Engineering DNA nanotubes for resilience in an E. coli TXTL system

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Abstract

Deoxyribonucleic acid (DNA) nanotechnology is a growing field with potential intracellular applications. In this work, we use an Escherichia coli cell-free transcription–translation (TXTL) system to assay the robustness of DNA nanotubes in a cytoplasmic environment. TXTL recapitulates physiological conditions as well as strong linear DNA degradation through the RecBCD complex, the major exonuclease in E. coli. We demonstrate that chemical modifications of the tiles making up DNA nanotubes extend their viability in TXTL for more than 24 h, with phosphorothioation of the sticky end backbone being the most effective. Furthermore, we show that a Chi-site double-stranded DNA, an inhibitor of the RecBCD complex, extends DNA nanotube lifetime significantly. These complementary approaches are a first step toward a systematic prototyping of DNA nanostructures in active cell-free cytoplasmic environments and expand the scope of TXTL utilization for bioengineering.

Key words: DNA nanostructure; in vitro systems; cell-free systems; DNA nuclease; synthetic biology.

1. Introduction

Several attempts to transition deoxyribonucleic acid (DNA) nanotechnology from ideal test tube conditions to living systems have been carried out in recent years (1). Interfacing DNA nanostructures with biology, however, faces many challenges, a major one being conferring resilience to the nanostructures for in vivo operation. In this work, we engineered synthetic DNA nanostructures and developed methods to increase their lifetime in an all Escherichia coli cell-free transcription–translation (TXTL) system. TXTL, used as an active cytoplasmic environment (2, 3), recapitulates the physiological conditions found in cells (Figure 1b), as well as harsh linear DNA degradation through the RecBCD complex.

Synthetic DNA can be designed to fold into unique nanoscale structures, which can perform specific tasks, such as drug delivery, intracellular scaffolding and synthetic-cell design (4–7). The diversity of DNA nanostructures, reported in the literature, illustrates the benefits of working with this versatile self-assembling material. Synthetic-DNA nanotubes assemble from DNA tiles via programmed interactions of the tile sticky ends (8, 9). Their geometry and mechanical properties are close to those of actin filaments and microtubules, suggesting that these nanotubes could serve as an artificial cytoskeleton in synthetic cells (8). They could operate as scaffolds to localize components, actively transport material and influence cell morphology. Further, DNA nanotubes tagged with fluorophores are easily observed with epifluorescence microscopy (Figure 1a), making characterization easier than for other nanometer-scale DNA structures.

Despite reports of DNA nanostructure stability in cell lysate and serum (10), we found that these nanotubes degrade within...
a few hours in active E. coli TXTL. This deficiency renders DNA nanotubes impractical for both synthetic-cell and intracellular applications that require operational viability for timescales exceeding 10 h (1). Previous reports on the stability of DNA origami in cell lysates incorporated sodium dodecyl sulfate (SDS) and deoxycholic acid (DCA), rendering the lysate inactive (10). We found that SDS and DCA inactivate TXTL as well, suppressing GFP expression (Supplementary Figure S1). This suggests that cellular lysates, which include SDS and DCA are not a good model system for a live cytoplasmic environment.

Herein, we explore the utility of modifying oligonucleotide strands to improve the resilience of DNA nanotubes and of modifying the composition of E. coli cell-free extract. We were able to increase the lifetime of nanotubes incubated in TXTL to more than 24 h by combining two approaches: inclusion of Chi6 dsDNA as a decoy for the complex RecBCD (11) and phosphorothioate (PS) bonding between bases of sticky ends of the tiles. By achieving lifetimes of over 24 h, we approach the ability to integrate tile-based DNA nanotubes with intracellular applications and gene expression systems. Our results suggest that two dominant contributors to degradation of DNA in E. coli cell extract are RecBCD and deoxyribonucleases (DNases) (12).

2. Materials and methods

2.1 TXTL preparation and reactions

The all E. coli cell-free TXTL system was prepared using BL21 Rosetta two competent cells from Novagen using procedures described previously (2, 3, 13, 14). Reactions are composed of 33% crude lysate with the remaining 67% being amino acids, salts, an energy buffer and other cofactors. A typical TXTL reaction is composed of 50 mM HEPES pH 8, 1.5 mM ATP and GTP, 0.9 mM CTP and UTP, 0.2 mg/ml tRNA, 0.26 mM coenzyme A, 0.33 mM NAD, 0.75 mM cAMP, 0.068 mM folinic acid, 1 mM spermidine, 30 mM 3-PGA, 2% PEG8000, 10–15 mM maltose or 20–40 mM maltodextrin, 1.5–3 mM of each of the 20 amino acids, 40–120 mM K-glutamate and 2–7 mM Mg-glutamate. Plasmid DNA concentration range from 0 to 2 nM while DNA nanotube concentration was kept at 200 nM. Chi6 dsDNA were used at 0 or 10 μM. The TXTL system used in this work is commercially available from Arbor Biosciences, under the name myTXTL.

