DNA nanotubes for NMR structure determination of membrane proteins

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Finding a way to determine the structures of integral membrane proteins using solution nuclear magnetic resonance (NMR) spectroscopy has proved to be challenging. A residual-dipolar-coupling–based refinement approach can be used to resolve the structure of membrane proteins up to 40 kDa in size, but to do this 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. We describe here a protocol for robust, large-scale synthesis of detergent-resistant DNA nanotubes that can be assembled into dilute liquid crystals for application as weak-alignment media in solution NMR structure determination of membrane proteins in detergent micelles. The DNA nanotubes are heterodimers of 400-nm-long six-helix bundles, each self-assembled from a M13-based p7308 scaffold strand and >170 short oligonucleotide staple strands. Compatibility with proteins bearing considerable positive charge as well as modulation of molecular alignment, toward collection of linearly independent restraints, can be introduced by reducing the negative charge of DNA nanotubes using counter ions and small DNA-binding molecules. 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. Production of sufficient nanotubes for four or five NMR experiments can be completed in 1 week by a single individual.

INTRODUCTION

The last three decades have witnessed the rapid development of DNA as a molecular engineering material to create nanostructures with controlled geometries, topologies and periodicities of increasing complexity[1,2]. The method of DNA origami, in which a long 'scaffold' strand is mixed with hundreds of short 'staple' strands to form a parallel bundle of double helices of custom shape, has proven particularly well suited for the robust self-assembly of two- and three-dimensional nanostructures up to ~10 MDa in size[3–5]. One of the most remarkable applications for this technology is in NMR-based membrane-protein structure determination[6,7].

Analysis of integral membrane proteins by NMR

In spite of the importance of integral membrane proteins (IMPs)[8–12], the structural biology of this class of proteins remains underdeveloped, with only a few hundred high-resolution structural models of membrane proteins determined by crystallography and NMR deposited into the RCSB Protein Data Bank as of summer 2012. However, advances in solution-state NMR spectroscopy are leading to its increased importance in the study of the structure and dynamics of IMPs[13–18]. There has recently been major progress in sample preparation protocols for membrane proteins, and these techniques[19] now enable studies of much larger structures through the application of the principles of transverse-relaxation-optimized spectroscopy[20].

The classical paradigm for protein structure determination by solution-state NMR spectroscopy is to extract and assign a dense network of ^{1}H-^{1}H nuclear Overhauser effects (NOEs) in order to define the three-dimensional fold of a protein. This still presents great challenges for liquid-state NMR-based structural investigations of membrane proteins because of substantial peak overlap in the spectra caused by large line widths, limited chemical-shift dispersion, and poor diversity of the amino acids in transmembrane regions of α-helical proteins.

An alternative to ^{1}H-^{1}H NOEs as a route to high-resolution structural restraints is found in the controlled re-introduction of anisotropic residual dipolar couplings (RDCs)[21–23]. RDCs constitute an excellent source of structural and dynamic information. The dipolar coupling between two atoms, *j* and *k*, or D*jk*, is related to the internuclear distance r*jk*, which is typically known in advance (e.g., bond length for covalently linked nuclei), and to the angle between the vector connecting the interacting nuclei and the static magnetic field by the relation $<3\cos 2\theta - 1>$, where the brackets indicate time-averaged sampling. These couplings can be a valuable source of angular structural data for NMR studies of macromolecules. This is because direct information on the orientations of the corresponding bond vectors relative to the protein's steric alignment vector is provided. However, molecular tumbling averages these interactions to zero in conventional isotropic solutions[24]. It has been shown that RDCs can be measured by using some type of anisotropic medium to allow for partial alignment and, therefore, non-vanishing dipole-dipole interactions[22]. Such incomplete directional averaging of macromolecules in liquid crystalline medium would allow routine measurement of RDCs while retaining conditions essential for high-resolution solution-state NMR (i.e., rapid tumbling).

A highly effective method for inducing weak-alignment of proteins is through mixing them with a dilute liquid-crystalline medium, such that the interaction between the protein and the medium is weak and highly transient (<1 ns lifetime). A number of liquid-crystal alignment media have been developed to measure accurate RDCs, including filamentous phage[25], 1,2-dimyristoyl-sn-glycero-3-phosphorylcholine (DMPC)/1,2-diheptanoyl-sn-glycero-3-phosphorylcholine (DHPC) bicelles[22], C12E5 polyethylene glycol[26], ternary mixtures of cetylpyridinium Cl/Br, hexanol and sodium Cl/Br (refs. 26,27), cellulose crystallites[28] and a highly hydrated anisotropically compressed polyacrylamide gel[29].

