop aptamers with novel functionalities.^[12] Nucleobases in aptamers are essentially the amino acid residues in antibodies – they play an indispensable role in epitope recognition. The number of distinct nucleobases available in aptamers, inherently limits the chemical variations of aptamers and hence their target diversity. One way to deal with this shortcoming is to append new functional moieties to nucleobases. The C5 position of pyrimidines and C8 position of purines have shown to be the most optimal.^[12] Chemical modifications at these positions protrude from the major groove (Figure 4a). Protein-like or hydrophobic moieties are common chemical appendages (Figure 4b).^[41-43] As a corollary, these modified aptamers build fewer polar and charged interactions

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with their targets. Instead, they engage with their target epitopes with a full range of hydrophobic, π -system, polar and charged interactions.^[18] A good example to illustrate the potential of these aptamers are the slow off-rate modified aptamers (SOMAmers) characterized with their C5-functionalized uridines.^[12] Despite fewer polar or charged interactions with their targets, the binding interface area of SOMAmers is generally larger and the resulting complex is more stable, as indicated by more negative ΔG values upon binding. Another positive effect of nucleobase modification is nuclease resistance. For instance, SOMAmers exhibit considerably lengthened half-life (nearly 9-folds) in serum than their unmodified counterparts.

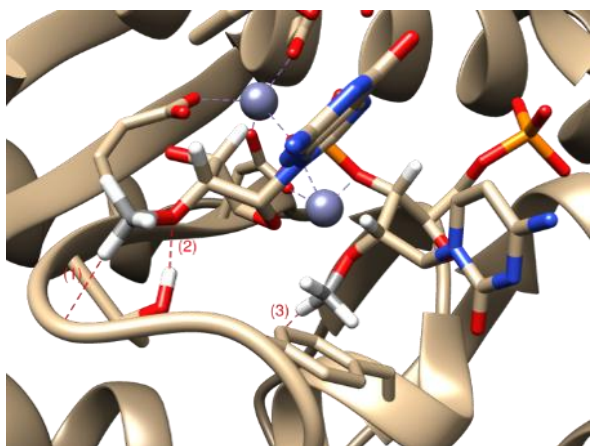


Figure 2. Crystal structure of a 2'-O-methoxyribose nucleotide binding to the Klenow fragment. This structure is modified from Klenow fragment (PDB: 2KZZ) by replacing the 2'-hydrogen by an O-methoxy group. (1): 2.23 Å, (2): 1.96 Å and (3): 0.94 Å. This crystal structure suggests significant steric clashes between the 2'-O-methoxy group and the exonuclease, thereby explaining the prolonged half-life of these 2'-modified aptamers.

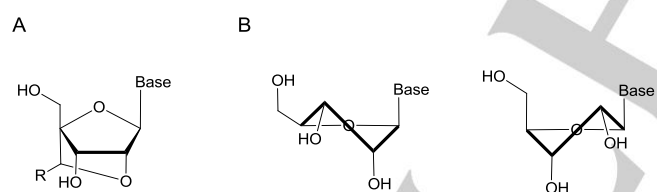


Figure 3. Structure and conformation of LNA. A) Chemical structure of LNA. B) Left: C3'-endo conformation. Right: C2'-endo conformation. For LNA, since its 2'-carbon is covalently linked to the C4'-carbon, the ribose has to adopt a C3'-endo conformation, instead of C2'-endo conformation, which is the conformation of ribose in B-DNA. LNA also confers structural rigidity to oligonucleotide and this preorganised structural rigidity contributes to the rise in LNA-DNA/RNA duplex melting temperature.

2.3. Genetic alphabet expansion

The introduction of novel base pairs is another method to overcome the challenge of having inadequate distinct nucleobases. These unnatural base pairs do not follow conventional Crick-Watson pairing.^[44] Decades of research on genetic alphabet expansion have yielded many unnatural base pairs, but not all of them can be utilized in aptamers. In order to be utilized in aptamers, this base pair should demonstrate high efficiency and fidelity in polymerase chain reaction (PCR). Moreover, hydrophobic nucleobases are more preferred since natural nucleobases are already highly hydrophilic. There are

several examples of such base pairs, for instance, the Ds-Px and 5SICS-NaM developed by Hirao's group and Romesberg's group respectively (Figure 5a and 5b).^[45-46] Compared with canonical base pairs, these novel base pairs are hydrophobic, and they do not rely on H-bonding complementarity when they are replicated by polymerases, indicating that shape complementarity is also a vital prerequisite for efficient nucleobase incorporation during polymerase-mediated replications.^[47] Take the Ds-Px base pair for further discussion. PCR trial suggests high efficiency and fidelity of Ds-PX pair in DNA amplification.^[48] Containing thiophene and alkyne groups, the Ds-PX pair is reasonably hydrophobic.^[21] In 2013, Hirao's group included the unnatural Ds nucleobase in an oligonucleotide library to select aptamers against vascular endothelial growth factor-165 (VEGF-165) and interferon- γ (IFN- γ).^[21] Post-SELEX surface plasmon resonance (SPR) assay indicated higher binding affinities of D nucleobase containing aptamers against VEGF-165 and INF- γ than existing aptamers with only natural base pairs. Therefore, expanded genetic alphabet is also a powerful tool in diversifying the functionalities of aptamers.

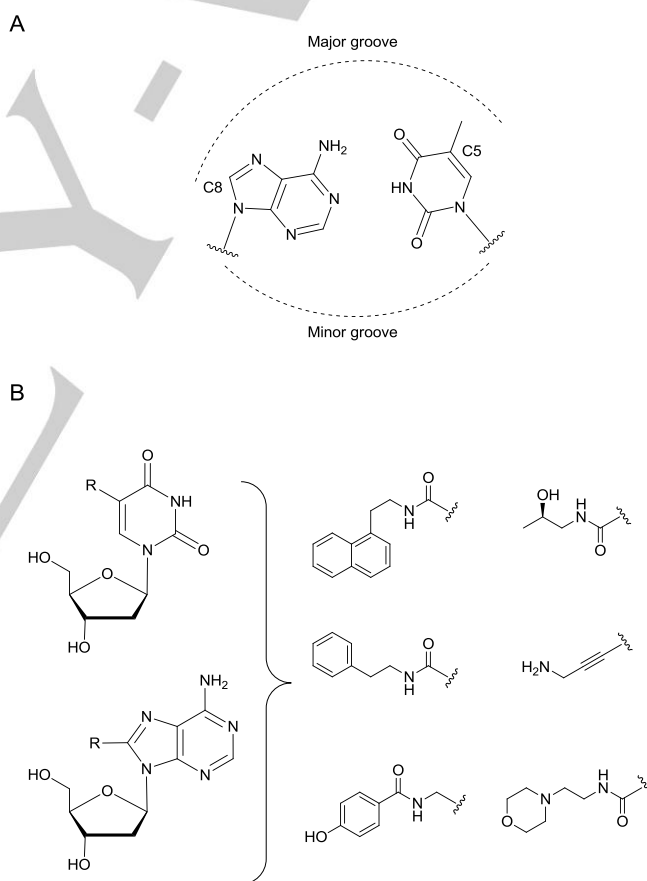


Figure 4. Common nucleobase modifications. A) Major and minor groove of a double helix. Modifications on C5 position of pyrimidines and C8 of purines protrude from the major groove. B) The C5 of pyrimidines and C8 of purines are the most common positions for chemical modifications on nucleobases. Hydrophobic or protein-like side chains can be appended to nucleobases to provide novel functionalities and binding modes to aptamers. Examples of such moieties are shown on the right side of the figure.

In another genetic alphabet expansion approach developed by Benner (Figure 5c), the hydrogen bonding pattern of

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nucleobases is modified so that the resulting analogs specifically form specific hydrogen bonding interactions.^[49-51] These unnatural base pairs are fully orthogonal to the canonical, Watson-Crick base pairs. This versatile method, coined artificially expanded genetic information systems (AEGIS), has been exploited to generate highly potent modified aptamers against cell cancer lines^[22, 52-53] as well as protein^[54] targets. Recently, a highly expanded AEGIS system has been reported but has not been used to generate aptamers as yet.^[55]

Another emerging strategy for expanding the genetic alphabet in the context of aptamer selections is the enzymatic construction of metal base pairs.^[56] In this approach, two synthetic nucleotides serve as ligand for metal coordination and the hydrogen bonding interaction found in canonical base-pairs are substituted with coordinative bonds which ensure stability and orthogonality. No aptamers have been isolated using this strategy yet, but successful enzymatic synthesis have been reported.^[57-61]

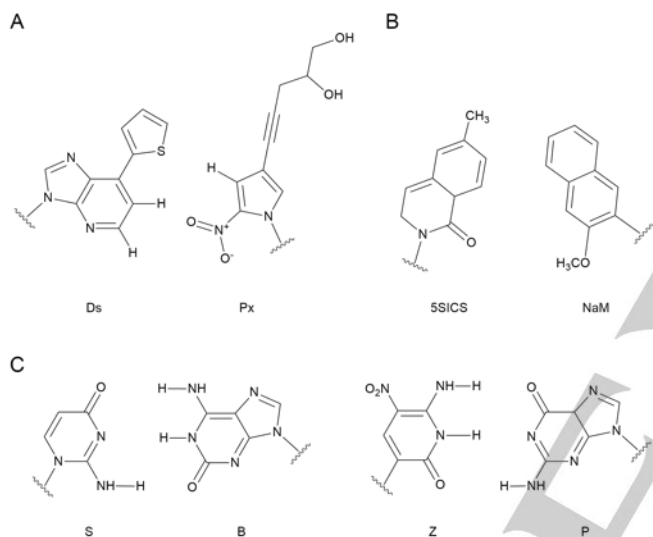


Figure 5. Unnatural base pairs. A) A Ds-Px base pair. B) A 5SISC-NaM base pair. For these unnatural and hydrophobic bases, instead of H-bonding, they form base pair largely because of shape complementarity. C) Modified nucleobases developed by Benner.

