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

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Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **Yes**.

   If **Yes**, can you record movies/images using your own microscope camera?

   Yes for fluorescence microscopy and No for TEM (we will only film talent using TEM)

   If your protocol involves microscopy but you are not able to record movies/images with your microscope camera, JoVE will need to use our scope kit (through a camera port or one of the oculars). Please list the make and model of your microscope.

   Nikon Eclipse Ti2 and Jeol JEM 1400

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Filming location:** Will the filming need to take place in multiple locations? **Yes**

   If **Yes**, how far apart are the locations? **1.1 miles (7-minute drive)**

Current Protocol Length

Number of Steps: 20
Number of Shots: 39, 5 reused

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Sriram Kumar:** This novel protocol describes the use of multiple, distinct sequences of the synthetic nucleic acid mimic γPNA to form nanostructures in specific organic solvent mixtures.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Sriram Kumar:** The assays described here show the need for adapting existing nucleic acid nanotechnology protocols towards organic solvents where little to no protocols have been published.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

OPTIONAL:

1.3. **Sriram Kumar:** Practitioners new to this technique would struggle to adapt protocols developed for aqueous environments into organic solvent-rich environments due to the propensity of the nanomaterial PNA to aggregate.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Protocol

2. Self-assembly Protocol for Multiple Distinct γPNA Oligomers

2.1. Begin by preparing the γPNA *(pronounce 'gamma-P-N-A')* stocks **[1]**. After obtaining HPLC-grade purified γPNA strands from a commercial manufacturer, resuspend each strand in deionized water to 300 micromolar concentrations **[2]**. Store the γPNAs at -20 degrees Celsius for up to several months **[3]**. *Videographer: This step is important!*

2.1.1. WIDE: Establishing shot of talent taking the γPNAs out of a storage or a shipping box.

2.1.2. Talent adding water to the γPNAs and resuspending them.

2.1.3. Talent putting the stock solutions in the freezer and closing the door.

2.2. When ready to perform self-assembly, prepare anneal batch samples in 75% DMSO, 75% DMF, and 40% 1,4-Dioxane. First, prepare 20 micromolar sub-stocks of each oligomer by aliquoting 0.67 microliters from the main stock and adding deionized water for a final volume of 10 microliters **[1]**. *Videographer: This step is important!*

2.2.1. Talent preparing sub stocks of the oligomers, with the DMSO, DMF, and 1,4-Dioxane containers in the shot.

2.3. Add 1 microliter of each oligomer from the 20-micromolar sub-stocks to a 200-microliter PCR tube, which will come to a total volume of 9 microliters for 9 oligomers **[1]**. Add 30 microliters of anhydrous DMSO or DMF with an additional 1 microliter of deionized water for a final volume of 40 microliters **[2]**. *Videographer: This step is important!*

2.3.1. Talent adding several oligomers to the PCR tube. *Videographer: Obtain multiple usable takes, this will be reused in 5.1.2.*

2.3.2. Talent adding DMSO or DMF to the tube.

2.4. To prepare the 40% 1,4-Dioxane batch, add 16 microliters of 1,4-Dioxane and bring the volume to 40 microliters with deionized water **[1]**.

2.4.1. Talent adding 1,4-Dioxane to a different PCR tube with oligomers, then adding water to the tube.

2.5. Anneal the samples for 22.5 hours in a thermal cycler cooling from 90 to 20 degrees Celsius. Typically, melting temperatures obtained for 2-oligomer and 3-oligomer γPNA subsets lie in the range of 40 to 70 degrees Celsius for different solvent conditions **[1]**. *Videographer: This step is important!*

2.5.1. Talent putting the PCR tubes in the thermocycler and closing the lid. *Videographer: Obtain multiple usable takes, this will be reused in 5.2.2.*

2.6. Program the thermal cycler according to manuscript directions **[1]**. Once annealing is complete, samples can be stored at 4 degrees Celsius for 12 to 24 hours before characterization **[2]**. *Videographer: This step is important!*

2.6.1. Talent programming the thermocycler.

2.6.2. Talent taking the samples out of the thermocycler.

3. Total Internal Reflection Fluorescence (TIRF) Microscopy Imaging

3.1. Make a humidity chamber from an empty pipette tips box by filling the box with approximately 5 milliliters of water **[1]**. Prepare a 20-fold dilution of commercially available 2% collodion in amyl acetate with isoamyl acetate solvent to create the nitrocellulose solution **[2]**. *Videographer: This step is important!*

3.1.1. Talent filling the pipette tips box with water.

3.1.2. Talent diluting 2% collodion in amyl acetate with isoamyl acetate solvent.

3.2. Use tweezers to dip a coverslip into the nitrocellulose solution and allow it to air-dry **[1]**. Then, make a flow chamber out of a microscope slide, 2 double-sided tape strips, and the nitrocellulose-coated coverslip **[2]**. *Videographer: This step is important!*

3.2.1. Talent dipping the slide into the nitrocellulose solution.

3.2.2. Talent assembling the flow chamber.

3.3. Prepare the biotin-BSA solution by weighing 1 milligram of biotin-BSA and dissolving it in 1 milliliter of PBS **[1]**. Pipette 15 microliters of the biotin-BSA into the flow channel at an angle **[2]** and incubating it for 2 to 4 minutes at room temperature in the humidified chamber **[3]**. *Videographer: This step is important!*

3.3.1. Talent dissolving the biotin-BSA in the PBS.

3.3.2. Talent adding the biotin-BSA to the flow channel.

3.3.3. Talent putting the flow channel in the humidified chamber. *Videographer: Obtain multiple usable takes, this will be reused in 3.4.2.*