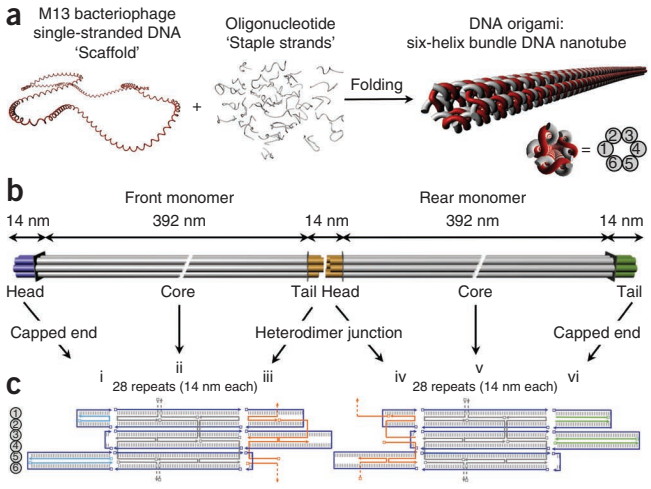

Figure 1 | DNA nanotubes design. (**a**) Schematic illustration of six-helix bundle DNA nanotube folding. In red, single-stranded M13-based vector p7308 scaffold. In gray, single-stranded staple oligonucleotides of length 42 bases, programmed with complementarity of the scaffold. Right, scaffold-plus-staples schematic view of the folded six-helix bundle DNA nanotube and a cross-sectional view of the DNA nanotube. (**b**) Three-dimensional cartoon view of 800 nm-long six-helix bundle heterodimer (not to scale). Left, six-helix bundle front monomer with core module in gray, capped head module in blue and connector tail module for heterodimerization in orange. Right, six-helix bundle rear monomer with core module in gray, connector head module for heterodimerization in orange and capped tail module in green. (**c**) Scaffold-plus-staples schematic view of the heterodimer junction of front and rear monomer. One strand of each double helix is contributed by the scaffold shown in blue, and the other strand is contributed by a staple. Base pairs are depicted as short vertical lines. Helices 1–6 are labeled on the left. In the orientation displayed, the outside surface of the nanotube is facing the viewer. (i) Front monomer head module. Three staple strands serve to cap the front monomer head (shown in cyan). (ii, v) Core module. There are 28 repeats of 42-bp modules for each monomer. A scaffold crossover connecting helix 2 to helix 3 occurs in the 15th repeat and one connecting helix 4 to helix 5 occurs in the 14th repeat (not shown). (iii) Front monomer tail module. Three staple strands with a total of 26 unpaired bases decorate the tail. The scaffold strand is unpaired for 36 bases. (iv) Rear monomer head module. Three staple strands with a total of 36 unpaired bases decorate the head. These unpaired regions are complementary to the corresponding 36 unpaired bases of the front monomer tail scaffold strand. The 26 unpaired bases in the rear monomer head scaffold strand are complementary to the 26 unpaired bases of the three staple strands that decorate the front monomer tail. In the DNA nanotube heterodimer, these unpaired regions match up to form the complete intermonomer junction. (vi) Rear monomer tail module. Four staple strands serve to cap the rear monomer tail (shown in green).

With the exception of the polyacrylamide gel (in some cases[30–33]) and the fd bacteriophage (in one case[34]), most of these media have been shown to be incompatible with the detergents and lipids needed to solubilize membrane proteins. With the compressed gel, it has generally been difficult to soak membrane proteins to higher than 0.1–0.2 mM because of the inhomogeneous pore size of randomly cross-linked gel matrices as well as the limiting accuracy and signal-to-noise ratios for NMR measurements. Because of this, we have never succeeded in measuring accurate RDCs for a single-chain protein longer than 150 residues; thus far, our only success has been with homomultimeric proteins[31]. In addition, for large systems the polyacrylamide gel may not be practical because of strong interactions with the acrylamide mesh, which can reduce the molecular tumbling rate. This can be tuned by the gel concentration, but acrylamide gels cannot be used at lower concentration than about 4% (wt/vol) because of mechanical instability.

More recently, two new detergent-compatible liquid crystals have been reported, one based on collagen[35], and the other based on nucleic-acid G-tetrad structures[36]. For these two new media, the reduction in molecular tumbling rates for large systems is much less problematic and they are easy to produce and non-expensive. However, both methods still have issues with signal-to-noise ratios and/or general detergent and buffer compatibility. The alignment induced by collagen gels is quite small when compared with other alignment media. The d(GpG)-based G-tetrad stacks require an excess of potassium, which enables effective stacking of pyrene moieties on the exposed guanine tetrads, but some specific detergents can be incompatible with the presence of potassium. Thus, measuring RDCs for membrane proteins has remained a difficult challenge.

DNA nanotubes and NMR of IMPs

Inspired by the architecture of the established phage-based alignment method[22,37] and facilitated by the magnetic susceptibility anisotropy of DNA, we designed DNA nanotube liquid crystals[6] as the first detergent-compatible liquid crystals generally suitable for high-resolution NMR study of membrane proteins (**Fig. 1**). Our six-helix DNA nanotube technique represents a simple, versatile and stable method for aligning macromolecules for the measurement of dipolar-coupling interactions. The ability to tune the alignment of molecules over a large selection of detergents and buffers as well as a large pH (stable from pH 4 to 9) and temperature range (stable until 60 °C) makes the use of DNA nanotubes especially attractive for studies of membrane proteins, as compared with