2.4 Other Chemical Modifications

In addition to the aforementioned modifications, phosphate backbone modifications and additions of tags are also reported in nucleic acid aptamers.^[62-63] Examples of phosphate backbone modifications include substitution of an oxygen atom on phosphate group with a sulphur atom (i.e., phosphorothioate) or a methyl group (i.e., methylphosphonate).^[64] In general, such substitutions can significantly improve the resistance of nucleic acid aptamers against nucleases.^[65-66] While phosphate backbone modifications can ameliorate the stability of aptamers against nuclease, incorporation of tag moieties to aptamers can slow down their renal clearance.^[67] Due to the relatively small molecular size of aptamers, they are subject to rapid glomerular filtration. Installing tag moieties, such as cholesterol, polyethylene glycol (PEGylation), etc. on the terminus of aptamers can increase the molecular size of aptamers, and as a result, the renal clearance can be reduced.^[68-69] Pegaptanib, the

first FDA approved therapeutic aptamer, is also PEGylated to increase its half-life.^[70]

3. Synthesis of modified nucleotides

Regardless of synthesising aptamers with solid phase synthesis (SPS) or by using polymerases, the parent nucleosides are needed as the fundamental building blocks. These unnatural nucleosides, although undoubtedly important constituent members amongst the diversity of aptamers, are less accessible. Traditional methods to acquire these nucleotides require multistep organic synthesis. Organic synthesis sometimes has low stereoselectivity and requires multiple protection-deprotection steps, resulting in unsatisfactory yield and higher cost.^[71] In recent years, however, there have been some breakthroughs in synthetic chemistry, opening new pathways to synthesize unnatural nucleotides. One of them is biocatalytic synthesis, which uses enzymes to catalyse chemical reactions.^[72] Enzyme catalysis is highly stereoselective and chemoselective, ensuring high yield and reducing the cost. Aside from biocatalytic synthesis, novel synthetic techniques, for example, copper (I) catalysed azide-alkyne cycloaddition (CuAAC), etc. CuAAC is also known as click chemistry.^[73] With these techniques, non-canonical nucleosides can be acquired at lower cost and higher efficiency.

3.1. Biocatalytic synthesis

Unnatural nucleosides are of growing importance in biomedical research owing to their promise as drug candidates and as fluorescent nucleotides in next-generation sequencing (NGS). Their nucleobases are extensively modified i.e. attached to fluorophores or functional moieties. However, chemical coupling of these unnatural moieties to the anomeric position (1'-position of ribose) poses difficulty in terms of reaction yield and β -selectivity.^[74] One of the solutions towards this problem is biocatalysis. Enzymes in living organisms have high efficiency and stereoselectivity. Recently, purine nucleoside phosphorylase (PNP) was found to be able to synthesize unnatural nucleosides with high yield and 100% β -selectivity in a phosphate buffer.^[72] In living organisms, PNP is a metabolic enzyme that catalyses the phosphorolysis of purine nucleosides to ribose-1 α -phosphate (Figure 6a). Thanks to the low substrate selectivity of PNP, it can also catalyse a base-exchange reaction between a pyrimidine nucleoside and a nucleobase analogue (Figure 6b). Notably, a variety of nucleobase analogues, such as 6-(N,N-di-n-propylamino) adenine, 5-(coumarin-7-oxyhex-5-yn) uracil, 5-halouracil, 2-selenopyrimidine^{[72] [75]} can be the substrates of PNP (Figure 6c). For reactions involving hydrophobic analogues, the phosphate buffer should contain 30-40% DMSO so as to enhance the solubility of these hydrophobic moieties. Subsequent docking analysis suggests the active site of PNP is located at the surface of the enzyme, which is rather large and loose. This implies the lack of substrate selectivity in PNP.

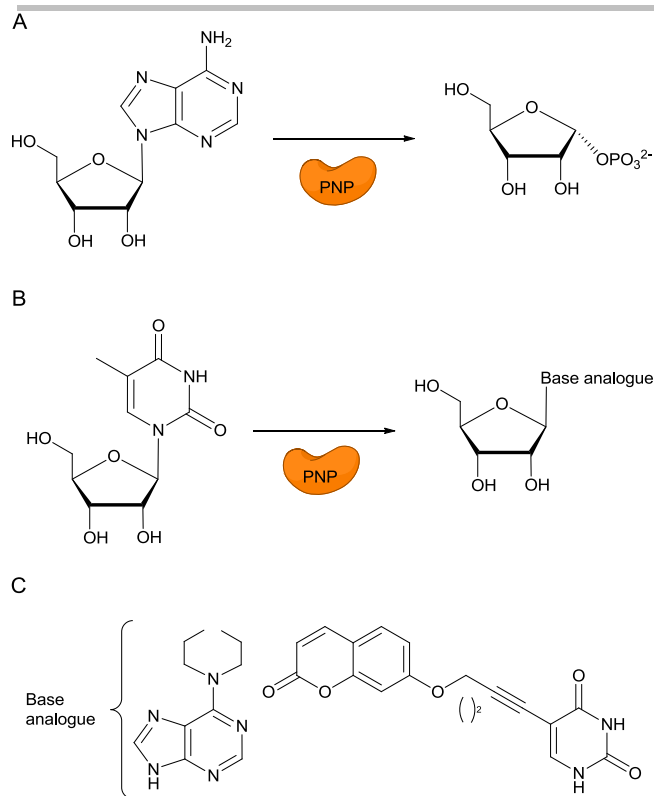


Figure 6. Biocatalytic synthesis by PNP. A) The metabolic reaction catalysed by PNP in vivo. A purine nucleoside is phosphorylated by PNP into purine and ribose-1 α -phosphate. B) PNP can also catalyse a base-exchange reaction. A thymidine is converted to a base analogue with high efficiency and 100% β -selectivity. C) Examples of some base analogues. Left: 6-(N,N-di-n-propylamino) adenine. Right: 5-(coumarin-7-oxylhex-5-yn) uracil.

3.2. Click chemistry

The concept of click chemistry was proposed by Barry Sharpless in 1998.^[73] In order to classify as click chemistry, the reaction must adhere to a set of strict criteria – it has to be modular, high reaction yield, stereoselective, generate non-toxic by-products and to be biorthogonal. One of the most notable examples of click chemistry is the CuAAC, which is a reaction between an azide, and an alkyne catalysed by copper (I). It offers an alternative pathway to synthesise unnatural nucleosides with novel functionalities.^[76-77] Due to its bioorthogonal nature, CuAAC can be applied to synthesise nucleic acids with extra functional moieties.^[78] Firstly synthesised by either phosphoramidite chemistry or primer extension reaction, using 5-ethynyl-dU (EdU) as building blocks, nucleic acids are then conjugated to their azido-moieties with CuAAC.^[79] The synthesis of EdU is well defined and thus it is a readily accessible reagent.^[80] This post-synthetic ligation, however, has low efficiency.^[81] Due to steric hindrance, EdU has low reactivity to couple to certain azido-partners.^[82] One way to cope with this problem is to increase the length of the linker group between the nucleobase and the alkynyl function group such that it can protrude from the surface of the oligonucleotide.^[82-84] Long alkyl group is not suitable to be the linker since it is too hydrophobic and has poor solubility.^[85] Trial and error indicate the O-propargyl-PEG (Figure 7) is the best linker for the subsequent CuAAC – it is long enough to circumvent the steric hindrance while hydrophilic enough to maintain its solubility.^[86]

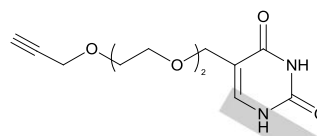


Figure 7. Structure of the O-propargyl-PEG group linked to a thymine base.