3.4. Flow 15 microliters of the wash buffer, which consists of 1 milligram of BSA in 1 milliliter of PBS, into the channel to wash away excess biotin-BSA **[1]**, then passivate the surface by incubating the flow channel for 2 to 4 minutes in the humidified chamber **[2]**. *Videographer: This step is important!*

3.4.1. Talent flowing the wash buffer through the channel. *Videographer: Obtain multiple usable takes, this will be reused in 3.5.3.*

3.4.2. *Use 3.3.3.*

3.5. Measure 0.5 milligrams of streptavidin and 1 milligram of BSA, then dissolve both in 1 milliliter of PBS. Pipette 15 microliters of the streptavidin solution into the flow channel and place it in the humidified chamber for 2 to 4 minutes **[1]** and wash the

channel by flowing 15 microliters of wash buffer **[2]**. *Videographer: This step is important!*

3.5.1. Talent adding the streptavidin solution to the channel.

3.5.2. *Use 3.4.1.*

3.6. Next, flow 15 microliters of the annealed batch of γPNA *(pronounce 'gamma-P-N-A')* oligomers in the humidified chamber **[1]**, then wash the unbound nanostructures by flowing 15 microliters of 1 millimolar Trolox in the same solvent composition as the nanostructures **[2]**. *Videographer: This step is difficult and important!*

3.6.1. Talent adding the annealed PNAs to the channel and putting it in the humidified chamber.

3.6.2. Talent adding wash solution to the flow channel.

3.7. Transfer the flow channel to the slide holder and image it with a fluorescence microscope equipped with TIRF imaging, a 60 X oil-immersion objective, and a 1.5x magnifier **[1]**. Scan the flow channel at either 60 or 90 X magnification while monitoring the Cy3 *(pronounce 'sigh-3')* channel **[2]**. *Videographer: This step is difficult and important!*

3.7.1. Talent positioning the flow channel on the microscope stage.

3.7.2. Talent imaging the flow channel. *Videographer: Obtain multiple usable takes, this will be reused in 5.3.1.*

4. Different Morphologies for γPNA Nanofibers in Varying Concentrations of SDS

4.1. Prepare 20 and 6% SDS stocks according to manuscript directions **[1]**, then anneal the γPNA oligomers by adding 1 microliter of each 20 micromolar oligomer to a 200 microliter PCR tube **[2]**.

4.1.1. Containers of the prepared SDS stocks, labeled with the appropriate concentrations.

4.1.2. *Use 2.3.1.*

4.2. Add 30 microliters of anhydrous DMSO and 1 microliter of 6% or 20% SDS to the PCR tubes for a final volume of 40 microliters, which will result in a final SDS concentration of 5.25 or 17.5 millimolar, respectively **[1]**. Anneal the γPNAs in the thermocycler as previously described **[2]**.

4.2.1. Talent adding DMSO and SDS to a PCR tube.

4.2.2. *Use 2.5.1.*

4.3. Characterize γPNA nanostructures in the presence of SDS using the TIRF or TEM protocol **[1]**.

4.3.1. *Use 3.7.2.*

Results

5. Results: TIRF and TEM Imaging of γPNA Nanofibers

5.1. TIRF imaging of γPNA *('gamma-P-N-A')* oligomers annealed in 75% DMSO showed well-organized architectures **[1]**, while annealing in 75% DMF resulted in spicule-shaped or needle-like nanostructures **[2]**. The 40% 1,4 dioxane condition produced a sparse decoration of filamentous nanostructures **[3]**.

5.1.1. LAB MEDIA: Figure 4 A.

5.1.2. LAB MEDIA: Figure 4 B.

5.1.3. LAB MEDIA: Figure 4 C.

5.2. Furthermore, the samples of γPNA nanotubes formed in 75% DMSO demonstrated bundling of nanofibers at high magnification or nanoscopic resolutions during TEM imaging. Quantitative analyses of the width of the nanostructures showed a median width of 16.3 nanometers with maximum values beyond 80 nanometers **[1]**.

5.2.1. LAB MEDIA: Figure 5.

5.3. Isosequential DNA oligomers that replace contiguous γPNAs form straight filamentous structures **[1]**, whereas replacement of crossover γPNAs results in stellate structures **[2]**. The nanostructures that were replaced with contiguous DNA oligomers had median widths of around 19 nanometers **[3]**.

5.3.1. LAB MEDIA: Figure 6. *Video Editor: Emphasize A and C.*

5.3.2. LAB MEDIA: Figure 6. *Video Editor: Emphasize B and D.*

5.3.3. LAB MEDIA: Figure 6.

5.4. At high SDS concentrations, γPNA nanofibers also adopt highly networked morphologies in comparison to its critical micelle concentrations when viewed with TIRF imaging **[1]**. TEM imaging indicates that γPNA assembly in 5.25 millimolar SDS has the most substantial reductions in structural bundling **[2-TXT]**.

5.4.1. LAB MEDIA: Figure 7.

5.4.2. LAB MEDIA: Figure 7 C. **TEXT: 8 – 12 nm**

Conclusion

6. Conclusion Interview Statements

6.1. **Sriram Kumar:** When attempting this protocol, it is important to remember that the solvent compositions mentioned here should be consistently maintained to avoid aggregation of the nanostructures.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

6.2. **Sriram Kumar:** These methods encourage practitioners to pursue other unexplored avenues of different solvent compositions, surfactants, and hybrid γPNA-DNA nanostructures.

6.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.