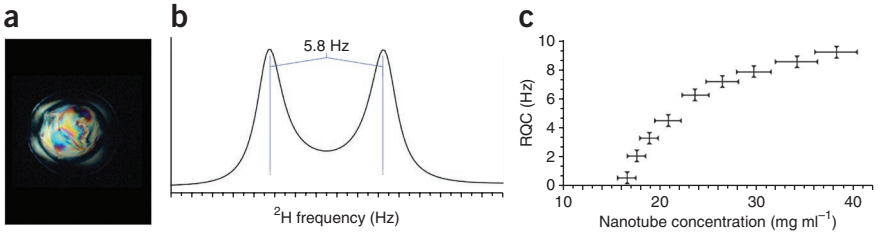

Figure 2 | Characterization of DNA nanotubes liquid crystal. (**a**) One-microliter drop solution birefringence shown between crossed polarizers by DNA nanotube heterodimers at 25 mg ml^{-1}. (**b**) One-dimensional NMR spectrum of D_2O at ^{2}H frequency of the six-helix bundle sample 25 mg ml^{-1} in 2 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.5, 100 mM DPC, 90%/10% D_2O. The 1D spectrum was recorded at ^{2}H frequency of 600 MHz at 25 °C on a Bruker 600 MHz spectrometer. (**c**) Concentration dependence of the D_2O residual quadrupolar coupling (RQC). Three nanotube preparations were tested, and the vertical error bars represent the s.d. of the splitting measurement. Horizontal error bars represent the standard s.d. in nanotube concentration between the three samples.

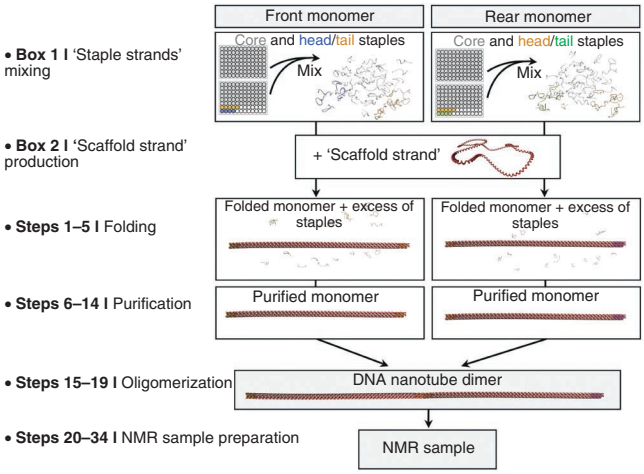

Figure 3 | A flowchart diagram summarizing the steps involved in and time required for setting up a large-scale synthesis of detergent-resistant DNA nanotubes. Step-by-step guide through molecular self-assembly of scaffolded DNA origami nanotube for NMR structure determination of membrane proteins. **Boxes 1** and **2** represent starting material preparation. DNA nanotubes are illustrated as simplified, not to scale. **Box 1** involves pooling staple oligonucleotides according to the structural modules, rear and front monomers (from 96-well plates). **Box 2** involves producing the single-stranded M13-based vector p7308 scaffold. Steps 1–5 involve folding. Self-assembly reactions are prepared. Front and rear monomers are folded in separate chambers by heat denaturation, followed by cooling for renaturation. Steps 6–14 involve purification. Each folded monomer sample is purified separately from excess staple strands via gravity-flow ion-exchange column. Steps 15–19 describe oligomerization. Nanotube heterodimers are self-assembled by combining purified front and rear monomer mixtures together. Steps 20–34 describe NMR sample preparation. The nanotube heterodimer mixture are PEG-precipitated and concentrated for NMR sample preparation.

other alignment media that align membrane proteins in a more limited range of conditions.

DNA nanotubes maintain a liquid crystalline phase in the presence of zwitterionic or negatively charged detergents that are typically used to solubilize membrane proteins for structural study. In our hands, DNA nanotube liquid crystals have yielded successful weak alignment of a number of membrane or membrane-associated proteins in the presence of different types of detergents.

Negatively charged detergents have been used to enable measurement of RDCs for a 40-kDa tetrameric BM2 channel, which includes a 20-residue membrane anchor and a soluble coiled-coil tetramerization domain, reconstituted in 1-myristoyl-2-hydroxy-sn-glycero-3-[phospho-rac-(1-glycerol)] (LMPG) detergent micelles[15]. Negatively charged detergents have also been used in mixed detergent micelles to enable measurement of RDCs for the human ζ-ζ transmembrane domain of the T cell receptor[6] embedded in a 5:1 molar ratio of dodecylphosphorylcholine (DPC) and sodium dodecyl sulfate (SDS) mixed-detergent micelles, for the human immunoreceptor DAP12TM dimer fragment, and for a covalently linked NKG2C-DAP12 trimeric n-tetradecyl phosphorylcholine (TDPC)-SDS mixed-detergent micelles[38]. DNA nanotube liquid crystals are also stable in the presence of zwitterionic detergents and have been used to align a tetrameric M2 channel[6] reconstituted with DHPC and several mitochondrial carriers reconstituted in DPC or in 1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine (LMPC) (J.J.C., unpublished data). However, we never attempted to use DNA nanotubes to align a membrane protein reconstituted with a positively charged detergent. DNA nanotubes have a net negative surface charge, and thus positively charged detergents might interact too strongly with the nanotube.

