Self-Assembly of Gamma-Modified Peptide Nucleic Acids into Complex Nanostructures in Organic Solvent Mixtures

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Abstract

Current strategies in DNA and RNA nanotechnology enable the self-assembly of a variety of nucleic acid nanostructures in aqueous or substantially hydrated media. In this article, we describe detailed protocols that enable the construction of nanofiber architectures in organic solvent mixtures through the self-assembly of uniquely addressable, single-stranded, gamma-modified peptide nucleic acid (γPNA) tiles. Each single-stranded tile (SST) is a 12-base γPNA oligomer composed of two concatenated modular domains of 6 bases each. Each domain can bind to a mutually complimentary domain present on neighboring strands using programmed complementarity to form nanofibers that can grow to microns in length. The SST motif is made of 9 total oligomers to enable the formation of 3-helix nanofibers. In contrast with analogous DNA nanostructures, which form diameter-monodisperse structures, these γPNA systems form nanofibers that bundle along their widths during self-assembly in organic solvent mixtures. Self-assembly protocols described here therefore also include a conventional surfactant, Sodium Dodecyl Sulfate (SDS), to reduce bundling effects.

Introduction

Successful construction of numerous complex nanostructures[1,2,3,4,5,6,7,8,9,10,11,12] in aqueous or substantially hydrated media made using naturally occurring nucleic acids such as DNA[1,2,3,4,5,6,7,8,9,10] and RNA[11,12] has been shown in previous works. However, naturally occurring nucleic acids undergo duplex helical conformational changes or have reduced thermal stabilities in organic solvent mixtures[13,14].

Previously, our lab has reported a method towards the construction of 3-helix nanofibers using gamma-position modified synthetic nucleic acid mimics called gamma-peptide nucleic acids (γPNA)[15] (**Figure 1A**). The need for such a development and potential applications of the synthetic nucleic acid mimic PNA has been discussed within the field[16,17]. We have shown, through an adaptation of the single-stranded tile (SST) strategy presented for DNA nanostructures[18,19,20], that 9 sequentially distinct γPNA oligomers can be designed to form 3-helix nanofibers in select polar aprotic organic solvent mixtures such as DMSO and DMF. The γPNA oligomers were commercially ordered with modifications of (R)-diethylene glycol (mini-PEG) at three γ-positions (1, 4 and 8 base-positions) along each 12-base oligomer based on methods published by Sahu *et al*.[21] These gamma-modifications cause the helical pre-organization that is associated with the higher binding affinity and thermal stability of γPNA relative to unmodified PNA.

This article is an adaptation of our reported work in which we investigate the effects of solvent solution and substitution with DNA on the formation of γPNA-based nanostructures[15]. The aim of this article is to provide detailed descriptions of the design as well as detailed protocols for solvent-adapted methods that were developed for the self-assembly and characterization of γPNA nanofiber. Thus, we first introduce the modular SST strategy, a general platform for nanostructure design using the synthetic nucleic acid mimic PNA.

The helical pitch for PNA duplexes has been reported to be 18 bases per turn in comparison to DNA duplexes, which undergo one turn per 10.5 bases (**Figure 1B**). Therefore, the domain-length of the demonstrated γPNA SSTs was set at 6 bases to accommodate one third of a full turn or 120° of rotation to enable interaction between three triangularly arrayed helices. Also, unlike previous SST motifs, each SST contains just 2 domains, effectively creating a 1-dimensional ribbon-like structure that wraps to form a three-helix bundle (**Figure 1C**). Each 12-base γPNA oligomer is gamma modified at the 1, 4 and 8 positions to ensure uniformly spaced distribution of mini-PEG groups across the overall SST motif. Additionally, within the motif, there are two types of oligomers: “contiguous” strands that exist on a single helix and helix-spanning “crossover” strands (**Figure 1D**). In addition, oligomers P8 and P6 are labelled with fluorescent Cy3 (green star) and biotin (yellow oval), respectively (**Figure 1D**), to enable detection of structure formation using fluorescence microscopy. Altogether, the SST motif is made of 9 total oligomers to enable the formation of 3-helix nanofibers through programmed complementarity of each individual domain to the corresponding domain on a neighboring oligomer (**Figure 1E**).

Protocol

1. γPNA sequence design

1. Download the DNA Design Toolbox[22] developed by the Winfree Lab at Caltech[23] into the folder containing programming scripts for designing sequences.
2. Within that sequence design folder, open a fourth-generation programming language compatible with the file extension “.m”, and then add the previously downloaded “DNAdesign” folder to the path using the following command:
   ```
   >> addpath DNAdesign
   ```
3. Subsequently run the following script named “PNA3nanofiber.m” (see **Supplementary Figure 1**) using the following command:
   ```
   >> PNA3nanofiber
   ```
4. Run this script to create variables called “thisSeqs” and “thisScore.” The variable “thisSeqs” contains the sequences of the designed oligomers, and “thisScore” is the penalty score.

5. Run the script multiple times to obtain the most minimal score. A sample of 20 such runs are shown in **Table 1**.
6. Manually confirm the desired Watson-Crick complementarity of each domain of the generated sequences for the specified SST structural motif. Sequence specifications for the 3-helix nanofiber structure are shown in **Table 2**.
7. Verify the following for generated sequences.
    1. Avoid using four consecutive C and G bases.
    2. Manually select specific sequences for N-terminal functionalization with fluorescent dye and biotin molecules to enable fluorescent microscopy studies.
    3. Include a minimum of 3 mini-PEG gamma-modifications on the backbone to enable pre-organized helical conformation in the single-stranded oligomers as described by Sahu *et al*.[21]

2. Preparation of γPNA stock strands

1. Obtain γPNA component strands of specified sequences at 50 nmol scale synthesis with high performance liquid chromatography (HPLC)purification from a commercial manufacturer.
2. Resuspend each strand in deionized water to 300 µM concentrations. Store the resuspended strands in -20 °C freezer up to several months until needed for experimentation.