3.3. Chemoenzymatic synthesis

Although biocatalysis can generate a desired product in high yield and stereoselectivity, there can be some challenges.^[87] In particular, there is significant effort to screen for a suitable enzyme. Sometimes, extensive protein engineering is needed to produce an enzyme with desirable properties. Besides, enzymes are unstable when they are not inside the cells. It is unlikely that biocatalysis can completely replace conventional synthetic methodology in near future. Thus, synthetic chemists nowadays are trying to integrate standard synthetic strategy with biocatalysis. This is known as chemo-enzymatic synthesis.^[88] The use of chemo-enzymatic synthesis reduces the number of steps needed and hence improves the overall yield when making complicated molecules. Owing to structural complexity of some modified nucleosides, chemo-enzymatic synthesis becomes an alternative in synthesising these nucleosides. One example of chemo-enzymatic synthesis of modified nucleosides is the application of Novozyme-435 to produce LNA.^[89] Conventional synthetic pathway is a 7-step synthesis with a yield of ~ 51% (Figure 8a). Novozyme-435 is a lipase derived from *Candida antarctica B*. The 5'-OH of the furanose diol (starting material of the synthesis in figure 8a) is selectively acetylated by this enzyme (Figure 8b). Therefore, this synthesis avoids the deprotection of this 5'-OH (as in step 5 and 6 of figure 8a). With the use of Novozyme-435, the synthetic steps are reduced to 5 with a yield of ~ 65% (Figure 8c).

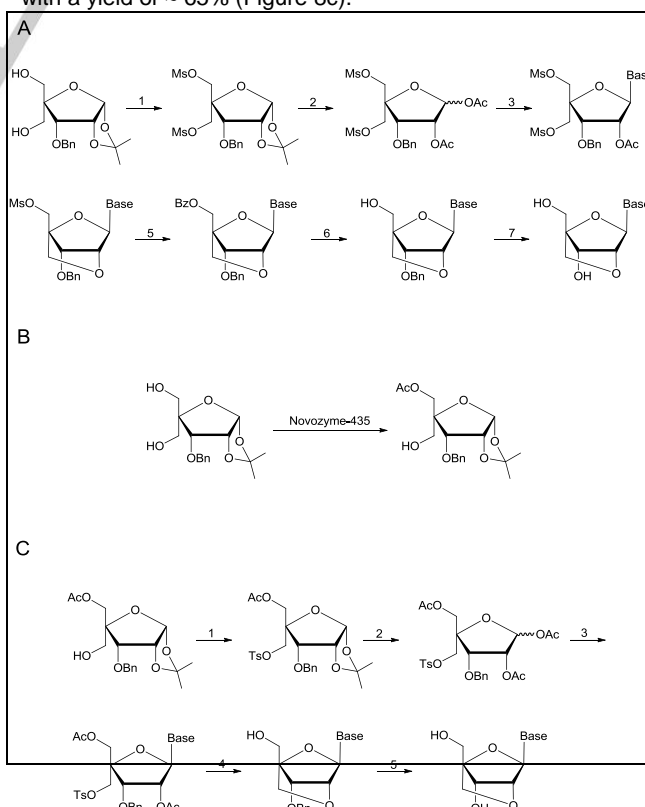


Figure 8. Different synthetic strategies to generate LNA. A) Standard chemical synthesis of LNA using 4'-C-hydroxymethyl furanose as starting material. Note that different nucleobases can result in different yields. The 51% yield shown in the maintext was calculated when adding thymine in step 3 of the synthesis. B) An enzymatic selective acetylation of 4'-C-hydroxymethyl furanose by Novozyme-435. This enzymatic reaction can achieve 100% regioselectivity and almost 100% yield. Novozyme-435 can be reused up to 10 times with yield > 95%. C) Chemical synthesis of LNA using 5-O-acetyl-3-O-benzyl-4-C-hydroxymethyl-1,2-O-isopropylidene- α -D-ribofuranose (the product of the enzymatic reaction) as the starting reagent. Higher yield is achieved than standard synthesis. The 65% yield shown in the maintext was calculated when adding thymine in step 2 of the synthesis.

4. SELEX of modified aptamers

Systematic evolution of ligands by exponential enrichment (SELEX) is the universal protocol for the selection of aptamers.^[2] SELEX is an iterative process made up of multiple evolutionary rounds. It seeds SELEX, an oligonucleotide library is first constructed, usually by solid phase synthesis. The library composes of up to $\sim 10^{14}$ to 10^{16} oligomers of random sequences. To start the SELEX round the library is incubated with the target to allow binding. Those unbound or loosely bound oligomers are then removed via washing steps, while those remain bound will be eluted. Eluted oligomers will be amplified using PCR. The enriched pool then seeds the next SELEX round and is incubated with the target again, with increasing selection pressure. This incubation-elution-amplification cycle will continue until aptamers of high binding affinity are identified. In addition, counter-selection is needed to ensure the specificity of aptamers. When these aptamers are obtained, they are characterized by sequencing. This standard protocol works very well for selecting canonical aptamers. When it comes to the selection of aptamers with unnatural nucleotides, SELEX becomes more challenging. The first issue concerns the incompatibility of some polymerases with modified nucleoside triphosphates (TPs).^[90] These nucleoside TPs, regardless of their modifications, are poor substrates for many polymerases. Providing an enriched pool of oligomers for subsequent rounds of selection, amplification by PCR is a crucial step in SELEX. Another troubling issue concerns the incapability of NGS to sequence aptamers containing non-canonical base pairs i.e. Ds-Px pair.^[21] NGS relies on "sequencing by synthesis" on a flow cell to determine the sequence of nucleic acid samples. This step requires 3'-capped nucleoside TPs conjugated to cleavable fluorescent dyes, which are only available for the two canonical base pairs A-T and G-C.

4.1. Choices of polymerases

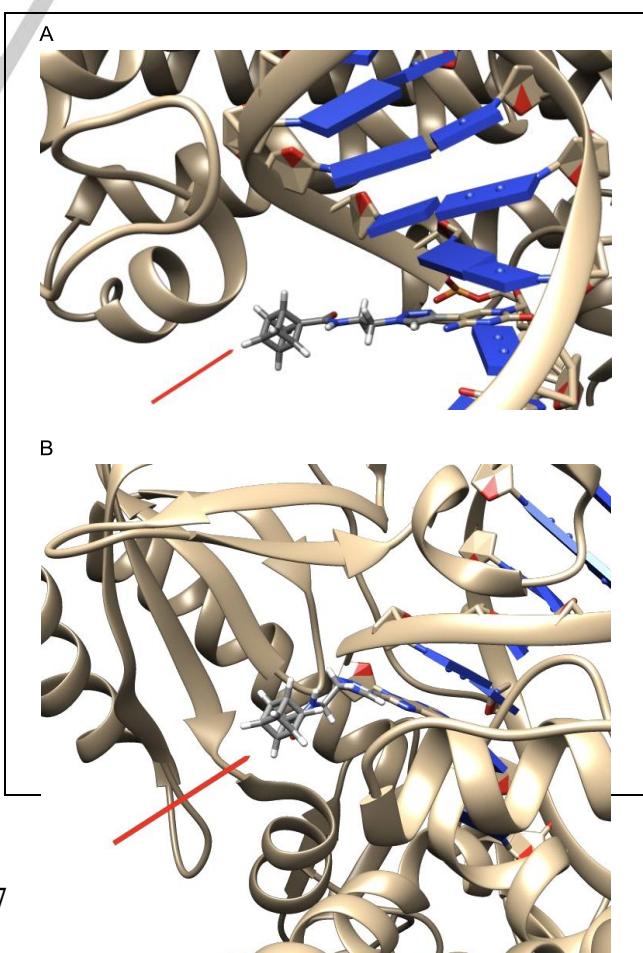
4.1.1. Modified nucleobases

Although many DNA polymerases are unable to accommodate modified nucleoside TPs, B family polymerases are found to possess the ability to incorporate modified nucleoside TPs, including LNA,^[91] C5-modified pyrimidines or purine nucleobases containing modifications at the N7 or N4^[92] positions into the growing strand in the primer extension (PEX) reaction.^[90, 92] Some commercially available polymerases, such as KOD, Phusion, Deep Vent, RB69, Terminator fall into this

category.^[93-94] Molecular modelling provides some insights to understand why these polymerases are tolerant to modified nucleoside TPs.^[90] For a polymerase to accept C5 or C8 modified nucleobases, the major groove of the DNA duplex must be accessible. In an A family DNA polymerase (KlenTaq), its thumb domain directly points towards the major groove of the DNA duplex, thereby sterically hindering the access of C5 or C8 modified nucleoside TPs to the active site of the enzyme (Figure 9a). In a B family polymerase (KOD), its thumb domain does not obstruct the major groove, ensuring the protrusion from nucleobases are not sterically blocked (Figure 9b).