2.2 Fluorescence anisotropy assays

Anisotropy and deGFP expression experiments were carried out in 2 μl reactions assembled using a Labcyte Echo Liquid Handler 550 in Costar 96 well, V-bottom plates. Kinetic experiments were carried out at 29°C or room temperature in a Biotek Synergy Neo2. To monitor GFP fluorescence, monochromators were set to excitation 485 nm and emission 528 nm. Parallel and perpendicular polarization was measured using a filter cube with an excitation filter of 540 nm and emission filter of 590 nm. The dichroic mirror in the filter cube had a cut-off of 540 nm.

2.3 Nanotube assembly

Lyophilized DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA, USA), resuspended in water, quantitated by UV absorbance at 260 nm using a Thermo Scientific Nanodrop 2000c Spectrophotometer, and stored at –20°C. All samples were stored or mixed using DNA Lo-bind tubes (# 022431021).

Nanotubes were annealed at either 3 or 1.8 μM tile concentration by mixing each tile strand at 3 or 1.8 μM (final concentration), in Tris-Acetate-EDTA (TAE) and 12.4 mM MgCl₂. Nanopure water was added to achieve the appropriate concentration of...
components. All nanotube designs, except those which were to be ligated, were annealed using an Eppendorf Mastercycler PCR machine by heating the sample to 90°C, and cooling it to 25°C over a 6h period. Nanotubes which were to be ligated were annealed by heating the sample to 90°C, and cooling it to 25°C over a 54h period.

Nanotubes were ligated at 1.2 μM using New England Biolabs (NEB) T4 DNA Ligase (# M0202S; 20,000 units) at 1.0 units/μl and NEB T4 DNA Ligase buffer (# B0202S, 10×, 10 mM MgCl₂, 50 mM Tris-HCl, 1 mM ATP, 10 mM DTT, pH 7.5 at 25°C) at 1×. Nanotubes were incubated at room temperature for at least 2 days in ligation solution preceding experiments.

### 2.4 Fluorescence microscopy

Nanotube samples were imaged using an inverted microscope (Nikon Eclipse Ti-E) with 60×/1.40 NA oil immersion objectives. Samples containing nanotubes were imaged at 200 nM tile concentration in E. coli cell extract with either 0 or 10 μM Chito-Toehold (11). Samples were imaged in chambers made using a Bio-Rad frame seal (# SLF0601; size: 15 × 15 mm, 65 μl) placed on Fisherbrand microscope cover glass (# 12-545E No. 1, thickness: 0.13–0.17 mm; size: 50 × 22 mm); VWR Micro Slides (Plain, Selected, Pre-cleaned, 25 × 75 mm, 1.0 mm thick, # 48300-025) were placed on the frame seal to complete the imaging chamber. Each sample chamber was imaged throughout the duration of the experiments. The fluorescence microscopy image in Figure 1a was prepared as described by Rothemund et al. (8). The sample preparation method described by Rothemund et al. (8) results in images of distinct, individual nanotubes in one focal plane, as the nanotubes are adhered to the surface of the coverslip. In contrast, imaging DNA nanotubes in crowded environments, such as TXTL, results in bundling of nano-tubes in one focal plane, as the nanotubes are adhered to the surface of the coverslip. To test the bundling effect of TXTL, we compared the fluorescence microscopy images of nanotubes in TXTL with those of nanotubes in E. coli cell extract, as described by Rothemund et al. (8).

Gel electrophoresis was done at 100 V for 90 min, followed by 20 min of gel staining in 1× TBE with 0.2× Syber Gold (Invitrogen, S11494). Imaging was done using a ChemiDoc MP Imaging System (Bio-Rad, Universal Hood III).

