DNA Nanotechnology

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Folding and Imaging of DNA Nanostructures in Anhydrous and Hydrated Deep-Eutectic Solvents**

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Abstract: *There is great interest in DNA nanotechnology, but its use has been limited to aqueous or substantially hydrated media. The first assembly of a DNA nanostructure in a water-free solvent, namely a low-volatility biocompatible deep-eutectic solvent composed of a 4:1 mixture of glycerol and choline chloride (glycholine), is now described. Glycholine allows for the folding of a two-dimensional DNA origami at 20°C in six days, whereas in hydrated glycholine, folding is accelerated (≤3 h). Moreover, a three-dimensional DNA origami and a DNA tail system can be folded in hydrated glycholine under isothermal conditions. Glycholine apparently reduces the kinetic traps encountered during folding in aqueous solvent. Furthermore, folded structures can be transferred between aqueous solvent and glycholine. It is anticipated that glycholine and similar solvents will allow for the creation of functional DNA structures of greater complexity by providing a milieu with tunable properties that can be optimized for a range of applications and nanostructures.*

Assemblies based on DNA programming[1] provide a method to create custom-designed shapes[2] at the nanometer scale. Common implementations are DNA origami,[2b,c] in which a long DNA strand (scaffold) is folded by hundreds of complementary base-paired oligonucleotides (staples), and systems based on single-stranded tails (SSTs),[2d,e] in which short DNA strands that contain four domains are able to fold into a target shape. DNA nanostructures have been successfully utilized to create two-dimensional[2b] and three-dimensional[2c] devices with applications in lithography,[3] photonics,[4] electronics,[5] and the fabrication of inorganic materials.[6] Despite the ever-expanding applications of DNA nanotechnology,[7] to date, DNA nanostructure designs and applications have been limited to aqueous[1b] or substantially hydrated milieus.[8] Reduced water activity or transfer to organic solvents typically results in alterations of the DNA helical structure or even the loss of base pairing.

In aqueous solution, DNA duplexes adopt the B-form helix. Therefore, all DNA devices have thus far been designed according to B-form helical parameters.[2b,9] Previously, our laboratory demonstrated that DNA can form a stable duplex in reline,[10] an anhydrous deep-eutectic solvent (DES) composed of choline chloride and urea in a 1:2 ratio.[11] This DES is from a family of non-aqueous solvents with improved properties for nanotechnology applications[12] in comparison with water-based media, including enhanced electrodeposition[13] of nanometallic and semiconducting materials, improved inorganic nanoparticle shape control and stability,[14] and low volatility. However, DNA duplexes adopt a more A-form helical structure in reline (Figure 1a), and the degree of structural change is sequence-dependent.[10] Thus, reline would not be compatible with existing DNA nano-

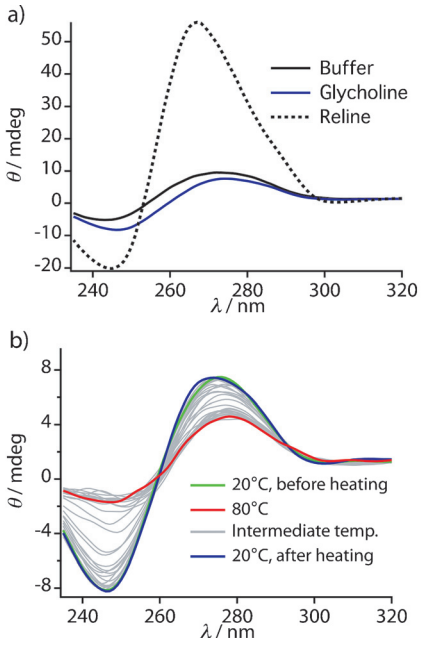

Figure 1. CD analysis of a 32-mer DNA duplex in aqueous and non-aqueous solvents. a) CD spectra of the 32-mer DNA duplex at 20°C in aqueous solution and in the non-aqueous solvents glycholine and reline. b) CD spectra of the 32-mer in glycholine after thermal denaturation by heating to 80°C (red) and cooling to 20°C (blue) in 2°C steps (gray) over a total time of 165 min. The complete recovery of the CD spectrum upon cooling to 20°C (compared with the spectrum before heating; green) demonstrates the reversibility of thermal duplex denaturation in glycholine.

