J Nanotechnol Nanomaterials. 2023;4(1)11-18.

Journal of Nanotechnology and Nanomaterials

Review Article

A Comprehensive Review of DNA Origami Stabilization Techniques

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Received date: April 29, 2023, **Accepted date:** May 19, 2023

Citation: Yan L. A Comprehensive Review of DNA Origami Stabilization Techniques. J Nanotechnol Nanomaterials. 2023;4(1):11-18.

Abstract

In recent years, DNA has emerged as a powerful tool in the field of nanotechnology. The DNA origami technique is largely responsible for this, revolutionizing nanofabrication due to its controllability, precision, and ability to leverage DNA's unique properties. The technique consists of folding a long, single-stranded DNA (called a scaffold strand) by binding it with shorter staple strands to create almost any shape desired. With a desired structure in mind, researchers can design and assemble scaffold and staple strands using computer software like Cadnano or Tiamat. This is possible because of the Watson-Crick base pairing of DNA strands, which allows for programmable self-assembly of DNA nanostructures and therefore, the synthesis of arbitrary 2D and 3D shapes. Because DNA is a biomolecule,the nanostructures are also biocompatible and can be employed in biological applications including drug delivery. DNA origami nanostructures are not only limited to biological applications; they have also found uses in nanophotonics, plasmonics, and electronics. However, DNA origami still faces many challenges before it can be widely adopted. One such challenge is ensuring stability, and thus guaranteeing the performance of the DNA origami, in the presence of heat, nuclease in organic bodies, and chaotropic agents. This warrants the question: what methodologies can be employed to best stabilize DNA origami structures? This paper further focuses on two methods: covalently binding various molecules by cross-linking and non-binding encapsulation. Detailed analysis and comparison between various molecules used to bind and coat DNA nanostructures is used to evaluate performance and applicability of each method. In the end an oligolysines coating cross-linked with glutaraldehyde was found to have the strongest biological stability, thymine cross-linking had the strongest thermal stability, a silica coating had the best stability against the largest number of factors, and both graphene and Al_3O_2 coatings had the best mechanical stability.

Keywords: DNA origami, Nanotechnology, DNA nanostructures, Cross-linking, Coating, Stabilization, Denature

Introduction

The concept of DNA nanostructures was first proposed by Nadrian Seeman, who used tile-based assembly to create DNA nanostructures. Then in 2006, Paul Rothemund published his work detailing the DNA origami technique which would simplify the process and allow for larger, more stable structures [1]. Today, DNA origami has become the dominant method in DNA nanotechnology because of its flexibility of producing any shape, ease of implementation due to its programmable nature, and nanometer precision which allows DNA to be utilized in nanotechnology. DNA origami has already found numerous applications in various industries ranging from lithography and nanofabrication for nanophotonics and electronics to biomedical applications including drug delivery and biosensing [2-7].

However, DNA origami is still a new and emerging technology that is not yet commercially available. One of the main reasons for this is the lack of stability within these applications that causes the DNA structures to denature. As a result, stabilization techniques have become necessary in many uses of DNA origami and have become an important step to consider for scientists. Because of this, numerous experiments have been conducted in order to improve stability of these structures in various applications. As a result, countless methods were published, each detailing a unique method for stabilization. However, all of these papers lead to difficulty in selecting and keeping track of the most effective stabilization techniques. Work has been done in an attempt to organize and summarize these methods. Ramakrishnan *et al.* has analyzed the stability of DNA origami in various applications and stabilizing methods [6]. Manuguri *et al.* also reviewed various stabilizing techniques [7]. However, all of these papers mainly serve to provide a list of various stabilizing techniques, and detailed, quantitative comparison and analysis of these techniques has yet to be done. Due to this, selecting the strongest stabilization technique and choosing a technique to improve upon still remains a challenge for researchers.

