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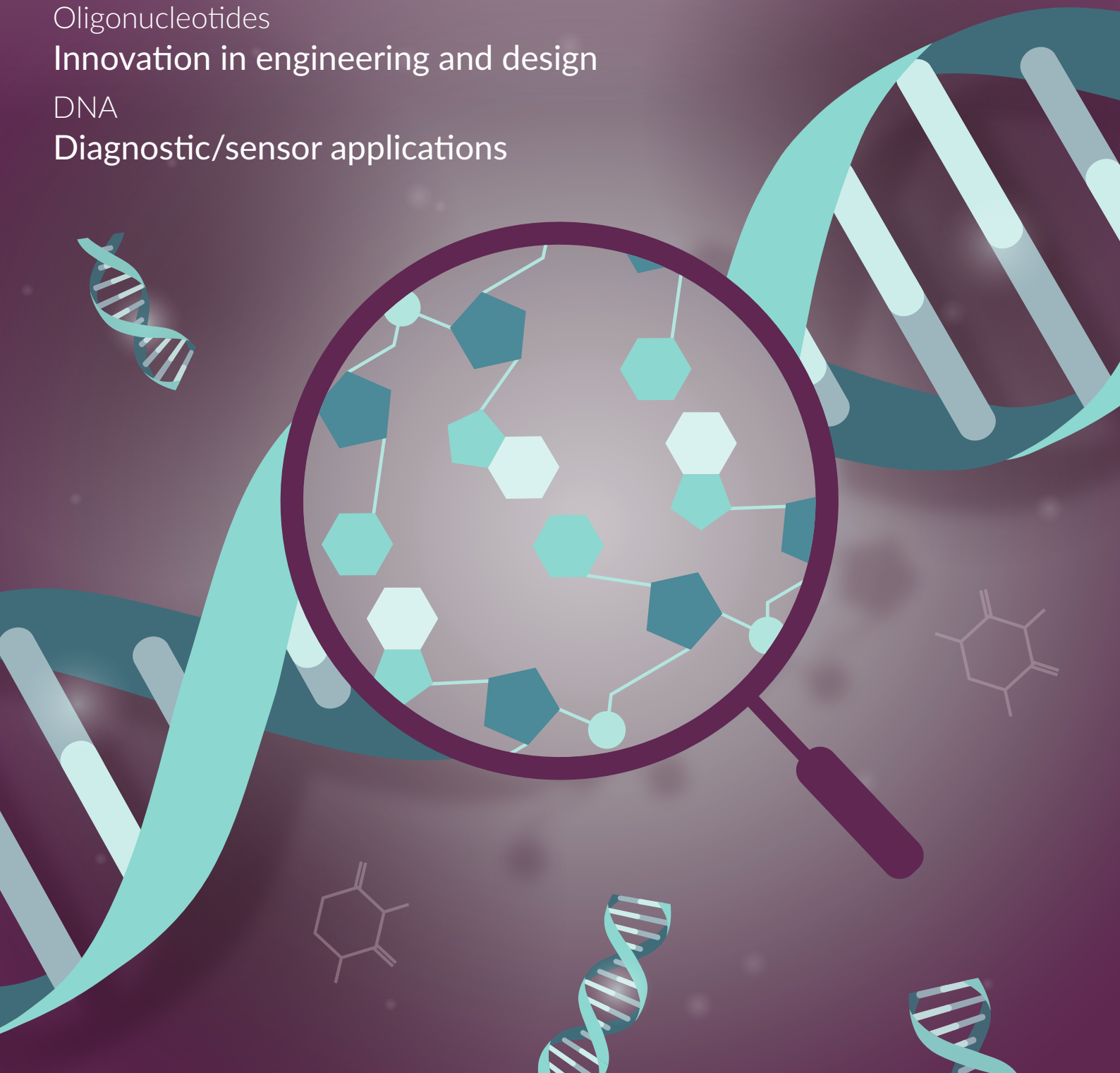

NUCLEIC ACID INSIGHTS

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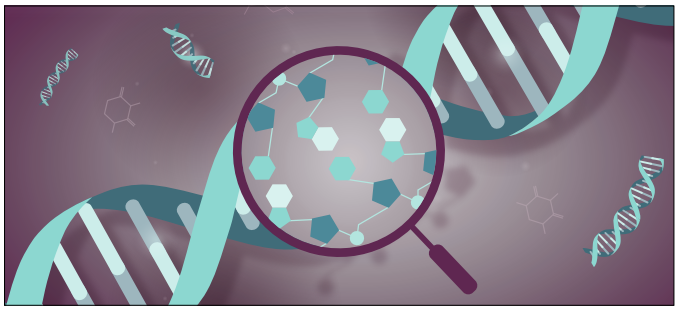
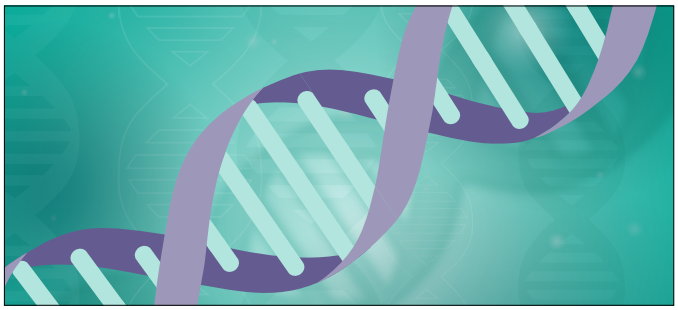




NUCLEIC ACID INSIGHTS

OLIGONUCLEOTIDES
INNOVATION IN ENGINEERING AND DESIGN

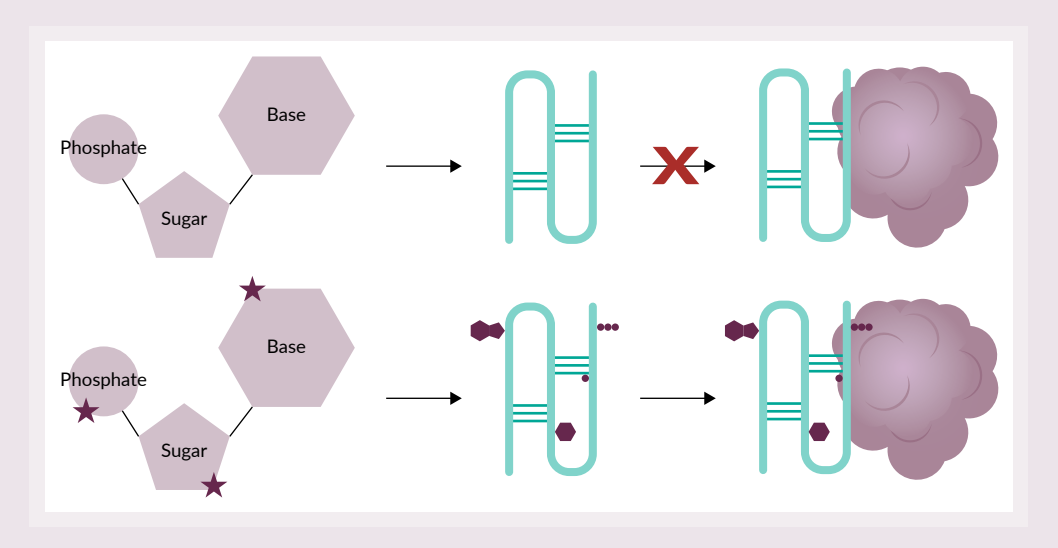
SPOTLIGHT

REVIEW

Chemical modifications for therapeutic aptamers

Henry W Rose, Aasif Ansari, Essraa A Hassan, Jie Tang, Rihe Liu, and Christopher J Serpell

Oligonucleotide aptamers, with their ability to bind diverse biological targets, may be promising successors to therapeutic antibodies in clinical applications due to their reduced production times, ease of synthesis and *in vitro* discovery protocol which does not require animal use or target immunogenicity. However, progress has stalled due to challenges of nuclease degradation, renal filtration, and binding thermodynamics. Some aptamers have overcome these hurdles, reaching FDA approval, and chemical modifications have played a pivotal role in their success. Chemical modifications give improvements to the binding affinity and selectivity, stability, and pharmacokinetics of aptamers, over natural nucleotides. However, changes made to monomers can also alter the overall 3D structure, having significant impact on an aptamer's effect. Identifying the best set of modifications for each therapeutic need will play a key role in the future of therapeutic aptamers.



Graphical abstract. Structure of natural versus modified nucleotides, the resulting aptamers and their binding to a target protein.

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INTRODUCTION

Aptamers are single stranded oligonucleotides which fold to create a 3D structure capable of strong and selective binding of biological targets (proteins, small molecules, or cells). They have had more than 30 years of development since their initial discovery by Ellington and Szostak [1], and Tuerk and Gold [2] in 1990. The Systematic Evolution of Ligands by Exponential Enrichment (SELEX) process [2], which works through rounds of affinity selection followed by enzymatic amplification, has allowed many different variations of single-stranded DNA (ssDNA) [3] and RNA [4] aptamers to be discovered.

These single-stranded nucleic acids have been investigated as novel diagnostic tools [5] and as alternatives to therapeutic antibodies. Therapeutic targets of aptamers include peptides [6], proteins [7] and whole-cells [8], with applications in indications such as cancer [9,10] and neurodegenerative disease [11], among others [12]. To date, two aptamers have been approved by the US FDA for clinical use, both for ocular administration: Pegaptanib, targeting wet age-related macular degeneration (AMD) [13], and avacincaptad pegol, treating geographic atrophy, a complication of AMD [14].

Many companies are capitalizing on aptamers through different commercial offerings. Creative Biolabs are offering aptamer drug delivery systems [15], PentaBind is an AI based aptamer discovery company [16], Aptamer Group are providing optimized aptamers for a range of different uses [17] and SomaLogic provide aptamers to enable proteomics studies [18].

Why aptamers?

As evidenced by these companies' interest in aptamers, there is great potential for therapeutic applications. The ability to discover aptamers without the need for an animal's immune response—in contradistinction to antibodies, the current standard—means that a much wider range of targets are available while also lowering cost, time, and ethical burden. The standardized phosphoramidite synthesis is both reliable and versatile, giving the ability to chemically modify aptamers in a much more comprehensive way than is possible with antibodies [19–21]. Aptamer therapeutics could also address major global issues. Their simple storage and transport requirements mean that their use as therapeutics could be implemented in less economically developed countries, in remote locations or where cold chain is a problem [22]. Sustainability is another point in aptamers' favor over antibody therapies, due not just to reduced storage/transport requirements, but also the roughly 50% reduction of input mass per kilogram produced [23,24].

