Mechanistic Insights into the Stability of DNA Nanostructures: Effects of Structural Positioning, Chemical Modifications, and Crosslinking Strategies

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Abstract. DNA nanotechnology enables the programmable construction of nanoscale architectures with high precision and has enabled applications in drug delivery, biosensing, and nanofabrication. However, the limited stability of DNA nanostructures under physiological conditions, particularly due to nuclease-mediated degradation and ionic fluctuations, remains a major challenge. In this work, we combine literature analysis with experimental validation to investigate the mechanisms governing the stability of DNA nanostructures. We focus on structural positioning, chemical modifications, and crosslinking strategies. Representative systems, including DNA double-crossover (DX), paranemic crossover DNA (PX), and DNA origami, are discussed to highlight structural influences on stability. Experimentally, we constructed DX and PX motifs and square DNA origami structures to evaluate stability under enzymatic conditions. Using exonuclease I/III and DNase I/FBS assays, we quantitatively compared the effects of chemical modifications and structural features on degradation behavior. We demonstrate that spatial heterogeneity plays a critical role, where exposed strand termini are more susceptible to enzymatic degradation, while internal regions are relatively protected. Furthermore, chemical modifications such as terminal modifications, 2′-O-methyl substitutions, and phosphorothioate backbone modifications significantly enhance nuclease resistance, with their effectiveness strongly dependent on positional distribution. Crosslinking strategies further improve structural rigidity and environmental tolerance. These findings provide a mechanistic framework for the rational design of stable DNA nanostructures and support their development for advanced biomedical applications.

1 Introduction

DNA nanotechnology has emerged as a powerful platform for constructing precisely defined nanoscale architectures by exploiting the programmability of Watson-Crick base pairing. Since its conceptual foundation was established, DNA has evolved from a genetic material into a versatile engineering substrate for nanoscale design.[1] Early studies demonstrated that synthetic DNA strands could be assembled into well-defined junctions and lattices, laying the groundwork for structural DNA nanotechnology.[2] These advances enabled the rational design of increasingly complex architectures with controllable geometry and mechanical properties.

Among the various developments in this field, the introduction of DNA origami marked a major breakthrough, allowing the folding of a long scaffold strand into arbitrary two- and three-dimensional shapes with the assistance of numerous short staple strands.[3] This strategy significantly improved assembly efficiency and structural complexity, making it possible to construct sophisticated nanostructures with high precision. Subsequent advancements in design tools and methodologies, such as computer-aided design platforms, further accelerated the development of DNA-based nanostructures.[4] As a result, DNA nanotechnology has found widespread applications in areas including biosensing, molecular computation, drug delivery, and nanofabrication.[5-7]

Despite these promising applications, the practical implementation of DNA nanostructures is still hindered by their limited stability under physiological and environmental conditions. In biological systems, DNA nanostructures are exposed to nucleases, low ionic strength environments, and various physicochemical stresses that can lead to rapid degradation or structural deformation.[8,9] This instability is particularly problematic for biomedical applications, where prolonged circulation time and structural integrity are essential for functionality.

To address these challenges, extensive efforts have been made to enhance the stability of DNA nanostructures through multiple strategies. Chemical modification of nucleic acids has been widely explored as an effective approach to improve nuclease resistance

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and structural robustness.[10] In parallel, structural design optimization, including increasing crossover density and minimizing structural defects, has been shown to enhance mechanical stability.[11] Importantly, emerging evidence suggests that the stability of DNA nanostructures is not solely determined by their overall design or modification type, but is also strongly influenced by spatial heterogeneity within the structure. Different regions, such as exposed strand termini, flexible domains, and densely packed cores, exhibit distinct susceptibilities to degradation. Therefore, understanding how structural positioning and chemical modifications interact to influence stability is essential for the rational design of robust DNA nanostructures.

In this work, we combine a literature-based analysis with experimental investigation to elucidate the mechanisms governing DNA nanostructure stability, focusing on representative structural systems and key stabilization strategies. Specifically, we experimentally evaluate the effects of nick positioning in DX structures, compare the nuclease resistance of 2′-O-methyl and phosphorothioate modifications, and analyze the influence of helix bundle design (2HB vs 6HB) on DNA origami stability. By integrating insights from structural design, chemical modification, and crosslinking approaches, we aim to establish a comprehensive framework for improving the stability of DNA-based nanomaterials.

2 Preparation of DNA nanostructures

DNA nanostructures are constructed through the programmable hybridization of complementary DNA strands, enabling precise control over geometry and topology across multiple structural scales. In this study, both motif-based structures and DNA origami were prepared using controlled thermal annealing protocols to ensure reproducible assembly. Representative architectures, including DNA double-crossover (DX), paranemic crossover DNA (PX), and DNA origami, are illustrated in Figure 1, highlighting their structural diversity and increasing complexity.