### 3.2 Nanotube tile structure influences degradation rates

Nanotube tile structure, for example sticky end length or inclusion of additional binding sites, can be designed to suit different purposes. To thoroughly characterize degradation of nanotubes with different tile structures in TXTL, we worked with four different tile variants. Increasing the length of the sticky ends results in an increased melting temperature of nanotubes. We considered nanotubes with either 5-base (5b) or 8-base (8b) sticky ends (Supplementary Figures S2 and S3). For each case, we considered an additional tile variant that includes a toehold region on one of the sticky ends (Supplementary Figure S4). The toehold region can be used to link other ligands to the nanotubes (17), or to incorporate strand displacement reactions at the sticky ends (18). We examined degradation of 5b and 8b nanotubes both with (5bT, 8bT) and without (5bNT, 8bNT) toeholds.

Of the four designs studied, the 8b nanotubes outlast the 5b nanotubes at both room temperature and 29°C (Figure 2 and Supplementary Figures S5–S7). At room temperature, 8b nanotubes degrade in under 3 h (Figure 2a), while 5bT and 5bNT nanotubes degrade in under an hour (Supplementary Figure S5a). Degradation occurs more quickly at 29°C, with 8bNT nanotubes degrading within 3 h (Supplementary Figure S7 and Supplementary Table S4). The time constant $b_1$ is reported in Supplementary Table S4 for all variations of nanotubes, temperature and Chi concentration. To quickly assess nanotube robustness in TXTL, we first tested two nanotube variants in which tiles have 8 base-long sticky ends, with and without a single stranded extension (toehold) (Figure 2 and Supplementary Figure S7a). The rapid degradation of the DNA nanotubes observed by fluorescence microscopy was confirmed by fluorescence anisotropy. The decrease in anisotropy begins immediately, suggesting digestion of the DNA nanotubes begins within 5 min of their addition to the extract (Figure 2b). In general, we observe that the anisotropy signal of nanotubes incubated in TXTL decreases reaching a steady-state comparable to that of a control sample of DNA nanotubes incubated with DNAse I at room temperature in TAE buffer (Supplementary Figure S10). Nanotubes assembled from tiles without a toehold (8bNT) were slightly more robust than the toeholded variant based on microscopy data. The effect was insignificant in anisotropy assays. We hypothesize that the toehold provides an easier access to RecBCD for degradation.
Table S4), and 5bT, 5bNT and 8bT nanotubes degrading in under an hour (Supplementary Figures S6 and S7, Table S4).

3.3 Nanotube lifetime increased in presence of Chi and chemical modification of the tiles

We hypothesized that by adding double stranded (ds) DNA molecules containing six repeats of the Chi sequence (5’-GCTGGTGG-3’) to TXTL reactions, we would see an increase in DNA nanotube lifetime (11). We recently demonstrated that Chi6 inhibits the degradation of linear dsDNA in TXTL by sequestering RecBCD, with the optimal concentration of Chi6 for protein expression being 2–4 μM. We choose to use 10 μM Chi6 in order to maximize nanotube stability at the cost of slightly depressed protein expression (Supplementary Figure S11).

At room temperature, 8bT and 8bNT nanotubes last up to 10 h in the presence of 10 μM Chi6 in the cell-free extract (Figure 2c). Anisotropy assays indicate that the nanotube degradation is not complete, evidenced by the fact that the absolute values of anisotropy for 8bT and 8bNT nanotubes with 10 μM Chi do not converge with those of the 8bT and 8bNT in the absence of Chi (Figure 2d). This suggests that aggregates not visible in microscopy experiments might remain for time periods greater than 10 h.

Robustness of nanotubes under different environmental stresses, such as chemical or thermal stress, is improved with ligation (19). Ligation seals the breaks, or nicks, in the sugar-phosphate backbone found at the four corners of inter-tile junctions (Figure 3a). Although the ligation efficiency for the 5’ end of strand SE2 was lower than that of the other strands, we verified ligation of all four corner nicks via denaturing gel (Supplementary Figure S12). Both the microscopy images (Figure 3b, Supplementary Figure S8), and the time constants obtained from the fluorescence anisotropy observations (Figure 3c) suggest that ligation enhances nanotube robustness in TXTL (Supplementary Table S4). Increasing the ligation efficiency could lead to even greater improvement in the resilience of the DNA nanotubes in TXTL.

Nucleases, such as DNase I, are inhibited by PS bonded DNA. PS bonds connect the sugar-phosphate backbone of one base to another, much as a phosphodiester bond does in native DNA, with sulfur replacing the native oxygen (Supplementary Figure S4b). Exonucleases are inhibited by PS bonds located at the ends of DNA, while endonucleases are inhibited by incorporating PS bonds into the entire backbone of a DNA structure (20).