4.1.2. Modified ribose

Apart from the issue arisen from modified nucleobases, modified nucleoside TPs containing most sugar modifications can pose difficulties for polymerase-mediated amplification. In LNA, the ribose adopts a C3'-endo conformation, whereas in B-DNA the ribose adopts a C2'-endo conformation.^[39] Moreover, LNA is conformationally constrained due to the additional tetrahydrofuran ring. Surprisingly, B family DNA polymerases demonstrate excellent compatibility with LNAs.^[94] Crystal structure of RB69 (a B family DNA polymerase) provides some clues to explain the compatibility between B family DNA polymerases and LNA. When canonical nucleoside TP enters the active site of RB69, the ribose exhibits a C3'-endo conformation (Figure 10a), which has the same conformation as an LNA nucleoside TP. This suggests why B family DNA polymerases are compatible with LNAs. *In silico* analysis further indicates the incorporation of an LNA nucleoside TP into the active site of RB69 does not result in any unfavourable steric clashes with the amino acid residues of the enzyme (Figure 10b). In addition, variants from the B family member KOD can be employed to synthesize >1 kilobase long sequences, hence opening up new possibilities in aptamer selections.^[95]



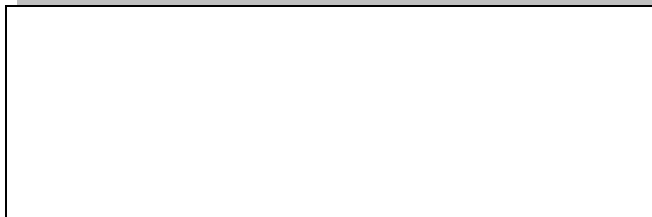


Figure 9. Crystal structures of modified nucleotides binding to DNA polymerases. A) This structure is modified from the A family KlenTaq DNA polymerase (PDB: 3SZ2) by attaching a hydrophobic cubane moiety (refer to figure 12a for the structure) at C5 position of cytosine. Major groove is quite inaccessible. B) This structure is modified from B family KOD DNA polymerase (PDB: 4K8Z) by attaching a hydrophobic cubane moiety (refer to figure 12a for the structure) at C8 position of adenosine. The figure suggests that the major groove is accessible and modified nucleobase does not cause steric hindrance.

Besides, some of the 2'-modified nucleoside TPs can be polymerised with the aid of mutant polymerases. Y639F T7 RNA polymerase is the most commonly used one.^[96] Tyr-693 recognises and binds to the 2'-OH of ribonucleoside TPs via a magnesium ion (Figure 11a). Mutation of tyrosine to phenylalanine at this position completely abolishes the 2'-OH discriminatory ability of the polymerase due to a loss of the ion-dipole interaction. As a result, 2'-halogenated ribose and even deoxyribose can bind to the active site of this mutant polymerase with high affinity (Figure 11b). The activity of this mutant is approximately the same as the wild-type one. It should be noted that this mutant T7 polymerase fails to incorporate modified nucleoside TPs with large 2'-moieties.^[97] A large moiety at the 2'-position of the incoming nucleoside TP is likely to induce steric clashes with the neighbouring groups. For instance, the activity of this polymerase reduces by 80% if 2'-methoxy is added.

The use of TNA (α -L-threofuranosyl nucleic acid) modification protects against nuclease degradation and increases thermal stability. Using a XNA display technique TNA aptamers were selected against HIV reverse transcriptase with KDs of \sim 0.4–4.0 nM.^[98-99] Another ribose modification used to select aptamers is 2'-fluoroarabinonucleic acid (FANA). FANA was used to select aptamers against HIV-1 integrase with KDs of \sim 50–100 pM.^[100] One FANA aptamer inhibited HIV-1 integrase activity and intasome formation in vitro. Hexitol nucleic acids (HNA) is a modification in which the deoxyribose or ribose is replaced with hexitol. The NU172 aptamer against Thrombin was modified to contain HNA at position 9 (NU172-TH9) resulting in a higher binding affinity toward human α -thrombin and improved serum stability with a half-life over 2-fold higher.^[101] HNA was used in the selection of aptamers were selected against rat vascular endothelial growth factor 164 (rVEGF164).^[102] The isolated HNA aptamers had higher specificity for rVEGF164 and human VEGF165 compared to rat VEGF120. Additionally, the HNA aptamers had greater stability to degradation in both serum and DNase I solutions.

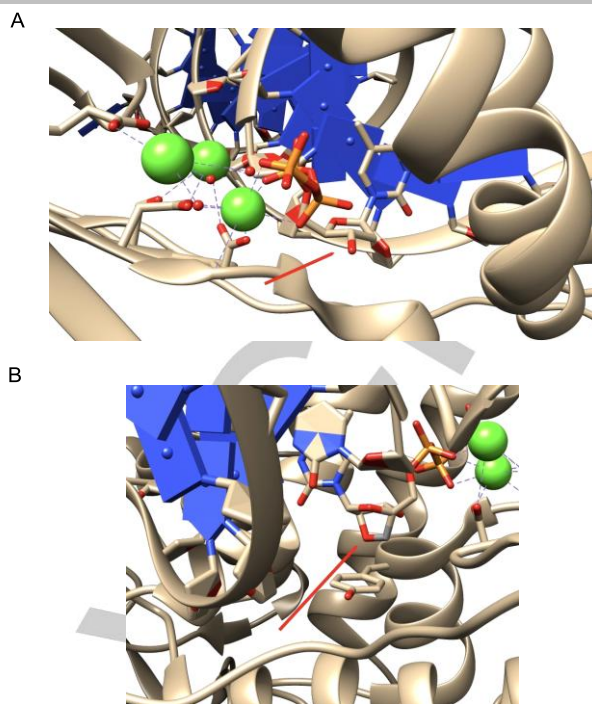


Figure 11. Crystal structures of active sites of RB69 DNA polymerase. A) This structure is from the B family RB69 DNA polymerase (PDB: 1IG9). The crystal structure clearly shows the C3'-endo conformation of the ribose. B) This structure is modified from the B family RB69 DNA polymerase (PDB: 1IG9) by connecting the 2'-carbon to 4'-carbon (refer to figure 3a for LNA structure).

4.2. Sequencing of modified aptamers

The last step of SELEX is to characterise the aptamers obtained by sequencing. NGS works quite well with oligonucleotides containing modified ribose and nucleobase as long as a compatible DNA polymerase is used to convert modified DNA into cDNA. However, this is not the case in aptamers containing Ds-Px base pairs. As mentioned, there is no 3'-capped nucleoside TPs conjugated to cleavable fluorescent dye available for Ds-Px base pair. The fluorescent dye is crucial for the “sequencing by synthesis” step in NGS. Yet, it is still possible to devise a strategy to sequence aptamers with Ds-Px base pairs.^[21] The initial oligonucleotide library can be divided into several subgroups. Each of their 5'-ends has a unique sequence of di/trinucleotides i.e. AA, AT, CAG, CAT, etc. that serves as a tag to identify the subgroup. Each subgroup contains a few Ds-Px base pairs at the defined position. SELEX is then performed to these subgroups of oligonucleotides. After selection, the remaining sequences will be amplified with replacement PCR in which the reaction contains canonical dNTPs and dPa'TP only. Ds nucleotides in these sequences are forced to be replaced by either A or T. Nonetheless, non-specific pairings of Dx to G or to A hamper the fidelity of the PEX reaction, thereby compromising the efficiency of this replacement PCR. dPa'TP is introduced to the reaction mixture because it is less selective and binds better to A or G than dPx'TP, thereby rescuing the efficiency of this PCR.^[103] After PCR, all non-canonical bases will be removed from the aptamers and routine NGS can be carried out. Although NGS can still be carried out, this strategy limits the positional variety of the unnatural base pairs. The best solution is to develop

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sequencing dyes that are compatible with these unnatural base pairs.

Alternatively, nanopore sequencing, which recognizes DNA bases from their individual physico-chemical properties, might be used in the future to sequence oligonucleotides containing ribose-modifications not compatible with polymerase or unnatural base pairs.^[104]

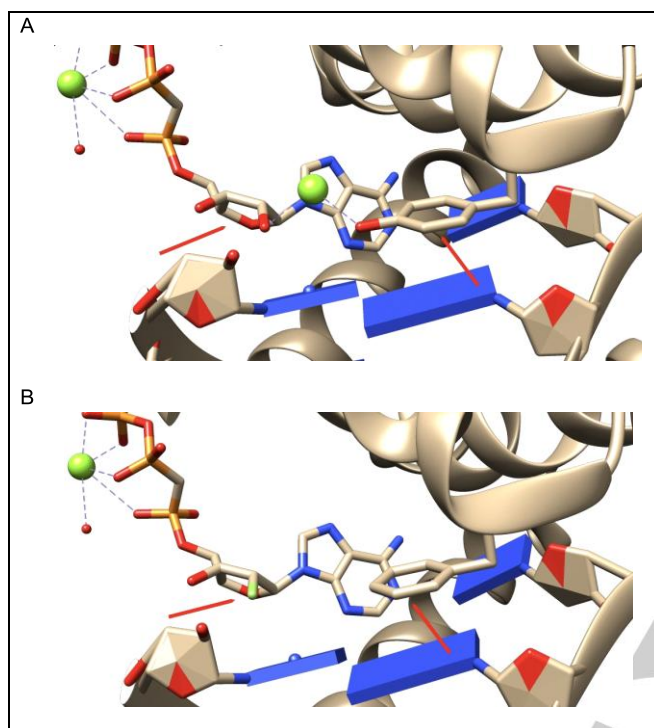


Figure 11. Crystal structures of active sites of WT & mutant T7 RNA polymerases. A) This structure is from the WT T7 RNA polymerase (PDB: 1S0V). Mg^{2+} ion links 2'-OH of RNA and -OH of tyrosine by ion-dipole interactions. This explains by 2'-OH is crucial for the binding to the WT T7 RNA polymerase. B) This structure is modified from the WT T7 RNA polymerase (PDB: 1S0V) by mutating Tyr-639 to Phe-639 and changing the 2'-OH to 2'-F. This mutation abolishes the ion-dipole interactions linking 2'-OH of RNA and -OH of tyrosine. Thus, the selectivity of 2'-OH is lost.