More recently, we described a new solution-NMR method, heavily relying on weak alignment by our DNA nanotube medium, for structural characterization of mitochondrial uncoupling protein 2 (UCP2) (ref. 7), a 33-kDa protein with six transmembrane domains reconstituted in mixed detergents (150 mM DPC, 1 mM cardiolipin and 2 mM DMPC). This method combined orientation restraints derived from NMR RDCs and semiquantitative distance restraints from paramagnetic-relaxation-enhancement measurements. We determined the local and secondary structures of the protein by piecing together molecular fragments from the Protein Data Bank that best fit experimental RDCs from samples weakly aligned in a DNA nanotube liquid crystal.

DNA nanotube design

Although a detailed understanding of DNA nanotube design is not required for success in the weak-alignment application, we have included a complete description for users who may wish to modify the design. Our DNA nanotube design involves the self-assembly of a parallel array of six double helices, for which every set of three adjacent helices frames an angle of 120° (**Fig. 1a**). Adjacent double helices are held together by Holliday-junction crossovers that occur every 42 bp. In order to build nanotubes of an 0.8-μm uniform length, an assembly strategy was conceived to link two unique 0.4-μm monomers ('front' and 'rear') first assembled separately using the DNA-origami method[4,5] (**Fig. 1b**). For each monomer a 7,308-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. 1c**). The staples self-assemble with the scaffold into the shape of six parallel double helices curled into a tube. Each DNA nanotube monomer is divided into three structural modules: core, head and tail (**Fig. 1b**,**c**). The inclusion of these three modules in the design allows for the linkage of monomers in a head-to-tail fashion. Three extra staple strands block the head of the front monomer, and four extra staple strands block the tail of the rear monomer (**Fig. 1c**). To facilitate heterodimerization, three extra staple strands with unpaired bases decorate the tail of the front monomer, and three extra staple strands with unpaired bases decorate the head of the rear monomer (**Fig. 1c**). After folding, purification and heterodimerization, the aqueous suspension of DNA nanotube dimers should be concentrated to approximately 25 mg ml^{-1}, well above the nematic threshold at which they spontaneously align to form a stable liquid crystal, as indicated by strong birefringence observed through crossed polarizers (**Fig. 2a**).

When the liquid crystal is aligned in an 11.4-T magnetic field in the presence of 100 mM DPC and 10% D_2O, the weakly oriented HDO yields ^2H quadrupolar splitting ranging from 4–8 Hz (**Fig. 2b**). This variation in alignment performance depends on the concentration and the quality of the DNA nanotube preparation. **Figure 2c** shows the quadrupolar splitting of the deuterium line, which is proportional to the extent of alignment. A nanotube concentration on the lower end of this spectrum (~4 Hz) has proven sufficient for most of our membrane-protein applications. The ^2H quadrupolar splitting can be determined on a 10% (vol/vol)

Box 1 | Hydrating and pooling oligonucleotide staple strands ● TIMING ~2 h

The DNA nanotubes are designed as heterodimers of two independent six-helix bundle monomers joined together in a head-to-tail fashion (**Fig. 1b**,**c**). Each monomer is folded with the p7308 scaffold and unique pools of oligonucleotide staple strands. To generate these pools, we purchase desalted and lyophilized DNA oligonucleotides in 96-well plates on the 200-nmol scale from Invitrogen. Oligonucleotide staple strands are listed in **Supplementary Table 1**. For cost-saving reasons, we generally request the entire synthesis of each staple strand; the amounts we receive vary within a twofold range between any pair of strands. Once hydrated, equal volumes of each oligonucleotide are pooled into two groups corresponding to the necessary staple strands for each monomer (**Fig. 3**). 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. 1b** and **Supplementary Table 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** and **Supplementary Table 1**). 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, achieving an exact excess of each strand is not needed.

PROCEDURE

1. To achieve near-complete resuspension of each staple strand, first apply 200 μl of dH_2O to each lyophilized oligonucleotide well on 96-well plates purchased from Invitrogen.
2. Seal each 96-well plate and allow to settle for at least 30 min at room temperature. Vortex at 700 r.p.m. for 2 min to actively resuspend every 10 min.
3. Spin down the plates to collect all liquid at 500*g* and 21 °C for 2 min.
4. Recover the material from each well with a multichannel pipette into a common reservoir (multichannel pipetter basin), either for the front monomer or for the rear monomer (**Supplementary Table 1**). The front monomer pool will contain staple strands for the front head cap, front core and front tail connector, whereas the rear monomer pool will contain staple strands for the rear head connector, rear core and rear tail cap.
5. Apply 50 μl of dH_2O to the empty wells from the 96-well plates in order to collect additional material.
6. Seal each plate and allow to settle for at least 30 min at room temperature. Vortex at 700 r.p.m. for 2 min to actively resuspend every 10 min.
7. Spin down the plates to collect additional material, pool the wells appropriately as before and mix the solution with the original solution from step 4.
8. Estimate the concentrations of the pooled staple stocks using a UV spectrophotometer (A260 = 1 for 30 μg ml^{-1} oligonucleotides in 1 cm path length). If necessary, add water to achieve a desired target concentration. If the estimated concentration decreases below the desired ~5 μM average, a greater volume of each pool can be used for folding.
9. Label the appropriate pools 'rear monomer staples stock' and 'front monomer staples stock.'