This study aims to address the existing knowledge gap by presenting 21 distinct stabilizing techniques for DNA origami. Moreover, it seeks to identify and highlight the most promising techniques with significant potential for commercial adoption. By doing so, this research endeavors to contribute to the understanding of DNA origami stability and facilitate the advancement and widespread application of this emerging technology.

Applications

Drug delivery may be the most promising application of DNA origami [5-7]. This is accomplished by designing nano-sized drug carriers using DNA origami. Having the ability to accurately deliver drugs to targeted regions of the body will greatly advance the medical field, but several challenges prevent use currently. The first challenge is the natural enzymes that actively degrade DNA, also known as nuclease. Additionally, DNA origami requires high concentrations of cations in order to prevent dissociation from electrostatic repulsion. Most DNA origami is folded in high concentrations of Mg^{2+} in order to prevent this, but most applications including drug delivery do not have the required cation concentration. DNA origami can also be used as substrates in biosensing to visualize single molecule reactions. However, high temperatures and/or denaturants may be required to catalyze reactions. Denaturants which can lower the melting temperature of DNA origami as well as these high temperatures means DNA origami also requires thermal stability.

The final primary application of DNA origami is in nanofabrication, either as templates in lithography or nanoparticle synthesis or as a tool to fabricate precise nanostructures in nanophotonics, plasmonics, and electronics [2-4,6,7]. For example, Acuna *et al.* constructed a nanoantenna using DNA origami and gold nanoparticles to increase fluorescence intensity in a plasmonic hotspot [3]. However, many nanofabrication techniques require harsh conditions such as exposure to deionized water, high temperatures, and repeated mechanical forces, all of which can damage the DNA origami. As such, it is imperative to stabilize it against these various factors in order to actively utilize it.

Stabilization Methods

DNA origami structures can be stabilized in multiple ways, but this paper will mainly focus on two. The first method involves chemical modifications by covalently cross-linking different molecules through chemical reactions or UV light irradiation. Many have also attempted to stabilize DNA origami nanostructures coating the structures non-covalently with other molecules using atomic layer deposition (ALD), electrostatic interactions, and biomineralization.

The few methods discussed will be focused on thermal stability. In 2011, Rajendran *et al.* first used a cross-linking technique by exposing DNA origami tiles to 8-methoxypsoralen (8-MOP), which forms covalent bonds with pyrimidine bases in DNA when irradiated with 365 nm UVA light [8]. They found that additional covalent bonds increased the thermal stability of the tiles by 30°C allowing the tiles to retain their structure at 85°C.

Near the same time, Tagawa *et al.* used a similar approach except they introduced 3-cyanovinylcarbazole (CNVK) instead of 8-MOP where the CNVK would crosslink to adjacent pyrimidine bases through 366 nm UV irradiation [9]. The DNA structures were then absorbed onto mica surfaces in order to test the thermal stability. The bare structures showed signs of degradation at 45°C whereas the crosslinked structures were stable at 70°C and did not start degrading until 75°C.

A year later, Gerrad *et al.* used a combination approach, utilizing strain-promoted azide-alkyne cycloaddition (SPAAC, also known as "copper-free click") to form covalent bonds in DNA hexagonal nanostructures while also photocrosslinking them with 3-cyanovinylcarbazole moieties [10]. The melting temperatures of the DNA hexagons were then tested under various denaturant concentrations. At 20% (v/v) formamide, the stabilized structures showed minimal damage while bare structures were completely denatured [10]. Stefano *et al.* used disulfide bonds to crosslink DNA structures and demonstrated increased heat and denaturant resistance. The crosslinked structures could withstand at least 60°C and a denaturing polyacrylamide gel electrophoresis (PAGE) [11].

