Cryopreservation of DNA Origami Nanostructures

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Although DNA origami nanostructures have found their way into numerous fields of fundamental and applied research, they often suffer from rather limited stability when subjected to environments that differ from the employed assembly conditions, that is, suspended in Mg$^{2+}$-containing buffer at moderate temperatures. Here, means for efficient cryopreservation of 2D and 3D DNA origami nanostructures and, in particular, the effect of repeated freezing and thawing cycles are investigated. It is found that, while the 2D DNA origami nanostructures maintain their structural integrity over at least 32 freeze–thaw cycles, ice crystal formation makes the DNA origami gradually more sensitive toward harsh sample treatment conditions. Whereas no freeze damage could be detected in 3D DNA origami nanostructures subjected to 32 freeze–thaw cycles, 1000 freeze–thaw cycles result in significant fragmentation. The cryoprotectants glycerol and trehalose are found to efficiently protect the DNA origami nanostructures against freeze damage at concentrations between $0.2 \times 10^{-3}$ and $200 \times 10^{-3}$ m and without any negative effects on DNA origami shape. This work thus provides a basis for the long-term storage of DNA origami nanostructures, which is an important prerequisite for various technological and medical applications.

1. Introduction

The DNA origami technique, which was introduced by Rothemund in 2006,[1] has shown enormous potential for applications in medicine,[2] (bio)chemical sensing,[3] materials synthesis,[4] and device fabrication.[5] While many of these applications of DNA origami depend on an intact and well defined nanoscale shape, DNA molecules and especially DNA origami nanostructures are known to be sensitive toward many environmental factors,[6] such as pH,[7] temperature,[7c,8] ionic strength,[7c,9] and even oligonucleotide age.[10] In order to minimize the potentially negative effects of such environmental conditions in a laboratory setting, experiments are usually carried out using freshly synthesized DNA origami samples. On the contrary, for technological and medical applications, the synthesized DNA origami nanostructures are often desired to maintain their structural and shape integrity under appropriate storage conditions for extended periods. Among the various preservation techniques, lyophilization has been discussed as a promising method for long-term storage of DNA origami nanostructures at ambient temperatures.[11] While lyophilization has resulted in drastically improved stability of DNA origami nanostructures during storage at room and elevated temperatures compared to storage in buffer solution,[11] it requires cryogenic temperatures and the resuspension of the lyophilized DNA origami before use. In many laboratory environments and

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technological applications, a cheaper and more straightforward storage method is desirable.

In this work, we thus investigate the stability of suspended DNA origami nanostructures subjected to repeated freeze–thaw cycles. Freezing has been shown to reduce the lifetime of DNA duplexes under tension, and may thus also reduce the stability of DNA origami nanostructures which often exhibit a high degree of twist strain. Furthermore, since DNA origami nanostructures behave rather like rigid particles than soft polymers, ice crystal formation may result in their rupture and breakage. We therefore also investigate the potential of the well-established cryoprotectants glycerol and trehalose to enhance DNA origami stability during repeated freezing and thawing by suppressing the formation of crystalline ice.

2. Results and Discussion

Triangular 2D DNA origami nanostructures in folding buffer were frozen at −20 °C for 40 min to 12 h and subsequently thawed at room temperature. The thawed DNA origami triangles were then adsorbed to a freshly cleaved mica substrate. After washing with water and blow-drying in a stream of ultrapure air, the structural integrity of the immobilized DNA origami nanostructures was characterized by atomic force microscopy (AFM) in air. As can be seen in Figure 1a, b, the DNA origami triangles remained completely intact after freezing and thawing with no apparent differences to freshly synthesized ones, which indicates that a single freezing and thawing step does not affect the structural integrity of the triangular DNA origami nanostructures. This is in agreement with previous observations. Repeated freezing and thawing, however, is known to progressively degrade suspended DNA molecules, with duplexes >100 kb in size being most susceptible toward freezing and thawing-induced degradation. To further investigate the effects of repeated freezing and thawing on DNA origami stability, we therefore subjected the sample to several rounds of the freeze–thaw cycle described above and evaluated their structural integrity with AFM after each cycle. As can be seen in Figure 1a–d, during the first 23 cycles the majority of the DNA origami nanostructures remained intact with well-preserved triangular shapes. Most significantly, only few broken or denatured DNA origami nanostructures are observed. After 24 cycles (Figure 1e), however, some deformed DNA origami nanostructures appear. These deformed DNA origami triangles are characterized by one or more of the edges of the trapezoids that compose the triangle being bent or curved. In addition, these deformed triangles have a more bulky and somewhat granular morphology. The number of deformed triangles increases with the number of freeze–thaw cycles until after 32 cycles, virtually all DNA origami triangles are deformed (see Figure 1f). This trend is also reflected in the statistical analysis of the AFM images shown in Figure 1g. Whereas about 90% of the DNA origami nanostructures maintain their structural integrity in the first 23 cycles, the fraction of deformed triangles increases steeply after the 24th cycle and reaches 100% after 32 freeze–thaw cycles.