3. Melting curve studies of γPNA oligomer subsets

1. Obtain melting temperature ranges for different combinations of complementary 2-oligomer and 3-oligomer subsets by running a melt curve study in aqueous buffers such as 1x phosphate buffered saline (PBS) or preferred polar organic solvent such as Dimethylformamide (DMF) or Dimethyl sulfoxide (DMSO) (see **Figure 2**).

NOTE: Thermal melting curves at >50% of DMF start to lose the upper baseline and show severe disturbances partly because of high absorbance of DMF at the wavelength required for the experiments. This is a noted phenomenon in the literature[24]. It is however possible to obtain the melting curves at 5 µM per strand concentrations in these solvent conditions.

1. Aliquot 16.7 µL from the 300 µM stock of each oligomer and make the final volume to a 1000 µL either in 1x PBS or 100% (v/v) DMSO or 100% (v/v) DMF effectively obtaining 5 µM final concentration per oligomer. Transfer this oligomer subset mixture to a 1 cm optical path, quartz cuvette.
2. Perform variable temperature UV-Vis experiments in a spectrophotometer equipped with a programmable temperature block.
3. Collect data points for the melting curves over a temperature range of 15–90 °C for both cooling (annealing) and heating (melting) cycles at a rate of 0.5–1 °C/min. Keep the samples for 10 min at 90 °C before cooling and at 15 °C before heating. Determine the melting temperature (T_m) from the peak of the first derivative of the heating curve.
4. Verify that melting temperature ranges for 2-oligomer subsets are above 35 °C in all solvent cases. Additionally, verify that the corresponding 3-oligomer subsets that contain an additional strand to their equivalent 2-oligomer subset shows a considerable increase in T_m due to increased co-operativity.

NOTE: This would verify that a single pot self-assembly of multiple oligomers can co-operatively fold into the desired nanostructure with reasonable thermal stability.

4. Self-assembly protocol for multiple distinct γPNA oligomers

NOTE: To devise a self-assembly thermal ramp protocol for γPNA nanostructures, slow-ramp annealing is desirable.

1. In the case of oligomer sequences generated, anneal the samples for 22.5 h in a thermal cycler cooling from 90 to 20 °C. Typically, melting temperature obtained for 2-oligomer and 3-oligomer γPNA subsets lie in ranges of 40–70 °C for different solvent conditions.
2. Program the thermal cycler as follows: hold at 90 °C for 5 min, ramp down from 90 to 70 °C at a constant rate of 0.1 °C/min, ramp down from 70 to 40 °C at a rate of 0.1 °C/3 min, ramp down from 40 to 20 °C at a rate of 0.1 °C/min and hold at 4 °C (see **Table 3**). Samples can be stored in 4 °C for 12–24 h before characterization.

   NOTE: While 2- and 3- oligomer subsets of our nanofiber system can form in 1x PBS, the full micron-scale nanofibers aggregate in 1x PBS. Therefore, solvent conditions should be optimized based on the scale and size of structure being formed as well as the type and density of gamma modifications.
3. For micron scale long 3-helix nanofibers, prepare anneal batch samples in 75% DMSO: H_2O (v/v), 75% DMF: H_2O (v/v), 40% 1,4-Dioxane: H_2O (v/v) based on solvent optimization studies[15].
4. Prepare anneal batches as follows (see **Table 4**). First, prepare 10 µL sub-stocks at 20 µM concentrations from the 300 µM main stocks for each oligomer by aliquoting 0.67 µL from the main stock and making the volume to 10 µl using deionized water.
5. Aliquot 1 µL from the 20 µM sub-stocks for each oligomer and add it to a 200 µL PCR tube. This accounts for a total volume of 9 µL for 9 oligomers.
6. Add either 30 µL of anhydrous DMSO/DMF for the 75% DMSO and 75% DMF cases with an additional 1 µL of deionized water to make a final volume of 40 µL with each oligomer at 500 nM final concentrations. Add 16 µL of 1,4-Dioxane and make the volume with deionized water to 40 µL for the 40% Dioxane solvent condition.
7. Load the anneal batches on to the thermal cycler and anneal using the protocol mentioned in step 4.2.

5. Total internal reflection fluorescence (TIRF) microscopy imaging

1. Prepare a humidity chamber from an empty pipette tips box. Fill the box with approximately 5 mL of water to prevent drying of the sample flow channels described as follows (see **Figure 3**).
2. Prepare flow chamber with a microscope slide, 2 double-sided tape strips, and nitrocellulose-coated coverslip. To coat the coverslip with nitrocellulose, dip the coverslip in a beaker containing 0.1% collodion in amyl acetate and air-dry.
   1. Prepare the nitrocellulose solution by making a 20-fold dilution from commercially available 2% collodion in amyl acetate with isoamyl acetate solvent.
3. Prepare biotinylated bovine serum albumin (Biotin-BSA) solution by weighing 1 mg of Biotin-BSA and dissolving it in 1 mL of 1x PBS. Flow 15 µL of 1 mg/mL Biotin-BSA in 1x PBS buffer by placing the flow channel at an

angle. Incubate the flow channel for 2–4 min at room temperature in a humidified chamber.