In terms of increasing thermal stability by coating, Wu *et al.* introduced a method to biomineralize DNA structures with calcium phosphate [12]. The group dispersed DNA structures into a pretuned calcium phosphate solution that allows the even growth of CA-P layers on the helices. The biomineralized structures were able to withstand a temperature of 70°C and also their mechanical stability improved; the Derjaguin-Muller-Toporov (DMT) modulus was doubled (100 MPa to 200 MPa) and the Young's modulus increased by 1.5 times [12]. These metrics measure how easily an object is deformed by comparing the force per unit area to extension per unit of length. Having a much higher modulus means the structures can withstand higher forces without being deformed.

Wang *et al.* also used a coating technique but instead took advantage of the negatively charged phosphate backbone of DNA to electrostatically coat them [13]. They designed a variety of artificial peptoids composed of positively charged *N*-(2-aminoethyl)glycine (Nae) and neutral *N*-2-(2-

(2-methoxyethoxy)ethoxy)ethylglycine (Nte) arranged in different combinations and lengths [12]. The peptoids were tested against a variety of destabilizing conditions that included heat, low Mg^{2+} concentrations, and nuclease. They found that the brush type PE2 peptoids which consisted of 12 Nae and 12 Nte arranged as an alternating chain provided the strongest stabilization overall [12]. The stabilized structures had an increase in melting temperature from 44°C to 50°C and a low cation solution with a Mg^{2+} concentration of 1.25 mM [12]. The structures were also stable under DNase I concentrations of 20 µg/mL for 30 minutes. Wang *et al.* additionally tested the DNA structures in cell media by incubating for 24 hours at 37°C in a low Mg^{2+} Dulbecco's modified Eagle medium (DMEM) and Roswell Park Memorial Institute (RPMI) 1640 medium media. The stabilized structures were intact while bare structures completely degraded. However, in the presence of 10% fetal bovine serum (FBS) combined with the DMEM, the coated structures decreased in number after 24 hours.

Further variations of coating and crosslinking have also been developed to stabilize DNA origami against multiple factors in addition to heat. Gerling *et al.* used photocrosslinking by strategically placing thymine at strand crossovers and termini in DNA origami bricks before irradiation with 310 nm UVB light [13]. The result is the formation of cyclobutane pyrimidine dimers (CPDs) which serve as additional bonds that reinforce the weak points and "nicks" of the DNA origami. The resulting structures were able to withstand temperatures of 90°C, a 40°C increase compared to bare structures. Additionally, the structures remained intact in double distilled water containing no cations for over 24 hours. Finally, the crosslinked structures survived for 1 hour in 4 U/ml DNase I. Cassinelli *et al.* modified a DNA 6-helix nanotube by replacing select strands with special 3'-alkyne, 5'-azide-modified oligonucleotides [14]. Copper ions were then introduced to catalyze the azide-alkyne cycloaddition to form ring-like structures across the tube resembling chainmail. These structures were shown to be completely stable in buffers containing no Mg^{2+} but the duration in the buffer isn't stated. The cross-linked structures were also measured to have their melting temperature increased by 6.3°C and tested in cell media and nuclease exposure. The structures withstood being incubated in DMEM media for 24 hours at physiological temperature and exonuclease I for 3 hours.

The majority of stabilizing strategies for DNA origami, however, focus on its biological applications and stabilizing against nuclease and low cation concentrations. Kim *et al.* introduced a unique coating strategy by hybridizing dendritic oligonucleotides to DNA bricks [15]. Dendritic oligonucleotides were synthesized by incorporating a trebler phosphoramidite that allowed the oligonucleotide to branch off into 3 separate strands, each of which could be further functionalized with the phosphoramidite to result in 9 strands protruding from each oligonucleotide. By hybridizing several of these dendritic oligonucleotides, the result was a fur-like coating that still allowed the inner DNA to be accessed for post stabilization modification unlike most coating strategies. The coated structures could withstand up to 30 hours in 10% FBS and 1 hour in 50 U/ml Dnase I.