In our previous work, we observed a similar type of damage, that is, deformed and distorted DNA origami triangles, resulting from prolonged storage of the staple strands. We found that these shape distortions appeared only during washing of the adsorbed DNA origami, whereas AFM imaging in liquid revealed fully intact DNA origami triangles without any shape distortions. In order to test whether the damage observed in above AFM images is present already in the suspended state in solution, we also imaged the DNA origami triangles after 32 freeze–thaw cycles in buffer solution, thereby avoiding any washing or drying-induced damage. As can be seen in the corresponding AFM images in Figure 2a, b, the majority of DNA origami triangles are completely intact in liquid, even after undergoing 32 freeze–thaw cycles. Therefore, we conclude that repeated freezing and thawing leads to the accumulation of damage that does not affect the global shape and structure of the DNA origami triangles in solution but makes them more susceptible to environmental conditions such as washing and drying. Indeed, we found that the observed damage mostly results from the blow-drying step and can be minimized by applying an air stream of lower pressure during drying (see Figure S1, Supporting Information).

Interestingly, we did not observe a significant increase in the fraction of DNA origami fragments with the number of

![Figure 1. AFM images of triangular DNA origami nanostructures a) before and after b) 1, c) 15, d) 23, e) 24, and f) 32 freeze–thaw cycles. AFM images have a size of 1.5 × 1.5 μm² and height scales are 2.3 nm. Insets show zoomed images of selected DNA origami triangles. g) Fractions of intact and deformed DNA origami nanostructures as a function of the number of freeze–thaw cycles. The values have been averaged over eight AFM images and the error bars represent the standard deviations.](image-url)
denatured in 4 M urea before AGE. Unfortunately, we could not detect any scaffold fragments (see Figure S3, Supporting Information). However, freezing-induced DNA strand breakage is typically attributed to the growth of ice crystals, which physically shear the strands.[15] In order to test whether the observed damage can be attributed to ice crystal formation, we subjected the DNA origami triangles to 32 freeze–thaw cycles using flash freezing in liquid nitrogen to avoid the formation of macroscopic ice crystals. Indeed, flash freezing yielded about 80% of intact DNA origami triangles even after 32 cycles (see Figure S4, Supporting Information). This observation thus renders nick formation due to physical shearing by growing ice crystals the most likely damage type to contribute to the limited DNA origami stability during repeated freezing and thawing.

The formation of crystalline ice during freezing can largely be suppressed by the addition of cryoprotectants.[16] However, since DNA origami nanostructures are often more sensitive toward chemical denaturation than genomic DNA,[17] it is important to carefully choose a cryoprotectant that does not inflict any damage on the DNA origami nanostructures at the concentration required to induce vitrification. Therefore, we investigated the stability of the DNA origami triangles in the presence of different concentrations of glycerol, which is one of the most widely used cryoprotectants for biological systems.[18] As can be seen in Figure 3a–d, the integrity of the DNA origami nanostructures was completely unaffected by the addition of glycerol at concentrations ranging from $0.2 \times 10^{-3}$ to $200 \times 10^{-3}$ M. Statistical analysis of the AFM images further yielded 85% to 90% of intact DNA origami (Figure 3i). After 32 freeze–thaw cycles, intact DNA origami triangles are observed for all glycerol concentrations (Figure 3e–h). Furthermore, we found that blow-drying does not affect the integrity of the DNA origami nanostructures in the presence of glycerol, indicating effective protection against freeze damage. For $0.2 \times 10^{-3}$ M glycerol, the fraction of intact DNA origami nanostructures after 32 freeze–thaw cycles structures is only slightly reduced by $\approx 3.5\%$ with respect to the freshly prepared DNA origami. Increasing the glycerol concentration, however, does not show a significant improvement. Therefore, glycerol is an effective cryoprotectant against freezing-induced DNA origami damage already at $0.2 \times 10^{-3}$ M concentration.