However, although aptamers demonstrate many advantages over antibody therapies, there still remains issues of biological lifetime and target engagement, where aptamers fall short [25,26]. The binding affinity shortfall is related to the lack of chemical diversity in natural nucleic acids compared to antibodies (four quite similar nucleotides versus twenty diverse amino acids). Unlike antibodies, these issues can be addressed in aptamers through chemical modifications.

Need for modifications

Aptamers which use the natural DNA or RNA chemistry are susceptible to rapid degradation by nucleases in serum [27]. Resistance to both exonucleases and endonucleases is paramount to an aptamer's therapeutic success [28]. The main

strategy for increasing nuclease resistance is the use of non-natural components in an aptamer, either in-SELEX or post-SELEX. One study showed an *in vivo* half-life of only 10 minutes for an unmodified aptamer, but this could be increased ten-fold when the aptamer had been modified [29].

The second reason for modifying aptamers is to improve their binding thermodynamics. To produce a therapeutic effect, the aptamer must be able to successfully bind to its target and cause a response from the protein/peptide etc. Modifying an aptamer by appending functional groups mimicking those of amino acid side-chains has shown an increase in selection success (in terms of binding strength and selectivity) from ~30 to 80% [30].

Renal filtration is a third obstacle to use of aptamers in therapeutics. Due to their high hydrophilicity and small size (compared to antibodies), the 6–30 kDa aptamers [31] are easily excreted and circulation time is minimal [32]. To overcome this, methods have emerged to functionalize aptamers with polyethylene glycol (PEG) and other bulky groups [33]. As a result of these modifications, the PEGylated aptamers have avoided renal filtration and displayed longer circulation times [34–36].

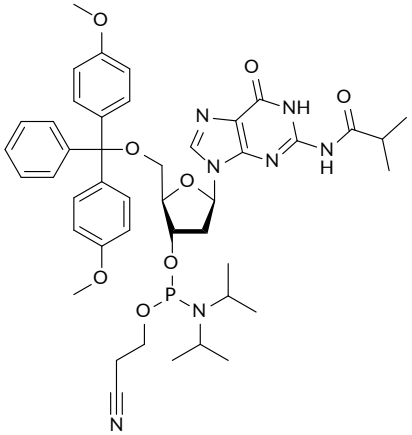
Synthesis and selection strategies

The use of modification strategies must be considered within the context of the requirements laid down by synthesis and selection processes.

Solid-phase phosphoramidite synthesis was invented in 1981 [19] and has enabled aptamer synthesis to evolve rapidly. Using protected nucleoside phosphoramidites (**Figure 1**), an oligonucleotide can be constructed a single nucleotide at a time to a specifically designed sequence. To produce libraries for SELEX screens, a portion of the oligonucleotide sequence is randomised by administering a mix of nucleoside phosphoramidite monomers at each step.

FIGURE 1

DMT-protected guanosine (iBu protected) phosphoramidite for solid-phase nucleic acid synthesis.



Using solid-phase synthesis allows modifications to be made pre-SELEX (i.e., starting with a modified library; this will also require modified NTP monomers in the enzymatic amplification steps) and post-SELEX (i.e., making modifications to a previously selected sequence), giving more freedom in investigation. It has been found that performing SELEX with modified monomers is a generally more reliable strategy since post-SELEX modifications could negatively impact the structure of an aptamer, and hence, its activity [37]. Conversely, it has also been demonstrated that a modification to the sugar post-SELEX can positively impact the structure and enhance activity [38].

However, using modified libraries and monomers in SELEX proves provides limited benefits as it changes the chemistry of the four monomers available, rather than increasing diversity (c.f. the 20 amino acids used in antibodies). Even if multiple modifications are available to the base in the selection step, these will be overwritten in the amplification step [39].

Non-enzymatic methods of aptamer selection have also been investigated,

including magnetic-assisted rapid aptamer selection (MARAS) [40] and selection of optimized ligands by fluorescence-activated bead sorting (SOLFABS) [41]. Both MARAS and SOLFABS, among other methods [42,43], have improved access to non-natural modifications to nucleotides because they are independent of PCR.

Regardless of the order of modifications (pre/post SELEX), it is evident that modifications are essential to generate clinical candidates (Table 1) [13,14,44–48].

TYPES OF NON-NATURAL MODIFICATIONS

Non-natural modifications can be made to each of the three parts of a nucleotide: the base, the sugar, and the phosphate, each of which affect an aptamer's properties differently. As well as modifications to the nucleotides, conjugation to heterospecies and changes in connectivity are also possible and have proven advantageous in aptamer development from a biodistribution and circulation standpoint.

Nucleobase

The primary gain which can be achieved by modifying nucleobases is enhancement of the binding (affinity and selectivity) of the aptamer. The C8 position of the purines and the C5 position of the pyrimidines are the most commonly chemically modified due to synthetic accessibility (Figure 2) [49,50]. This has been shown best, specifically, by 5-methylcytidine) due to their use in FDA-approved antisense oligonucleotide (ASOs) drugs [51,52].

Modifications to a nucleobase can alter the aptamer's selectivity and binding affinity by introducing new intermolecular interactions, similar to those available to proteins [53]. A plethora of base modifications beyond 5-methylation have been made as nucleoside triphosphates (NTPs), but these have yet to be effectively applied in oligonucleotide aptamer therapies [44]. For example, a large number of hydrophobic modifications have been made to uridine bases, with some of the resultant aptamers being termed slow off-rate modified aptamers (SOMAmers) [44,54]. Examples of these include amino acid side-chain related groups [55,56] such as phenols which mimic tyrosine [57].

As well as affinity, aptamers with modified nucleobases have been found to increase nuclease resistance, demonstrating for example a nine-fold half-life increase in serum [58].

TABLE 1

Clinically investigated modified therapeutic aptamers.

Aptamer therapeutic		Modification(s)	Phase	Reference
Pegaptanib		2′-fluoropyrimidines; 2′-*O*-methylpurines; 3′-inverted dT; 40 kDa PEG	Approved	[13]
Avacincaptad		2′-fluoropyrimidines; 2′-*O*-methylpurines; 3′-inverted dT; 43 kDa PEG	Approved	[14]
E-10030		2′-*O*-methylpurines; 3′-inverted dT; 40 kDa PEG	3	[44]
ARC1779		3′-inverted dT; 2′-*O*-methyl with a single phosphorothioate linkage; 20 kDa PEG	2	[45]
ARC1905		2′-fluoropyrimidines; 2′-*O*-methylpurines; 3′-inverted dT; 40 kDa PEG	2	[46]
REG1	RB006 (drug)	2′-ribopurine or 2′-fluoropyrimidine	2	[47]
	RB007 (antidote)	2′-*O*-methyl; 40 kDa PEG	2	[47]
ARC19499		2′-*O*-methylpurine; 3′-inverted dT; 40 kDa PEG	1	[48]

Nucleobase modification in aptamers remains an area to be exploited within medicinal chemistry. Further work allowing more diverse functional group modifications could show improvements in target-binding properties and hence become beneficial for therapeutic aptamers.

Sugar

Substitutions can be made to the (deoxy) ribose sugar ring, altering the structure of the subsequent aptamer synthesised. An aptamer can be synthesised using DNA or RNA backbones, each with their differing sugar functional groups. As well as these natural options, chemical modifications allow a much greater diversity for aptamer selection. The main outcome of ribose sugar modifications is circumvention of aptamer degradation by nucleases in serum and *in vivo* [25,59,60].

The most common modifications to the ribose sugar are at the 2′ position **(Figure 3)** [61], with the first 2′-amino-ribonucleic acid produced in 1994 [62]. 2′-fluoro-ribonucleic acids [63,64] and 2′-methoxy-ribonucleic acids [65] have been used in aptamers and proved valuable for stability. Use of fluorine substitutions in nucleic acids can also allow F—H hydrogen bonding with the target and improve stability by replacing hydrogen or hydroxy groups (in deoxyribose and ribose respectively) due to their smaller steric size but higher electronegativity. There are multiple examples of (non-aptamer) 2′-fluoro-ribonucelic acids that are FDA-approved drugs themselves, used to treat various diseases [66]. In an enzymatically selected aptamer, even using a few 2′-fluoro modifications optimized specific interactions with the target protein as well as nuclease resistance [67].

Locked nucleic acids (LNAs), a class of ribose sugar rings that have a covalent linkage between the 2′-C and 4′-C, have also been synthesized and shown efficacy in increasing *in vivo* stability [68–70].

FIGURE 2

Modification of C8 of purines and C5 of pyrimidines [49-52].

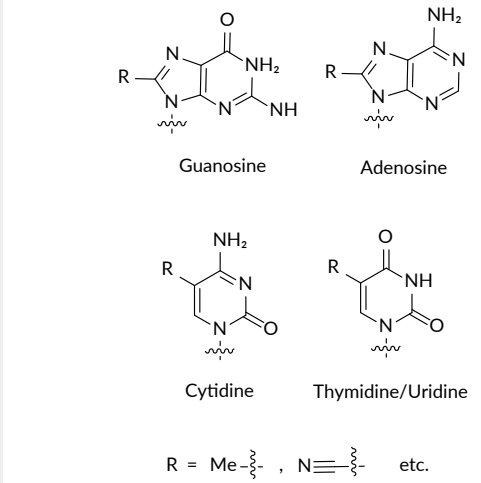
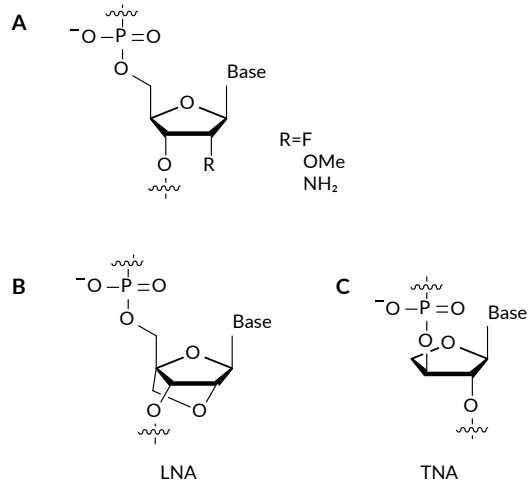


FIGURE 3

Non-natural ribose modifications. (A) 2′-fluoro, 2′-methoxy, 2′-amino modifications. (B) Locked nucleic acid. (C) Threose nucleic acid.



Another alternate sugar is threose, used in threose nucleic acid (TNA) which utilizes 3′-2′ phosphate binding, altering the structure of the aptamer's backbone [71]. A TNA-backbone aptamer provides a very distinct structure in comparison to other ribose-sugar aptamers and demonstrates

outstanding stability in serum, remaining entirely undigested even after 7 days of simulated physiological conditions [72,73]. The change in sugar structure prevents recognition by natural enzymes. In addition to stability, aptamers utilizing TNA backbones have demonstrated high binding affinity in a study on HIV and therefore show promise for further application in a therapeutic context against infectious diseases [74].