4.3. Chemical modifications: pre-SELEX or post-SELEX?

With various chemical modifications, the diversity of aptamers can be greatly expanded. However, these modifications inevitably deviate from the natural chemistry of nucleic acids, posing challenges to conventional SELEX. Advancements in biotechnology and protein engineering partially alleviate the problem. Decisions typically need to be made to modify pre- or post-SELEX.

pre-SELEX modifications mainly involve chemical changes that are vital for the functions of the aptamers.^[2] Modifications on nucleobases and genetic alphabet expansion usually fall into the category. The aims of introducing these novel chemical moieties or bases to aptamers are to enrich their functionalities and allow them to interact with more targets. These modifications also have a direct impact on the three-dimensional structure of aptamers.^[18] Therefore, additions of these chemical groups after SELEX may completely abolish the activity of aptamers. Moreover, pre-SELEX modifications are not limited to the

forementioned changes. Ribose modifications can sometimes be done before SELEX. For instance, in the development of Pegaptanib,^[16] aptamers were selected from a 2'-fluoropyrimidine RNA library using a mutant T7 RNA polymerase, as mentioned above, which can catalyse the synthesis of RNA containing 2'-fluororibonucleotides.^[96] Thanks to the aid of these engineered polymerases, more pre-SELEX modifications are now possible. Hopefully, more advanced techniques and tools can be developed to aid and simplify the selection of modified oligonucleotides. After all, many aptamers will be chemically modified, and pre-SELEX modification is the most effective way to prevent the loss of activity.

post-SELEX modifications often concern the stability of an aptamer. Terminal and ribose modifications usually fall into this category.^[2] The aim of these modifications is to improve the pharmacokinetic parameters of aptamers by protecting aptamers from a nucleolytic environment and enhancing their half-life in the body. These modifications neither provide novel functionalities to aptamers nor affect their three-dimensional structures. The most common terminal modifications involve conjugation of a polyethylene glycol (PEG) group and an inverted dT nucleotide. Recently, addition of other modifications such as Ruthenium-based photosensitizers^[105] or small hydrophobic residues^[106] improved the therapeutic efficiency of treatment modalities and prolonged the in vivo lifetime of aptamers by binding to albumin. These modifications typically have little impact on the activity of aptamers, and thus can be done after SELEX. Ribose modification is another common post-SELEX chemical change. Though some 2'-substituents are compatible to T7 RNA polymerase, some remain to be poor substrates, such as the O-methoxy group.^[97] In the case of Pegaptanib, all 2'-O-methoxy groups are added to purine nucleotides after SELEX.^[16] Sometimes, it is important to elucidate the structure-activity relationship (SAR) before ribose modifications since some of these modifications may alter the conformation of an aptamer and reduce their binding affinity.^[17] For instance, in the development of a Tenascin-C binding aptamer, TTA1, the secondary structure of this aptamer was first predicted. Based on this structure, it was hypothesised that the duplex region (stem I) did not involve in target binding. Subsequently, some of the ribose at that region was replaced with LNA without any compromise in binding affinity.

While most post-SELEX modifications aim at improving the stability of aptamers, some post-SELEX modifications can enhance the affinity of aptamers towards their targets, for instance, by limiting the conformational flexibility of nucleic acid aptamers.^[107] Similar to proteins, the three-dimensional structure of aptamers is the underlying basis for target recognition and binding. Therefore, only the properly folded aptamers can bind to their targets with high affinity. However, given that nucleic acids are conformationally flexible molecules, facilitating the proper folding of aptamers remains challenging. In 2019, a research group proposed a novel post-SELEX strategy to overcome this problem.^[107] A triple helix structure was installed at the termini of an anti-lysozyme aptamer. This significantly stabilised and increased the structural rigidity of this aptamer. Hence, its affinity towards lysozyme was raised by 10 folds. This facile method demonstrated it is possible to enhance the affinity of aptamers after SELEX.

5. Applications of modified aptamers

5.1. Clinical applications

As a technology emerged in the 1990s, clinical applications of aptamers are quite limited. Pegaptanib is the only FDA approved aptamer-based drug. It is also the only marketed clinical application of aptamers. Until 2018, there have been 11 aptamers entering clinical trials or marketed and developments are further ongoing.^[63, 108]

5.2. Protein detection with SOMAMers

Traditional approach of biomarker profiling relies on antibody-epitope interactions, but the use of antibody detection system has several shortcomings – they are less stable, more expensive and difficult to produce.^[11] With regards to these limitations, SOMALogic, an American based biotechnology company, developed their own protein affinity assay using SOMAMers (Figure 12), known as SOMAscan.^[12, 109-110] In 2014, the company reported that this high throughput system could measure up to 1,129 proteins with magnificent sensitivity and precision. The system has successfully profiled the biomarkers of many diseases, such as chronic kidney disease, mesothelioma, etc. With the unique merits of aptamers, SOMAscan is a very appealing tool in proteomic research.

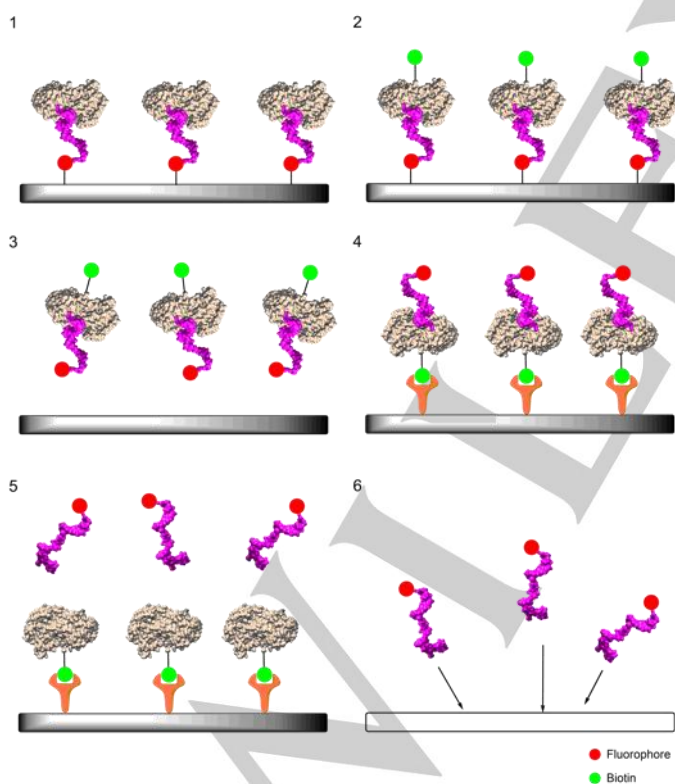


Figure 12. SOMAmer affinity assay. Green: biotin. Red: fluorophore. 1) the analyte (containing a mixture of proteins) is incubated with beads. The surface of these beads is immobilised with SOMAMers. Unbound or loosely bound proteins are washed away. 2) every protein bound to a SOMAmer is tagged with a biotin. 3) these SOMAmer-protein complexes are released from the bead surface by photocleavage. 4) the biotinylated SOMAmer-protein complexes are captured by beads with streptavidin coated on their surface. 5) SOMAMers are released from the protein complexes by denaturing all proteins.

6) these SOMAMers are dotted in a microarray chip to hybridise with sequences that are complementary to them. By reading the fluorescence on the chip, one can determine the identity and quantity of the proteins found in the analyte.

5.3. Abiotic base modifications for ultra-specific protein recognition

In 2020, a chemically modified aptamer specific for *Plasmodium vivax* lactate dehydrogenase (PvLDH) was reported which could function in malaria diagnostics (Figure 13a).^[18] Discovered after 15 rounds of selection, this diagnostic aptamer contained five uracil nucleotides with a cubane moiety at their C5 positions (Figure 13b), thereby known as cubamer. The addition of this abiotic cubane moieties introduces novel functionalities to this aptamer and displays a unique mode of binding – the total interaction between the cubamer and PvLDH is the summation of hydrophobic effect and H-bonding at the binding pocket (Figure 13c).