■ **PAUSE POINT** The DNA oligonucleotide pools are very stable and can be stored at −20 °C for at least 12 months.

D_2O DNA nanotube sample via a simple 1D experiment. The concentration of ordering nanotube medium needed for optimum alignment may be different for positively charged proteins, as DNA nanotubes have a net negative surface charge. If the protein of interest carries a net positive charge, the alignment could be stronger than for a neutral or negatively charged protein. The magnetic-susceptibility anisotropy of the DNA nano-rod is dominated by the diamagnetic purine bases, which have the lowest energy when the external magnetic field is parallel to the plane of the base. Thus, the DNA nanotubes align with their long axes orthogonal to the field.

This protocol aims 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 RDCs with a high level of accuracy.

Overview of the procedure

The workflow for building DNA-origami nanotubes is illustrated in **Figure 3**. A single-stranded (ss) M13-based p7308 scaffold DNA molecule is self-assembled into a nanotube shape using a set of mostly 42-nt staple oligodeoxyribonucleotides. The workflow starts with pooling equal amounts of the 168 concentration-normalized staple strands for each monomer (front monomer and rear monomer; **Fig. 3**, **Box 1** and **Supplementary Table 1**) and, in parallel, producing the p7308 scaffold (**Fig. 3**, **Box 2**). Once scaffold DNA is prepared and staple oligonucleotide pooling is performed, the self-assembly can be accomplished. Front and rear monomers can be assembled in separate reaction vessels by mixing a sixfold excess of pooled staples (front monomer staples or rear monomer staples) with p7308 ssDNA scaffold in magnesium-containing aqueous buffer (Step 1). After the mixing step, front and rear monomers can be folded separately by heat denaturation, followed by cooling for renaturation (Steps 2–5). Each folded monomer sample can be purified separately from excess staple strands via gravity-flow ion-exchange chromatography (Steps 6–14). Finally, nanotube heterodimers can be self-assembled by combining purified front and rear monomer mixtures together (Steps 15–19). The nanotube heterodimer mixture can then be polyethylene glycol (PEG) precipitated and concentrated (Steps 20–34). At this point, the sample is ready for mixing with the target protein of interest, further concentration and subsequent NMR characterization.

Limits of applicability and practical considerations

There are two main considerations in implementing the presented protocol. First, we must consider the reproducibility and

Box 2 | Nanomole-scale production of M13 bacteriophage ssDNA scaffold

● TIMING 2 d

We use a modified 7,308-base bacteriophage M13 genome as described previously for DNA origami[13] (http://www.pnas.org/content/suppl/2007/04/02/0700930104.DC1/00930SuppAppendix2.pdf). To achieve sufficient quantities of this single-stranded DNA scaffold, production of the bacteriophage that bears the modified 7,308-base 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.

Additional materials

- Luria Broth (Research Products International, cat. no. L24041-500.0)
- Bacto agar (General Stores, cat. no. 4236)
- Petri dishes, 100 × 15 mm. ▲ CRITICAL All the equipment used for growing cells should be sterilized.
- JM109 bacteria (New England Biolabs, cat. no. E4107S)
- M13mp18 ssDNA (New England Biolabs, cat. no. N4040S)

PROCEDURE

1. Transform the recombinant M13 bacteriophage RF dsDNA into JM109 *E. coli* cells. Add 20 ng of recombinant M13 bacteriophage RF dsDNA into 50 µl of JM109 *E. coli* competent cells. Place the tube on ice for 30 minutes and heat-shock the cells for 45 seconds in a 42 °C water bath. Add 450 µl of 25 °C SOC medium to transformation reaction. Incubate for 1 h at 37 °C and ~250 r.p.m. Spread on an LB agar plate and incubate the plate at 37 °C overnight.
2. Prewarm an LB agar plate at 37 °C for ~30 min.
3. Streak the prewarmed LB agar plate for single colonies and incubate the LB agar plate overnight at 37 °C.
4. After overnight incubation, pick a single colony from the LB agar plate and use to inoculate 50 ml of LB culture. Incubate for 8 h at 37 °C and ~250 r.p.m.
5. Collect bacteria by centrifugation at 6,000*g* for 20 min at 4°C.
6. Recover the supernatant (bacterial pellet can be discarded) and precipitate the bacteriophage that bears the 7,308-base scaffold by adding PEG (average MW = 8,000) and NaCl to final concentrations of 4% (wt/vol) and 0.5 M, respectively.
7. Incubate on ice for 30 min, and then collect the precipitated bacteriophage by centrifugation at 4 °C and 6,000*g* for 20 min.
8. Resuspend pelleted bacteriophage in 100 ml of 10 mM Tris (pH ~8.5), 1 mM EDTA. This is the preinoculation bacteriophage that will be used in the following steps to scale up production of the 7,308-base scaffold.

■ PAUSE POINT The preinoculation bacteriophage can be stored at −20 °C for at least 12 months.

