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DNA origami nanostructures in biomedicine and the issue of stability

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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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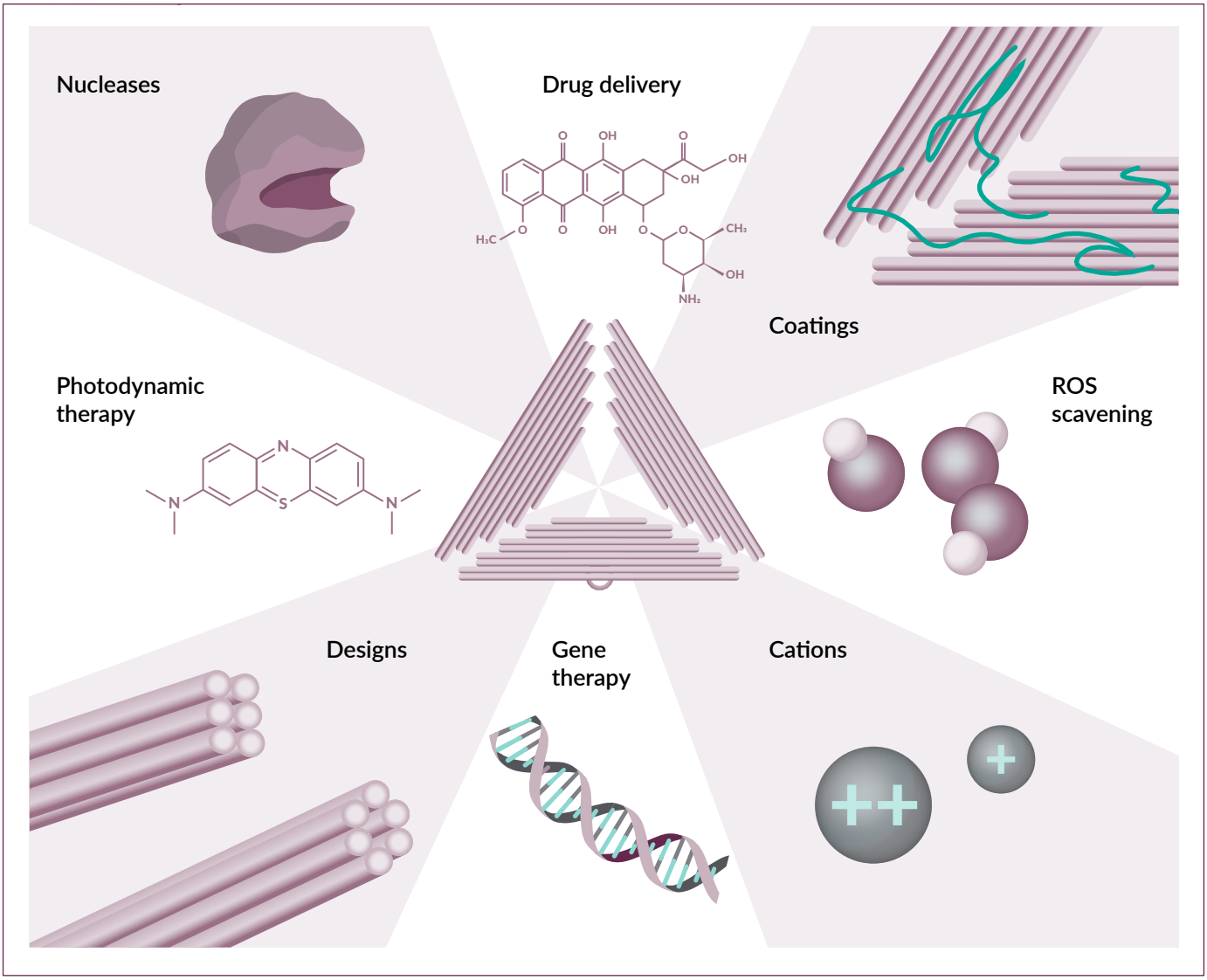