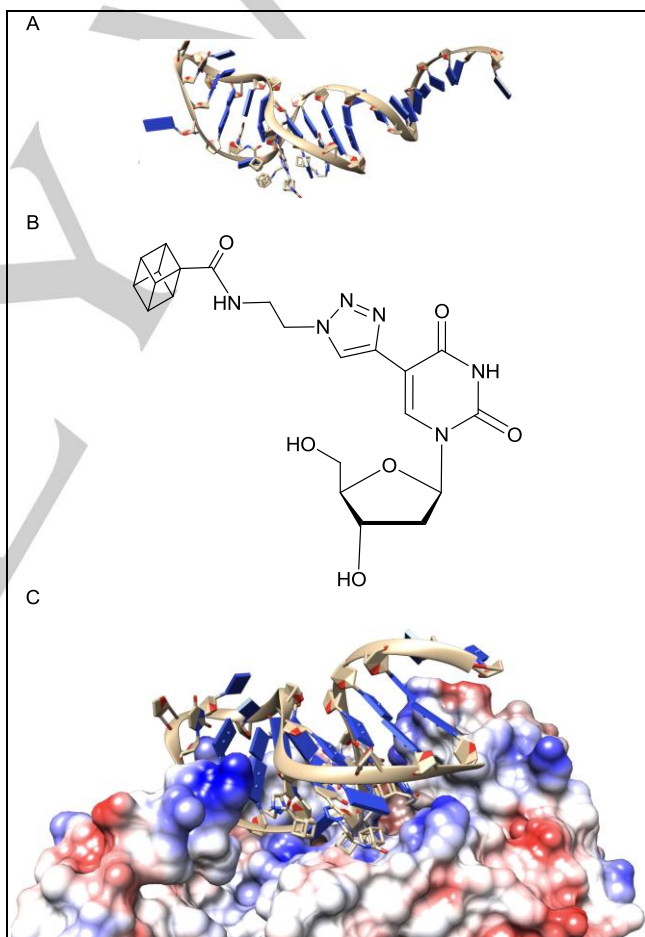


Figure 13. Structure of the novel cubamer and its mode of binding. A) Crystal structure of cubane aptamer (cubamer). B) The cubane moiety is attached to 5-EdU by CuAAC reaction. C) Crystal structure of the cubamer binding to PvLDH (PDB: 6TXR). Coulombic colouring of the binding interface suggests a unique mode of interaction. The cubamer forms hydrophobic interactions with PvLDH at the binding pocket. This is further strengthened by hydrogen bonding

The cubane moieties of the aptamer bind to PvLDH with hydrophobic interaction and H-bonding at the same time.

Cubane is a highly strained ring system. The unusually small bond angle of cubane (90°) combined with high ring strain results in high p-character in all its C-C bonds. Hence, there is marked s character in its C-H bonds which explains the relatively high acidity of the cubyl protons.^[111-112] Therefore, these hydrogens can participate in H-bonding. This hybrid mode of binding (i.e., hydrophobic interaction and H-bonding) serves as the basis for this cubamer to discriminate PvLDH against PfLDH. PfLDH is the lactate dehydrogenase of *Plasmodium falciparum* and it shares high protein sequence similarity with PvLDH (> 90 %).^[113] Canonical DNA aptamers are unable to differentiate PvLDH from PfLDH. The fact that a chemically modified aptamer can differentiate two very similar proteins suggests it is possible that an aptamer, with careful design, to achieve highly specific binding, even at the presence of very similar targets.

This ultra-specific protein recognition has a magnificent implication to malaria diagnosis – both *Plasmodium vivax* and *Plasmodium falciparum* cause malaria, but infections by *Plasmodium falciparum* lead to the most lethal malaria.^[114] This cubamer can work as a quick, yet precise, method to diagnose malaria of different severities such that appropriate treatments.

6. Conclusion & Future Perspectives

Although chemical modifications have greatly expanded the functions of aptamers, two fundamental problems of aptamers remain to be addressed – poor oral bioavailability and cell delivery.^[12] When taken orally, only a limited amount can pass through the plasma membrane of intestinal endothelial cells due to their highly hydrophilic and charged nature. This limited amount of aptamers will be metabolically transformed into inactive compounds in the liver. Therefore, parenteral administration of aptamers is the current therapeutic route. Moreover, the highly charged and hydrophobic nature of aptamers contribute to their poor cell delivery, as shown in many aptamer-based clinical studies focus on extracellular targets. Overcoming these hurdles remains a difficult task, though some promising suggestions have been proposed.^[11, 115]

The introduction of exotic chemistries to nucleic acid aptamers has diversified their chemical properties and widened the scope of their applications in both clinical and research settings. In the process of searching for the most appropriate aptamer, many state-of-the-art tools in both chemistry and biology are employed, namely, click chemistry, biocatalytic synthesis, protein engineering and structural biology.

Structural elucidation is a powerful tool for intelligent design of systems in chemical biology. Furthermore, structural biology can be used to analyse inventions using these new or altered chemical biology systems. Aptamer modification in the ribose, nucleobase or via alphabet expansion can give rise to higher stability, tighter binding and more specific aptamers. Initially, chemical modifications have tried to mimic the chemical repertoire of proteins. The adaptability of these chemical biology systems means that the chemical repertoire of proteins can be surpassed, by using abiotic modifications not seen in biology.

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Keywords: Aptamers • Chemical modifications • SELEX • XNA • nucleic acids

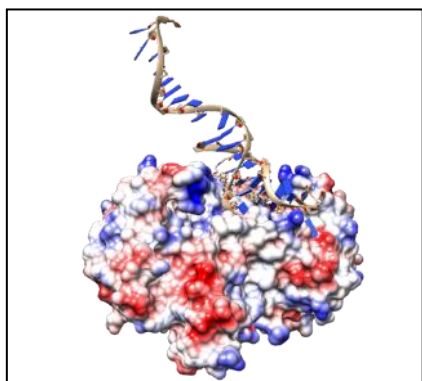
- [1] A. D. Ellington, J. W. Szostak, *Nature* **1990**, *346*, 818-822.
- [2] P. Röthlisberger, M. Hollenstein, *Advanced Drug Delivery Reviews* **2018**, *134*, 3-21.
- [3] P. Fernandez-Millan, A. Autour, E. Ennifar, E. Westhof, M. Ryckelynck, *RNA* **2017**, *23*, 1788-1795.
- [4] R. Sullivan, M. C. Adams, R. R. Naik, V. T. Milam, *Molecules* **2019**, *24*, 1572.
- [5] C. M. Dollins, S. Nair, B. A. Sullenger, *Human gene therapy* **2008**, *19*, 443-450.
- [6] C. Tuerk, L. Gold, *Science* **1990**, *249*, 505-510.
- [7] Y. Wang, K. Nguyen, R. C. Spitale, J. C. Chaput, *Nature Chemistry* **2021**, *13*, 319-326.
- [8] L. Ma, J. Liu, *Science* **2020**, *23*, 100815.
- [9] R. Micura, C. Höbartner, *Chemical Society Reviews* **2020**, *49*, 7331-7353.
- [10] L. K. McKenzie, R. El-Khoury, J. D. Thorpe, M. J. Damha, M. Hollenstein, *Chemical Society Reviews* **2021**, *50*, 5126-5164.
- [11] Y. Zhang, B. S. Lai, M. Juhas, *Molecules* **2019**, *24*, 941.
- [12] J. C. Rohloff, A. D. Gelinas, T. C. Jarvis, U. A. Ochsner, D. J. Schneider, L. Gold, N. Janjic, *Molecular Therapy-Nucleic Acids* **2014**, *3*, e201.
- [13] J. M. Berg, J. L. Tymoczko, L. Stryer, *Biochemistry*, International 7th ed. ed., W.H. Freeman/Palgrave Macmillan, New York, **2012**.
- [14] L. A. Moran, H. R. Horton, K. G. Scrimgeour, M. D. Perry, *Principles of biochemistry*, 5th ed. ed., Pearson, Boston, **2012**.
- [15] L. Gold, D. Ayers, J. Bertino, C. Bock, A. Bock, E. Brody, J. Carter, V. Cunningham, A. Dalby, B. Eaton, *Nature Precedings* **2010**, 1-1.
- [16] C. A. Trujillo, A. A. Nery, J. M. Alves, A. H. Martins, H. Ulrich, *Clinical ophthalmology (Auckland, NZ)* **2007**, *1*, 393.
- [17] K. S. Schmidt, S. Borkowski, J. Kurreck, A. W. Stephens, R. Bald, M. Hecht, M. Friebe, L. Dinkelborg, V. A. Erdmann, *Nucleic acids research* **2004**, *32*, 5757-5765.
- [18] Y.-W. Cheung, P. Röthlisberger, A. E. Mechaly, P. Weber, F. Levi-Acobas, Y. Lo, A. W. Wong, A. B. Kinghorn, A. Haouz, G. P. Savage, *Proceedings of the National Academy of Sciences* **2020**, *117*, 16790-16798.
- [19] M. Ondruš, V. Sýkorová, L. Bednářová, R. Pohl, M. Hocek, *Nucleic acids research* **2020**, *48*, 11982-11993.
- [20] A. Espinasse, H. K. Lembke, A. A. Cao, E. E. Carlson, *RSC chemical biology* **2020**, *1*, 333-351.
- [21] M. Kimoto, R. Yamashige, K.-i. Matsunaga, S. Yokoyama, I. Hirao, *Nature biotechnology* **2013**, *31*, 453-457.
- [22] L. Zhang, Z. Yang, K. Sefah, K. M. Bradley, S. Hoshika, M.-J. Kim, H.-J. Kim, G. Zhu, E. Jiménez, S. Cansiz, *Journal of the American Chemical Society* **2015**, *137*, 6734-6737.
- [23] M. Kimoto, I. Hirao, *Chemical Society Reviews* **2020**, *49*, 7602-7626.
- [24] N. Freund, M. J. L. J. Fürst, P. Holliger, *Current opinion in biotechnology* **2022**, *74*, 129-136.
- [25] D. Kodr, C. P. Yenice, A. Simonova, D. P. Saffić, R. Pohl, V. Sýkorová, M. Ortiz, L. k. Havran, M. Fojta, Z. J. Lesnikowski, *Journal of the American Chemical Society* **2021**, *143*, 7124-7134.
- [26] S. Arangundy-Franklin, A. I. Taylor, B. T. Porebski, V. Genna, S. Peak-Chew, A. Vaisman, R. Woodgate, M. Orozco, P. Holliger, *Nature chemistry* **2019**, *11*, 533-542.
- [27] K. M. Beck, M. B. Krogh, M. Hornum, P. T. Ludford, Y. Tor, P. Nielsen, *Organic & biomolecular chemistry* **2020**, *18*, 7213-7223.
- [28] Q. Li, V. A. Maola, N. Chim, J. Hussain, A. Lozoya-Colinas, J. C. Chaput, *Journal of the American Chemical Society* **2021**, *143*, 17761-17768.