4. Prepare the wash buffer by dissolving 1 mg of BSA in 1 mL of 1x PBS. Wash the excess Biotin-BSA by flowing 15 µL of the wash buffer. To passivate the surface, incubate the flow channel for 2–4 mins at room temperature in a humidified chamber.

5. Prepare the streptavidin solution by measuring 0.5 mg of streptavidin and 1 mg of BSA and then dissolving using 1 mL of 1x PBS. Flow 15 µL of 0.5 mg/mL streptavidin in 1x PBS containing 1 mg/mL BSA. Incubate the flow channel for 2–4 min at room temperature in a humidified chamber. Flow 15 µL of the wash buffer to wash away unbound Streptavidin.

6. Flow 15 µL of previously annealed batch of γPNA oligomers at 500 nM concentration per strand in either 75% DMSO, 75% DMF or 40% 1,4-Dioxane condition. Incubate the flow channel for 2–4 mins at room temperature in a humidified chamber.

7. Prepare 1 mM Trolox in preferred solvent conditions by diluting 10-fold from a 10 mM stock of Trolox in DMSO (measure 2.5 mg of Trolox and dissolve in 1 mL of DMSO). Wash the unbound nanostructures in the flow channel with 15 µL of 1 mM Trolox having the same solvent composition as the nanostructures.

   NOTE: The >50% DMSO content in the flow channel would produce minor signal disturbances due to the different index of refraction of the solvent condition during TIRF which is typically calibrated for coverslip-water TIRF angles. This can be rectified by washing with 1 mM Trolox made by diluting the 10 mM Trolox 10-fold using deionized water. This technique provides clearer images but is only useful for assessing whether formation occurred, however, because it produces two-phase micro-bubbles in the flow channel. As a result, nanostructures might be visible at the interface of the two-phases as shown in **Figure 4D**.

8. Transfer the flow channel on to the slide holder and image using a fluorescence microscope equipped with TIRF imaging using a 60x oil-immersion objective and a 1.5x magnifier. Scan the flow channel at either 60x or 90x magnification by monitoring the Cy3 channel (see **Figure 4**).

6. Transmission electron microscopy (TEM) imaging

1. Weigh 0.5 g of uranyl acetate in 50 mL of distilled water to prepare a 1% aqueous uranyl acetate stain solution. Filter the 1% aqueous uranyl acetate stain solution using a 0.2 µm filter attached to a syringe.

   NOTE: Alternatively, 2% aqueous uranyl acetate could be purchased from a commercial manufacturer.

2. Purchase commercially available formvar support layer coated Copper grids with 300-mesh size.

   NOTE: It is important to note that the formvar support layer could be dissolved away by solvents like DMSO beyond 2 min. For longer sample incubation, commercially available formvar stabilized with silicon monoxide on copper grids are available which allow the grid to be more hydrophilic than carbon-coated grids and can withstand vigorous sample conditions and electron beam as shown in **Figure 5C**.

3. Pipette 4 µL of sample onto the grid for 15 s. Use a piece of filter paper to wick off the sample by bringing the filter paper in contact with the grid from the side.

4. Immediately add 4 µL of the stain solution onto the grid for 5 s.

5. Wick off the stain as before and hold the filter paper against the grid for 1–2 min to make sure the grid is dry. Samples should be typically imaged within 1–2 h after staining. Alternatively, if the grid is ensured to be completely dry, grids can be stored in a TEM grid storage box for up to 3 days before imaging.
6. Transfer the grid to a TEM specimen holder and image using a transmission electron microscope operated at 80 kV with magnification ranging from 10 K to 150K (see **Figure 5**).

7. Different morphologies for γPNA-DNA hybrids based on selective replacement with DNA

1. Obtain DNA oligomers of specified sequences (see **Table 5**) from commercial oligonucleotide manufacturers synthesized at 25 nmol scale using standard desalting. Resuspend these DNA sequences using RNase-free deionized water at 20 µM stock concentrations.
2. For contiguous γPNA strand replacements with DNA, sequences D3, D5 and D9 can replace strands p3, p5 and p9. Similarly, for crossover γPNA strand replacements with DNA, sequences D1, D4 and D7 can replace strands p1, p4 and p7.
3. Aliquot 1 µL from the 20 µM sub-stocks for each γPNA or DNA oligomer and add it to a 200 µL PCR tube as in step 2.7. Add 30 µL of anhydrous DMSO and 1 µL of RNase-free deionized water to make the final volume to 40 µL (see **Table 6**).
4. Load the anneal batches on to the thermal cycler and anneal using the protocol mentioned in step 4.2.
5. Characterize γPNA-DNA hybrid nanostructures using the TIRF protocol following steps in section 5 or using TEM imaging protocol mentioned in section 6 (see **Figure 6**).

8. Different morphologies for γPNA nanofibers in varying concentrations of SDS

1. Prepare a 20% (wt/v) SDS main stock by measuring 20 mg of SDS and dissolving it in 100 µL of deionized water.
2. Prepare a 6% (wt/v) SDS sub-stock by aliquoting 3 µL from the 20% SDS stock and making the volume to 10 µL using deionized water.
3. Anneal the γPNA oligomers in final concentrations of 5.25 mM and 17.5 mM SDS as follows. Aliquot 1 µL from the 20 µM sub-stocks for each γPNA oligomer and add it to a 200 µL PCR tube as in step 2.7. Add 30 µL of anhydrous DMSO and 1 µL of 6% and 20% SDS to make the final volume to 40 µL to achieve SDS final concentrations of 5.25 mM and 17.5 mM, respectively (see **Table 7**).
4. Load the anneal batches of varying SDS concentrations on to the thermal cycler and anneal using the protocol mentioned in step 4.2.
5. Characterize γPNA nanostructures in the presence of SDS using the TIRF protocol following steps in section 5 or using TEM imaging protocol mentioned in section 6 (see **Figure 7**).