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structures or with existing software for the design of new structures, as altered DNA helical structures would necessitate a new set of design parameters. Furthermore, even if reline-specific parameters were developed, the resulting structures would not be compatible with aqueous solvents, thereby limiting the amount of water that can be added to reline without disrupting an assembled DNA structure.

Herein, we report the first assembly of a DNA nanostructure in a water-free solvent composed of a 4:1 molar ratio of glycerol and choline chloride (with no added divalent cations). This solvent, referred to as "glycholine", is a DES inspired by recent work with solvents of similar compositions.[15] Glycholine allows the folding of 2D DNA origami structures under isothermal conditions. Moreover, we show that tuning the solvent composition with water leads to more rapid folding of the same structure and relatively fast folding of a more complex 3D DNA origami structure and a 2D SST system under isothermal conditions. These results indicate that glycholine reduces the problem of kinetic traps typically encountered during folding in aqueous buffer, and that tuning the viscosity of water/DES mixtures provides a means to control the kinetics of DNA nanostructure self-assembly.

After performing a systematic study with numerous non-aqueous solvents, we found that DNA duplexes maintain a B-form helical structure in glycholine (Figure 1a; see the Supporting Information for details). Duplex stability is also considerable in glycholine in comparison with aqueous buffer, with a 32 bp duplex of mixed AT/GC sequence exhibiting a melting temperature (T_m) of 52 °C (see the Supporting Information, Table S1). Furthermore, circular dichroism (CD) spectra of the 32 bp duplex acquired before and after heating above its T_m confirmed the reversibility of DNA duplex melting in glycholine (Figure 1b).

To test the compatibility of glycholine with DNA structures developed for aqueous solvents, we initially selected the "tall rectangle" DNA origami,[2b] a structure used extensively for its versatility as a display platform.[16] An atomic force microscopy (AFM) image of the tall rectangle, folded and imaged in aqueous buffer, is shown in Figure 2a. As an initial test of the stability of this structure in glycholine, the aqueous buffer of the tall rectangle on a mica substrate was replaced with glycholine. AFM images confirmed the stability of this structure in glycholine (Figure 2b). We also tested the stability of this DNA origami motif under high-vacuum conditions while solvated in glycholine by placing mica substrates with the tall rectangles under vacuum ($\leq$150 mT) for up to four days. AFM images confirmed the persistence of this DNA structure in glycholine (Figure S10), demonstrating the potential utility of glycholine as a solvent for DNA nanotechnology applications where low volatility and low water activity (less than 0.1% water, see the Supporting Information) would be advantageous.

We then proceeded to fold the 2D DNA origami in the water-free solvent. First, a glycholine solution containing the M13mp18 scaffold and a 30-fold excess of staple strands was prepared (see the Supporting Information). Initially, it was expected that a slow thermal ramp would be necessary to fold this structure in glycholine owing to the high viscosity of the solvent (0.43 Pa s at 20 °C).[15] However, well-formed tall

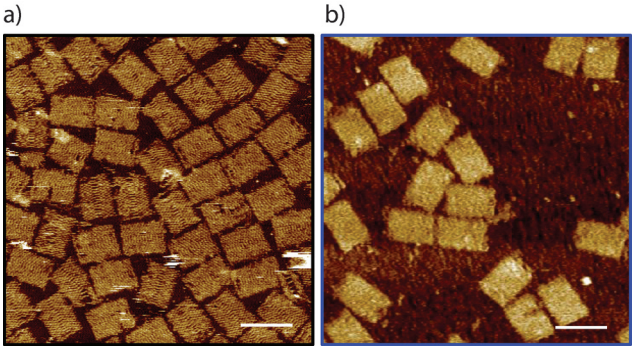

Figure 2. AFM imaging of the tall rectangle in buffer and glycholine. a) Image of the tall rectangle in aqueous buffer on a mica substrate. b) Image of the tall rectangle after the solvent above the mica surface had been replaced by glycholine. For both samples, the tall rectangle was annealed in buffer solution prior to deposition on the mica surface using an adapted version of the Rothemund procedure (see the Supporting Information). Scale bars: 100 nm.

