Chapter 14

Designing DNA Nanotube Liquid Crystals as a Weak-Alignment Medium for NMR Structure Determination of Membrane Proteins

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Abstract

Thirty percent of the human proteome is composed of membrane proteins that can perform a wide range of cellular functions and communications. They represent the core of modern medicine as the targets of about 50% of all prescription pharmaceuticals. However, elucidating the structure of membrane proteins has represented a constant challenge, even in the modern era. To date, only a few hundred high-resolution structural models of membrane proteins are available. This chapter describes the emergence of DNA nanotechnology as a powerful tool for the structural characterization of membrane protein using solution-state nuclear magnetic resonance (NMR) spectroscopy. Here, we detail the large-scale synthesis of detergent-resistant DNA nanotubes that can be assembled into a dilute liquid crystal to be used as a weak-alignment media in solution NMR structure determination of membrane proteins.

Key words DNA origami, Membrane protein, Nuclear magnetic resonance, Structural biology, Residual dipolar coupling

1 Introduction

Membrane proteins help coordinate pretty much everything a cell does, including signaling, energy generation, transport, and recognition. Today, about 50% of approved therapeutics target human membrane proteins [1, 2]. Given their biological importance, it is surprising that our understanding of membrane proteins molecular mechanisms is still in its infancy. This significant gap in knowledge can be partially attributed to the extreme hydrophobic nature of most membrane protein, as well as the slow pace in the development of structural biological tools to study them. Today, only a few hundred high-resolution structural models of membrane proteins have been deposited into the RCSB Protein Data Bank by X-ray cryptographers and NMR structural biologists. However, recent advances in solution-state NMR spectroscopy are leading to its increased importance in the study of the structure and dynamics of

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membrane proteins, especially those with multiple transmembrane-spanning alpha-helices.

One of these recent advances in particular makes possible the accurate measurement of residual dipolar couplings for a wide array of membrane proteins via a new DNA origami technology [3–5]. The residual dipolar couplings method introduced in 2000 involves weak alignment of proteins [6–9]. This alignment is aided by large molecules that form liquid crystals at low concentration, which can provide global orientation restraints that greatly facilitate NMR structure determination and facilitate the de novo NMR structure determination of large proteins that cannot be determined using classical NMR techniques [9–14]. A residual dipolar coupling-based refinement approach can be used to resolve the structure of proteins up to 40 kDa in size. However, to do this on membrane proteins you need a weak-alignment medium that is detergent resistant and it has thus far been difficult to obtain such a medium suitable for weak alignment of membrane proteins [15–18]. To remedy this, in the William Shih laboratory, we have developed a new method for a robust, large-scale synthesis of the first detergent-resistant liquid crystals of DNA nanotubes that enable weak alignment of detergent reconstituted membrane proteins [4, 5]. Inspired by the architecture of the well-established phage-based alignment method and facilitated by the magnetic susceptibility anisotropy of DNA, we designed 0.8-μm-long DNA nanotube liquid crystals suitable for high-resolution NMR study of membrane proteins (Fig. 1) [4, 5, 19].