Ponnuswamy *et al.* electrostatically coated barrel shaped DNA origami with oligolysines conjugated to polyethylene glycol by simply mixing appropriate stoichiometric ratios of DNA and oligolysines and incubating at room temperature [16]. The length of the oliglysine molecules was experimented, since shorter chains had weaker binding while longer chains led to aggregation. In the end, oligolysines containing 10 lysine monomers were found to have the best balance, and the structures maintained structural integrity overnight in a zero Mg^{2+} buffer. The structures also had a measured half-life of 36 hours in 10% FBS, a 400 fold increase compared to bare structures and showed no signs of degradation after 1 hour in 500 U/ml-1 Dnase, which is a thousandfold increase compared to bare structures.

Additionally, Ponnuswamy *et al.* demonstrated effective transfection into mouse primary bone marrow-derived dendritic cells which bare structures could not achieve as well as improved circulation times when injected into mice. The bare structures were quickly filtered out of the bloodstream and had a half-life of 9 minutes while coated structures had a half-life of 45 minutes, hypothesized to be due to the higher nuclease resistance. Anastassacos *et al.* improved Ponnuswamy *et al.*'s method by further cross linking the oligolysine-coated DNA with glutaraldehyde because the stability has not yet reached the degree required by some biomedical applications [18]. The oligolysine polyethylene glycol combination coating produced strong results since lysines serve as substitutes for Mg^{2+} in screening electrostatic repulsion and polyethylene glycol had been previously shown to increase nuclease resistance.

However, the electrostatic bonds between the coating and DNA were weak so Annastassacos *et al.* cross-linked glutaraldehyde to the coated DNA structures in order to decrease dislocation of the oligolysines and increase stability. The newly stabilized structures were incubated for 14 days in 1 U/µL DNase I, which is a 2600 times higher concentration than natural blood. Bare structures completely degraded in less than 1 minute and oligolysine-coated structures lasted for 3 hours. However, cross-linked oligolysine structures had a half-life of 66 hours with 16% of the structures still intact after 14 hours. Additionally, glutaraldehyde cross-linked structures showed over double transfection efficiency when compared to coated structures when introduced to HEK293T cells diluted in standard DMEM + 10%FBS (~0.7 mM $MgCl_2$) for 24 hours. At 10 nM DNA concentration, the transfection efficiency for cross linked structures was about 65% while coated structures had an efficiency of 30%.

Auvinen *et al.* coated DNA origami bricks with a protein dendron conjugate. Bovine serum albumin (BSA) protein was first attached to dendrons by cysteine-maleimide bond and the dendron electrostatically binds to the DNA [19]. The coating fully protected the samples when exposed to 10 U/ml DNase I for 1 hour. Additionally, the coating increased the transfection efficiency of the DNA 2.5 times and had a reduced immune response rate when injected into mice.

Garcia *et al*. designed a protein-based polymer coating called C4-BK12 that contains a lysine binding domain [20]. The coated and uncoated structures were exposed to high concentrations of nuclease where the uncoated structures denatured in 2 minutes while coated structures lasted 10 minutes with a half-life of 3 minutes.

Agarwal *et al.* electrostatically coated DNA structures with a cationic poly(ethyleneglycol)–polylysine block copolymer. DNA samples were incubated for 16 hours at 37°C in a buffer containing DNase I or RPMI media supplemented with 10% fetal bovine serum (FBS) and coated structures were stable throughout both while bare structures fully degraded [21]. Next, the structures were tested in buffers containing no Mg^{2+} but 30mM NaCl for 16 hours and the coated structures again were stable while bare structures degraded.

Ahmadi *et al.* tested two different coatings by mixing DNA structures with linear polyethyleneimine (LPEI) and Chitosan oligosaccharide lactate [22]. Both polyplex structures were shown to withstand the zero Mg^{2+} buffer containing 30mM NaCl for 24 hours. Both coatings were also stable in 10 U/ml^{-1} DNase I.