9. To generate the inoculation phage, prewarm an LB agar plate at 37 °C for ~30 min.
10. Streak JM109 *E. coli* cells on the prewarmed LB agar plate to generate single colonies.
11. Incubate the LB agar plate overnight at 37 °C.
12. After overnight incubation, pick a single colony from the LB agar plate and use to inoculate 3 ml of 2× YT culture. Incubate overnight at 37 °C and ~250 r.p.m.
13. Use all 3 ml of the overnight culture from the previous step to inoculate a 2-liter Erlenmeyer flask containing 300 ml of 2× YT medium supplemented with 5 mM $MgCl_2$.
14. Shake at 280 r.p.m. and 37 °C until optical density (OD) at 650 nm = 0.5.
15. Add 50 ml of the preinoculation phage stock. Continue shaking at 37 °C for 4 h at 280 r.p.m.
16. Recover the bacteriophage as described in steps 5–8 above. Resuspend the pelleted bacteriophage in 3 ml of 10 mM Tris (pH ~8.5), 1 mM EDTA and store as 50-µl aliquots at −20 °C. This is the inoculation phage.
17. For nanomole-scale production of phage, obtain a single JM109 colony as described in steps 9–11 and use the colony to inoculate 50 ml of 2× YT culture. Incubate overnight at 37 °C and shake at 250 r.p.m.
18. Using 3 ml of the starter culture from step 17, inoculate each of 12 2-liter Erlenmeyer flasks containing 300 ml of 2× YT cultures supplemented with 5 mM $MgCl_2$.
19. Shake at 280 r.p.m. and 37 °C until the OD at 650 nm = 0.4.
20. To one 50-µl aliquot of inoculation phage from step 16, add 600 µl 10 mM Tris (pH ~8.5), 1 mM EDTA and add 50 µl of the resulting solution to each of the 12 cultures for a multiplicity of infection (MOI) of 1.
21. Continue shaking for 4 h at 37 °C and 280 r.p.m.
22. After the 4-h incubation, harvest the bacteriophage by centrifuging the cultures in four 1-liter bottles (~900 ml per bottle) at 6,000*g* and 4 °C for 15 min.
23. Recover supernatant (bacterial cells can be discarded) to fresh centrifuge bottles. Again, there should be ~900 ml of supernatant in each bottle. Directly to these centrifuge bottles, add dry NaCl to 30 g per liter and dry the PEG8000 to 40 g per liter.
24. Mix with a magnetic stir bar until all PEG8000 has dissolved.

! CAUTION At this point, the supernatant should be a cloudy suspension. If it is still clear, it is likely that there is little or no phage present.

25. After mixing, incubate the supernatant on ice for 30 min.
26. Collect the precipitated phage by centrifugation at 4,500 *g* and 4 °C for 15 min.

! CAUTION The pelleted phage is the fraction of interest, but it is best to save the supernatant in case some of the pellet is dislodged into this fraction.

(continued)

Box 2 | (Continued)

27. Allow bottles to sit at an angle for a few minutes, and then remove any additional supernatant with a pipette.
28. Actively resuspend pelleted phage into 1/100 of the original culture volume (9 ml) with 10 mM Tris (pH ~8.5), 1 mM EDTA. To maximize bacteriophage yield, first resuspend the pellet in each bottle with 5 ml of Tris-EDTA buffer, and then rinse with an additional 4 ml of buffer.
29. Transfer resuspended phage to two 40-ml centrifuge tubes (there will be roughly 18 ml per tube) and spin for 15 min at 6,000*g* and 4 °C to remove residual bacterial cells.
30. Recover supernatant to a 50-ml conical tube.

■ **PAUSE POINT** The sample can be stored at −20 °C overnight.

31. Thaw the harvested phage. When thawed, split it into two 250-ml centrifuge bottles.
32. Add 2 volumes of PPB2 per bottle to strip phage protein. PPB2 is 0.2 M NaOH, 1% (wt/vol) SDS.
33. Mix gently by inverting the tube three successive times.
34. Add 1.5 volumes of PPB3 to each bottle to neutralize the NaOH. PPB3 is 3 M potassium acetate (pH 5.5).
35. Again, mix gently by inverting the bottles three successive times.
36. Incubate the centrifuge bottles on ice for 15 min.
37. Spin down the bottles at 16,000*g* and 4 °C for 10 min to remove precipitated SDS and proteinaceous bacteriophage components.
38. Transfer the supernatant to two fresh 250-ml centrifuge bottles.
39. Precipitate the 7,308-base scaffold by adding 1 volume of 200-proof ethanol. Mix by swirling.
40. Incubate on ice for 30 min, and then pellet the DNA scaffold by spinning at 16,000*g* and 4 °C for 30 min.
41. Remove the supernatant with pipette to minimize loss.
42. Wash each DNA pellet with 20 ml of 75% (vol/vol) ethanol.
43. Pellet DNA once more at 16,000*g* and 4 °C for 10 min.
44. To each pellet, apply 10 ml of 10 mM Tris (pH ~8.5) and 1 mM EDTA.
45. Allow the pellet to sit in buffer for 20–30 min, and then actively resuspend any remaining pellet by pipetting.
46. Wash each bottle with an additional 5 ml of 10 mM Tris (pH ~8.5), 1 mM EDTA to collect residual DNA. Note that residual DNA can cling to the side wall of the bottle facing away from the center of the rotor.
47. Estimate the concentrations of the resuspended scaffold DNA using a UV spectrophotometer (absorbance at 260 nm = 1 for 37.5 μg ml^{-1} p7308 in a 1-cm path length).
48. The resuspended scaffold DNA (p7308) can be stored at −20 °C. Scaffold DNA is very stable and can be stored at −20 °C for at least 12 months.