Representative Results

The protocols discussed in the sections above describe the design of an adapted SST motif from DNA nanofibers for the robust generation of self-assembled nanofibers structures using multiple, distinct γPNA oligomers. This section describes the interpretation of data obtained from the successful recreation of the protocols described.

Following the protocol described in section 5 for TIRF imaging of samples of γPNA oligomers annealed in 75% DMSO: H_2O (v/v) most readily provides evidence of well-organized

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architectures under microscopic observations as shown in **Figure 4A**. 75% DMF: H_2O (v/v) solvent condition results in spicule-shaped or needle-like nanostructures (**Figure 4B**) and, in our experience, the 40% 1,4 dioxane: H_2O (v/v) condition shows sparse decoration of filamentous nanostructures when viewed using TIRF microscopy (**Figure 4C**). Furthermore, the samples of γPNA nanotubes formed in 75% DMSO: H_2O (v/v) demonstrate bundling of nanofibers at high magnification or nanoscopic resolutions during TEM imaging following steps mentioned in section 6 (**Figure 5**). Quantitative analyses of the width of the nanostructures showed a median width of 16.3 nm with maximum values beyond 80 nm[15] .

For devising a scheme towards generating γPNA-DNA hybrid nanostructures in organic solvent mixtures to enable further functionalization using DNA, it is important to consider the oligomeric position in the SST motif being replaced with isosequential DNA oligomers. Adapting steps mentioned in section 7 for the case of the nanotube construct described here, isosequential DNA oligomers that replace the contiguous γPNA oligomers forms straight filamentous structures whereas replacement of the crossover γPNA oligomers forms stellate structures when viewed using TIRF and TEM imaging (**Figure 6**). Quantitative analyses of the width of the nanostructures that were replaced with contiguous DNA oligomers showed median widths around 19 nm[15] .

Finally, upon adapting steps from section 8, γPNA nanofibers adopt thinner morphologies consistent with reduced bundling in the presence of SDS concentrations below its critical micelle concentrations (CMC, 8.2 mM). γPNA nanofibers also adopt highly networked morphologies at high SDS concentrations in comparison to its CMC when viewed using TIRF imaging (**Figure 7**). TEM imaging of γPNA oligomers self-assembled in the presence of SDS indicate that the 5.25 mM SDS condition indicate the most substantial reductions in structural bundling with widths ranging 8–12 nm as shown in (**Figure 7C**).

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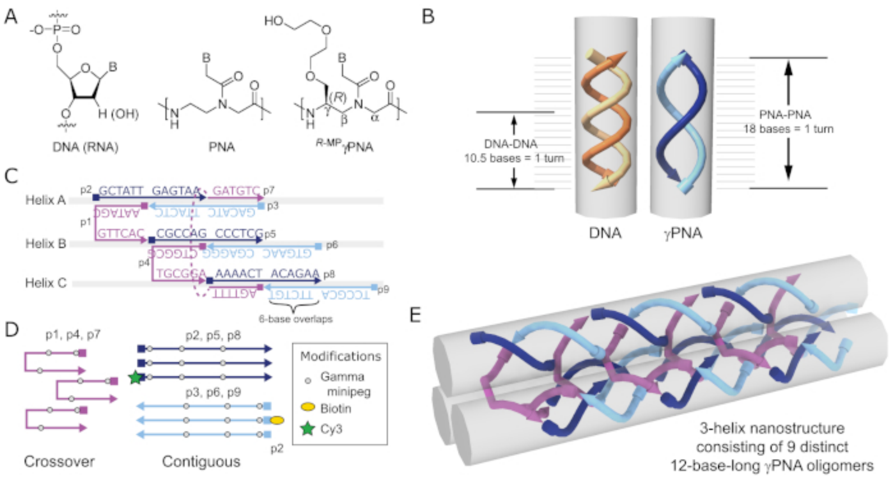

Figure 1: Peptide nucleic acid oligomers as building blocks for complex nanostructures.

(**A**) Chemical structures of DNA (PNA), PNA and MP-containing γPNA units. (Figure has been reprinted with permission from Ref[21] .) (**B**) PNA-PNA helices are less twisted than DNA-DNA helices, having 18 bases per turn instead of 10.5. (**C**) This three-helix structural single stranded tile (SST) motif consists of 9 distinct 12-base-long γPNA oligomers, each having two six-base domains. (**D**) Within the structure there are two types of oligomers: "contiguous" strands that exist on a single helix and helix-spanning "crossover" strands. (**E**) This 18-base-long structural motif can polymerize to form micron-scale filaments. Panels B, C and E have been modified with permission from Ref[15] . Please click here to view a larger version of this figure.