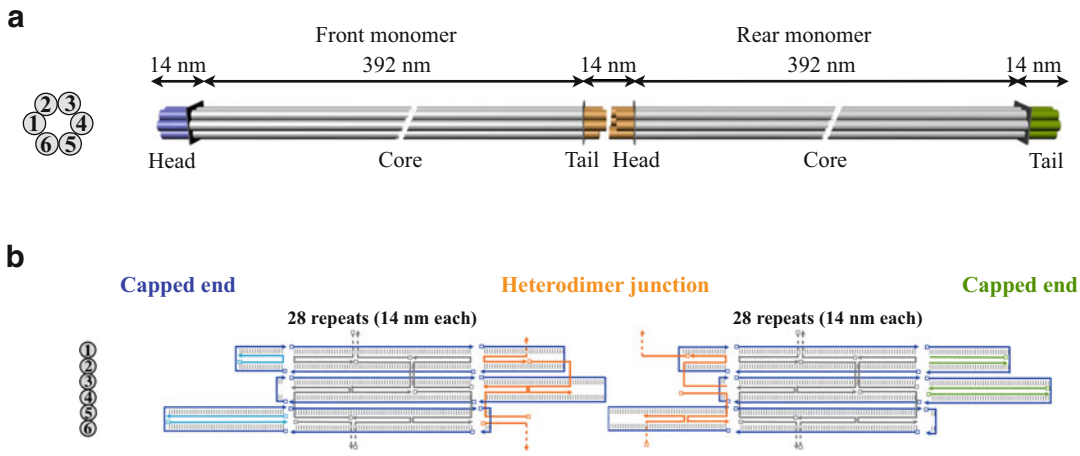

Fig. 1 DNA-nanotube design overview. (**a**) Schematic illustration of the 800-nm-long six-helix bundle heterodimer (not to scale). *Left*, a cross-sectional view. Front monomer with capped head module in *blue*, core module in *grey*, and connector tail module for heterodimerization in *orange*. Rear monomer with capped tail module in *green*, core module in *grey*, and connector head module for heterodimerization in *orange*. (**b**) Design schematics of the DNA six-helix bundle. Scaffold-plus-staple schematic view of the heterodimer junction of front and rear monomer

The emergence of this detergent-resistant liquid crystals has facilitated the accurate measurement of residual dipolar couplings on immunoreceptors, channels, and membrane transporters [20–25]. This detergent-resistant liquid-crystal medium offers a number of properties conducive for membrane protein alignment, including high-yield production, thermal stability, buffer compatibility, and structural programmability. The detailed protocol here describes a method to generalize the use of DNA nanostructures as a detergent-resistant liquid crystal for membrane protein NMR study by offering a user-friendly method for the measurement of membrane protein residual dipolar couplings with a high level of accuracy (Fig. 2).

2 Materials

2.1 Nanomole-Scale Production of M13 Bacteriophage ssDNA Scaffold

1. Luria Broth medium.
2. 2× YT broth capsule microbial medium.
3. Bacto agar.
4. Petri dishes, 100 × 15 mm. All the equipment used for growing cells should be sterilized.
5. Ampicillin sodium salt.
6. Isopropyl β-D-1-thiogalactopyranoside.
7. JM109 bacteria.
8. M13mp18 ssDNA.
9. Shaker incubator, 37 °C.

2.2 Large-Scale Synthesis of DNA Origami Assembly and Agarose Gel Electrophoresis

1. Desalted and lyophilized DNA oligonucleotides.
2. Folding buffer, 20×: Folding buffer contains 100 mM Tris (pH ~8.0), 20 mM EDTA, and 200 mM $MgCl_2$. Folding buffer can be stored at room temperature for up to 6 months.
3. EDTA.
4. Magnesium chloride hexahydrate, 99.995%.
5. BioProducts 96-well PCR plate.
6. Aluminum sealing tape for 96-well plates.
7. Disposable multichannel pipettor basins.
8. Gilder fine bar grids.
9. Thermal cycler.
10. UltraPure agarose.

2.3 Nanomole-Scale Purification of DNA Nanotube

1. Qiagen-tip 10000: The maximum DNA binding capacity is 10 mg.
2. Wash buffer QC: Wash buffer QC contains 50 mM MOPS (pH 7.0), 1 M NaCl, and 15% (vol/vol) isopropanol. Wash buffer can be stored at room temperature for up to 6 months.