cost-effectiveness of nanomole-scale nanotube production. Any researcher with experience in protein purification should find this protocol quite easy to follow and should be able to obtain reliable results on the first try; all steps besides bacterial growth at 37 °C can be carried out at room temperature (25 °C), and no knowledge of DNA nanotechnology is required. With this version of the protocol, enough M13-based ssDNA scaffold can be produced for a dozen NMR samples by expression in *Escherichia coli* by a researcher working for about 2 d full-time. This scaffold can be stockpiled and frozen for later use. Assembly and purification of DNA nanotubes sufficient for about five NMR samples requires another 5 d by a researcher working half-time. Thus, the total hands-on labor time required per NMR sample, including scaffold preparation and DNA nanotube assembly and purification, averages in the long run to about two-thirds of a day. The most expensive material component is the set of oligodeoxyribonucleotide staple strands at a cost of approximately $300 per NMR sample (in USD, according to list pricing for 200-nmol-scale synthesis from Invitrogen).

Second, as with other negatively charged alignment systems such as the Pf1 filamentous phage, high pI proteins can bind nonspecifically to DNA nanotubes. The negatively charged DNA nanotubes may slow down the tumbling rate of positively charged protein-micelle complexes. Six-helix DNA nanotubes have ~24 surface-exposed phosphates per nanometer of length. With a 7-nm diameter, the surface-charge density then is ~1.76×10^{-19} C nm^{-2}. This compares with a surface-charge density of 8.01×10^{-20} C nm^{-2} for the Pf1 phage[37]. Slowdown of tumbling due to charge: charge attraction can reduce the accuracy of RDC measurement. Clearly, multiple strategies and new sample conditions to further generalize the method and to allow compatibility with highly positively charged protein-micelle complexes would be advantageous. Toward this goal, we took advantage of a large library of highly specific DNA-binding molecules that were previously developed for medicinal purposes and that can be used to shield the negative charge of the DNA nanotubes. Reduction in surface negative charge can increase compatibility with protein-micelle complexes that carry substantial positive charge; it can also lead to the induction of alignment tensors that are linearly independent to that induced by the original medium[39], as has been shown with Pf1 phage alignment of protein G at low versus high ionic strengths[40]. We have found two approaches that, in some cases, can help alleviate these two concerns.

Measure RDCs at higher-salt concentrations to shield charge-charge interactions. Even with a highly positively charged protein such as ubiquitin or UCP2 membrane protein, it is possible to obtain a reasonable spectrum in the presence of a liquid crystal of DNA nanotubes when salt concentrations are raised above 100 mM. However, some amount of slowdown of protein tumbling may occur, thus compromising the resolution of the acquired RDCs. Furthermore, high ionic strength has an adverse effect on the sensitivity gains of NMR experiments in general. To maximize the advantage of cryogenic probes, the salt concentration should be less than 50 mM (ref. 41). The nature of this effect is attributed to the increased ionic and dielectric conductivity of the sample, which leads to dissipation of the RF (radiofrequency) power and

TABLE 1 | Alignment tensor parameters[a].

LC medium conditions	D_a (Hz)	R	Tensor orientation[b]	Q factor
DNA nanotubes with 200 mM NaCl	5.64	0.107	107.41, −11.85, −8.97	0.093
DNA nanotubes with 20 mM $MgCl_2$	3.09	0.115	112.03, −6.30, −29.14	0.082
DNA nanotubes with Hoechst 33258	4.16	0.0918	120.91, 2.40, 0.52	0.072

[a]Tensor parameters based on NH RDC measurements. Data recorded at 25 °C on a Bruker 600-MHz spectrometer.
[b]Euler angles for rotation into the principal axis frame using a rotation about three-dependent axes, between the ubiquitin molecular frame and the alignment frame.

appearance of ring currents in the sample tube[42–44]. We observed that the negative charge of the DNA can be screened as effectively by 20 mM divalent cations (e.g., Mg^{2+}) as by 200 mM monovalent cations (Na^+), in terms of eliminating nonspecific protein binding to DNA nanotubes. The advantage is that 20 mM $MgCl_2$ leads to much lower dissipation of RF power and therefore to higher signal-to-noise ratios. Here we show how to perform this method by applying it to the protein ubiquitin. Ubiquitin carries a substantial positive charge, and thus is an especially good test case for assessing compatibility between DNA nanotubes and positively charged proteins (ubiquitin has a positive net charge at pH 7, $q = 0.53 \times 10^{-19}$ C). It has been shown that ubiquitin interacts nonspecifically with negatively charged media such as the Pf1 phage[45]. In our experience, interactions between ubiquitin and DNA nanotubes can be adequately minimized with either 20 mM $MgCl_2$ or 200 mM NaCl. However, the acquisition time of couplings with the same signal-to-noise ratio takes twice as long in 200 mM NaCl compared with 20 mM $MgCl_2$. In addition, the alignment tensor induced under 200 mM NaCl was slightly different than that under 20 mM $MgCl_2$. The parameters describing the alignment tensors are reported in **Table 1**. The normalized scalar product was calculated for the tensor with 20 mM $MgCl_2$ relative to the tensor with 200 mM NaCl. The normalized scalar product is 0.97 between the different alignment medium conditions, indicating a co-linearity between the alignment tensors. In this case, the difference was not large enough to enable the extraction of independent information.