2.1 DNA double-crossover structures

DNA double-crossover (DX) motifs are among the most fundamental building units in DNA nanotechnology. A DX structure consists of two adjacent double helices connected by crossover junctions, which constrain their relative orientation and significantly enhance structural rigidity compared to single duplex DNA.[12] Several canonical forms exist, including antiparallel even (DAE), antiparallel odd (DAO), parallel even (DPE), and parallel odd (DPO) configurations, as shown in Figure 1A. These structural variations arise from differences in strand orientation and crossover spacing, which influence the mechanical properties of the motif.

DX structures are typically assembled from a small number of synthetic oligonucleotides via thermal annealing. In our experiments, each strand was mixed at an equimolar ratio, heated to 95°C, and rapidly annealed to room temperature over approximately 1.5 hours to form DX motifs. The incorporation of crossover points reduces local flexibility and suppresses branch migration, resulting in improved structural stability. Owing to their simplicity and well-defined geometry, DX motifs have been widely used as model systems for studying DNA nanostructure assembly and stability, as well as for constructing periodic two-dimensional lattices.[13]

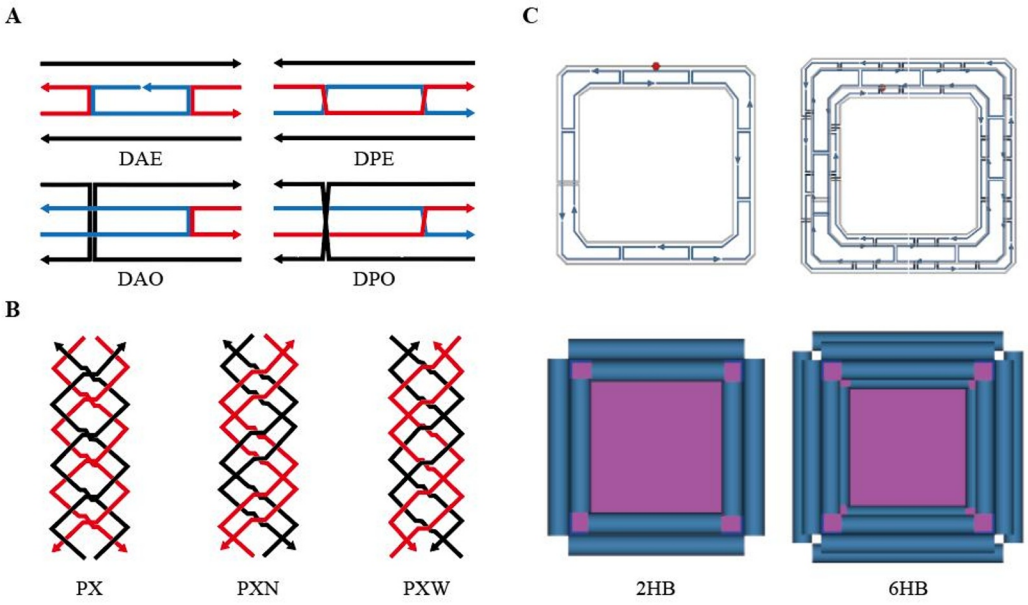

Figure 1. Representative DNA nanostructures.(A) Classical configurations of DNA double-crossover (DX) motifs, including antiparallel even (DAE), antiparallel odd (DAO), parallel even (DPE), and parallel odd (DPO) arrangements. (B) Representative paranemic crossover (PX) DNA structures and their variants (PX, PXN, and PXW), illustrating increasing crossover density and strand interweaving. (C) Design and structural models of square DNA origami. Top: routing diagrams showing scaffold and staple strand organization. Bottom: corresponding cylindrical models. Left: origami constructed from two-helix bundles (2HB); right: origami constructed from six-helix bundles (6HB).

2.2 Paranemic crossover DNA structures

Paranemic crossover (PX) DNA represents a more complex structural motif characterized by a high density of crossover points between two adjacent helices. In contrast to DX structures, PX motifs involve continuous strand exchange along the helical axis, forming a tightly interwoven topology.[11] Variants such as PXN and PXW further modify crossover frequency and arrangement, as illustrated in Figure 1B.

The assembly of PX structures requires precise sequence design to ensure correct alignment of multiple crossover sites. Similar to the DX assembly process, each chain of the PX structure is mixed in an equal molar ratio, and undergoes a rapid temperature reduction from 95°C to room temperature within approximately 1.5 hours for thermal annealing treatment. Sequence symmetry has been shown to facilitate proper assembly and reduce structural defects.[14] The dense crossover architecture significantly enhances rigidity and reduces conformational flexibility, which in turn improves resistance to nuclease degradation.

Because of these properties, PX structures are often regarded as intrinsically stable DNA motifs. Their reduced enzymatic accessibility makes them particularly useful for investigating how structural topology influences degradation pathways and for designing robust DNA-based nanomaterials.