rectangles could be obtained using a much simpler isothermal folding procedure. Specifically, the mixture was heated to 70 °C for 20 min (to denature initial structures), cooled to 20 °C within approximately 40 s, and then maintained at 20 °C for up to eight days. The process of DNA assembly at 20 °C was followed by AFM imaging and agarose gel mobility analysis (Figure 3). To perform AFM imaging, the reaction mixture was added to a mica substrate at the desired time point during the folding process, and then an excess of aqueous buffer was added to facilitate DNA adhesion to the mica substrate. Incubation at 20 °C for 24 hours revealed almost complete folding of most DNA structures, and after six days (144 h), a high yield of fully formed tall rectangles was achieved (93%, $n = 223$).

Increased electrophoretic mobility in agarose gels has been correlated with the folding of DNA nanostructures.[2c,17] The progression of DNA assembly by isothermal folding at 20 °C in glycholine was therefore also monitored by this method (Figure 3b, 0–192 h). The fastest migrating band, denoted as F, corresponds to a well-formed DNA origami structure, as verified by AFM, whereas the slowest migrating band, denoted by U for the sample after 6 h at 20 °C, corresponds to the unfolded state. We noted that the DNA at the start of isothermal folding (0 h), designated as U*, consistently exhibited a mobility that was faster than that observed at 6 h, but slower than that of the fully folded structures observed at 144 h and 196 h (Figure 3b). AFM imaging of the same samples at 0 h indicated that the faster mobility is due to M13m18 being partially structured by staple strands and partially compacted by intramolecular associations (Figure 3a, 0 h). An important additional observation is that even after eight days in glycholine, the DNA origami does not aggregate, as indicated by the lack of bands with reduced mobility (Figure 3b); lower mobility bands were attributed to aggregated and multimerized DNA structures.[2c,17]

It has been shown that in buffer and in the presence of magnesium ions, a rectangular 2D DNA structure folds