Phosphate

The third kind of synthetic chemistry modification of aptamers is the modification of the phosphate backbone which links the nucleoside monomers. As with sugars, this modification is usually made to improve stability. The use of these phosphate substitutions allows subversion of nuclease hydrolysis while still maintaining many of the same properties as the P-O bond they have replaced [75]. The opportunities for phosphate modifications have been more limited than those of the nucleobase or sugar. Phosphorothioate and methylphosphonate analogues of the native phosphate have been investigated most commonly in aptamers and found to be more stable than non-modified DNA and RNA oligonucleotides with the same sequence [76]. In addition, phosphorodithioate substitutions have been synthesized and demonstrated a greatly increased target binding affinity in comparison with the natural aptamer [77]. The phosphorothioate substitution has been used in some FDA-approved oligonucleotide drugs due to the demonstrated improvement of nuclease resistance and cell permeability [78]. Although the stability and nuclease resistance are increased in oligonucleotides with phosphorothioate, there is evidence that modifying the phosphate linkage can negatively impact the affinity to the target, and other modifications may be required to retain satisfactory binding [76,79]. As well as methylphosphonate and sulphur-based modifications, boranophosphates and phosphoramidates have been produced and have proven compatible with enzymatic selection methods **(Figure 4)** [80,81].

Alternative connectivity, cyclization, and multivalency

Endonuclease enzymes recognize natural patterns of nucleotide connectivity, and exonucleases work only at the termini of the oligonucleotide. Changing the connectivity of nucleotides in an aptamer can therefore bolster biostability **(Figure 5)**.

The use of 3′-inverted-deoxythymidine (dT) [82] has proven the most common among aptamers in clinical trials **(Table 1)**, giving the oligonucleotide two 5′ ends from the perspective of a nuclease. Inverted base capping has been shown to have modest effects on serum stability and has proven more stable than a 2′-fluoro modified counterpart [27]. Other studies have investigated using inverted bases at both ends of an aptamer which was found to have noteworthy improvements to the original aptamer's properties, including thermal denaturation [83,84].

Functionalizing the termini of an aptamer should mean that the original binding properties are not significantly impaired, and this is indeed usually the case [85]. However, the thrombin binding aptamer (TBA) has been modified with inversion of polarity in this way, and it resulted in a switch from anticoagulative to antiproliferative properties [83].

Cyclization is also a promising strategy to enhance aptamer stability. Engineering aptamers into circular forms can prevent degradation caused by plasma exonucleases, which cleave phosphodiester bonds at either 3′ or 5′ terminals. As an example, a circular DNA aptamer targeting glutamate dehydrogenase from *Clostridium difficile* exhibited a much higher affinity and stability (half-life of >3 h) compared to its linear

counterpart (functional half-life 0.35 h), providing valuable insights into circular aptamer stability against nucleases [86]. The use of different linker molecules to bind the 5′ and 3′ ends of an aptamer to make a cyclic oligonucleotide has been studied with regard to improving stability, where in serum half-life was found to increase up to eight times [87].

Multivalency, i.e., combining two or more identical aptamer motifs can enhance overall target-binding affinity [88,89]. So far, this has been demonstrated for detection, but the principle could readily be translated to therapeutic uses: Luo *et al.* demonstrated that a multivalent aptamer probe, named DNA nanocreeper (Zy1-DNC), designed for tumor cell detection in liquid biopsy, showed high stability in the presence of nuclease or in human serum; and a reduced dissociation constant 0.75 ± 0.14 nM (1/10th) compared with Zy1 monomers [90].

Conjugation

In order to bypass rapid renal clearance, aptamers can be conjugated to larger molecular units such as polymers, peptides, or nanostructures [44,91]. This has the primary aim of improving pharmacokinetic parameters.

The use of polyethylene glycol (PEG) has been common in clinically investigated therapeutic aptamers (Table 1) and has proved effective in increasing their *in vitro* and *in vivo* half-life [45–47]. Currently, it seems PEG is the front-runner when conjugating an aptamer to a polymer, but other structures have also proven beneficial [90,92,93]. Polyphosphoesters provide an alternative while remaining biocompatible conjugation structures [94]. Phos4nova is a company that produces polyphosphoesters with the aim of replacing PEG [95]. Although PEGlyation has been effective in aptamer therapeutics, it has also resulted in a Phase 3 trial failing: Pegnivacogin (RB006 from Table 1) was part of the REG1 drug system and had been investigated for use as an anticoagulant [48]. It had been effective in its treatment and so was advanced to a Phase 3 trial [96]. However, during this stage, it became clear that anti-PEG antibodies caused severe allergic reactions in some participants and the study failed to proceed [97,98]. As well as the potential to trigger an immune response, PEGylation also restricts subcutaneous dosing.

Zhang *et al.* conjugated aptamers with low molecular weight coupling agents (LMWCAs), which bind to human serum albumin (HSA) and extend the circulation half-life of the aptamer. They demonstrated that sclerostin aptamers conjugated with two LMWCAs showed a significantly longer half-life (12 days)

FIGURE 4

Methylphosphonate, phosphorothioate and phosphorodithioate, boranophosphate and phosphoramidate modifications to the phosphate backbone of an aptamer.

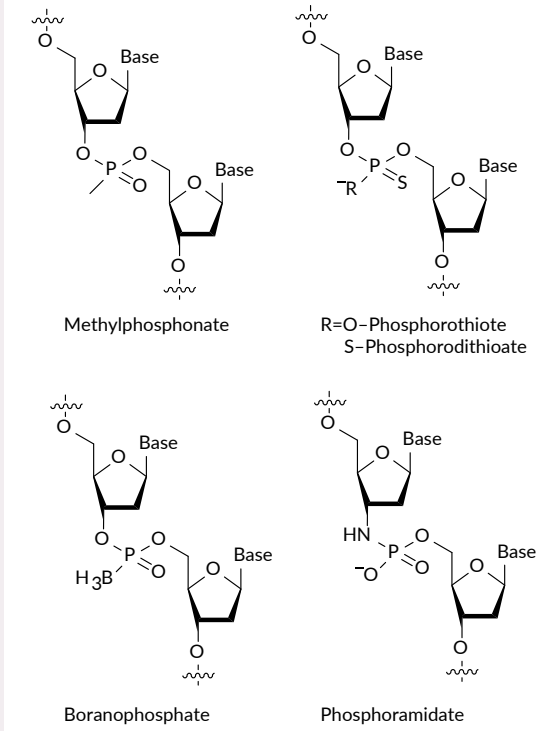
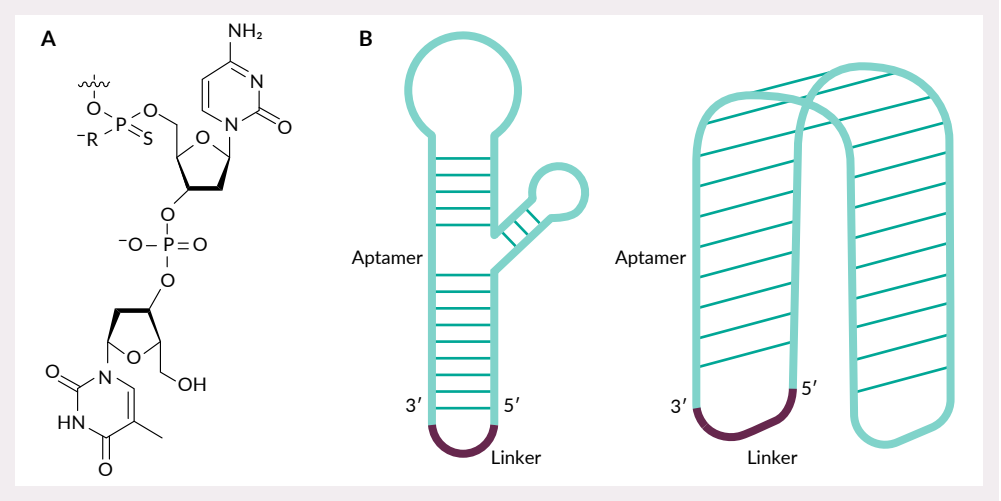


FIGURE 5

(A) 3′-inverted deoxythymidine (dT) modification to the 3′-terminus of an aptamer. (B) Graphical depictions of a linker molecule binding the 5′ and 3′ termini to form a cyclic aptamer.



compared to PEGylation (2.7 days), thereby enhancing the therapeutic efficacy of the aptamer [99]. Cholesterol exhibits a strong affinity for low-density lipoprotein (LDL). Chemically attaching cholesterol to the 5′-end of an aptamer enables the formation of cholesterol-aptamer LDL complexes, which exhibit significant resistance to serum nucleases, therefore extending the aptamer's half-life by tenfold compared to the unmodified version [100]. Lee *et al.* reported that a 2′-F-modified aptamer conjugated with cholesterol, when administered intravenously, exhibited an extended half-life (cholesterol-conjugated versus non-cholesterol-conjugated, 11 ± 12 versus 5.8 ± 2.1 h) and reduced clearance (0.26 ± 0.025 l·hour−1 kg^{-1} for the cholesterol-conjugated aptamer compared to 2.4 ± 0.86 l·hour−1 kg^{-1} for the non-conjugated form). Additionally, the cholesterol-conjugated aptamer demonstrated significantly higher AUClast (382 ± 40 μg·hour·ml^{-1}) compared to the non-conjugated version (46 ± 17 μg·hour·ml^{-1}), indicating better pharmacokinetics for the cholesterol conjugated aptamer [101].

Nanomaterial and nanoparticle (NP) conjugation enhances aptamer stability by protecting them from nuclease degradation and clearance without altering their target-binding properties. For example, DNA strands have been attached to single-walled carbon nanotubes (SWNTs), however, target binding had been seen to interrupt the interaction between the aptamer and carbon nanotube [102]. Another example is conjugation to gold nanoparticles, which has resulted in increased resistance to enzymatic degradation [103]. This is attributed to the high local ion concentrations on the NP surface and conformational changes that make aptamers less recognizable by nucleases [104]. Xia *et al.* reported the development of gold nanocluster (GNC)-aptamer self-assemblies (GNCs@aptamer), which showed resistance to nuclease degradation for up to 48 hours, compared to just 3 hours for unmodified aptamers. Additionally, GNCs@aptamer showed nine-fold higher tumor retention compared to the aptamers alone [105].

Aptamers (ca. 12 kDa) can even be conjugated with an antibody (150 kDa). The

antibody component increases the aptamer's molecular size, reducing rapid renal clearance and extending its circulation half-life. Heo *et al.* investigated a cotinine-conjugated aptamer combined with an anti-cotinine antibody. The antibody part of 'oligobody' resulted in extended *in vivo* pharmacokinetics of the aptamer with $t_{½}$ = 15 h for the cotinine conjugated aptamer in contrast to the aptamer in the absence of the antibody ($t_{½}$ = 0.56 h) without influencing its binding affinity [106].