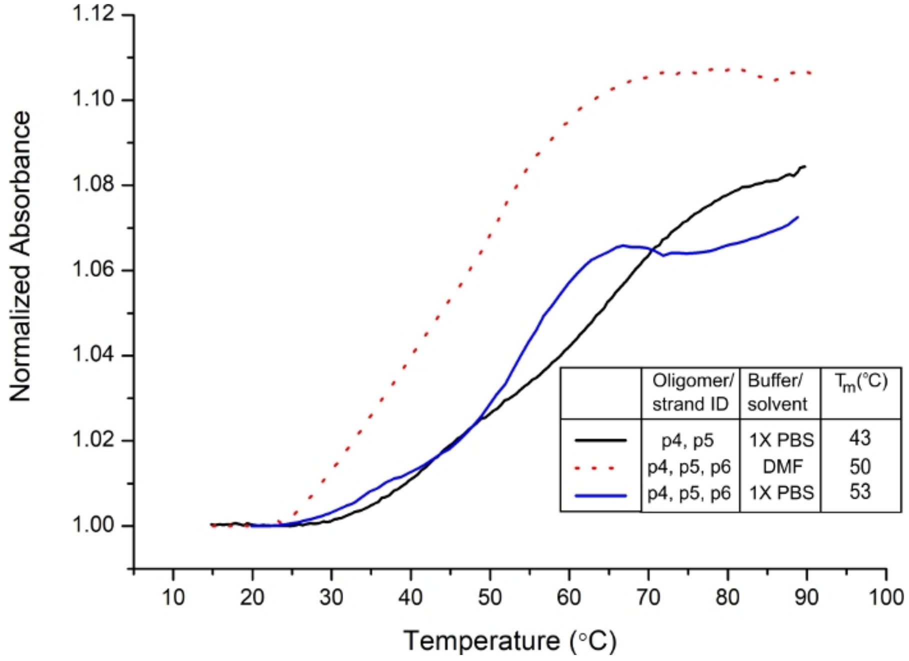

Figure 2: Representative melt curves of select γPNA oligomers in different solvent conditions.

2-oligomer (p4, p5) subset showing 6-base domains can bind with reasonable thermal stability (black curve) in 1x PBS. 3-oligomer (p4, p5, p6) subset shows increased thermal stability due to cooperativity in 1x PBS (blue curve) and shows little to no instability with respect to T_m when solvent is changed to organic solvents like DMF (red dotted curve). Figure has been modified with permission from Ref[15] . Please click here to view a larger version of this figure.

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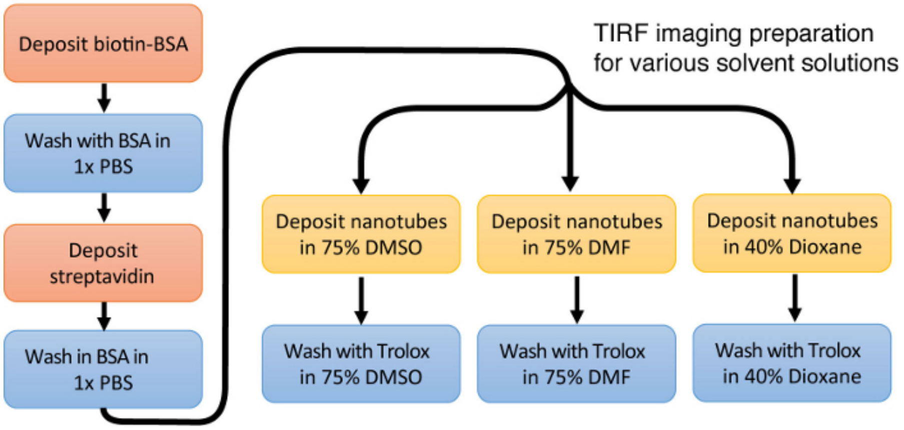


Figure 3: Flow chart for fluidic flow channel for sample TIRF imaging.

A step-by-step workflow indicating steps involved in sample preparation for TIRF imaging of γPNA nanofibers after flow channel assembly. Please click here to view a larger version of this figure.

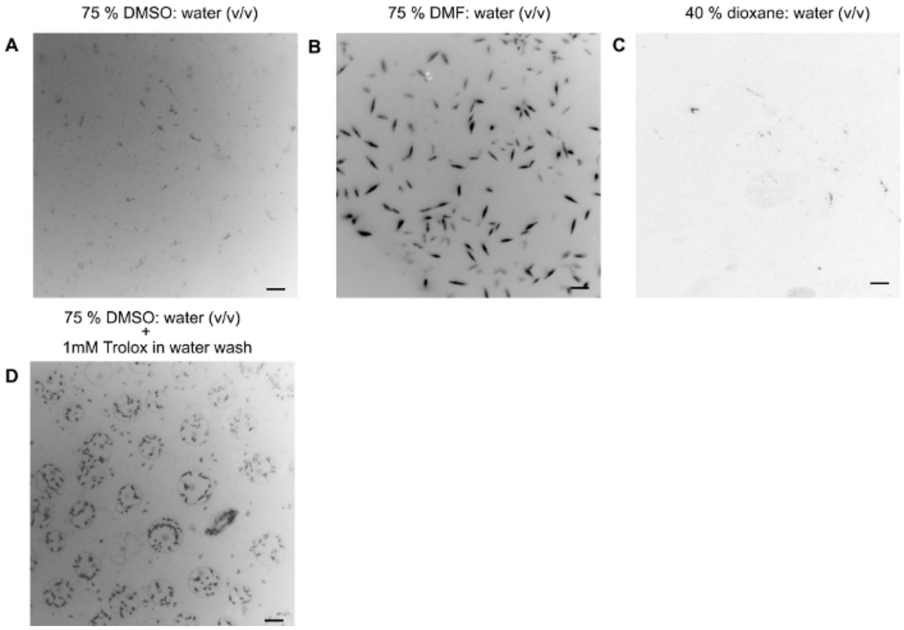
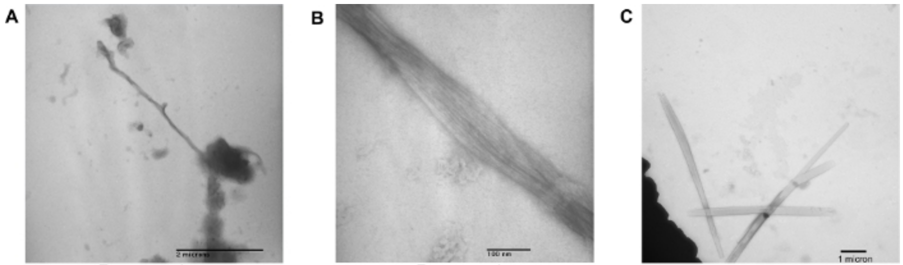