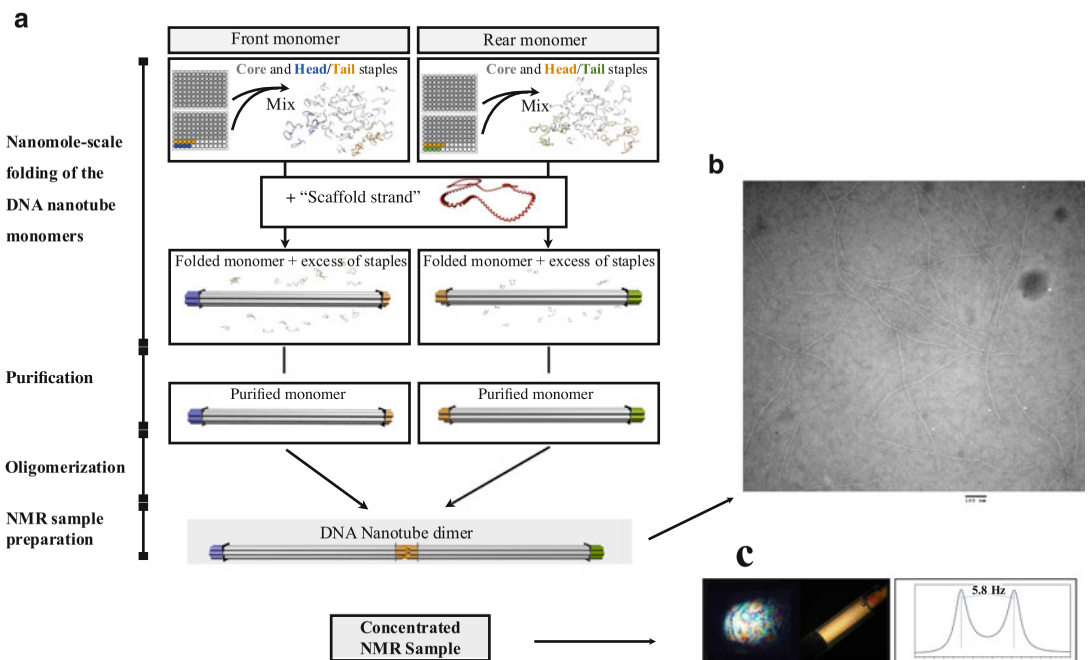

Fig. 2 A flowchart diagram summarizing the steps involved in setting up a large-scale synthesis of the DNA six-helix bundle. (**a**) Step-by-step guide through molecular self-assembly of scaffolded DNA origami nanotube for NMR structure determination of membrane proteins. (**b**) 1 μl drop solution exhibited birefringence between crossed polarizers by DNA-nanotube heterodimers at 25 mg/ml. 1D NMR spectrum of $2H_2O$ at 2H frequency of the six-helix bundle sample 25 mg/ml in 2 mM $MgCl_2$, 20 mM Tris–HCl pH 7.5, 100 mM DPC, 90 %/10 % H_2O/D2O. The 1D spectrum was recorded at 2H frequency of 600 MHz at 25 °C on a Bruker 600 MHz spectrometer. (**c**) Negative-stain transmission electron micrograph of a purified sample of six-helix bundle heterodimer

3. Elution buffer QF: Elution buffer QF contains 50 mM Tris (pH 8.5), 1.25 M NaCl, and 15% (vol/vol) isopropanol. Elution buffer can be stored at room temperature for up to 6 months.
4. Loading buffer QBT: Loading buffer QBT contains 50 mM MOPS (pH 7.0), 750 mM NaCl, 15% (vol/vol) isopropanol, and 0.15% (vol/vol) Triton X-100. It can be stored at room temperature for up to 6 months. It is highly recommended that all buffers used for chromatography applications be filtered.
5. Centricon-100 concentrators.
6. Sodium phosphate dibasic anhydrous.
7. Sodium phosphate monobasic anhydrous.
8. Polyethylene glycol 8000 (PEG8000).
9. Triton X-100.
10. Sodium chloride.
11. Tris base.
12. MOPS.
13. Glacial acetic acid.
14. Isopropanol.
15. Ethanol, 200 proof.

2.4 Preparation of NMR Protein Samples with DNA Nanotubes and Data Analysis

1. Teflon tube, fluorinated ethylene propylene (FEP).
2. Shigemi NMR tube.
3. Low-DNA-affinity Teflon tube.
4. Microscope with polarizer and rotating analyzer.
5. NMR spectrometer equipped with a triple-resonance probe head [26].
6. NMRPipe and nmrDraw software for processing and analyzing NMR spectra [27–30].

3 Methods

3.1 Nanomole-Scale Folding of the DNA Nanotube Monomers

In order to build six-helix bundle nanotubes of 0.8 μm uniform length, an assembly strategy was conceived to link two unique 0.4 μm six-helix bundle monomers in a head-to-tail fashion (Fig. 1b). For each monomer a 7308-nucleotide (nt) M13-derived single-stranded circle of DNA is used as a "scaffold" and 168 single strands of DNA (of length 42 nt, programmed with complementarity to three separate 14-nt regions of the scaffold) are used as "staples" (Fig. 1b).

11. Each monomer is folded with the p7308 scaffold and unique pools of oligonucleotide staple strands. In a multichannel pipette basin, prepare a 37.8 ml master mix containing the following for each monomer: 120 nM scaffold p7308 (*see* **Note 1**), 720 nM (average) each staple (*see* **Note 2**), 20 mM $MgCl_2$, 1 mM EDTA, and 5 mM Tris (pH 8.0). This volume is intended for 240 folding reactions at 150 μl per reaction and includes a 5% excess to account for pipetting error. *See* **Notes 3** and **4**.
12. After preparing the master mix, use a multichannel pipette to distribute 150 μl aliquots into 96-well plates.
13. Fill the central 60 wells in each of the four 96-well plates for a total of 240 reactions; *see* **Note 5**.
14. Seal the plates with aluminum sealing covers for 96-well plates. *See* **Note 6**.
15. Load the 96-well plates into the thermal cycler and set up the thermal annealing ramp as follows: hold at 80 °C for 5 min, then decrease by 1 °C every 5 min to 65 °C, and then decrease by 1 °C every 40 min to 20 °C. Use a heated lid to minimize evaporation.