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- [29] M. Luo, E. Groaz, M. Froeyen, V. r. Pezo, F. Jaziri, P. Leonczak, G. Schepers, J. Rozenski, P. Marlière, P. Herdewijn, *Journal of the American Chemical Society* **2019**, *141*, 10844-10851.
- [30] B. Janik, M. Kotick, T. Kreiser, L. Reverman, R. Sommer, D. Wilson, *Biochemical and biophysical research communications* **1972**, *46*, 1153-1160.
- [31] J. Hobbs, H. Sternbach, M. Sprinzl, F. Eckstein, *Biochemistry* **1973**, *12*, 5138-5145.
- [32] R. S. Geary, T. A. Watanabe, L. Truong, S. Freier, E. A. Lesnik, N. B. Sioufi, H. Sasmor, M. Manoharan, A. A. Levin, *Journal of Pharmacology and Experimental Therapeutics* **2001**, *296*, 890-897.
- [33] E. W. Ng, D. T. Shima, P. Calias, E. T. Cunningham, D. R. Guyer, A. P. Adamis, *Nature reviews drug discovery* **2006**, *5*, 123-132.
- [34] S. A. Vineros, *International journal of nanomedicine* **2006**, *1*, 263.
- [35] Q. Shao, T. Chen, K. Sheng, Z. Liu, Z. Zhang, F. E. Romesberg, *Journal of the American Chemical Society* **2020**, *142*, 2125-2128.
- [36] S. K. Singh, A. A. Koshkin, J. Wengel, P. Nielsen, *Chemical communications* **1998**, 455-456.
- [37] S. Obika, D. Nanbu, Y. Hari, K.-i. Morio, Y. In, T. Ishida, T. Imanishi, *Tetrahedron Letters* **1997**, *38*, 8735-8738.
- [38] S. Obika, D. Nanbu, Y. Hari, J.-i. Andoh, K.-i. Morio, T. Doi, T. Imanishi, *Tetrahedron letters* **1998**, *39*, 5401-5404.
- [39] A. A. Koshkin, S. K. Singh, P. Nielsen, V. K. Rajwanshi, R. Kumar, M. Meldgaard, C. E. Olsen, J. Wengel, *Tetrahedron* **1998**, *54*, 3607-3630.
- [40] M. A. Campbell, J. Wengel, *Chemical Society Reviews* **2011**, *40*, 5680-5689.
- [41] B. N. Gawande, J. C. Rohloff, J. D. Carter, I. von Carlowitz, C. Zhang, D. J. Schneider, N. Janjic, *Proceedings of the National Academy of Sciences* **2017**, *114*, 2898-2903.
- [42] M. Renders, E. Miller, C. H. Lam, D. M. Perrin, *Organic & biomolecular chemistry* **2017**, *15*, 1980-1989.
- [43] T. R. Battersby, D. N. Ang, P. Burgstaller, S. C. Jurczyk, M. T. Bowser, D. D. Buchanan, R. T. Kennedy, S. A. Benner, *Journal of the American Chemical Society* **1999**, *121*, 9781-9789.
- [44] E. Ereemeeva, P. Herdewijn, *Current opinion in biotechnology* **2019**, *60*, 259-267.
- [45] M. Kimoto, R. Kawai, T. Mitsui, S. Yokoyama, I. Hirao, *Nucleic acids research* **2009**, *37*, e14-e14.
- [46] D. A. Malyshev, Y. J. Seo, P. Ordoukhanian, F. E. Romesberg, *Journal of the American Chemical Society* **2009**, *131*, 14620-14621.
- [47] T. J. Matray, E. T. Kool, *Nature* **1999**, *399*, 704-708.
- [48] R. Yamashige, M. Kimoto, Y. Takezawa, A. Sato, T. Mitsui, S. Yokoyama, I. Hirao, *Nucleic acids research* **2012**, *40*, 2793-2806.
- [49] Z. Ouaray, S. A. Benner, M. M. Georgiadis, N. G. Richards, *Journal of Biological Chemistry* **2020**, *295*, 17046-17059.
- [50] S. H. Alamudi, M. Kimoto, I. Hirao, *RSC Medicinal Chemistry* **2021**.
- [51] E. Biondi, S. A. Benner, *Biomedicines* **2018**, *6*, 53.
- [52] K. Sefah, Z. Yang, K. M. Bradley, S. Hoshika, E. Jiménez, L. Zhang, G. Zhu, S. Shanker, F. Yu, D. Turek, *Proceedings of the National Academy of Sciences* **2014**, *111*, 1449-1454.
- [53] H. Zurnut, Z. Yang, N. Williams, J. Arizala, S. Batool, S. A. Benner, P. Mallikaratchy, *Biochemistry* **2019**, *59*, 552-562.
- [54] E. Biondi, J. D. Lane, D. Das, S. Dasgupta, J. A. Piccirilli, S. Hoshika, K. M. Bradley, B. A. Krantz, S. A. Benner, *Nucleic acids research* **2016**, *44*, 9565-9577.
- [55] S. Hoshika, N. A. Leal, M.-J. Kim, M.-S. Kim, N. B. Karalkar, H.-J. Kim, A. M. Bates, N. E. Watkins Jr, H. A. SantaLucia, A. J. Meyer, *Science* **2019**, *363*, 884-887.
- [56] M. Flamme, C. Figazzolo, G. Gasser, M. Hollenstein, *Metallomics* **2021**, *13*, mfab016.
- [57] M. Flamme, P. Röthlisberger, F. Levi-Acobas, M. Chawla, R. Oliva, L. Cavallo, G. Gasser, P. Marlière, P. Herdewijn, M. Hollenstein, *ACS Chemical Biology* **2020**, *15*, 2872-2884.
- [58] T. Nakama, Y. Takezawa, M. Shionoya, *Chemical Communications* **2021**, *57*, 1392-1395.
- [59] T. Funai, C. Tagawa, O. Nakagawa, S.-i. Wada, A. Ono, H. Urata, *Chemical Communications* **2020**, *56*, 12025-12028.
- [60] C. Kaul, M. Müller, M. Wagner, S. Schneider, T. Carell, *Nature chemistry* **2011**, *3*, 794-800.
- [61] T. Nakama, Y. Takezawa, D. Sasaki, M. Shionoya, *Journal of the American Chemical Society* **2020**, *142*, 10153-10162.
- [62] S. Ni, H. Yao, L. Wang, J. Lu, F. Jiang, A. Lu, G. Zhang, *International journal of molecular sciences* **2017**, *18*, 1683.
- [63] S. Ni, Z. Zhuo, Y. Pan, Y. Yu, F. Li, J. Liu, L. Wang, X. Wu, D. Li, Y. Wan, *ACS Applied Materials & Interfaces* **2020**, *13*, 9500-9519.
- [64] B. Sacca, L. Lacroix, J.-L. Mergny, *Nucleic acids research* **2005**, *33*, 1182-1192.
- [65] G. Pozmogova, M. Zaitseva, I. Smirnov, A. Shvachko, M. Murina, V. Sergeenko, *Bulletin of experimental biology and medicine* **2010**, *150*, 180-184.
- [66] R. S. Quartin, C. L. Brakel, G. Wetmur, *Nucleic acids research* **1989**, *17*, 7253-7262.
- [67] K. D. Kovacevic, J. C. Gilbert, B. Jilma, *Advanced drug delivery reviews* **2018**, *134*, 36-50.
- [68] C. H. Lee, S.-H. Lee, J. H. Kim, Y.-H. Noh, G.-J. Noh, S.-W. Lee, *Molecular Therapy-Nucleic Acids* **2015**, *4*, e254.
- [69] J. M. Healy, S. D. Lewis, M. Kurz, R. M. Boomer, K. M. Thompson, C. Wilson, T. G. McCauley, *Pharmaceutical research* **2004**, *21*, 2234-2246.
- [70] P. G. Schmidt, K. M. Campbell, K. D. Hinds, G. P. Cook, *Expert opinion on biological therapy* **2007**, *7*, 1427-1436.
- [71] R. Wohlgenuth, *Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology* **2007**, *82*, 1055-1062.
- [72] A. Hatano, H. Wakana, N. Terado, A. Kojima, C. Nishioka, Y. Iizuka, T. Imaizumi, S. Uehara, *Catalysis Science & Technology* **2019**, *9*, 5122-5129.
- [73] H. C. Kolb, M. Finn, K. B. Sharpless, *Angewandte Chemie International Edition* **2001**, *40*, 2004-2021.
- [74] J. Guo, X.-S. Ye, *Molecules* **2010**, *15*, 7235-7265.
- [75] K. F. Hellendahl, F. Kaspar, X. Zhou, Z. Yang, Z. Huang, P. Neubauer, A. Kurreck, *ChemBioChem* **2021**, *22*, 2002.
- [76] F. Pfeiffer, F. Tolle, M. Rosenthal, G. M. Brändle, J. Ewers, G. Mayer, *Nature protocols* **2018**, *13*, 1153-1180.
- [77] P. M. Gramlich, C. T. Wirges, J. Gierlich, T. Carell, *Organic letters* **2008**, *10*, 249-251.
- [78] D. Ganz, D. Harijan, H.-A. Wagenknecht, *RSC Chemical Biology* **2020**, *1*, 86-97.
- [79] D. Baraniak, J. Boryski, *Biomedicines* **2021**, *9*, 628.
- [80] T. Efthymiou, W. Gong, J.-P. Desaulniers, *Molecules* **2012**, *17*, 12665-12703.
- [81] M. Slavičková, M. Janoušková, A. Šimonová, H. Cahová, M. Kambová, H. Šanderová, L. Krásný, M. Hocek, *Chemistry—A European Journal* **2018**, *24*, 8311-8314.
- [82] P. M. Gramlich, S. Warncke, J. Gierlich, T. Carell, *Angewandte Chemie International Edition* **2008**, *47*, 3442-3444.
- [83] A. Panattoni, R. Pohl, M. Hocek, *Organic letters* **2018**, *20*, 3962-3965.
- [84] J. Gierlich, *Org. Lett* **2006**, *8*, 3639-3642.
- [85] A. Baccaro, A. Marx, *Chemistry: A European Journal* **2010**, *16*, 218-226.
- [86] C. Dutton, E. Allen, M. J. Thompson, J. H. Hedley, H. E. Murton, D. M. Williams, *Molecules* **2021**, *26*, 2250.
- [87] R. A. Sheldon, D. Brady, *Chemical Communications* **2018**, *54*, 6088-6104.
- [88] J. Li, A. Amatuni, H. Renata, *Current opinion in chemical biology* **2020**, *55*, 111-118.
- [89] V. K. Sharma, M. Kumar, C. E. Olsen, A. K. Prasad, *The Journal of organic chemistry* **2014**, *79*, 6336-6341.
- [90] K. Bergen, K. Betz, W. Welte, K. Diederichs, A. Marx, *ChemBioChem* **2013**, *14*, 1058-1062.
- [91] R. N. Veedu, B. Vester, J. Wengel, *Organic & biomolecular chemistry* **2009**, *7*, 1404-1409.
- [92] J. Jakubovska, D. Tauraitė, L. Birštonas, R. Meškys, *Nucleic acids research* **2018**, *46*, 5911-5923.
- [93] Y. Hikida, M. Kimoto, I. Hirao, S. Yokoyama, *Biochemical and biophysical research communications* **2017**, *483*, 52-57.
- [94] V. Poongavanam, P. K. Madala, T. Højland, R. N. Veedu, *Plos one* **2014**, *9*, e102126.
- [95] H. Hoshino, Y. Kasahara, M. Kuwahara, S. Obika, *Journal of the American Chemical Society* **2020**, *142*, 21530-21537.