Aptamer-drug conjugates can also be created, analogous to antibody-drug conjugates, in which the aptamer acts as the targeted delivery vessel itself [107,108]. This allows the benefits of target-specific binding without the need to modify the aptamer to induce a therapeutic effect.

OUTLOOK

The 3D structure of an aptamer is dictated by the arrangement of potential supramolecular interactions in its sequence. Like other biomolecules, this 3D structure determines its function [109,110]. Modifying the structure of any part of a nucleic acid monomer has the potential to alter the overall 3D structure and can cause a once promising aptamer to fail. However, modification can also greatly improve aptamers as therapeutics and these substitutions have proven integral clinical successes [111,112]. Post-selection modifications also allow screening of a much larger number of aptamers against the same target and so can provide data indicating beneficial alterations to the structure and activity of the therapeutic. An increase in the number of modification types as well as modifiable positions leads to an exponentially larger number of post-SELEX modified aptamers.

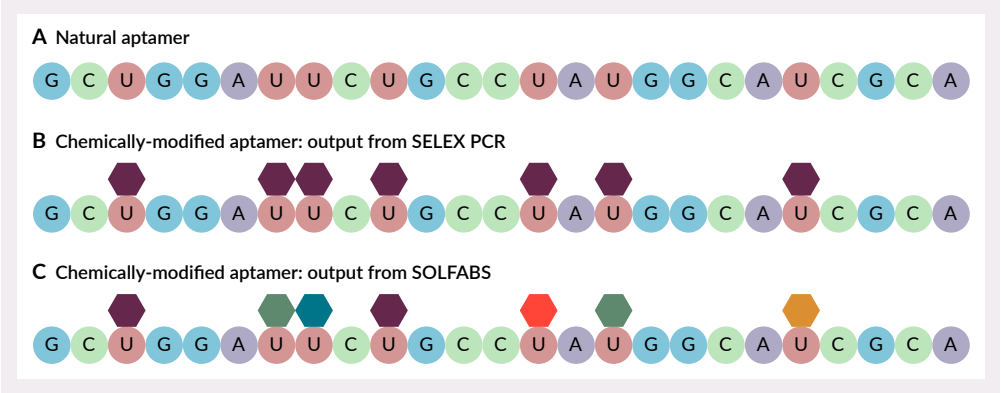
However, as mentioned, modifications can be beneficial in one respect but detrimental in another, so it can be a challenge to quantify how much a specific substitution improves an aptamer overall, out of the huge variety of possibilities, and requires consideration on a per-target basis. Modifications post-SELEX can prove more difficult and could have negative effects on the aptamer, while in-SELEX modifications are built into the resultant base sequence and cannot be readily compared with the unmodified aptamer. In the development of pegaptanib, addition of the PEG chain decreased affinity for the target by factor of four *in vitro*, but improved inhibition of its target *in vivo* by 83% [13].

There is still much potential to expand current pre-SELEX modification capacity through functionalization of different groups or potentially even modifying a nucleotide in an entirely new location or process. In addition, combining different types of modification to a single nucleotide (e.g., sugar and base modification [41]) would allow greater variety and possibly improvement due to the synergistic effects of their substitutions. The combination of a modified base and modified sugar could give eight different varieties of a selected base and so, using the example of a non-enzymatically selected, 40-base aptamer with 10 uridine bases, this would allow over 1 billion (8^{10}) aptamers to be screened.

The next steps are to move beyond the uniform modifications which can be generated using SELEX **(Figure 6A and B)**, to select multiple beneficial modifications to a single aptamer simultaneously **(Figure 6C)** [113]. A number of alternative methods to SELEX have been demonstrated but each has its drawbacks. One such method enhanced SELEX with negative selection through the use of a competitor [53]. This resulted in high binding affinity, slow dissociating SOMAmers being selected, however, the manual screening of modification positions to replace incompatible 2′-*O*-Me modifications proved very laborious. Another alternate method modified the C5 position of the pyrimidines and through the modifications, the selected nucleic acid polymers' binding affinity was

FIGURE 6

(A) An example unmodified aptamer. (B) Chemically modified example aptamer, losing modification information through the SELEX PCR replication stage. (C) The same chemically modified aptamer, retaining all modifications through SOLFABS.



improved [114]. But the method used a codon system meaning that only one type of modification was present for the three-base sequences. As such this limited the ability of modifications due to sequence dependence. A key emerging method that has allowed selection of multiple modifications is SOLFABS [41]. SOLFABS gives the ability to chemically modify a nucleobase with different functional groups, synthesize aptamers with a mixed combination of these non-natural nucleotides, and then select the top fraction of binders, repeating at increased stringency until there are just a small number of aptamers displaying the strongest binding affinity. While conducting SOLFABS, different chemical modifications made to the same type of nucleobase are retained, contrary to enzymatic methods **(Figure 6)** and it was shown that the mix of modifications was superior to uniform modification. This provides much greater diversity to the aptamer chemistry available and so provides a huge advantage during selection, since it moves closer to the range of functionalities displayed by antibodies.

Renal clearance is still an issue for aptamers, and a new approach could be to overcome it through modification-mediated binding to albumin [115]. Albumin is the most common protein in serum and so any increase in albumin binding could prove positive in a longer half-life and reduced renal clearance [116]. However, currently even a modified, nuclease resistant aptamer can have as little as <10 min *in vivo* half-life when intravenously administered [29].

Modification chemistry may also need to be combined with delivery methods related to the target (i.e., lung-targeted therapy using an inhaler) for improved pre-clinical and clinical stages. Lei *et al.* [117] studied the effect of HBA7 aptamers with 2′fluoro-pyrimidine modifications when administered via oropharyngeal aspiration for the treatment of acute lung injury. The lung distribution study showed that the aptamer retention at 24 h was less than 15% of that at 4 h highlighting the need for an effective delivery system to increase the aptamer's residence time and to deliver it with a clinically translatable device (via nebulizers or dry powder inhalers rather than oropharyngeal or intratracheal administration). Nucleic acid therapeutics have the potential to revolutionize many areas of medicine through ASOs, siRNA, mRNA, and aptamers. Aptamers are distinct in this grouping, in that they interact with non-nucleic acid

species, rather than interfacing with the genetic code at the DNA or RNA level. This orthogonal behavior means that a new role for aptamers could be solving some of the targeting problems which ASOs and siRNAs have, if they are combined into a single unit either covalently, through hybridization, or in a delivery vehicle [118].

As well as the clinical and investigational advantages of aptamers over antibodies, the environmental impact of aptamers and less demanding storage and transport requirements (mentioned earlier) must also be considered as an area in which aptamers can make serious strides forward.

TRANSLATION INSIGHT

Aptamer therapeutics provide many different commercial routes and benefits for industry, partly due to the wide availability of solid-phase synthesizers that can produce at a larger scale [119]. These enable R&D teams to develop synthetically modified nucleotides while identifying and selecting the key improvements from the natural aptamer. Large batches of aptamers can then be synthesized, enabling clinical studies either through CRO or an in-house investigation. The reliable chemical synthesis is both faster and more readily fine-tuned than production of antibodies in cells. Naturally, any synthetic process still has an environment impact, and it is true that aptamers are more demanding than small molecules, but intense efforts are afoot to increase sustainability and reduce cost [23]. Aptamers provide many benefits over antibodies due to cost reductions in improved stability and hence less pressure on storage conditions and shelf-life. Aptamer therapeutics still have some way to go to reach their development potential, but it is possible that they could provide a valuable alternative or even replace antibodies in terms of effectiveness as well as commercial opportunity.

Even though there appear to be few fundamental issues with manufacturing aptamers, challenges are still present in commercially viable therapeutics. PEGlyation is a common solution to renal clearance but this has previously resulted in a failed clinical study. On the other hand, PEG has been a component of many formulations which have resulted in successful clinical trials and so should not be ruled out for aptamer conjugation. Nonetheless, development for other polymers or aptamer delivery vessels could make a big difference in therapeutic applications. As a new approach, aptamer-drug conjugates could pose an alternative solution to aptamer therapies due to their targeting capabilities. This could encourage a direction change from aptamers as therapeutics themselves to delivery vessels of drugs. In addition, due to the difficulties of renal clearance and nuclease degradation, alternate delivery systems may need to be considered rather than the current intravenous route. This could include, for example, an inhaler form targeting lung-related conditions and so provides another obstacle in terms of formulation for inhalation.

In summary, there are significant hurdles to development of aptamer therapeutics, particularly in comparison to antibodies, but chemical modification provides a route through which these challenges can be addressed. Once these barriers have been overcome, aptamer therapeutics could prove a huge success from healthcare, commercial, and sustainability perspectives.

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AUTHORSHIP & CONFLICT OF INTEREST

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NUCLEIC ACID INSIGHTS

OLIGONUCLEOTIDES: INNOVATION IN ENGINEERING AND DESIGN

SPOTLIGHT

For the many and the few: bringing ASO therapies to both mainstream and nano-rare indications

INTERVIEW

"...we have a 100% success rate to date in the patients that have been treated long enough to evaluate for benefit."

Roisin McGuigan, Commissioning Editor, *Nucleic Acid Insights*, speaks to **Stanley Crooke**, Chairman, Founder and CEO, n-Lorem, about his long career and pioneering role in the antisense oligonucleotide (ASO) space, how RNA-targeting therapies are set to enter the mainstream, and his latest focus: making personalized, free-for-life ASO therapies available to nano-rare patient populations.

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Q Could you tell me a bit about your career, from entering the oligonucleotide space to founding the n-Lorem Foundation?

SC I have been involved in basic research and drug discovery and development for many decades now. I did my MD–PhD and house staff training at Baylor College of Medicine and was on the faculty for over 25 years. In the first 5 years of my career, I led the building of the first broadly successful line of cancer programs at

Bristol Myers, and then was president of R&D at what is now GSK. In 1989, I founded Ionis Pharmaceuticals in order to create a new, more efficient drug discovery technology: antisense oligonucleotide (ASO) technology. Having succeeded at doing this, in 2020 I retired.

Then, in response to the desperate need of patients with extremely rare mutations—what I have dubbed nano-rare—I established n-Lorem as a non-profit to provide experimental ASO treatments to patients with nano-rare diseases.

Q How has the antisense field evolved over the time you've been active in this space, and what would you pick out as the biggest milestones to date?

SC We spent 30 years creating the technology and advancing it. The fact that it exists is an extraordinary achievement, particularly since it was to a large extent done in a single company. At Ionis, we advanced the chemistry and the understanding of the molecular mechanisms by which these drugs produced their pharmacological, toxicological, and immunological effects, and we learned how to manage that. To date, there are now 17 RNA-targeted drugs that have been approved. That includes the first blockbuster, which is our antisense drug for spinal muscular atrophy, Spinraza®.