Perrault *et al.* encapsulated DNA origami structures with a lipid bilayer that was inspired by viruses in order to protect DNA structures in physiological conditions [23]. The encapsulation was done by annealing lipid–oligonucleotide and fluor–oligonucleotide conjugates to the nanostructure in a surfactant buffer and then purified and dialyzed. The 1.5 µg of DNA were incubated with 20 units of DNase I for 24 h at 37 °C, and 84.6 ± 7.2% remained in the encapsulated group. The encapsulated structures were also injected into mice to measure their circulation time. The encapsulated groups had approximately a 6-minute half-life compared to bare structures with a 50-minute half-life.

Lacroix *et al.* first conjugated dendritic alkyl chains to DNA which have high binding affinity to human serum albumin in order to coat the DNA with the protein [24]. When incubated in DMEM supplemented with 10% FBS the coated structures had a half-life of 22 hours. Multiple different groups all tested using silica to coat DNA.

Linh Nguyen *et al.* used the Stöber method to condense silica onto DNA. They used N-trimethoxysilyl-propyl-N,N,N-trimethylammonium chloride (TMAPS) as a positive co-structure directing agent to address the issue of both the silica and DNA being negatively charged [25]. The structures were heated to 100°C and then quickly cooled on ice. Bare structures completely degraded while the coated structures withstood the temperature fluctuations. The stability of the silica coated origami in DNase was tested by incubating them in 1 mgmL DNase I for 1.5 hours, after which the structures showed no sign of degrading. Additionally, they were able to coat 3D origami crystals which were observed in a salt-free dry state, showing that the coating also protects against low cation conditions.

Liu *et al.* used the exact same silica coating method stated above but tested the mechanical properties instead [26]. They measured a tenfold increase in the Young's modulus (E modulus) from 100 MPa to 1 GPa and improved rigidity to compression compared to bare structures. They also found the structures to have a degree of flexibility elasticity by returning to original height when repeatedly undergoing compressive forces between 1-3 nN.

Minh-Kha Nguyen *et al.* created a different method for the controllable homogenous growth of silica on DNA [27]. First, they electrostatically coated the DNA with a positively charged alkylalkoxysilane group which served as a coupling agent. Then, the silanol groups of the coupling agent acted as co-condensation sites for TEOS to form a silica shell around the DNA structures. They tested new silica coated structures in DI water and found that they were stable for at least 10 months compared to 1 week for bare structures. The structures were then tested in variable concentrations of DNase I for 3 hours. The bare structures were degraded at 4 U/ml while coated structures were completely stable at those concentrations.

Coating strategies have also been developed in order to increase stability in non-biological applications. Matkovic *et al.* coated DNA origami triangles with a single layer of exfoliated graphene through micromechanical cleavage [28]. The DNA was deposited onto silicon substrates, and the graphene layer was deposited on top of that. They showed that the morphology of the DNA was preserved by the graphene and could withstand forces up to 60 nN from AFM contact mode manipulation. In comparison, bare structures were deformed at only 2.7 nN. Additionally, the structures lasted at least 30 minutes against DI water exposure compared to 1 minute by bare structures.

Hyojeong Kim *et al.* similarly deposited DNA origami on silicon substrates but coated them with Al_2O_3 with atomic layer deposition instead [29]. They showed that a 5 nm coating of Al_2O_3 protects the DNA through many processes used in soft lithography including UV/O_3 treatment, washing DI water and drying with N_2 gas. Increased mechanical stability was shown through repeated pattern transfers using the coated DNA, which retained their shape. Finally, the authors theorized that Al_2O_3 coating additionally improves long term storage stability since bare DNA degrades after 30 days when exposed to atmospheric conditions.

Results

Given the vast number of stabilizing techniques, it is important to differentiate and identify the most effective methods. The methods here are assessed based on the degree of stabilization offered, the number of destabilizing conditions prevented, ease of implementation, and any unique advantages or disadvantages offered. The first method presented in 2011 by Rajendran *et al.* provides an easy effective stabilization method through photocrosslinking with 8-MOP [8]. However, this method was only shown to stabilize against heat, and subsequent methods improved the degree of stabilization. Tagawa *et al.* used a similar method that falls short for the same reasons. Their method was more difficult to implement due to use of 3-cyanovinylcarbazole, which is harder to synthesize than 8-MOP and has worse results.