3.2 Nanomole-Scale Purification of DNA Nanotube Monomer

1. Pool all 240 completed reactions for each DNA nanotube monomer into a multichannel pipette basin (i.e., one basin for each of the two monomers) and transfer the pooled reactions (37.8 ml) into designated 250 ml Erlenmeyer flasks.
2. Once pooled, bring each sample volume to 100 ml with Buffer QBT. *See* **Note 7**.
3. Remove 50 μl of each of the two pools for an analytical agarose gel.
4. Column equilibration step: Use one Qiagen-tip 10000 ion-exchange column per monomer. Label each column as rear and front monomer. Equilibrate each column with 75 ml of buffer QBT. Allow the buffer to flow through completely.
5. Column loading step: Apply 100 ml of each monomer to the appropriate column and allow to flow through completely.
6. Column washing step: After the nanotube pools have completely flowed through the column, wash the column six times with 100 ml of buffer QC. Save the washes. *See* **Note 8**.
7. Nanotubes elution step: Elute each monomer from the column with 100 ml of buffer QF. At this stage, the DNA solution should be homogenous and clear.
8. Remove 50 μl of each of the two eluted samples for an analytical agarose gel.
9. Add $MgCl_2$ to a final concentration of 25 mM. *See* **Notes 9** and **10**.

3.3 Hetero-dimerization of DNA Nanotube Monomers

1. The DNA nanotubes need to be heterodimerized prior to further purification. By using the material that was eluted from the Qiagen-tip 10000 ion-exchange columns, mix equal volumes of the rear and front monomer elutions. *See* **Notes 11** and **12**.
2. The heterodimerization is performed at 37 °C to improve the kinetics of the reaction. Warm the mixture by incubation in a 37 °C water bath for 15 min.
3. Incubate the mixture at 37 °C for an additional 1 h and 45 min for a total of 2 h at 37 °C. *See* **Note 13**.
4. Remove 50 μl of the mixture for an analytical agarose gel.

3.4 Concentration of DNA a Nanotubes and Formation of DNA Nanotube Liquid Crystals

1. Add 0.25 volumes of 20% (wt/vol) PEG8000 to the heterodimerized nanotubes.
2. Mix gently and incubate at room temperature for 15 min.
3. Spin down the nanotubes for 30 min at 15,000 × *g* and 4 °C.
4. Carefully decant the supernatant into another bottle. *See* **Note 14**.
5. Spin the pellet once more for only 1 min at 15,000 × *g* and 4 °C to collect additional supernatant.
6. Carefully remove all remaining supernatant with a pipette.
7. To the nanotube pellet, add sufficient 0.5× folding buffer to achieve a concentration of 3 mg/ml, assuming 80% recovery from the Qiagen-tip ion-exchange columns; this requires ~6 ml of buffer. *See* **Note 15**.
8. Once the pellet has dissolved, mix the nanotube sample gently and transfer to a 50 ml conical tube.
9. Estimate the concentration of the nanotubes. *See* **Note 16**.
10. Concentrate the nanotubes to ~30 mg/ml using Centricon-100 concentrator units. Prerinse the Centricon-100 concentrator units by adding 2 ml of water. Spin at 2000 × *g* and 15 °C for 5 min to achieve concentration.
11. Remove excess water by inverting tubes and spinning at 900 × *g* for 2 min.
12. Weigh the Centricon-100 concentrator units, then apply DNA-nanotube samples, and record the mass of the concentrator unit with the DNA nanotubes.
13. Spin the nanotubes in 15-min increments at 1500 × *g* and 15 °C. Estimate the concentration by periodically recording the mass of the concentrators with the DNA nanotubes. The starting concentration of the DNA (3 mg/ml) is ten times lower than the desired concentration (30 mg/ml); therefore, a ten times decrease in the mass of the sample gives a good approximation of the desired concentration. *See* **Note 17**.