I fully expect additional drugs to be approved this year. At Ionis they have ASOs to treat very large cardiometabolic indications that are completing: including an 8,000-patient cardiovascular outcome trial, a 5,000-patient Phase 3 trial, and still other large clinical trials. In the next couple of years, I anticipate that the technology will take its place in the mainstream of therapeutic options for patients.

However, after scaling up for 30 years at Ionis, in the last 5 years at an-Lorem we have been scaling down. This is because antisense technology is today the only technology that can address the needs of a meaningful number of these patients with nano-rare mutations.

Q Turning to the present, could you tell me a bit more about the current key activities and goals of the foundation?

SC The history of the foundation begins around 2017, when I was CEO and lead scientist at Ionis. I was visited by two sets of parents of two boys with mutations in SCN2A, that encodes the voltage-gated sodium channel alpha-subunit Na(V)1.2. Both of these boys were severely affected, as one might expect, with severe seizures, movement disorders, autonomic dysfunction, and a wide range of autistic symptomatology. In short, they were desperately ill, and progressing.

In that meeting, I had to tell these parents that the indication was simply too small for Ionis to pursue. However, I realized that the technology is efficient enough that I could probably make an ASO for them and give it to them for free. As I learned more about nano-rare mutations and the syndromes they cause, I began to recognize that with genomic sequencing we were identifying many of these patients. While it wasn't what I originally planned for my retirement, it seemed like something that I had to do.

I founded n-Lorem with the mission to industrialize a process via which we can respond to the needs of these patients by discovering, developing, manufacturing, and providing

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"We now have significant evidence of benefit in patients with eye disease and kidney disease."

ASOs. Our aim is to do this one patient at a time, with a personalized ASO for each patient, and to do that for free, for life.

That is our mission and we have been fortunate enough to raise sufficient funds to grow dramatically to respond to the need that we have—and I certainly didn't expect the demand that we have experienced. Further, we know that that demand is going to continue to grow exponentially every year.

Today we are privileged to be able to say we have a 100% success rate to date in the patients that have been treated long enough to evaluate for benefit. We have also observed pristine safety and tolerability, and that sets the stage for doing more tomorrow. And more is required, as every day more and more patients are identified and come to us for help.

Q You have published some very positive clinical data recently. Can you discuss some of those key clinical milestones to date, and what's next?

SC When I founded n-Lorem I thought by this stage we would have maybe a handful of applications, and might be treating a patient. In fact, we've now processed more than 330 applications for treatment and accepted more than 160 patients, with a wide range of different diseases and mutations. We are capable of filing about 20 INDs per year, and we have filed in four divisions of the US FDA: two neural divisions, the eye division, and cardiorenal. Although the clinical studies we conduct are not registrational studies, in our view, we now have significant evidence of benefit in patients with eye disease and kidney disease. In the CNS we've seen truly profound benefit in a wide range of patients, with dramatic reductions in seizures, significant improvement in movement disorders, substantial recovery of functions lost, and even the acquisition of new skills that the patient never had before. This causes us to rethink entirely how plastic the CNS is and how much we can recover from developmental delays. We've also seen significant benefit in autism symptoms, in patients with evidence of autonomic dysfunction, and improvement in pain syndromes.

Notably, we have done all this while having no ASO-related serious adverse events to date—we think that record can be continued, given the qualities of this technology and our experience in using it. It is very important to us to avoid imposing additional hardship on these patients in the form of drug-related side effects.

Another critical component of our efforts is the work that we did with the FDA, mostly in 2019, before I founded n-Lorem. This led to the unique guidance that the FDA has issued for ASOs for nano-rare patients, which supports us treating these extremely sick patients directly with only in vitro data and a single 3-month rodent toxicity study. That makes it cost-effective, and we can get to patients in 15–20 months. Most of these patients need that, because they are progressing rapidly to death or to loss of organ.

We are also investing in the future of antisense technology. I personally led most of the work that resulted in many of the advances in understanding the mechanisms by which

"We are also learning a great deal about why some phosphorothioate-modified ASOs activate innate immunity, and that's an area of active research..."

these work and then used that to make better ASOs. The technology is still continuing to advance at a rapid pace. We are working in a number of areas to make better ASOs for our patients, both for nano-rare patients and for more prevalent diseases.

We think that our mission is galvanic, and have been gratified by the response to it. I'm very pleased with the amount of funds that we have been able to raise, and excited by the progress that we've made in moving toward establishing more sustainable financing models. We are poised to do a lot more and do it a lot better in the coming years.

Q Where are the key opportunities as you see them to improve targeted delivery of ASO therapies?

SC There are still many opportunities to broaden the reach of the technology and of course, a large fraction of current research efforts are focused on targeted delivery to different organs. From my perspective the GalNAc conjugation was a major step forward, but it was fairly obvious. The liver's job is to scavenge molecules from blood, so we just asked the liver to do what it does.

What gives me more optimism about targeted delivery is that we showed that with a GLP-1 conjugated ASO, we could get ASOs in pharmacologic concentrations in pancreatic β cells. We get no ASO in the absence of the GLP-1 conjugate, and that receptor is not a scavenger receptor, but actually a G protein-coupled receptor.

Many of the programs are also focusing on transferrin to improve delivery to skeletal muscle and elsewhere, and there are many broader opportunities for targeted delivery involving different targets.

The work we have done to understand the mechanisms of cytotoxicity has yielded what we think of as the probable third-generation chemistry, in which we point-modify at specific sites with specific modifications to reduce the potential for cytotoxicity.

We are also learning a great deal about why some phosphorothioate-modified ASOs activate innate immunity, and that's an area of active research in my group. We are making real progress in learning to control that. There are multiple new chemistries coming along, some of which look potentially interesting. Of course, we've looked at many thousands of different chemical modifications through the years. You have to accept that for every 1,000 modifications you look at, maybe one of them will have some value in the long term.

The entry of ASOs into the major cardiometabolic and CNS diseases in the next two to three years is going to be very important, because that will take RNA-targeted drug discovery and put it in the center of therapeutics, which is where it belongs.

These are a few areas of opportunity for the space, and there are many others. For example, we are learning that 60% of our patients require allele selective ASOs, and developing means to do that in a much better way. There are multiple mechanisms to upregulate translation that we have already shown, and perfecting those so that we can do a better job for loss of function mutations is another major effort that is ongoing at n-Lorem.

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Q A challenge often cited in the oligonucleotide space is moving towards more environmentally sustainable manufacturing. What are your thoughts on how the space can improve in this area?

SC It's important to emphasize that this is still an extremely young technology. Small molecule drug discoveries have been around for 125 years. Monoclonal antibodies are now in their 50th year. The industry has spent 40-plus years on gene therapy, and well over $60 billion, and fundamental advances are still needed and there are plenty of things to work on.

For ASOs the next steps are fully integrating manufacturing to include both manufacture at all kinds of scales, and formulation, and having more in-process quality control systems. I think there are a variety of firms that are working on that now.

There are also no exotherms, no high-pressure reactions, and no difficult chemical reactions. The main environmental issue we face is that we have still not found a substitute for acetonitrile. Handling acetonitrile and the waste from it is an expensive and environmentally sensitive challenge. However, in the grand scheme of manufacturing drugs, it's fairly modest compared to many other things I've dealt with in my career. Simplifying the manufacturing process and weaning it from acetonitrile to the extend that is feasible would be an important step forward. It's certainly not that we and others haven't tried, but it has proven tough. I am optimistic that it will get resolved.

Q How do you predict the ASO space will develop over the next 5 years, and what will be your own key priorities in that timeframe?

SC The technology's next step will happen in the next couple of years as ASOs take their place in mainstream medicine in cardiometabolic indications, and CNS diseases like Parkinson's and Alzheimer's disease. We will continue to advance new mechanisms that will broaden the reach of the technology and importantly, learn how to make these more agonist-like drugs by altering translation of specific target proteins. We know how to do that, so now we need to do it better.

Advances in allele selectivity that depend on understanding RNase H1 are important, particularly for our patients. For any situation in which you have a heterozygous mutation and you'd like to alter only the mutant form of the RNA and protein, targeted delivery is an important effort. I am particularly anxious to see us do a better job in the heart and in some immune cells. We still have challenges with treating solid cancers, for a variety of reasons. Better control of innate immune activation is something that we are actively working on.

At the same time as this is happening, at n-Lorem we are downscaling the technology to treat a single patient. To my mind, the breadth of the appetite that we have for antisense is truly extraordinary to think about: treating millions, and treating one. There's never been a technology that could lend itself to even thinking about that.

We have demonstrated we can do this, and do it repeatedly and safely. We've demonstrated that a non-profit model can work. Our next task is to take this to the next level and provide our services to the many patients who still need our help. I don't make light of those challenges, which are very real, but they are mostly about money. From a technical perspective we have crossed all the hurdles and shown that none of this is impossible. It

just boils down to raising as much money as we can for n-Lorem over the next few years, and expanding our reach.

n-Lorem is a non-profit organization creating individual antisense oligonucleotide (ASO) treatments for patients with nano-rare diseases, for free, for life—learn more about the foundation, and ways to support it, at www.nlorem.org.

BIOGRAPHY

Stanley T Crooke is Founder, Chairman and Chief Executive Officer of n-Lorem, Carlsbad, CA, USA, a nonprofit foundation focused on providing treatments for patients with nano-rare disease patients (1–30 patients worldwide), which he initiated in January 2020. Prior to n-Lorem, Dr Crooke founded and was Chairman and Chief Executive Officer and Lead Scientist of Ionis Pharmaceuticals. Early in Dr Crooke's career, he led the creation of the first broad anticancer program in the industry at Bristol-Myers. He then assumed responsibility for worldwide R&D (President) at SmithKline Beckman (now GSK). Dr Crooke has also contemporaneously led a successful academic career becoming a full Professor at Baylor College of Medicine, Houston, TX, USA and the University of Pennsylvania Medical School, Philadelphia, PA, USA where he trained a number of PhD students and won several teaching awards. Dr Crooke has been an active scientist throughout his career as well. He has received a number of awards, most recently, the Steven C Beering Award for Advancement of Biomedical Science, Indiana University School of Medicine, Prix Galien Roy Vagelos Pro Bono Humanum Award, the American Chemical Society's EB Hershberg Award for Important Discoveries in Medicinally Active Substances, the Lifetime Achievement Award presented by the Oligonucleotide Therapeutics Society, the Scrip Lifetime Achievement Award, and the 2019 Massry Prize. Dr Crooke received his MD and PhD degrees and house staff training at Baylor College of Medicine, where he currently serves on the Board of Advisors. In 2021, Dr Crooke has been named Distinguished Alumnus of both Baylor College of Medicine's Graduate and Medical schools and named one of the 20 of the most influential biopharma R&D executives by Endpoints News. He has published over 600 scientific publications, edited more than 20 books, has numerous patents, and led the development of more than 23 drugs that have been commercialized.