Gerrad *et al.*'s method also used cyanovinylcarbazole, making their method difficult to implement and having poor thermal stability [10]. They did show improved stability in the presence of formamide, but formamide is not widely used in any major DNA origami application. The use of disulfide bonds presented by Stefano *et al.* also provided weak stabilization results with structures only withstanding 60°C and an unspecified concentration of denaturing PAGE [11]. Wu *et al.* biomineralized DNA origami with calcium phosphate and improves stability in more than one area, but the degree of stabilization is lacking [11]. The structures were only shown to withstand 70°C and had double the DMT modulus and 1.5 times Young's modulus compared to bare structures. However, the heat and mechanical stability demonstrated has been improved by other methods.

Copper-catalyzed bonds forming "chain-armor" proposed by Cassinelli *et al*. also stabilizes in a wide variety of conditions [14]. The improved thermal stability is low (only a 6°C increase in melting temperature), but the method provides moderate to substantial stability in biological conditions: 24 hours in cell media, 3 hours in exonuclease, and 24 hours in zero Mg^{2+} buffer. However, this stabilization method is more difficult to implement and has lower stabilization than other methods. Peptoid coating used by Wang *et al*. similarly increased melting temperature by 6°C while providing moderate biological stability: 1.25 mM Mg^{2+} concentration, 20 µg/mL DNase I for 30 minutes and 24 hours in cell media [12].

Thymine cross-linking introduced by Gerling *et al.,* however, does not have any of the problems previously mentioned [13]. The formation of CPDs yields the highest thermal stability out of any method. The DNA origami structures were stable up to 90°C which is a 40°C increase in melting temperature. Additionally, the method provides moderate to high stability in biological conditions: 24 hours in zero Mg^{2+} distilled water and 1 hour in 4 U/ml Dnase I. The method is also relatively easy to implement as the thymine can be easily incorporated into the DNA origami in the initial synthesis stage using software and the structures simply need to be exposed to UV light. This enables the method to be highly scalable as large amounts of DNA origami can easily be mass irradiated and stabilized, and thymine is a relatively cheap chemical ($3.54 per ml). Gerling *et al.*'s method should be the primary method used when thermal stability is the main issue in a DNA origami application due to having the best stabilization results and easy implementation. Due to its easy implementation, it can also be used in biological applications but some applications may require higher degrees of biological stabilization than this method allows.

In terms of determining the optimal stabilization technique for biological applications, the main factors to consider are the degrees of stabilization in both low salt and nuclease present conditions since both will be present simultaneously. Additionally, several methods have demonstrated improved circulation and transfection efficiency of the DNA structures into cells, which should also be taken into account. Coating with dendritic oligonucleotides by Kim *et al.* provides strong protection against nuclease degradation and has the additional advantage of allowing continued modification to the DNA origami after the coating [15]. However, the method primarily falls short because of the lack of protection in low cation conditions, meaning it cannot be used physiologically no matter how great the nuclease protection is.

The virus-inspired membrane encapsulation done by Perrault *et al.* similarly only provides nuclease protection [23]. They demonstrated improved circulation time when injected into mice, but it is unknown whether the structures were intact while in circulation. Their method is also more difficult to implement than others as it requires precise and extensive functionalization of the DNA after assembly. Different protein-based coatings from Auvinen *et al.,* Garcia *et al.,* and Lacroix *et al* [19,20,24] also have the same problem of only providing nuclease protection and requiring difficult dendrimer synthesis and protein synthesis.