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- [96] K. J. Durniak, S. Bailey, T. A. Steitz, *Science* **2008**, 322, 553-557.
- [97] J. Chelliserrykattil, A. D. Ellington, *Nature biotechnology* **2004**, 22, 1155-1160.
- [98] H. Mei, J.-Y. Liao, R. M. Jimenez, Y. Wang, S. Bala, C. McCloskey, C. Switzer, J. C. Chaput, *Journal of the American Chemical Society* **2018**, 140, 5706-5713.
- [99] M. R. Dunn, C. M. McCloskey, P. Buckley, K. Rhea, J. C. Chaput, *Journal of the American Chemical Society* **2020**, 142, 7721-7724.
- [100] K. M. Rose, I. Alves Ferreira-Bravo, M. Li, R. Craigie, M. A. Ditzler, P. Holliger, J. J. DeStefano, *ACS chemical biology* **2019**, 14, 2166-2175.
- [101] M. De Fenza, E. Ereemeeva, R. Troisi, H. Yang, A. Esposito, F. Sica, P. Herdewijn, D. D'Alonzo, A. Guaragna, *Chemistry-A European Journal* **2020**, 26, 9589-9597.
- [102] E. Ereemeeva, A. Fikatas, L. Margamuljana, M. Abramov, D. Schols, E. Groaz, P. Herdewijn, *Nucleic acids research* **2019**, 47, 4927-4939.
- [103] I. Hirao, M. Kimoto, T. Mitsui, T. Fujiwara, R. Kawai, A. Sato, Y. Harada, S. Yokoyama, *Nature Methods* **2006**, 3, 729-735.
- [104] S. Yan, X. Li, P. Zhang, Y. Wang, H.-Y. Chen, S. Huang, H. Yu, *Chemical science* **2019**, 10, 3110-3117.
- [105] L. K. McKenzie, M. Flamme, P. S. Felder, J. Karges, F. Bonhomme, A. Gandioso, C. Malosse, G. Gasser, M. Hollenstein, *RSC Chemical Biology* **2022**, 3, 85-95.
- [106] D. Ding, C. Yang, C. Lv, J. Li, W. Tan, *Analytical Chemistry* **2020**, 92, 4108-4114.
- [107] L. Zhao, X. Qi, X. Yan, Y. Huang, X. Liang, L. Zhang, S. Wang, W. Tan, *Journal of the American Chemical Society* **2019**, 141, 17493-17497.
- [108] H. Kaur, J. G. Bruno, A. Kumar, T. K. Sharma, *Theranostics* **2018**, 8, 4016.
- [109] J. Candia, F. Cheung, Y. Kotliarov, G. Fantoni, B. Sellers, T. Griesman, J. Huang, S. Stuccio, A. Zingone, B. M. Ryan, *Scientific reports* **2017**, 7, 1-13.
- [110] B. B. Sun, J. C. Maranville, J. E. Peters, D. Stacey, J. R. Staley, J. Blackshaw, S. Burgess, T. Jiang, E. Paige, P. Surendran, *Nature* **2018**, 558, 73-79.
- [111] F. A. Carey, R. J. Sundberg, *Advanced Organic Chemistry: Part A: Structure and Mechanisms*, 5 ed., Boston, MA: Springer US, Boston, MA, **2007**.
- [112] T. A. Reekie, C. M. Williams, L. M. Rendina, M. Kassiou, *Journal of medicinal chemistry* **2018**, 62, 1078-1095.
- [113] D. Turgut-Balik, E. Akbulut, D. K. Shoemark, V. Celik, K. M. Moreton, R. B. Sessions, J. J. Holbrook, R. L. Brady, *Biotechnology letters* **2004**, 26, 1051-1055.
- [114] S. Imtiaz, M. F. Drohlia, K. Nasir, M. Hussain, A. Ahmad, *Saudi Journal of Kidney Diseases and Transplantation* **2015**, 26, 1169.
- [115] K. Tanaka, T. Okuda, Y. Kasahara, S. Obika, *Molecular Therapy-Nucleic Acids* **2021**, 23, 440-449.

Entry for the Table of Contents

Cubane aptamer (cubamer) is a chemically modified nucleic acid aptamer published in 2020. It targets the malaria biomarker *Plasmodium vivax* lactate dehydrogenase (PvLDH) specifically, with high specificity relative to *Plasmodium falciparum* lactate dehydrogenase (PfLDH). Such selectivity has not been possible with conventional DNA aptamers. This example demonstrates the enhanced functionality of modified aptamers. Herein, we review the chemistry, synthesis, selection and application of such modified aptamers.