Figure 4: TIRF microscopy imaging of γPNA nanofiber self-assembled in different solvent conditions. γPNA nanofibers visualized using TIRF microscopy (5 µm scale bar) while monitoring the Cy3 channel when γPNA oligomers are self-assembled in (**A**) 75% DMSO: water (v/v), (**B**) 75% DMF: water (v/v) and (**C**) 40% Dioxane: water (v/v) solvent conditions. (**D**) When γPNA nanofibers self-assembled in 75% DMSO: water (v/v) bound to the flow channel is washed with 1mM Trolox in water, two-phase microbubbles of DMSO-water form with nanostructures aligning along the interface of the microbubble. Figure has been modified with permission from Ref[15] . Please click here to view a larger version of this figure.



Formvar only

Formvar only

Formvar-SiO

Figure 5: TEM imaging of γPNA nanofibers self-assembled in 75% DMSO: water. (v/v) using formvar support layer copper 300 mesh grids.

(**A**) TEM images of γPNA nanofibers visualized under low magnification (15x). (**B**) TEM images of γPNA nanofibers visualized under high magnification (150x) shows bundling of nanotubes along the width at nanoscopic resolutions. (**C**) TEM images of γPNA nanofibers visualized under low magnification (10x) using a Formvar-Silicon Monoxide support layer on a Copper grid allows imaging under vigorous specimen conditions. Figure has been modified with permission from Ref[15] .

Please click here to view a larger version of this figure.

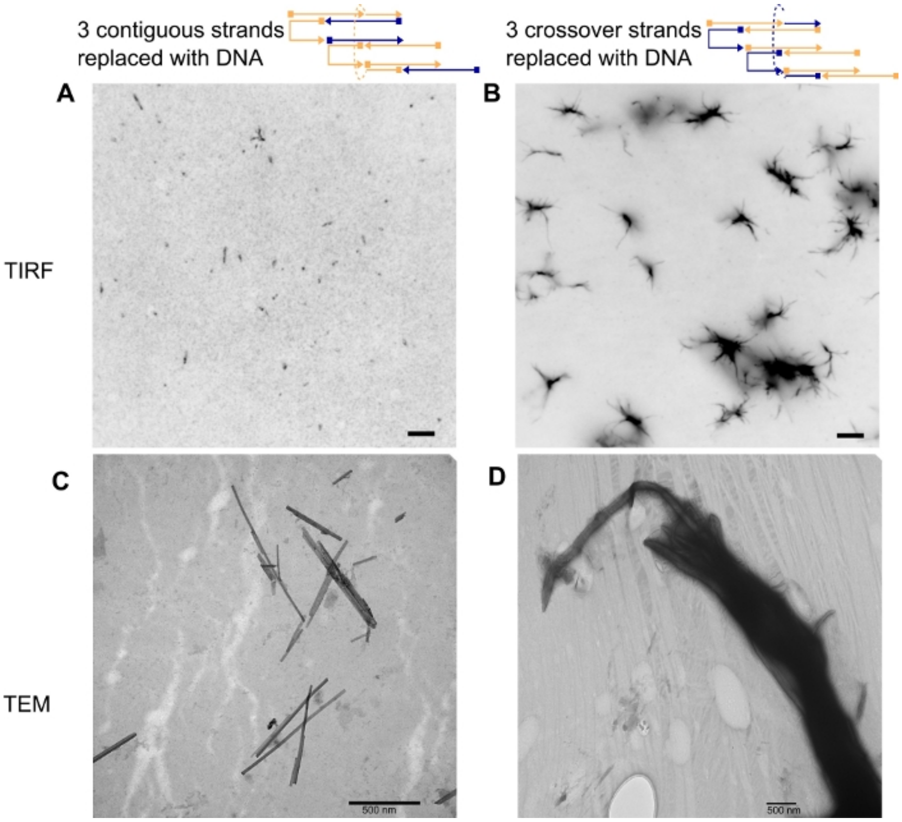


Figure 6: Different morphologies for γPNA-DNA hybrids based on selective replacement with DNA.

(**A**) TIRF (5 µm scale bar) and (**C**) TEM images (60x magnification) of self-assembly of γPNA-DNA hybrids upon replacing contiguous γPNA oligomers (p3, p5, p9) with isosequential DNA oligomers (D3, D5 D9) show nanotubes adopting a straight filament morphology. (**B**) TIRF (5 µm scale bar) and (**D**) TEM images (25x magnification) of self-assembly of γPNA-DNA hybrids upon replacing crossover γPNA oligomers (p1, p4, p7) with isosequential DNA oligomers (D1, D4 D7) show nanofibers adopting a stellate morphology. Figure has been modified with permission from Ref[15]. Please click here to view a larger version of this figure.