14. When a ten times decrease in sample mass has been achieved, recover DNA by inverting tubes into collection vials and spinning for 3 min, at 1000×*g*, at 20 °C. The final total volume will typically be between 1 and 1.5 ml. Concentrated to 30 mg/ml, the nanotube sample will be homogeneous, clear, and viscous. If the DNA nanotube solution does not appear viscous, it is recommended to check the birefringence, as described in **step 15**. If the sample is not birefringent, spin the nanotubes in 15-min increments at 1500×*g* and 15 °C until the sample appears viscous.
15. Place a 1 μl drop of DNA nanotube liquid crystal solution on a glass microscope slide. Examine the drop at room temperature using a dissecting microscope under normal and crossed polarized light. The nanotubes will appear birefringent between crossed polarizers with characteristic textures of the type shown in Fig. 2c. *See* **Note 18**.

3.5 Measuring Residual D2O Quadrupole Coupling in the Presence of DNA Nanotubes

1. Add D2O to 250 μl of the DNA nanotube liquid crystal to a final concentration of 10% (vol/vol). Mix slowly by pipetting.
2. Use a low-DNA-affinity Teflon tube to transfer 250 μl of the nanotube sample with 10% D2O into a Shigemi NMR tube. *See* **Note 19**.
3. Spin down the NMR sample at 500×*g* and 15 °C for 2 min and add the Shigemi plunger. *See* **Note 20**.
4. Record 1D NMR spectrum at 2H frequency.
5. Process 1D NMR spectra and measure D2O splittings.

3.6 Preparation of NMR Protein Samples with DNA Nanotubes

1. Prerinse a Centricon-100 concentrator unit.
2. Weigh the Centricon-100 concentrator unit while empty, and then apply 250 μl + 10% of the DNA nanotube sample at a concentration of ~25 mg/ml. Weigh the Centricon-100 concentrator unit with the DNA sample.
3. Exchange the DNA nanotubes into the desired protein buffer by diluting the nanotubes twofold with the protein buffer.
4. Mix the twofold-diluted sample slowly by pipetting up and down. Spin the nanotubes in 5-min increments at 1500×*g*, at 15 °C.
5. Between each spin, mix the sample slowly by pipetting up and down. Stop the concentration when the columns reach roughly the starting weight.
6. Repeat previous **steps 3–5** three times to achieve sufficient exchange.
7. Once the DNA nanotubes are in the appropriate buffer, an appropriate amount of protein is added to the DNA nanotube solution. The final NMR sample is then prepared by concentrating down

to the appropriate sample volume using a series of 5-min spins at 1500 × *g* and 15 °C. *See* **Note 21**.

8. Recover the NMR sample from the Centricon concentrator unit. *See* **Notes 22** and **23**.

4 Notes

1. Scaffold: We use a modified bacteriophage M13 genome that is 7308 bp in length, as described previously for DNA origami (http://www.pnas.org/content/suppl2007/04/02/0700930104.DC1/00930SuppAppendix2.pdf).

   To obtain sufficient quantities of this single-stranded DNA scaffold, production of the bacteriophage that bears the modified 7308 bp genome is progressively scaled-up in a series of steps that yield the "preinoculation" phage, then the "inoculation" phage, and finally the nanomole-scale phage. The inoculation phage is produced in two steps (preinoculation and inoculation) to ensure sufficient quality and quantity.

2. Staples: To generate staples pools, we purchased desalted and lyophilized DNA oligonucleotides in 96-well plates on the 200 nmol scale from Invitrogen. Exact sequences of the oligonucleotide staple strands are listed in supplementary Table 1. Once hydrated, equal volumes of each oligonucleotide are pooled into two groups corresponding to the necessary staple strands for each monomer (Figs. 1a and 2a). For the front monomer, the pool includes core staples, "caps" for the head of the monomer to prevent nonspecific oligomerization, and connector staples for programmed dimerization at the tail of the monomer (Fig. 1). For the rear monomer, the pool includes core staples, caps for the tail of the monomer to prevent nonspecific dimerization, and connector staples for programmed heterodimerization at the head of the monomer (Fig. 1b). Pools are hydrated to achieve an average concentration of ~5 μM per staple strand; individual strand concentrations therefore vary within a range of ~3.5–6.5 μM. Because we are adding a large excess of staple strands compared to scaffold strand, the folding reaction is fairly tolerant of the concentration variations between individual staple strands.