2.3 DNA origami nanostructures

DNA origami nanostructures enable the construction of complex nanoscale architectures by folding a long single-stranded scaffold into predefined shapes using numerous short staple strands.[3] The routing of scaffold and staple strands determines the final geometry, as illustrated in Figure 1C (top). Structural models based on cylindrical representations further reveal the three-dimensional organization of helices within the origami (Figure 1C, bottom). In this study, square DNA origami structures were designed using caDNAno and Athena software. The scaffold strand and staple strands were mixed at a ratio of 1:10, followed by slow thermal annealing from 95°C to room temperature over approximately 12 hours to ensure correct folding.

Depending on the design strategy, DNA origami structures can be assembled from different helix bundle motifs. For example, two-helix bundle (2HB) designs provide relatively simple architectures, while six-helix bundle (6HB) designs introduce higher packing density and mechanical rigidity, as shown in Figure 1C. The 6HB motif, originally developed as a robust tubular structure, significantly enhances structural stiffness and has been widely used in the construction of mechanically stable DNA nanostructures.[15]

Assembly typically involves thermal annealing in the presence of divalent cations such as Mg^{2+}, which stabilize the negatively charged DNA backbone. Advances in computational design tools have further enabled the rapid prototyping of complex origami geometries.[4] In addition, strategies such as staple strand redesign and enzymatic ligation have been explored to improve folding efficiency and stability.[16]

Despite their versatility and high addressability, DNA origami structures often contain numerous strand termini and nicks, making them more susceptible to nuclease attack compared to smaller motifs. This structural feature highlights the importance of integrating chemical modification and positional design strategies to enhance stability.

3 Mechanisms influencing the stability of DNA nanostructures

3.1 Position-dependent effects

DNA nanostructures exhibit pronounced spatial heterogeneity, in which different regions possess distinct susceptibilities to degradation. Among these, strand termini and discontinuities such as nicks are particularly vulnerable due to their increased accessibility and reduced structural constraints.

Previous studies have demonstrated that exposed DNA ends are the primary targets for exonucleases, initiating degradation that subsequently propagates into the interior of the structure. For example, research by Ramakrishnan et al. showed that DNA origami structures exposed to destabilizing environments undergo structural degradation beginning at flexible and exposed regions, highlighting the importance of local structural context in determining stability.[8] DNA origami also presents obvious regional differences in degradation when exposed to complex biological fluids such as cell lysate.[17]

In addition to strand termini, the presence and positioning of nicks, which are interruptions in the phosphodiester backbone, represent another critical factor. Nicks introduce local flexibility and weaken base stacking interactions, thereby facilitating enzymatic access. In our study, DX structures with different nick positions were designed and subjected to enzymatic degradation using a combination of Exonuclease I and Exonuclease III, followed by gel electrophoresis analysis. The results showed that degradation rates varied significantly depending on the placement of the nick. Structures with nicks located in more exposed or mechanically critical regions exhibited accelerated degradation, whereas those with nicks positioned in constrained regions showed enhanced resistance. These findings demonstrate that backbone continuity and its spatial distribution play a decisive role in determining the biostability of DX motifs.

Additionally, structural design at the bundle level influences stability. Six-helix bundle (6HB) architectures, which feature tightly packed helices and increased crossover density, exhibit significantly enhanced mechanical rigidity compared to two-helix bundle (2HB) designs. In our observations, DNA origami structures constructed from 6HB motifs showed improved resistance to enzymatic degradation relative to those based on 2HB configurations. This difference can be attributed to reduced accessibility and increased steric

hindrance in densely packed regions, which limit enzyme binding and cleavage.

3.2 Chemical modifications

Chemical modification represents one of the most effective and versatile strategies for enhancing the stability of DNA nanostructures, as it enables direct manipulation of the physicochemical properties of nucleic acids at the molecular level. A wide range of chemical approaches have been developed to improve nuclease resistance, regulate backbone flexibility, and stabilize local structures, as comprehensively reviewed by Gothelf.[18] Importantly, the effectiveness of these modifications is not only determined by their chemical nature but also strongly influenced by their spatial distribution within the nanostructure.

Among various strategies, terminal modification is widely recognized as a primary approach to suppress enzymatic degradation. Since strand termini are the most exposed and vulnerable regions in DNA nanostructures, blocking these sites can effectively inhibit exonuclease activity. By introducing modified nucleotides or functional groups at the 5′ or 3′ ends, steric hindrance is increased and enzyme binding is reduced, thereby delaying the initiation of degradation.[19] This approach is particularly efficient in large DNA origami systems, where numerous exposed termini collectively contribute to structural instability.