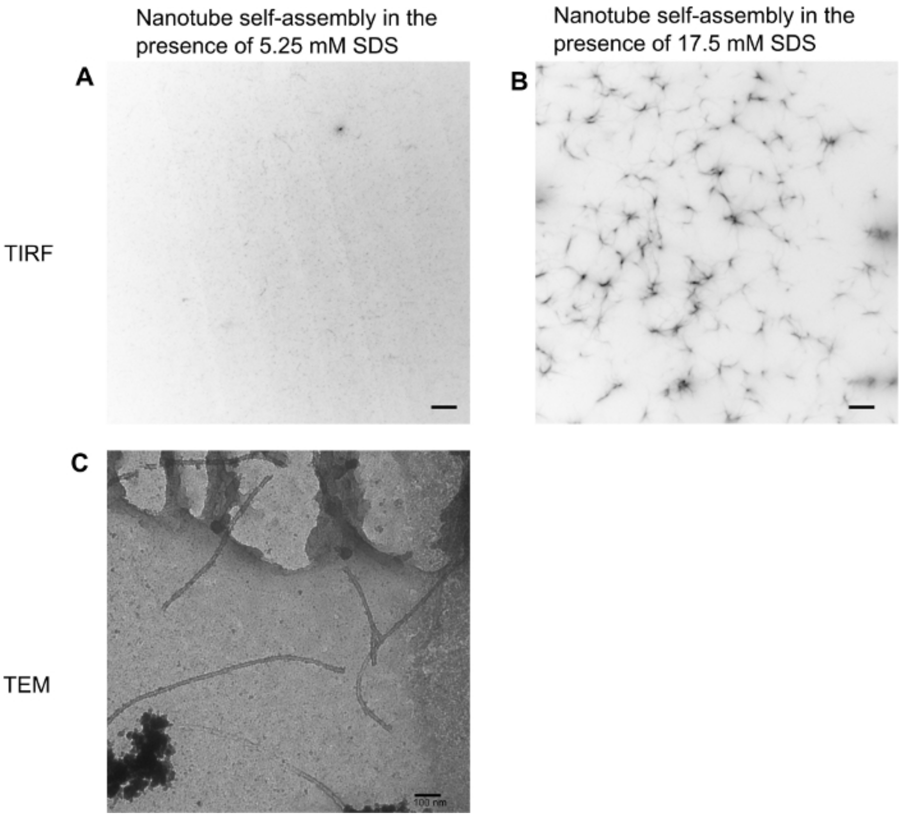


Figure 7: Different morphologies for γPNA nanofibers in varying concentrations of SDS.

TIRF images (5 µm scale bar) of self-assembly of γPNA nanofibers in the presence of SDS at concentrations of (**A**) 5.25 mM and (**B**) 17.5 mM. Self-assembly in the presence of SDS concentrations less than the CMC (8.2 mM) shows thinner morphologies from TIRF images based on fluorescence intensity. (**C**) This is verified by TEM images (100x magnification) of the system where median width for nanofibers lies in the range of 8–12 nm. At concentrations significantly above the CMC, γPNA nanotubes appear to form higher order assemblies through highly networked nanotube structures. Figure has been modified with permission from Ref[15] . Please click here to view a larger version of this figure.

MATLAB script string output "thisSeq"	Penalty Score "thisScore"
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'tgtagttggtct actacacccttg ggacatcaaggg ttcggcaggcgg gccgaacgctaa agaccattagcg acgagtatgtcc actcgtattttg ccgcctcaaaat '	0.0624
'tggaaccttgta gttccattcgca atcacctgcgaa ccacacttactg gtgtggtcggct tacaagagccga ggcgttggtgat aacgccctatct cagtaaagatag '	0.0656
'agaggtcgtatt acctctgtgagt cggtttactcac actttccagcaa gaaagtccatcc aatacgggatgg tagcccaaaccg gggctaaggcga ttgctgtcgcct '	0.0688
'cggcaaactatg ttgccgatttct acttacagaaat cgtgtcctaaag gacacgggatgg catagtccatcc tgaggcgtaagt gcctcacagcga ctttagtcgctg '	0.0704

Table 1: 20 sample algorithmic results for potential optimization of γPNA sequence design. 20 sample **algorithmic** results with sequence outputs in column 1 and their corresponding score in column 2. Repeated iterations of the script are performed to obtain the most minimal score.

γPNA Sequence ID	Sequence	Domain 1 complement	Domain 2 complement
p1	N-AATAGCGTTCAC-C	p2 domain 1	p6-Biotin domain 1
p2	N-GCTATTGAGTAA-C	p1 domain 1	p3 domain 2
p3	N-GACATCTTACTC-C	p7 domain 2	p2 domain 2
p4	N-CTGGCGTGCGGA-C	p5 domain 1	p9 domain 1
p5	N-CGCCAGCCCTCG-C	p4 domain 1	p6-Biotin domain 2
p6-Biotin	N-Biotin-GTGAACCGAGGG-C	p1 domain 2	p5 domain 2
p7	N-AGTTTTGATGTC-C	p8-Cy3 domain 1	p3 domain 1
p8-Cy3	N-Cy3-AAAACTACAGAA-C	p7 domain 1	p9 domain 2
p9	N-TCCGCATTCTGT-C	p4 domain 2	p8-Cy3 domain 2

Table 2: γPNA sequence design results for nanofibers. Individual oligomer sequences were generated as indicated in this table. Underlined bases indicate the gamma-position modifications with mini-PEG. Table has been modified with permission from Ref[15].