3. In order to prevent evaporation during the folding step, it is highly recommended to leave an empty "border" of wells on each plate. This is because the adhesive used on plate sealing covers rarely form a perfect seal when heated, allowing the boarder wells to evaporate. These border wells will be filled with water, leaving 60 wells per plate for nanotube folding reactions. This is done because the adhesive used on plate sealing covers rarely form a perfect seal when heated, allowing the boarder wells to evaporate.

4. We have observed that the precise magnesium concentration of the folding solution has a dramatic effect on the quality of nanotube folding. Optimal concentrations of $MgCl_2$ vary with the design of the structure and with the vendor of the oligonucleotide staple strands. For the six-helix bundle nanotube described in this protocol and for staple strands provided as described by Invitrogen, 20 mM $MgCl_2$ is optimal. Modified nanotubes or nanotubes folded with staple strands purchased from a different vendor may have slightly different optimal concentrations of $MgCl_2$. It is highly recommended to use pure magnesium chloride hexahydrate (99.995%) during the folding process. EDTA is added to 1 mM final concentration in the master mix to chelate divalent ion impurities that can compete with magnesium during the folding process.
5. When aliquoting the folding master mix into plates, make sure that there are no air bubbles trapped in the wells, as they could promote the formation of artifacts during folding. Bubbles can be removed after making aliquots by gently pipetting the wells up and down.
6. Ensure that the plates are very well sealed in order to prevent any evaporation during the thermal annealing step.
7. Be sure to mix the samples thoroughly after adding buffer QBT to ensure homogenous distribution of DNA.
8. To improve the efficacy of the column wash step, allow each wash to flow through entirely before applying subsequent washes.
9. The addition of magnesium to the eluted product stabilizes the DNA nanotubes. Some white precipitate may appear during this step but it does not interfere with subsequent steps.
10. The eluted sample can be stored at 4 °C for at least 2 days.
11. After combining the two monomers, be sure to mix the solution by gently swirling the flask.
12. We are assuming that the Qiagen-tip 10000 purification yields roughly equimolar quantities of each monomer, and thus we need to only consider volume. Equimolar amounts of each monomer have to be mixed to form 100% of the heterodimer. If one of the monomers is formed in excess, its amount should be reduced to a stoichiometric quantity before mixing. This can be estimated from the florescence intensity of each monomer's band when analyzed via agarose gel electrophoresis.
13. At this point, the heterodimerized mixture can be stored at 4 °C if it is necessary to return to the precipitation at a later time. After few hours at 4 °C the sample can turn turbid. This is a typical behavior of DNA nanostructure stored in buffer QF at 4 °C and does not harm the sample.

14. Save the supernatant in case the nanotube pellet becomes dislodged from the bottle.
15. Do not disturb the pellet initially. Simply add buffer to the tube and allow the buffer to diffuse into the pellet over time. Actively resuspend loose portions of the pellet periodically by swirling. Care should be taken to avoid extremely vigorous mixing at this step. It is highly recommended to let the buffer slowly dissolve the pellet to prevent damage to the nanotubes. This process can easily take one to several hours.
16. The nanotubes can be stored in 0.5× folding buffer at 4 °C for at least 6 days until one is ready to proceed with the concentration step.
17. To prevent damage to the nanotube structure it is recommended that all spins be at speeds less than 2000 × *g*. Between 15-min spins, mix the concentrated solution by pipetting up and down gently with a P1000 tip. This will help prevent the buildup of extremely high local concentrations of the nanotubes near the Centricon membrane .
18. DNA nanotubes are very stable and can be stored at 4 °C for at least 12 months.
19. To minimize the loss of DNA, transfer the DNA sample in several steps by pipetting only 40 μl into the NMR tube at a time.
20. At 30 mg/ml, the DNA nanotube liquid crystal solution appears viscous. Despite the viscosity, conventional pipettes or Teflon tube work well to transfer the liquid crystals to an NMR tube. A uniform and bubble-free sample is obtained by slow centrifugation (100–200 × *g*) after transferring the sample to the tube, inserting the plunger slowly to the bottom of the tube and pulling the plunger to the desired height.
21. During the course of concentration, a local concentration of both protein and nanotubes around the Centricon membrane may appear. As a consequence, there is a much more favorable environment locally for interaction between the nanotubes and the protein. It is recommended to periodically homogenize the DNA and protein concentration between each spin by pipetting up and down slowly.
22. DNA material may stick to the Centricon membrane. It is possible to recover more than 95% of the DNA sample by inverting tubes into collection vials and spinning for 3 min at 1000 × *g* and 20 °C.
23. Store at 4 °C or temperature appropriate for protein of interest.

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