Beyond terminal protection, internal chemical modifications further enhance stability by altering backbone properties and local conformational dynamics. One prominent example is the incorporation of 2′-O-methyl (2′-OMe) modifications, which increase the rigidity of the sugar–phosphate backbone and reduce susceptibility to nuclease cleavage.[20] Dantsu and Zhang demonstrated that 2′-OMe derivatization significantly improves both thermal and structural stability of nucleic acid systems, highlighting its effectiveness in stabilizing helical conformations.[21] These modifications are generally well tolerated within DNA nanostructures and can reinforce flexible or exposed regions without disrupting base pairing interactions.

In addition to sugar modifications, backbone modifications such as phosphorothioate (PS) substitution provide another important route to enhancing nuclease resistance. By replacing a non-bridging oxygen atom with sulfur, PS modifications alter the chemical environment of the backbone and reduce enzymatic cleavage efficiency. However, the introduction of PS linkages may also affect structural properties, including folding kinetics and mechanical stiffness, particularly when incorporated at high density.

Comparative analysis of different modification strategies further reveals the importance of selecting appropriate chemistries for specific structural contexts. In our study, square DNA origami structures were modified with either 2′-OMe or PS groups and incubated with DNase I and fetal bovine serum (FBS), followed by gel electrophoresis analysis to evaluate their stability. The results showed that 2′-OMe modification provided superior resistance to enzymatic degradation compared to PS modification. This enhanced performance is likely attributed to the increased backbone rigidity and reduced conformational flexibility conferred by 2′-OMe groups, which limit enzyme accessibility more effectively than PS substitution alone.

3.3 Crosslinking strategies

Crosslinking provides an additional level of stabilization by introducing covalent bonds between DNA strands, thereby reinforcing structural integrity and reducing conformational flexibility.

Recent work by Kalra et al. demonstrated that triplex-directed site-specific photo-crosslinking can be used to selectively stabilize DNA origami structures. In their study, UV-induced crosslinking between designed sites significantly improved resistance to thermal denaturation and enzymatic degradation.[22] This approach highlights the potential of precise, site-specific crosslinking for enhancing stability without compromising structural design.

Crosslinking strategies can be broadly categorized into photochemical and chemical approaches. Photochemical methods typically rely on UV irradiation to induce covalent bond formation, while chemical crosslinking involves reactive functional groups that form stable linkages under controlled conditions.

The introduction of crosslinks reduces structural flexibility and increases mechanical strength, which is particularly beneficial under harsh environmental conditions such as low ionic strength or high temperature. However, excessive crosslinking may limit dynamic behavior and reduce the responsiveness of DNA nanodevices.

4 Challenges and perspectives

Despite recent advances in stabilization strategies, several challenges continue to limit their practical applications. One major issue is the trade-off between structural stability and functional flexibility. While chemical modifications and crosslinking strategies can effectively improve resistance to specific nucleases such as DNase I and exonucleases, excessive stabilization may compromise dynamic behaviors required for applications such as targeted delivery and responsive nanodevices. Another challenge lies in the limited understanding of DNA nanostructure behavior in complex biological environments. Most studies are conducted under simplified in vitro conditions, whereas in vivo environments involve additional factors such as serum protein adsorption, nuclease diversity, immune recognition, and mechanical shear stress, all of which can influence structural integrity. Moreover, the inherent structural heterogeneity of DNA nanostructures makes it difficult to uniformly optimize stability across different regions. Future efforts should focus on integrated design strategies that combine structural engineering with site-specific chemical modifications. The development of novel nucleic acid chemistries, including non-natural and

mirror-image nucleotides, may further enhance resistance to degradation. In addition, computational modeling and machine learning approaches are increasingly being explored to predict degradation-prone regions and guide sequence-level optimization. These advances will be essential for translating DNA nanostructures into practical biomedical applications.

5 Conclusion

In conclusion, the stability of DNA nanostructures is governed by a combination of structural, chemical, and topological factors. This work highlights the importance of position-dependent effects, demonstrating that exposed termini and backbone discontinuities are primary sites of degradation. Experimental results further confirm that the positioning of nicks within DX structures significantly affects enzymatic degradation rates, underscoring the role of backbone continuity in maintaining structural stability.

Chemical modifications provide effective strategies for enhancing stability, with their performance strongly dependent on spatial distribution. Terminal modifications can suppress degradation initiation, while internal modifications such as 2′-O-methyl substitution improve backbone rigidity and nuclease resistance. Notably, comparative analysis shows that 2′-OMe modification provides more pronounced protection against nuclease and serum-induced degradation than phosphorothioate modification in square DNA origami structures. In addition, densely packed architectures, such as six-helix bundle-based designs, exhibit enhanced resistance due to reduced enzymatic accessibility. Overall, these findings provide a mechanistic framework for the rational design of stable DNA nanostructures and support their further development for applications requiring prolonged structural integrity, such as in vivo delivery systems and biosensing platforms.

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