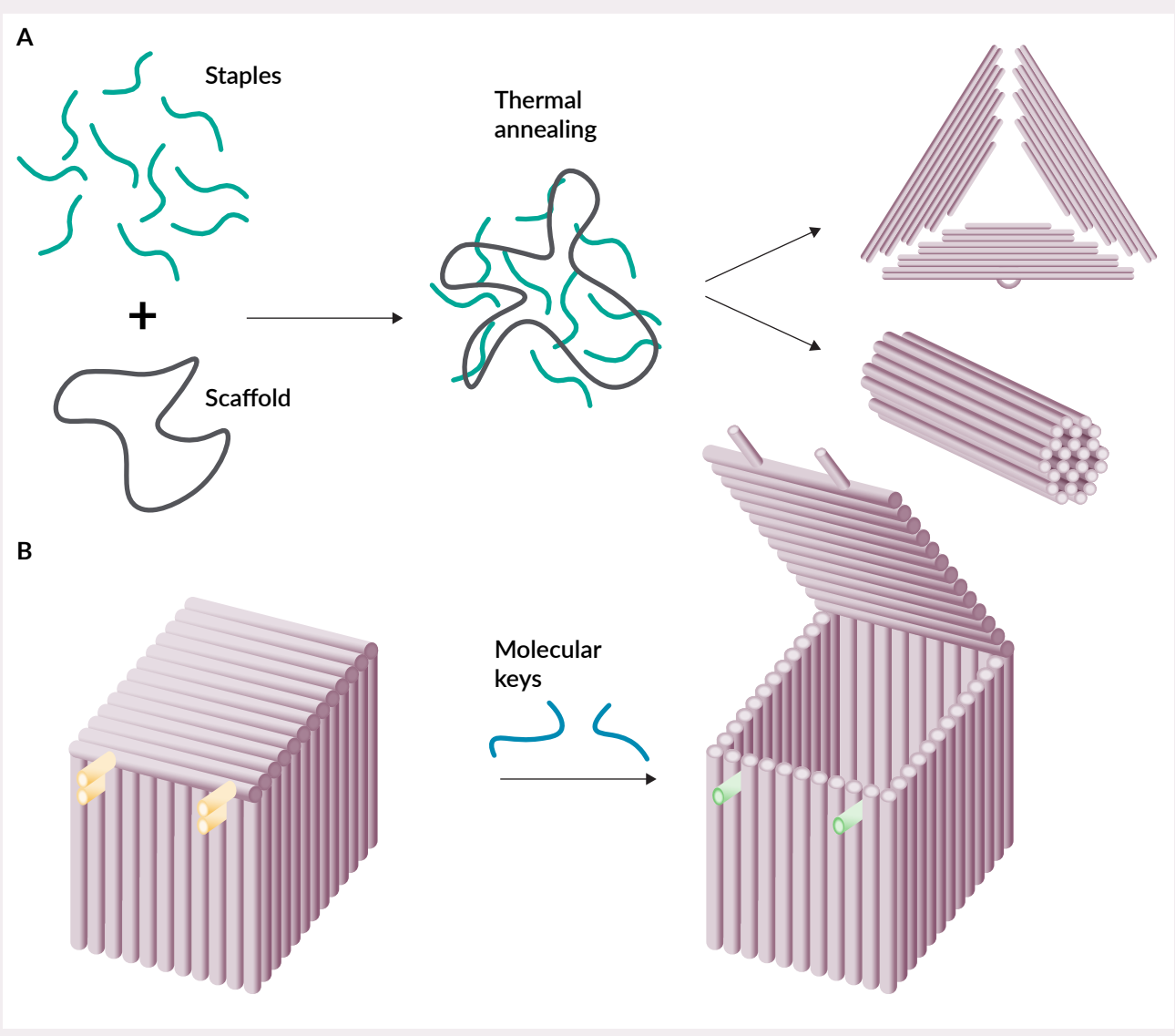


INTRODUCTION

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.

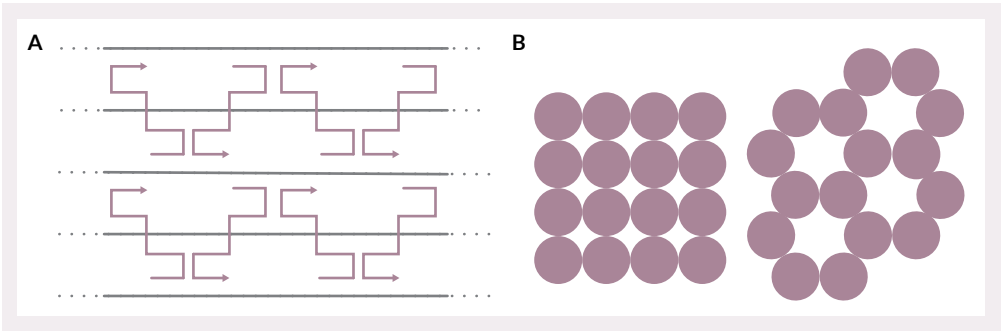
Design factors

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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