Stanley T Crooke MD PHD, Founder, Chairman and Chief Executive Officer, n-Lorem, Carlsbad, CA, USA

AUTHORSHIP & CONFLICT OF INTEREST

Contributions: The named author takes responsibility for the integrity of the work as a whole, and has given their approval for this version to be published.

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NUCLEIC ACID INSIGHTS

DNA
DIAGNOSTIC/SENSOR APPLICATIONS

SPOTLIGHT

EXPERT INSIGHT

DNA origami nanostructures in biomedicine and the issue of stability

Adrian Keller

During the last decade, DNA origami nanostructures (DONs) have evolved into molecular precision tools widely applied in the biomedical field and especially in targeted drug delivery. Numerous successful *in vivo* studies have demonstrated potential therapeutic applications in the treatment of cancer, autoimmune diseases, and bacterial infections, among others. Tremendous progress has been made toward the clinical application of DONs and several important hurdles have been overcome. As one of the last major challenges, efficient means for controlling the *in vivo* stability of DONs need to be developed that do not interfere with their anticipated functions. Although we are not quite there yet, numerous recent studies have approached this issue from different angles, uncovered the intrinsic and extrinsic molecular mechanisms that govern DNA origami stability in physiological environments, and developed strategies to stabilize DONs in the absence of cations and against digestion by nucleases. This contribution provides an overview of the recent advances in the field and tries to paint a coherent picture of the various processes and interdependencies that affect the structural integrity of DONs *in vivo*. The most promising strategies for the stabilization of DONs under those conditions and their current limitations are discussed in order to guide future research efforts.

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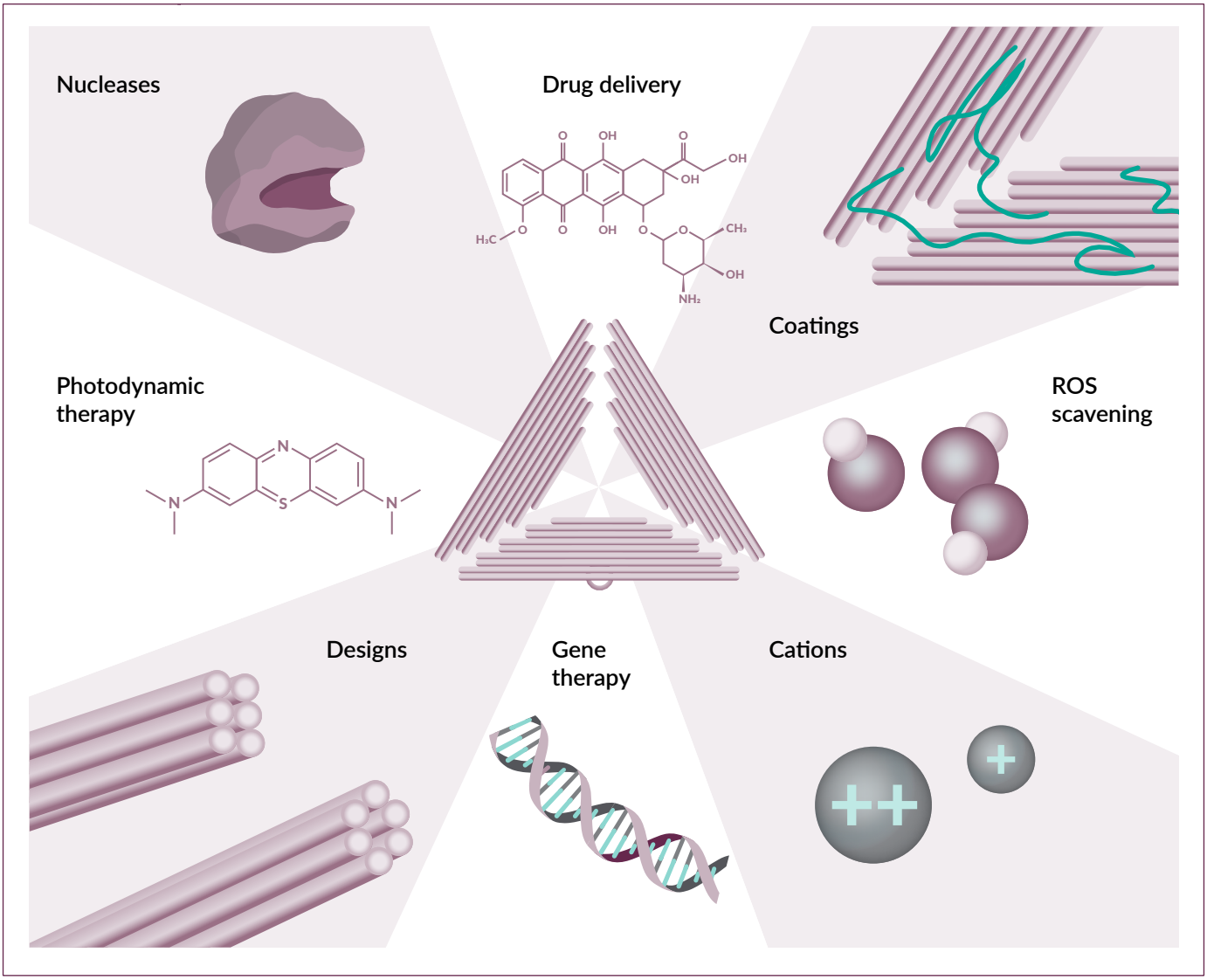
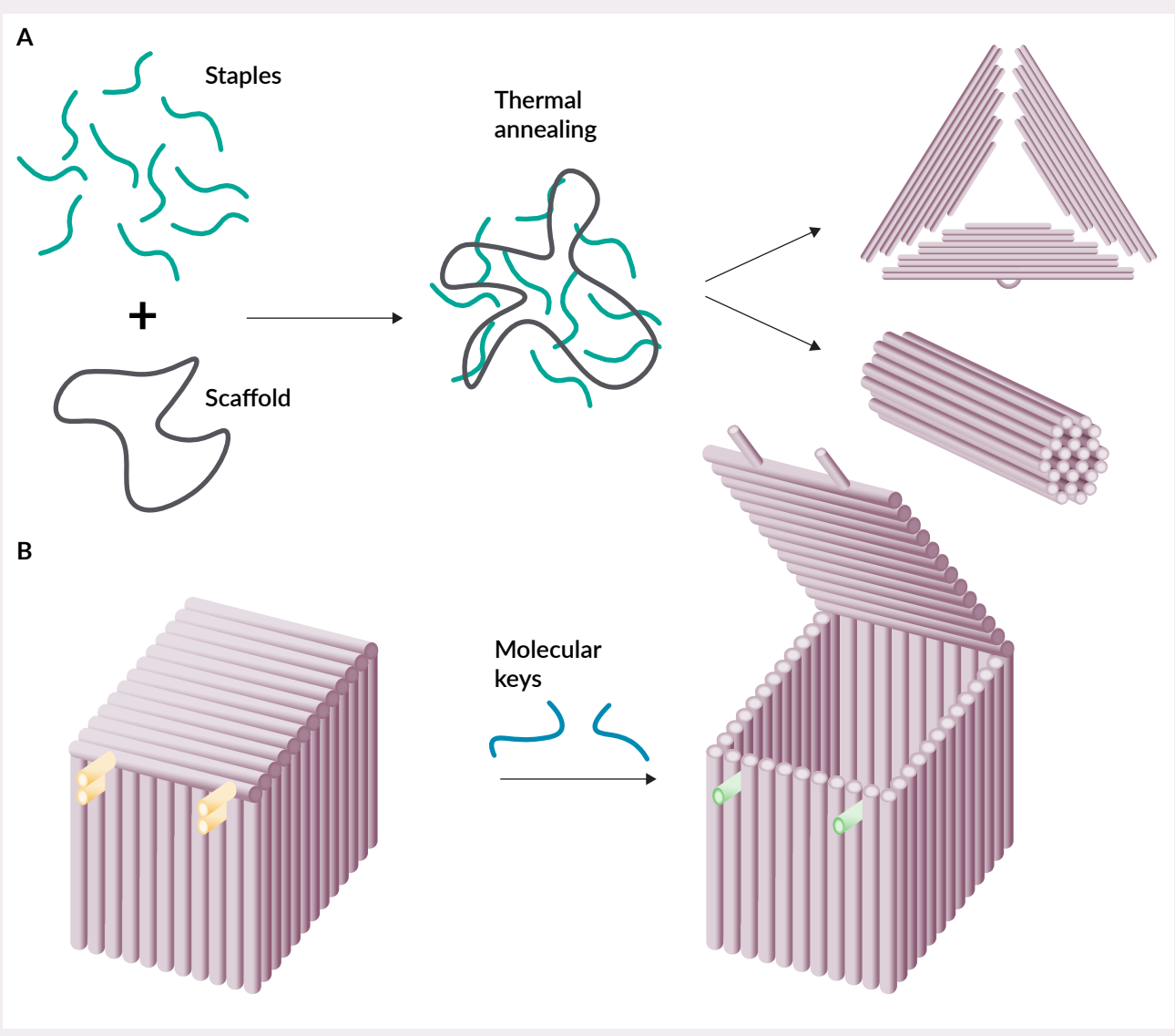


INTRODUCTION

In 2006, Rothemund presented a new method for the self-assembly of DNA nanostructures with almost arbitrary shapes called DNA origami [1]. It relies on the controlled folding of a long single-stranded scaffold by hybridization with a set of short oligonucleotides, so-called staple strands (Figure 1A). The total number of staples used for DNA origami assembly depends on the design and the employed scaffold and may range from few ten [2] to more than 200 staples [1]. Each staple consists of multiple domains that hybridize with different separated segments of the scaffold, thereby forcing the scaffold to adopt a predefined shape. This is usually achieved by first heating the scaffold and staple mixture to about 80 °C, followed by slow cooling to room temperature in the presence of cations that screen electrostatic repulsion between neighboring helices. Rothemund already demonstrated the assembly of a variety of 2D DNA origami shapes about 100 nm in diameter using the same scaffold (the M13mp18 phage genome) in combination with different sets of staple strands [1]. In 2009, four studies extended the DNA origami approach to the fabrication of bulky as well as hollow 3D shapes [3–6], including a box-shaped container with a controllable lid that could be opened by addition of two oligonucleotide keys (Figure 1B) [5]. This

FIGURE 1

Folding and transforming DONs.