Besides glycerol, we also studied the potential of trehalose to preserve the integrity of DNA origami nanostructures during freezing and thawing. Trehalose is commonly used to protect various cellular components such as DNA, proteins, and membranes from loss of activity under stress conditions.[19] On the other hand, trehalose is known to destabilize double-stranded DNA by lowering its melting temperature,[20] which may lead to DNA origami damage. As can be seen in Figure 4a–d, however, the addition of trehalose at concentrations ranging from $0.2 \times 10^{-3}$ to $200 \times 10^{-3}$ M has no notable effect on the structural integrity of the DNA origami triangles. Similar to glycerol, trehalose was also able to protect the DNA origami nanostructures during 32 freeze–thaw cycles (Figure 4e–h). The statistical analysis shown in Figure 4i reveals that the fraction of the intact DNA origami nanostructures decreased due to freezing and thawing only by 4–5% over the whole concentration range, rendering trehalose a similarly efficient cryoprotectant as glycerol.

Figure 2. AFM images of triangular DNA origami nanostructures after 32 freeze–thaw cycles measured a) in air and b) in liquid. AFM images of triangular DNA origami nanostructures assembled from scaffold and staple strands that were subjected to 32 freeze–thaw cycles measured c) in air and d) in liquid. Images have a size of $1.5 \times 1.5 \mu m^2$ and height scales are 2.3 nm.
Next, we set out to test whether above observations are also valid for 3D DNA origami nanostructures. To this end, 14 helix bundles (14HBs) were subjected to 32 freeze–thaw cycles, both at \(-20\) °C and in liquid nitrogen. In order to better visualize possible freeze damage, we turned to transmission electron microscopy (TEM). As can be seen in Figure 5a, three populations are observed, that is, intact, kinked, and broken DNA origami 14HBs. No significant deformations could be detected, which may either be a result of the gentler adsorption and washing conditions or the limited resolution of the TEM images. Furthermore, we did not observe any significant changes in the fractions of these populations due to the 32 freeze–thaw cycles, with 85 to 90% of the DNA origami nanostructures being intact (see Figure 5a–c). Therefore, we increased the number of freeze–thaw cycles to induce more damage. Due to time constraints, this was done using flash freezing in liquid nitrogen. Although flash freezing reduces the size of the formed ice crystals, it cannot completely inhibit crystallization. Indeed, after 1000 freeze–thaw cycles, the fraction of intact DNA origami 14HBs was decreased to 38%, with more than 50% of the 14HBs being broken (see Figure 5d). Addition of 200 \times 10^{-3} \text{ M} glycerol and trehalose, respectively, again resulted in about 85% of intact DNA origami after 1000 freeze–thaw cycles (see Figure 5e,f). This proves that the stabilizing effect of these cryoprotectants acts universally on 2D and 3D DNA origami nanostructures.

Finally, DNA origami stability under repeated freezing and thawing also depends on their overall shape and structure. 24HBs for instance, turned out more resistant against freeze damage than 14HBs. Even after 1000 freeze–thaw cycles in liquid nitrogen without any cryoprotectants added, almost 80% of the 24HBs remained intact (Figure S5, Supporting Information). Addition of 200 \times 10^{-3} \text{ M} glycerol resulted only in marginal improvement. This may be attributed to the fact that the 24HBs are shorter and more compact than the 14HBs, which reduces the probability of 24HBs being deformed and thereby damaged by growing ice crystals.
3. Conclusion