Temperature Range	Ramp rate
90 °C	Hold for 3 minutes
90-80 °C	0.1°C/minute
80-70 °C	0.1°C/minute
70-60 °C	0.1°C/3 minutes
60-50 °C	0.1°C/3 minutes
50-40 °C	0.1°C/3 minutes
40-30 °C	0.1°C/minute
30-20 °C	0.1°C/minute
4 °C	Hold indefinite

Table 3: Anneal ramp protocol for thermal cycler. Table has been modified with permission from Ref[15].

	Stock concentration	**75% DMSO: water (v/v) anneal sample**	**75% DMF: water (v/v) anneal sample**	**40% dioxane: water (v/v) anneal sample**	**Final Concentration**
γPNA oligomer (x9)	20 µM	9 µL (1 µL x 9)	9 µL (1 µL x 9)	9 µL (1 µL x 9)	500 nM
DMSO	-	30 µL	-	-	75 % (v/v)
DMF	-	-	30 µL	-	75 % (v/v)
1,4-dioxane	-	-	-	16 µL	40 % (v/v)
deionized water	-	1 µL	1 µL	15 µL	25 or 60% (v/v)
Total volume	-	40 µL	40 µL	40 µL	-

Table 4: Protocol for preparing anneal batches of γPNA oligomer in different solvent conditions.

DNA Sequence ID	**Sequence**
D1	5’-AATAGCGTTCAC-3’
D3	5’-GACATCTTACTC-3’
D4	5’-CTGGCGTGCGGA-3’
D5	5’-CGCCAGCCCTCG-3’
D7	5’-AGTTTTGATGTC-3’
D9	5’-TCCGCATTCTGT-3’

Table 5: Isosequential DNA sequences as replacement oligomers to γPNA. Table has been modified with permission from Ref[15] .

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	Stock concentration	DNA Contiguous strand replacements	DNA Crossover strand replacements	Final Concentration
γPNA oligomer (x6)	20 μM	6 μL (1 μL x 6)	6 μL (1 μL x 6)	500 nM
DNA oligomer (x3)	20 μM	3 μL (1 μL x 3)	3 μL (1 μL x 3)	500 nM
DMSO	-	30 μL	30 μL	75 % (v/v)
deionized water	-	1 μL	1 μL	25 % (v/v)
Total volume	-	40 μL	40 μL	-

Table 6: Protocol for preparing anneal batches of γPNA-DNA hybrid nanostructures in 75% DMSO: H_2O (v/v) **through replacement of contiguous or crossover γPNA oligomers with isosequential DNA oligomers.**

	Stock concentration	SDS concentrations below CMC	SDS concentrations above CMC	Final Concentration
γPNA oligomer (x9)	20 μM	9 μL (1 μL x 9)	9 μL (1 μL x 9)	500 nM
6% SDS	6% (wt/v)	1 μL	-	5.25 mM
20% SDS	20% (wt/v)	-	1 μL	17.5 mM
DMSO	-	30 μL	30 μL	75 % (v/v)
deionized water	-	-	-	25 % (v/v)
Total volume	-	40 μL	40 μL	-

Table 7: Protocol for preparing anneal batches of γPNA nanostructures in 75% DMSO: H_2O (v/v) **in the presence of SDS concentrations below and above CMC.**

Supplementary Figure 1: Programming script PNA3nanofiber.m for designing oligomer sequences. Please click here to download this figure.

Discussion

This article focuses on adapting and improving existing nucleic acid nanotechnology protocols towards organic solvent mixtures. The methods described here focus on modifications and troubleshooting within a defined experimental space of select polar aprotic organic solvents. There is yet unexplored potential for other established nucleic acid nanotechnology protocols to be adapted within this space. This could improve potential applications through integration in other fields such as polymer and peptide synthesis which typically are performed in similar organic solvents[25,26]. Additionally, we focus here on critical steps to be observed while practicing the above-mentioned protocols.

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During the preparation of sample for self-assembly, it is important to keep the volume percentages of water at 25% (v/v) for DMSO and DMF conditions. Accordingly, it is important to recognize that the organic solvent used for self-assembly should be from anhydrous stocks as well. The nanofiber structures are hydrophobic and aggregate in increased water content.

Unlike scaffolded DNA origami approaches that can create structures of discrete lengths, the current SST motif design for γPNA nanofibers and other previously established DNA SST nanotubes does not yield structures of discrete lengths. Nanotubes polymerize achieving a range of multi-micron lengths (up to 11 μm). For the same reason, yield associated with structure formation cannot be quantified.

However, due to the uncharged peptide backbone of γPNA, there are no dependencies on counter ion balance in relation to structure formation. Imaging buffers, therefore, do not need to include cations like Mg^{2+} typically required for the stabilization of nanostructures made from naturally occurring nucleic acids.

Lastly, γPNA nanostructure bundling and aggregation in different solvent conditions are also dependent on the individual oligomer concentrations introduced during self-assembly. Concentrations ranging from 1 μM and higher of individual oligomers increase the propensity for bundling and aggregation and affect clear imaging of nanostructures either during TIRF or TEM imaging.

Disclosures

The authors declare no competing financial interests.

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