Ahmadi *et al.* used two different coating techniques, both of which effectively stabilized DNA origami against both 10 U/ml DNase and zero Mg^{2+} conditions for 24 hours each [22]. This technique provides a strong degree of stability in physiological conditions since blood DNase concentrations were measured to be less than 1 U/ml, and 24 hours is sufficient time for most applications. Additionally, they found that the degree of stabilization is related to the N/P ratio (number of positive amines in the coating to negative phosphate in the DNA), meaning the degree of stabilization can be augmented to fit the application. They found that LPEI achieves the same stabilization as chitosan at a lower N/P ratio, indicating it is the more efficient coating of the two. Agarwal *et al*. achieved similar results by coating with poly(ethyleneglycol) –polylysine block copolymers. However, the duration of stabilization was only tested up to 16 hours compared to 24 hours by Ahmadi *et al* [21,22].

In terms of silica coating, Minh-Kha Nguyen *et al.* presented the most effective way to coat DNA origami [27]. The 5 nm silica coating provided essentially unlimited stability in low ion conditions lasting 10 months in DI water and moderate DNase stability by withstanding 4 U/ml for 3 hours. Although not specifically tested by Minh-Kha Nguyen *et al.,* it can be assumed that previous stability results can be applied as well. Linh Nguyen *et al.* demonstrated the structures could withstand large temperature fluctuations from 0°C to 100°C, but the thickness of the silica could not be measured [25]. Using a 3 nm silica coating, Liu *et al.* demonstrated increased mechanical stability as well due to a tenfold increase in the Young's modulus from 100 MPa to 1 GPa [26].

Annastassacos *et al.* present the only method that uses both cross-linking and coating simultaneously [18]. By further cross-linking already coated structures with glutaraldehyde, they achieved even greater stability against nuclease. DNA origami coated with just oligolysines had a half-life of 16 minutes and fully degraded after 3 hours in 1000 U/ml, whereas both coated and cross-linked structures had a half-life of 66 hours and were not fully degraded after 14 days. This shows a 250-fold improvement in stability after cross-linking and provides the highest degree of nuclease stability out of any method. It can also be assumed that the coated and cross-linked structures retain the low cation stability achieved by just the coating as well as the improved circulation times when injected into mice. Additionally, cross-linking was shown to improve transfection efficiency by 2.5 times compared to plain coated structures.

Kim *et al.* and Matkovic *et al.* coated with both Al_2O_3 and graphene to increase mechanical stability for lithographic applications [28,29]. Both methods were shown to preserve morphology, protect against exposure to DI water, and provide adequate mechanical stability for applications. The main differentiating factor would be the ease of implementation which would depend on the equipment available.

Discussion

Although numerous experiments were conducted to stabilize DNA origami nanostructures, the existence of a few, top-performing methods means most techniques likely will not be developed further. Out of the methods listed here, eight compelling stabilization methods show promise to be implemented in real world applications. However, they can be further down selected to determine the best stabilization technique for each application. Peptoid coating used by Wang *et al.* stabilizes against a variety of factors but the melting temperature increase is negligible, and the low salt stability is not sufficient for biological applications [12]. According to Annastassacos *et al.,* physiological Mg^{2+} concentrations are <1 mM, whereas the peptoid coating only stabilizes down to 1.25 mM. Agarwal *et al.* and Ahmadi *et al.* both created effective coatings using poly(ethyleneglycol)–polylysine and linear polyethyleneimine to stabilize against both nuclease and low salt conditions [18]. Both methods are also claimed to be "cost-effective," but still fall short in comparison to other methods remaining that have higher degrees of all-around stability. Glutaraldehyde cross-linking of oligolysines coating DNA origami used by Annastassacos *et al.* proved to be the most effective stabilization method for any biological applications with having unmatched degrees of stability in the presence

Table 1. Comparison of stabilization technique's performance in DNase I.