In conclusion, we investigated the structural stability of 2D and 3D DNA origami nanostructures subjected to repeated freezing and thawing cycles. Our results demonstrate that cryopreservation at −20 or −196 °C is an effective method for the long-term storage of DNA origami nanostructures. While 2D DNA origami triangles maintain their structural integrity over at least 32 freeze–thaw cycles, ice crystal formation seems to lead to the accumulation of nicks, which make the DNA origami gradually more sensitive to environmental conditions. In particular, significant structural deformations of adsorbed and dried DNA origami nanostructures were observed for samples subjected to 24 freeze–thaw cycles and more, caused by the blow-drying of the sample. After 32 freeze–thaw cycles, this led to all DNA origami nanostructures being deformed. For 3D DNA origami 14 HBs, no freeze damage could be detected after 32 freeze–thaw cycles. Subjection to 1000 freeze–thaw cycles, however, resulted in significant DNA origami fragmentation. Nevertheless, the DNA origami nanostructures can be efficiently protected against freeze damage by addition of the cryoprotectants glycerol and trehalose. At concentrations between $0.2 \times 10^{-3}$ and $200 \times 10^{-3}$ M, both cryoprotectants reduced the freezing-induced damage to a level that resulted in 85%–90% of intact DNA origami nanostructures. These cryoprotectants thus provide a cheap and straightforward means for the efficient long-term storage of DNA origami nanostructures, both at −20 and −196 °C.

4. Experimental Section

Assembly of 2D DNA Origami Nanostructures and Subjection to Freeze–Thaw Cycles: Triangular DNA origami nanostructures were assembled from the 7249-nt long M13mp18 scaffold and 208 staple strands (Metabion) in 1× Tris Acetate-EDTA (TAE) buffer (Carl Roth) containing $10 \times 10^{-3}$ M MgCl₂ (Sigma-Aldrich), as previously described. The purified samples were then frozen either for 40 min to 12 h at −20 °C or for 5 s in liquid nitrogen, and subsequently thawed at room temperature for 40 min.

Assembly of 3D DNA Origami Nanostructures and Subjection to Freeze–Thaw Cycles: Rod-shaped DNA origami nanostructures consisting of a 14HB were assembled from the 8634-nt long M13mp18 scaffold and 234 staple strands (Eurofins Genomics) as previously described. Structures were purified by AGE, extracted from the gel, and subsequently stored in 1× TAE buffer containing $11 \times 10^{-3}$ M MgCl₂. 24HB DNA origami nanostructures were adapted from ref. They were assembled from the 8064-nt long M13mp18 scaffold and 193 staple strands (Eurofins Genomics). After folding, the structures were purified.
by PEG precipitation[22] and re-dispersed in 1× TAE buffer containing 11 × 10⁻³ M MgCl₂. The nanostructures were then either frozen at −20 °C and thawed at room temperature or frozen for 15 s in liquid nitrogen and thawed in a 35 °C warm water bath for 75 s.

AFM Imaging: For imaging in the dry state, 3 µL of the DNA origami sample and 20 µL of 1× TAE containing 10 × 10⁻³ M MgCl₂ were deposited onto freshly cleaved mica surfaces. After 1 min incubation, the mica sample was vertically dipped into HPLC-grade water for 30 s and blow-dried in a stream of ultrapure air (~1 bar overpressure unless stated otherwise). AFM measurements were performed using an Agilent 5100 AFM in intermittent contact mode with HQC:NSC18/Al BS cantilevers (MikroMasch). Images were collected with scan sizes of 3 × 3 µm² with a resolution of 1024 × 1024 px.

For AFM imaging in liquid, 3 µL of the DNA origami sample were deposited onto freshly cleaved mica surfaces within a liquid cell. After 1 min incubation, the liquid cell was filled with 1 mL 1× TAE buffer containing 10 × 10⁻³ M MgCl₂. The measurements were performed using a JPK Nanowizard ULTRA Speed with USC F0.3-k0.3 cantilevers (NanoWorld). Images were collected with scan sizes of 3 × 3 µm² with a resolution of 1024 × 1024 px.

MALDI-TOF: An Autoflex Speed mass spectrometer (Bruker Daltonik) was used in linear positive mode and calibrated using the Oligonucleotide Calibration Standard (Bruker Daltonik). Samples were dissolved in water/acetonitrile (50:50) with 10 g L⁻¹ uranyl formate for 10 s.

Transmission Electron Microscopy: TEM imaging of DNA origamis was performed using a JEM-1011 transmission electron microscope (JEOL) operating at 80 kV. For sample preparation, 5 × 10⁻⁴ L of DNA origami structures were deposited on glow-discharged TEM grids (formvar/carbon-coated, 300 mesh Cu; TED Pella, Inc) for 2 min. For visualization, origami structures were negatively stained by briefly washing the grid with 5 × 10⁻⁶ L of a 2% uranyl formate solution followed by staining with uranyl formate for 10 s.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

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