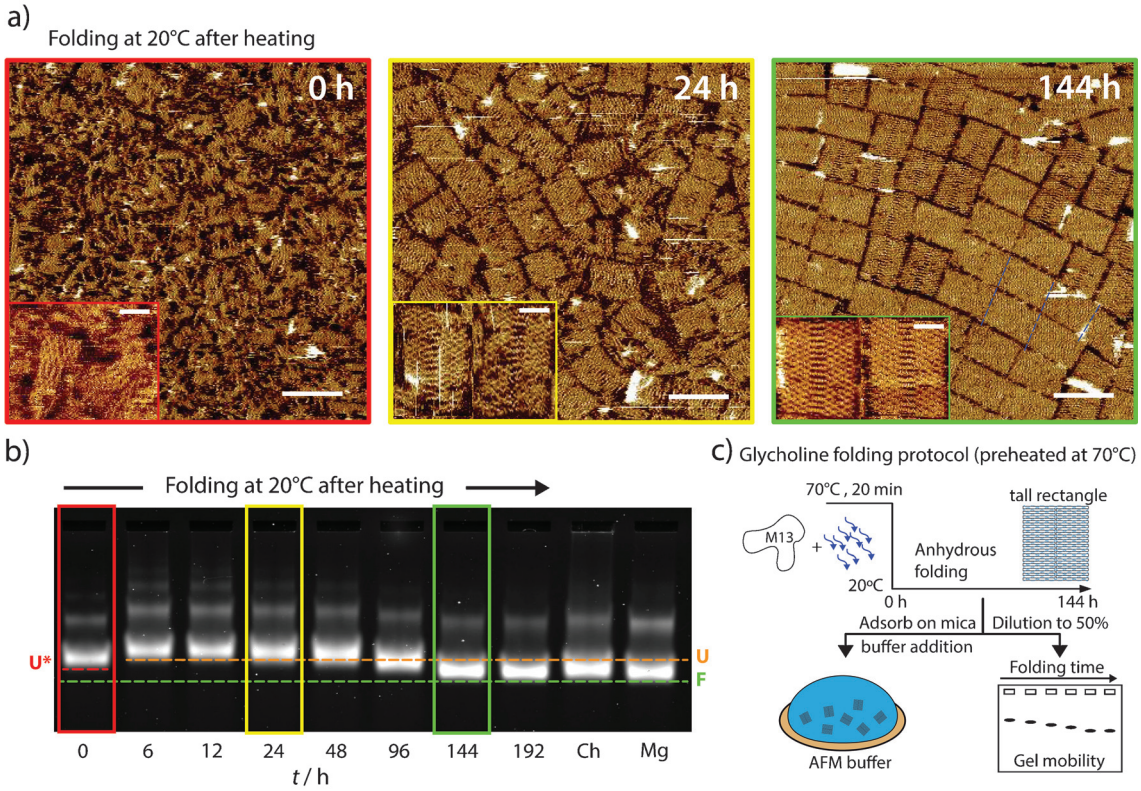

Figure 3. Folding of a DNA origami structure in anhydrous glycholine at 20 °C. a) AFM images showing the DNA origami folding process at 20 °C at selected time points. b) Agarose gel mobility analysis of the DNA origami showing the folding process under isothermal conditions. The colored rectangles in (b) correspond to the selected time points shown in the AFM images in (a). U and F indicate the gel migration of the unfolded and folded structures, respectively. U* indicates a band after cool-down that corresponds to M13 in a grossly assembled state. Lanes Mg and Ch correspond to DNA origami structures annealed in aqueous conditions using the adapted Rothemund method (see the Supporting Information), which are used as references for comparing the mobility of completely folded structures (see Figure S8 for AFM images of these control samples). Mg: DNA origami annealed in buffer solution containing Mg^{2+} (12.5 mM). Ch: DNA origami annealed in aqueous solvent containing choline chloride (2 M). c) Annealing and analysis methods used for isothermal folding in anhydrous glycholine. Scale bars: 100 nm, insets: 25 nm.

rapidly ($\leq$5 min) at a critical temperature.[17] We observed that using the same folding procedure that was used for glycholine, the tall rectangle could be folded with a high yield after cooling (Figure S7). Given that the diffusion-limited steps of DNA folding are substantially slowed down in viscous, anhydrous media,[18] we postulated that the extended time required for complete folding in glycholine at room temperature is the result of slow DNA diffusion and reorganization in this viscous solvent. To test this possibility, we also investigated DNA duplex stability and folding in hydrated versions of glycholine. For example, adding 10 % of water (w/w) substantially reduced the solvent viscosity (0.01 Pa s at 20 °C), but the stability of the 32 bp DNA duplex was not altered from that in a 100 % glycholine solution (T_m = 53 °C). We found that a 90 % glycholine solution allows for the assembly of the tall rectangle in a one-pot reaction after only three hours at 20 °C, based on gel electrophoresis and AFM (Figure 4a), a result that is not possible in aqueous buffer in the presence of magnesium ions (Figure S11). Specifically, AFM analysis revealed well-shaped structures with a yield of 92 % (n = 250), which is comparable to the yield reported for a rectangular DNA origami folded at its critical temperature,[17] and much higher than the yield obtained by folding the same type of structure with denaturing agents.[8b]