Because the 8bNT nanotube design is the most stable in TXTL, we designed 8bNT tiles with the 8 bases of the sticky ends of the tiles (Figure 3d). By modifying the sticky ends, we expected to impede nanotube degradation by RecBCD and other exonucleases. The lifetimes of PS bonded DNA nanotubes was more than double that of unmodified 8bNT nanotubes, as observed by fluorescence microscopy and fluorescence anisotropy (Figure 3e and f). At room temperature, 8bPS tubes lasted up to 10 h without the Chi6 DNA, with aggregates of degraded tubes visible at 10 h. In the presence of the Chi6 DNA, at room temperature, 8bPS nanotubes remained at least 24 h in TXTL. At 29°C, 8bPS nanotubes degraded in under 3 h without the Chi6 DNA and between 3 and 6 h with the Chi6 DNA (Supplementary Figure S9).

4. Summary and conclusions

DNA nanotubes are rapidly degraded in the TXTL environment. Incorporating Chi DNA into the cell-free system extends the lifetime of the DNA nanotubes, confirming the previous observations that RecBCD is the major actor responsible for degradation (11). Modifications to the DNA tiles themselves also affect the lifetime of the DNA nanotubes. Lengthening the tile sticky
ends increases nanotube stability, and eliminating toehold overhangs increases the lifetime even further. PS bonding on the sticky ends of the nanotubes was the most effective tile modification, while ligating these same domains had a small effect.

This study is limited to DNA nanotubes and does not consider what the chemical modifications to the cell-free extract or to the DNA self-assembling components may have on other DNA structures, such as DNA origami (7) or crystals (21). We conjecture that more tightly packed assemblies such as origami might be stable for several days with appropriate chemical modifications. It is unclear if other modifications to the extract could be used to prevent other nucleases from acting on DNA in the cytoplasmic environment as Chi DNA was used to specifically target RecBCD. Chi DNA is specific to RecBCD so it would not extend DNA nanotube lifetimes in cytoplasmic environments other than E. coli. However, in these cases we expect that it would still be possible to mitigate nanotube degradation by introducing or up-regulating the concentration of competitive binding sites for the corresponding enzymes.

While DNA nanostructures have a variety of potential applications in biological contexts, the stability of these structures has not been previously thoroughly demonstrated in cell-like environments mimicking cytoplasmic conditions. Many DNA structures are designed with the intention for intracellular use, as scaffolding, drug delivery or synthetic cell components, making the absence of characterization of these structures in cell-like environments a considerable impediment to these aims.

We demonstrated that TXTL can be used as an experimental platform to test the robustness of DNA nanostructures and to engineer new ones with longer lifetimes in cytoplasmic environments. This method could be used as a testbed to rapidly prototype robust DNA nanostructures for in vivo applications.

Supplementary data
Supplementary Data are available at SYNBIO Online.

Acknowledgements
We thank PWK Rothemund for discussions about DNA nanotubes.

Funding
U.S. Department of Energy under Award Number [DESC0010595 to EF], which paid for reagents and supplies used at UC Riverside; the Human Frontier Science Program [RG0037/2015 to V.N.].

Figure 3. Enhancing the stability of DNA nanotubes in TXTL systems with Chi-site DNA and chemical modifications (ligation or phosphorothioate bonding of sticky ends). (a) Cartoon of ligation. (b) Fluorescence microscopy kinetics of 5-base DNA nanotubes with a ligation of the sticky ends of the tiles incubated in E. coli TXTL reactions with and without Chi-site DNA present. (c) Fluorescence anisotropy kinetics, and exponential decay model prediction (solid lines), of a single stranded DNA oligo (SE3ss) and 5-base DNA nanotubes with (S1245P) and without (5bNT) a ligation of the sticky ends of the tiles incubated in E. coli TXTL reactions with and without Chi-site DNA present. (d) Cartoon of phosphorothioation. (e) Fluorescence microscopy kinetics of 8-base DNA nanotubes with phosphorothioate bonded sticky ends of the tiles incubated in E. coli TXTL reactions with and without Chi-site DNA present. (f) Fluorescence anisotropy kinetics of a single stranded DNA oligo (SE3ss) and 8-base DNA nanotubes with (PSE) and without (8bNT) the phosphorothioation of the sticky ends of the tiles incubated in E. coli TXTL reactions with and without Chi-site DNA present. The predictions of our exponential decay model are shown as solid lines (Supplementary Table S4). All the reactions were incubated at room temperature.
Conflict of interest: Noireaux laboratory receives research funds from Arbor Biosciences, a distributor of myTXTL cell-free protein expression kit.

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