Stabilization Method	**DNase I Concentration**	**Stabilization Duration**
Poly(ethyleneglycol)–polylysine coating	0.256 U/ml	16 hours
Human serum albumin coating	0.256 U/ml	22 hour half-life
Thymine cross-linking	4 U/ml	1 hour
Silica coating	4 U/ml	3 hours
LPEI and chitosan coating	10 U/ml	24 hours
Bovine serum albumin	10 U/mL	1 hour
Dendritic oligonucleotide coating	50 U/ml	1 hour
Peptoid coating	167 U/mL	0.5 hours
Oligolysine-coated	500 U/ml	1 hour
Glutaraldehyde cross-linking of oligolysines coated DNA Origami	1000 U/mL	~66h half-life
C4–BK12 protein coating	"high"	3 minute half-life
Virus–inspired membrane coating	20 U	24 hours

of nuclease, high degrees of stability in low-salt conditions, and improved circulation times and transfection efficiency. Annastassacos *et al.* hypothesize that the cross-linked and coated structures can survive for more than a year in 10% fetal bovine serum cell media. This stabilization method is also cost-effective and scalable.

Thymine cross-linking introduced by Gerling *et al.* has the easiest implementation out of any method since the thymine can be placed during the design phase of the DNA origami and the structures can be easily mass irradiated. This method provides moderate biological stability and can be used as an easier alternative method when lower degrees of stabilization are sufficient [13]. Additionally, it provides high degrees of thermal stability allowing structures to withstand up to a 90°C and 40°C increase in melting temperature. Silica coating using Minh-Kha Nguyen *et al.'s* method stabilizes against the widest variety of factors with improved mechanical stability, thermal stability and resistance to nuclease and deionized water [27]. Although more difficult than the two mentioned above, silica coating can still be used in situations where DNA needs to be stabilized in many situations. Both Al_3O_2 and graphene coating can be used to stabilize in lithographic applications and interchangeably depending on which method is easier with the given equipment [28,29]. In conclusion the five methods that remain each have distinct advantages and uses and should be the first tools to consider during the application of DNA origami.

Conclusions

DNA origami has demonstrated its potential across diverse fields but its practical usage is hindered by the susceptibility of DNA structures to low cation environments, nuclease activity, heat, and mechanical forces. To overcome these limitations, scientists have developed a multitude of coating and crosslinking methods aimed at stabilizing DNA origami. However, most of these methods suffer from flaws, resulting in only a few being effective for implementation. Existing techniques often exhibit limitations such as stability against a single factor, lower stabilization strength and duration compared to other methods. For instance, methods solely targeting nuclease stability may have limited applicability due to the requirement for low cation stability in biological applications. Nonetheless, our study has identified five promising methods that provide comprehensive stability against all instability factors, catering to a wide range of applications. Specifically, the findings reveal that an oligolysines coating cross-linked with glutaraldehyde exhibits the strongest biological stability, thymine cross-linking demonstrates the highest thermal stability, a silica coating showcases superior stability against multiple factors, while graphene and Al_3O_2 coatings offer the best mechanical stability [13,18,27-29]. These findings will significantly aid future applications of DNA origami by allowing scientists to easily select their stabilization method of choice. While we have identified the most effective stabilization methods out of the twenty one listed, limitations to this study still exist. Many of the methods reviewed reported different metrics in terms of stabilization and some methods weren't tested to their limit. This results in some methods being stronger in reality than what is reported. Additionally, there may be alternative approaches being developed during the time of this study warranting this form of research to be frequently updated. The future of DNA origami stabilization includes utilizing the five stabilization techniques listed here as benchmarks to improve upon. Future research should also aim to implore similar procedures and measurements when reporting stability for ease of comparison. Additionally, exploring novel strategies may uncover additional functions of DNA origami. Ultimately, by addressing the stability challenges, this study contributes to unlocking the full potential of DNA origami and paves the way for its broader utilization in diverse fields.

Acknowledgements

This research was made possible by the Polygence program that provided the resources, opportunity and mentorship. All research contained is my own.

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