We then explored the folding of DNA structures in glycholine that require a tailored annealing procedure in aqueous buffer. For these studies, we folded a triangle based on a single-stranded tail system[2d] composed of nine helices (9H triangle) and a 3D six-helix bundle DNA origami (6HB). These DNA structures require a one-day annealing ramp to fold in aqueous buffer (see the Supporting Information) owing to their more complex folding pathways. We therefore decided to use hydrated glycholine to study the folding of these nanostructures at room temperature with a time scale of one to two days. We found that the 9H triangle and 6HB can both be folded in 75 % glycholine as demonstrated by gel mobility assays and AFM (Figure 4b and c). After 24 hours at 20 °C, well-shaped 9H triangles were observed by AFM (Figure 4b). An analysis of the folded structure yield by AFM revealed that 87 % (n = 313) were well-shaped nanostructures as quantified directly from the crude reaction. For the 3D 6HB DNA origami, a folding time of 48 hours at 25 °C was necessary to obtain well-shaped nanostructures (Figure 4c) with a yield of 84 % (n = 159) directly from the crude reaction and with a yield of 93 % (n = 31) after purification by

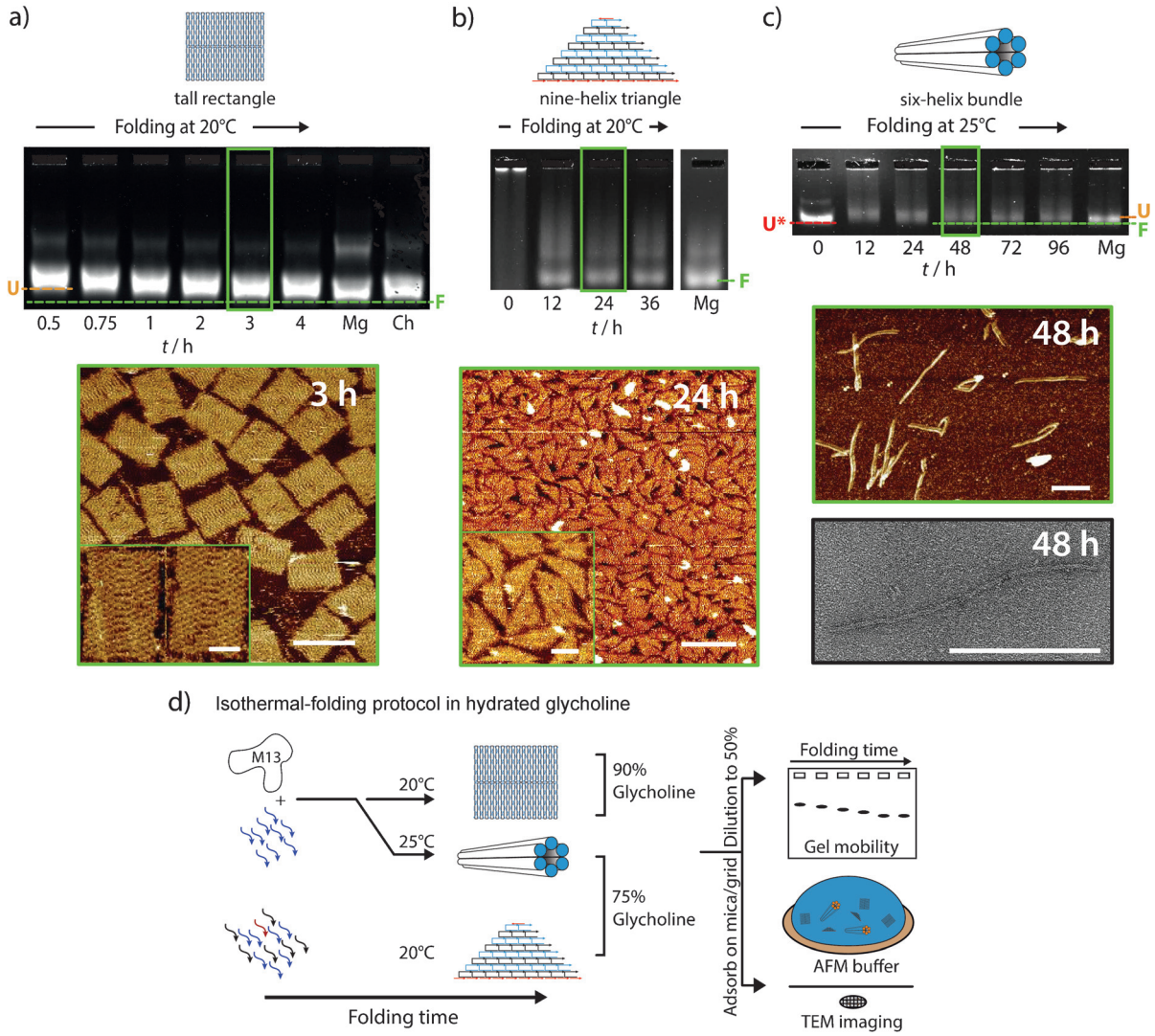

Figure 4. Folding of DNA nanostructures in hydrated glycholine. a–c) Agarose gel mobility analysis and AFM and TEM imaging of the three DNA structures showing the folding process. Lanes Mg and Ch correspond to the DNA nanostructures annealed in aqueous solution according to the standard procedure for each nanostructure (see the Supporting Information), which were used as references for the mobility of completely folded structures. Mg: DNA structure annealed in a buffer solution containing Mg^{2+}. Ch: DNA origami annealed in aqueous solvent containing choline chloride (2 M). U* indicates a band after cool-down that corresponds to M13 in a partially assembled state. U and F point to the gel migration of the unfolded and folded structure, respectively. The gel lanes framed by green rectangles correspond to the time points shown in the microscopy images; the time at which the imaging was performed is indicated in each panel. The bottom image in (c) is a TEM image of the 6HB DNA origami stained with 2% uranyl formate after purification by agarose gel electrophoresis. d) Annealing conditions and analysis method used for the folding of the structures in hydrated glycholine. Scale bars: 100 nm (a and b), inset: 25 nm, 200 nm (c).