(A) In DNA origami assembly, a long single-stranded DNA scaffold is folded into a predesigned 2D or 3D nanoscale shape upon hybridization with a set of oligonucleotides called staple strands. (B) A hollow 3D DNA origami box with two locks (yellow) that can be opened by two molecular keys (blue). DON: DNA origami nanostructure.

not only demonstrated the possibility of generating stimuli-responsive DNA origami nanostructures (DONs) with the capability to undergo defined shape transformations upon interacting with a preselected molecular trigger, but already hinted at possible applications as drug delivery vehicles for the controlled release of therapeutic cargos [5]. In the following 15 years, DONs have become widely applied molecular tools routinely employed in various research fields including synthetic [7] and chemical biology [8], super-resolution microscopy [9,10], biophysics [11,12], nanoelectronics [13,14], biosensing [15,16], optics [17,18], and especially biomedicine [19–22].

Compared to other nanomaterials, DONs have several key advantages that make them ideal candidates for biomedical applications:

- DONs can be assembled at high yields that may reach values of 90% and more [1];
- Complex, almost arbitrary shapes can be fabricated, ranging from quasi-1D fibers and 2D sheets to compact nanoparticles and wireframe-like cages [23];
- Being fully composed of DNA, DONs are biodegradable and nontoxic and illicit only a moderate immune response [24];
- The diverse chemistry of DNA enables the straightforward loading of DONs with drug molecules through various interactions, including intercalation [25], groove-binding [26], and electrostatic binding [27];
- Additionally, each staple strand has a unique location within the DNA origami shape, which enables the controlled arrangement of small molecules, DNA and RNA strands, enzymes, and proteins into complex patterns with nanometer and sub-nanometer precision [8];
- In a similar way, stimuli-responsive elements for the triggered release or display of therapeutic cargo can be incorporated in the form of switchable DNA motifs such as aptamers [28] and triple helices [29].

Because of these advantages, numerous potential applications of DONs in the biomedical field have been investigated, primarily in drug delivery [30] but also in drug discovery [31] and biomaterials science [32]. Tremendous advances toward the clinical application of DNA origami-based drug carries have been made in the past 10 years, including not only numerous successful *in vivo* treatments, but also their *in vivo* tracking at single-cell resolution [33], their biotechnological large-scale production [34] and the introduction of custom scaffolds that lack any potentially active genes [35] and thus face fewer regulatory challenges [19,20]. However, while many challenges have been faced and overcome, some challenges remain. Chief among them still is the limited stability of DONs in physiological environments, even though considerable progress has been made in the last few years toward elucidating and understanding the complex molecular mechanisms that govern DNA origami stability under relevant conditions. We are now beginning to understand how DNA origami stability can be controlled by rational design choices. Additionally, several strategies for the application of stabilizing molecular coatings and the introduction of covalent links have been developed. Nevertheless, there still are some open questions and unsolved issues that need to be addressed before therapeutic DONs can enter the clinic. Therefore, this contribution summarizes the recent advances in the field and tries to paint a coherent picture of the various processes and interdependencies that affect the structural integrity of DONs in the physiological environment. Promising strategies for the stabilization of DONs under those conditions and their current limitations are discussed to guide future research efforts.

APPLICATIONS OF DNA ORIGAMI NANOSTRUCTURES IN BIOMEDICINE

Many of the early studies exploring biomedical applications of DONs focused on cancer chemotherapy. To some extent, this was due to the fact that several cancer chemotherapeutic drugs in clinical use such as doxorubicin spontaneously bind to DNA, thereby enabling their rather straightforward loading into DONs [30,36,37]. Doxorubicin intercalates between the base pairs of the DNA duplexes and is released spontaneously upon transfer into doxorubicin-free media and/or DNA origami

degradation. This approach therefore relies on the accumulation of the drug-loaded DONs in the tumor tissue, either due to the enhanced permeability and retention (EPR) effect [30] or the incorporation of targeting entities such as aptamers [38]. Readers are advised, however, that the interaction between doxorubicin and DNA is much more complex than some of these studies assumed, so that the employed loading protocols may have led to severe doxorubicin aggregation and thus unreliable results [25].

As of today, DNA origami nanocarriers have been employed successfully *in vivo* in various cancer treatment strategies, including enzyme delivery [39], RNA interference [40], photothermal therapy [41], immunotherapy [42], and various combinations thereof [40,43,44]. In many of these studies, the DONs featured targeting entities on their surfaces that enabled their specific binding to cancer cells [39,40,42,44], as well as stimuli-responsive elements that triggered the release or display of the cargo [39,40,42,44].

More recently, the direct therapeutic potential of DONs beyond drug delivery has been explored as well. DONs are efficient scavengers of reactive oxygen species (ROS) and especially singlet oxygen [45], which opens up potential applications in the treatment of ROS-related diseases such as acute kidney injury [46–48], rheumatoid arthritis [49], sepsis under diabetic conditions [50], and atherosclerosis [51]. Also in these cases, the DONs are often equipped with additional functional entities to improve targeting or add another mechanisms of action [47–51]. These general strategies have also been applied in delivery concepts for the treatment of ocular diseases [52,53] and bacterial infections [54,55].

DNA ORIGAMI STABILITY UNDER PHYSIOLOGICAL CONDITIONS

All approaches discussed above rely on controlling the structural integrity of the DONs as unwanted degradation or denaturation will result in the loss of targeting capabilities and/or the premature release of the loaded cargo. Maintaining structural integrity in physiological environments, however, turned out rather challenging because DONs are more sensitive toward certain environmental factors than linear double-stranded DNA. Whereas nucleases represent the greatest threat to the *in vivo* stability of double-stranded DNA, DNA origami stability in physiological media is strongly affected also by the ionic composition. For a more detailed discussion of these phenomena and their underlying molecular mechanisms, the reader is referred to some recent reviews [56,57].

During DNA origami assembly, a large number of base pairs (~7500) are compacted into a small volume (~12000 nm^3), resulting in a large charge density of about -1.25 nm^{-3}. To facilitate efficient assembly, the resulting electrostatic repulsion between neighboring helices needs to be compensated. This is typically achieved by adding relatively high concentrations of Mg^{2+} ions (~10–20 mM) to the assembly reaction mixture. These Mg^{2+} ions then form salt bridges between the backbone phosphates of neighboring helices and thereby stabilize the overall assembly. Transferring the DONs into physiological media that have much lower Mg^{2+} concentrations may therefore lead to their disintegration due to electrostatic interhelix repulsion. For some time, this was considered a major factor restricting the application of DONs in biomedicine [58]. However, it is generally accepted now that DONs can be transferred into media with Mg^{2+} concentrations in the low µM range without any negative effects on their structural integrity [59–61]. Under such low-Mg^{2+} conditions, DNA origami stability depends critically on the presence of residual Mg^{2+} salt bridges [59], the removal of which will result in DNA origami denaturation. This can be caused for instance by the presence of ethylenediaminetetraacetic

acid (EDTA), which efficiently chelates Mg^{2+} ions. HPO_4^{2-} ions may elicit a similar effect by interfering with the phosphate-bound Mg^{2+} ions, thereby reducing their ability to compensate the electrostatic interhelix repulsion. In such cases, DNA origami stability can be maintained by monovalent Na^+ ions at physiological concentrations of 100–200 mM. However, design-specific factors play an important and sometimes even dominant role as well, as will be discussed in the next chapter.

In addition to the ionic environment, the fact that many biological fluids contain nucleases presents another threat to the *in vivo* stability of DONs [58]. Consequently, several studies have investigated DNA origami degradation by various nucleases [25,61–66]. The most relevant nuclease in the context of drug delivery is DNase I, a non-specific nuclease abundant in serum and various tissues. While DNase I rapidly digests linear double-stranded DNA, the situation is more complex for DONs. Here, their susceptibility toward DNase I digestion depends on several intrinsic (i.e., design-specific) and extrinsic (i.e., environmental) factors. The former encompasses the local and global mechanical properties of the DONs that will be discussed in the next chapter. The latter includes again the presence of Mg^{2+} ions, which are used as cofactors by DNase I to facilitate the catalytic cleavage of the DNA backbone. Low Mg^{2+} concentrations will thus result in diminished digestion efficiency. However, low Mg^{2+} concentrations may in turn destabilize DONs (see above), which can lead to a stronger impact of the strand breaks generated by limited DNase I activity on the overall integrity of the DON by promoting the dissociation of the generated fragments.

An important fact to consider is that the majority of the mentioned studies investigated the effects of ionic composition and nucleases on non-modified DONs. Loading them with chemotherapeutic cargos via intercalation or other methods may lead to altered sensitivities toward ionic effects and nuclease attack. Intercalation of doxorubicin, for instance, was found to slow down DNA origami digestion by DNase I dramatically [25]. This can be attributed to the unwinding of the DNA duplex upon intercalation, which results in less efficient binding of DNase I to the minor groove. DNA origami digestion could also be slowed down by blocking the minor groove with a minor groove binder [67]. While such effects may on the one hand be beneficial for stabilizing DONs *in vivo*, they will on the other hand also delay the release of the cargo.

In certain applications, ROS may play an important role as well. As discussed in the previous chapter, DONs are employed as ROS scavengers to treat ROS-associated diseases. However, ROS may also be created during the treatment of other conditions. For instance, DONs have been investigated as potential nanocarriers for the targeted delivery of photosensitizers in photodynamic therapy [26,68–70]. Additionally, DONs can also be decorated with ROS-producing DNAzymes [54]. These ROS will interact with and thereby damage the DONs, eventually leading to complete disintegration after prolonged exposure times. This may have adverse effects on the therapeutic outcome. When the DONs are loaded with ROS-producing entities, generated ROS are scavenged before they can damage any cellular components. This will reduce their therapeutic efficiency and may even completely suppress any therapeutic effect as recently demonstrated for antimicrobial photodynamic inactivation [45]. In the treatment of ROS-associated diseases, the DONs are sometime utilized not only as ROS scavengers but also as delivery vehicles for therapeutic proteins [48,71]. The limited structural stability of the DONs under high-ROS conditions thus may negatively affect their effectiveness as delivery vehicles. Also in such settings, ROS-induced structural damage will

generally be more severe in the absence of stabilizing Mg^{2+} ions, as electrostatic interhelix repulsion promotes the dissociation of those staple strands whose hybridization to the scaffold is weakened by oxidative base damage [70].

STABILIZING DNA ORIGAMI NANOSTRUCTURES IN PHYSIOLOGICAL ENVIRONMENTS

The stability issues discussed in the previous chapter have led to a large research effort aiming at stabilizing DONs under relevant physiological conditions. These efforts can be divided into three conceptually different approaches. The first approach tries to enhance the resistance of the DONs against adverse influences via rational design choices. Instead, the second approach tries to shield the DONs from adverse influences via the application of molecular coatings. The third approach tries to reinforce the DONs via the introduction of additional covalent links to make them more tolerant against adverse influences.