gel electrophoresis, as determined by AFM and transmission electron microscopy (TEM).

The results presented here demonstrate that DNA self-assembly is not limited to aqueous or substantially hydrated solvents. Deep-eutectic solvents provide a non-aqueous and non-volatile alternative. The miscibility of glycholine with water also allows for the isothermal folding of more complex DNA structures in glycholine (and its hydrates) at room temperature, which can then be transferred to aqueous buffers to provide DNA structures with greater thermodynamic and kinetic stability as well as improved compatibility with enzymes. Furthermore, deep-eutectic solvents reduce the DNA melting temperature, which may enhance the exploration of the thermodynamic landscape via an initial dynamic molten-globule structure,[19] permitting the system to reach its thermodynamic minimum (i.e., the folded structure with maximum base pairing).

This study is a proof of principle for the self-assembly of a DNA structure in a water-free solvent and the isothermal folding of a more complex 3D DNA origami and a 2D tail-based system in a hydrated deep-eutectic solvent. For applications where the use of a complex DNA structure in anhydrous solvent is required, it would be possible to perform accelerated folding in hydrated glycholine followed by rendering the solvent anhydrous simply by the removal of water from glycholine under vacuum.

Our results, in combination with rational sequence design, should facilitate the development of a more general method that exploits the properties of glycholine and its hydrated forms for the synthesis of more complex DNA structures with fewer local and global defects—a key challenge for DNA nanotechnology.[7] It will also be important to further study the tunability of this solvent and its control over the kinetics and thermodynamics of DNA self-assembly. Deep-eutectic solvents represent an advantage for applications in plasmonic and semiconductor nanomaterials synthesis, where a water-based solvent fails.[12b,c,13] Bringing the exquisite control of DNA to such applications opens the door for new approaches to biosensors, nanofabrication, and nanoelectronics.

Keywords: deep-eutectic solvents · DNA nanotechnology · isothermal folding · self-assembly

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