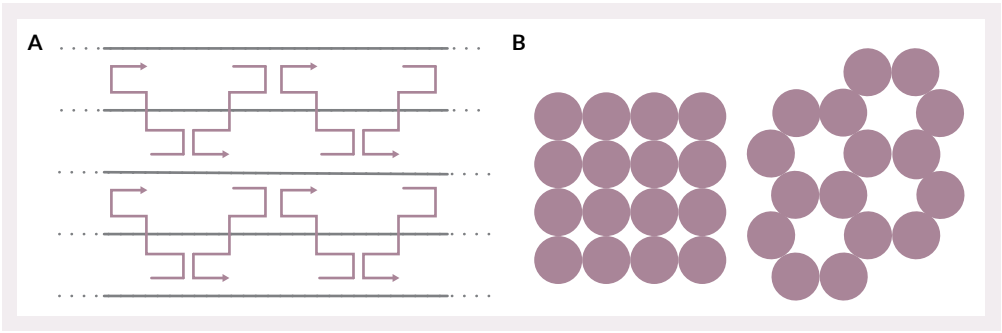
Design factors

It has been observed early on that the stability of DONs depends on their shape and internal structure, with some designs being more stable under physiological conditions than others [58]. DONs are largely composed of parallel double helices connected by backbone crossovers (**Figure 2A**). The double helices can be arranged using two different lattice types, the square lattice or the honeycomb lattice (**Figure 2B**). These lattices differ not just in the geometric arrangement of helices but also in the structure of the duplexes. While the honeycomb lattice maintains the 10.5 bp per helical turn of regular B DNA, the square lattice requires 10.67 bp per helical turn [62]. This is because in this lattice, the backbone crossovers have to be placed in the plane of the duplexes in order to create a flat sheet, which is hindered by the different dimensions of the major and minor grooves. The resulting artificial distortion of the base stack may lead to considerable strain. Therefore, the lattice type on which a certain DON is based may have an effect on its mechanical properties [72,73]. However, the mechanical properties of DONs are also influenced by other design factors and especially the density of staple crossovers. A higher crossover density in general leads to mechanically more rigid structures [61].

A considerable number of studies have been published in the last few years that

FIGURE 2

Internal structure of DONs.



(A) Routing of staple strands (purple) in the 2D DNA origami triangle designed by Rothemund on the square lattice [1] shown in **Figure 1A**. Scaffold is shown in grey. (B) Cross section of 16 parallel DNA duplexes arranged in the square (left) and the honeycomb lattice (right). DON: DNA origami nanostructure.

investigated the effects of different design factors on DNA origami stability under various destabilizing conditions, including at elevated temperatures [74], in the presence of chaotropic denaturants [75] and organic solvents [76], in electrolytes with different ionic compositions [59,61,66,74,77], and in the presence of ROS [70] and different nucleases [25,61,63–65]. From this bulk of studies, one general conclusion can be drawn. Mechanically flexible DONs are more stable than rigid ones under conditions that destabilize the base stack via unstacking or dehybridization. This particularly includes low-salt and oxidizing (ROS) conditions and can be explained by strain-induced melting. In a rigid DON, the staples experience more mechanical strain as a result of electrostatic interhelix repulsion. This promotes the melting of the strained staples under destabilizing conditions. In contrast, more flexible DONs can accommodate electrostatic interhelix repulsion by shape alterations, which lowers the strain experienced by their staples and makes them more tolerant toward destabilizing conditions.

When it comes to the effects of design factors on nuclease digestion, the situation is a bit more complex. Mechanical properties play an important role also in this context, with rigid DONs being more resistant toward digestion by DNase I [61]. This is because the binding of DNase I to duplex DNA results in groove widening and especially duplex bending. Rigid DONs resist this bending, which leads to reduced DNase I binding and thus lowers the digestion rate. However, such a clear correlation is usually observed only for rather simple shapes with homogeneous mechanical properties such as helix bundles [61]. Other shapes often feature a selection of structurally different design elements with different mechanical properties and thus different digestion rates. In those designs, it is often observed that more flexible elements are digested rapidly, while more rigid elements may survive for rather long times [63]. This may then lead to the structural collapse of the DNA origami shape, even though the majority of duplexes are still intact. In addition, DNase I has a diameter much larger than that of a DNA duplex. In the dense duplex arrangements found in many DONs, DNase I binding will be reduced substantially because of steric hindrance. In this case, the more densely packed square lattice should result in lower digestion rates [63]. This, however, is not always observed because other design factors may influence the mechanical properties of the DONs to such an extent that their effect on DNase I digestion is larger than that of the lattice type [25,63]. Because of its large size, DNase I cannot penetrate bulky 3D DONs, so that the helices buried in their interior are efficiently shielded. This in general leads to bulky 3D DONs having lower digestion rates than 2D shapes [25].

All this suggests that the design factor approach suffers from an intrinsic limitation. DONs with high stability under low-salt conditions are more susceptible to nuclease digestion, while those with high nuclease resistance denature easily in the absence of stabilizing Mg^{2+} ions. Unfortunately, most biological fluids feature low Mg^{2+} concentrations and nucleases. In such environments, additional stabilization strategies may be required.

Molecular coatings

The *in vivo* stability of DONs can be enhanced by the application of molecular coatings. This was first demonstrated by encapsulation of DNA origami cages in lipid membranes with the aid of lipid-DNA conjugates attached to the outer DNA origami surface [78]. Subsequently, the stabilizing potential of several other coating strategies has been evaluated, including polymer [79–82] and peptide [83,84] coatings as well as protein coatings based on

modified albumin [85,86] or virus capsid proteins [87,88]. In all these examples, coating was achieved via electrostatic interactions and resulted in enhanced nuclease resistance and/or enhanced stability under low-Mg^{2+} conditions. In some cases, the coatings also improved cellular uptake [79,85].

From a translational point of view, the most interesting coatings are oligolysine-PEG copolymer coatings [79] as these commercially available copolymers are fully synthetic and thus cheaper to produce under CMC and GMP regulatory compliance than proteins, which often face issues of sterilization, purity, and batch-to-batch consistency [89]. Furthermore, they offer some fine-tuning of their biological interactions. It has been demonstrated that the nuclease resistance of these coatings can be further enhanced by crosslinking of the lysines using the well-established amine-amine crosslinker glutaraldehyde to reduce the mobility and dissociation of the electrostatically adsorbed polymers [90]. In addition, such coatings are able to protect also DNA handles attached to the DNA origami surface [91] without interfering with their functionality [79] and can be used to control protein corona formation and cellular uptake [92,93].

While representing a powerful approach for the stabilization of DONs in physiological environments, applying a molecular coating to a DNA origami nanocarrier comes at a price. Most importantly, most if not all the discussed coatings will prevent the DONs from undergoing any shape transformations. This means that the triggered release of encapsulated cargo will not be possible. However, also passive release will be severely hindered due to the restricted transport across the coating, which is quite significant already for small molecules [82]. Even though some biomedical applications may be able to tolerate or even benefit from these tradeoffs, others may not.

Covalent links

A few alternative approaches to enhancing DNA origami stability have recently been developed, which introduce covalent links to reinforce the internal structure of the DONs. Early on, enzymatic ligation was adopted to seal the staple nicks within the DON, so that the several rather short staples are joined to form longer oligonucleotides with higher melting temperatures [94]. Unfortunately, ligation resulted only in moderate increases in DNA origami stability, presumably due to limited accessibility of the nicks within the dense arrangement of duplexes resulting in incomplete ligation [95]. Recently, however, it was demonstrated that this problem can be solved by either the addition of cosolvents that enhance enzyme activity or by using enzyme-free chemical ligation [96]. Both approaches enabled the near-quantitative ligation of 2D and 3D DONs with increased stability against low Mg^{2+} concentrations and DNase I digestion [96].

DNA origami stability in low-Mg^{2+} and nuclease-containing environments has been improved also by the UV-induced crosslinking of staple strands [97]. This approach utilizes the formation of cyclobutane pyrimidine dimers between thymine overhangs of neighboring staples under UV irradiation. By employing custom scaffolds, UV crosslinking can be achieved even without the introduction of staple overhangs [35].

All these covalent linking approaches can be combined with both the design factor and the coating approach, thus offering an additional means of fine-tuning DNA origami stability. However, they also come with some potential drawbacks. Reinforcing their internal structure increases DNA origami rigidity, which may affect drug loading and release. Also, they may hinder shape transformations in DONs by locking them in a fixed conformation. Avoiding

these issues will require extensive design optimization and may impose restrictions on other design factors.

TRANSLATIONAL INSIGHT

Many important advances have been made in the last years toward the clinical application of biomedical DONs. Important hurdles such as the initially high costs [34] or the reliance on genomic scaffolds [35] have already been overcome with the help of new biotechnological methods. Toxicity studies are showing very promising results [24,98] and the large bulk of successful *in vivo* studies is encouraging, highlighting numerous possible treatment targets ranging from cancer [30] to autoimmune diseases [49] to bacterial infections [54]. What remains as a last major challenge at the preclinical stage appears to be the reliable control of pharmacokinetics, biodistribution, and cellular uptake, which in turn requires efficient means of controlling the *in vivo* stability of DONs that do not interfere with their anticipated functions. Tremendous progress has been made in the past few years toward this goal by uncovering the fundamental mechanisms that govern DNA origami stability, elucidating the complex interplay between design and environmental factors, and developing a variety of stabilization methods. However, we now recognize the limitations of these different stabilization strategies. It appears rather unlikely that either of these approaches alone will be able to meet all requirements of the large number of different applications. Applications relying on shape transformations for the triggered release of the cargo may for instance utilize highly specific designs tailored toward high stability, whereas passive release strategies may rather employ protective coatings that not only increase DNA origami stability but also modify their drug release profile.

To aid in the selection of appropriate stabilization strategies for a given application, further insights are required regarding the effects of the different stabilization strategies on drug loading, drug release, and stimuli-responsive shape transformations. Design factors are known to affect drug loading and release [26,36], whereas drug loading can alter the nuclease resistance of DONs, which in turn modifies drug release profiles [25]. However, little is known regarding the impact of protective coatings on drug release, except that they may restrict transport in and out of the DON [82]. Additionally, many DNA-binding drugs are positively charged, so that their loading into DONs may affect the application of molecular coatings via electrostatic interactions. Furthermore, while it was demonstrated that oligolysine-PEG copolymer coatings do not impair the functionality of single-stranded DNA handles on the DNA origami surface [79], it is not clear at all whether this is also the case for more complex entities such as aptamers, triple helices, or DNAzymes. Especially aptamers are known to be highly sensitive toward changes in their immediate vicinity [99]. Future studies thus need to systematically investigate the interdependencies between the different stabilization strategies and the biomedical performance of DONs to enable their successful translation to the clinic.

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