Weak alignment in the presence of small DNA-binding molecules. Some specific detergents can be incompatible with the presence of magnesium. In order to generalize the approach, we screened different small positively charged molecules to replace the magnesium ion. Binding modes fall into two categories: intercalation and specific hydrogen-bonding interactions in the DNA grooves[46]. We screened different positively charged groove binders and intercalators that can potentially shield the negative charge of the nanotubes[47]. Of the four molecules tested (ethidium, 9-aminoacridine, DAPI and Hoechst 33258), we found the best performance with Hoechst 33258, a DNA groove binder[48,49]. As with Mg^{2+} screening, Hoechst 33258 screening markedly reduces line broadening within NMR spectra of ubiquitin, but more substantially alters the alignment tensors compared with the sample screened with 200 mM NaCl (**Table 1**). The normalized scalar product for the two alignment tensors measured in the presence of 200 mM NaCl and Hoechst 33258 is 0.83, indicating a lower co-linearity compared with the 200 mM NaCl screened sample, although this is still insufficient to enable independent structural information to be obtained. However, for some proteins with the appropriate surface-charge distribution, this difference may be large enough so that additional information can be obtained by repeating the screening under the two conditions.

Thus modification of charged surface DNA can be an effective approach to change molecular alignment with the same alignment media. This is notable because NMR structure determination is more accurate when multiple alignment media are used. For many difficult membrane protein targets, multiple alignment media will be needed for reliable structure determination. The detailed protocol here describes a method in which charge compatibility and variations in molecular alignment can be introduced merely by changing the charge distribution of DNA nanotubes via counter ions and small molecules.

Future challenges

Although the DNA nanotube technology is expected to be used for a wide variety of membrane proteins, it will be important to facilitate measurement of linearly independent restraints in order to obtain more structural information. Additional DNA nanostructure–based alignment media will be needed. Currently, covalently modified surface charge of nanotubes and the construction of different shape objects are being developed in our laboratories.

MATERIALS

REAGENTS

- Ampicillin sodium salt (Sigma, cat. no. A0166)
- Isopropyl β-D-1-thiogalactopyranoside (IPTG; Sigma, cat. no. I6758)
  ! CAUTION Do not breathe the dust. Avoid contact with skin and eyes.
- Triton X-100 (Sigma, cat. no. T8787) **! CAUTION** It is harmful; avoid contact with eyes.
- EDTA (Fisher Scientific, cat. no. E478-1)
- Magnesium chloride hexahydrate, 99.995% (Sigma-Aldrich, cat. no. 255777-25G)
- MOPS (VWR International, cat. no. BDH4522-500)
- 2× YT broth capsules microbial medium (Research Products International, cat. no. X15640-500.0)
- Sodium phosphate dibasic anhydrous (Fisher Scientific, cat. no. S375-500)
- Sodium phosphate monobasic anhydrous (Fisher Scientific, cat. no. S397-500)
- Polyethylene glycol 8000 (PEG8000) (Sigma-Aldrich, cat. no. P4463-1)
- Sodium chloride (Fisher Scientific, cat. no. S271-10)
- Tris base (Fisher Scientific, cat. no. BP152-10)
- UltraPure agarose (General Stores, cat. no. 7012)

- Glacial acetic acid (Fisher Scientific, cat. no. A491-212) ! CAUTION It is evaporative and corrosive. Wear goggles, lab coat and face mask during experiments. Handle acetic acid inside a hood.
- Isopropanol (Fisher Scientific, cat. no. A415-4) ! CAUTION Isopropanol is flammable. Perform all manipulations under a fume hood.
- Ethanol, 200 proof
- Hoechst 33258, 2′-(4-hydroxyphenyl)-5-[5-(4-methylpiperazine-1-yl)benzimidazol-2-yl]benzimadole, is a synthetic bis-(benzimidazole) derivative developed by Hoechst Pharmaceutical
- D_2O
- Loading buffer QBT
- Wash buffer QC
- Elution buffer QF
- Folding buffer, 20×
- Staple strands (**Box 1** and **Supplementary Table 1**)
- M13 bacteriophage ssDNA scaffold (**Box 2**)
- Protein sample conditions: typically protein samples in the range of 0.1–1 mM protein concentration are required. Detergents needed to solubilize membrane proteins must be compatible with 2 mM of $MgCl_2$. Positively charged detergent can be incompatible with DNA nanotubes. Large ranges of pH (stable from pH 4–9) and temperature (stable until 60 °C) can be used. Proteins that have already analyzed successfully using this protocol: a mitochondrial uncoupling protein 2 (UCP2) protein[7], a 40-kDa tetrameric BM2 channel[15], a homologous transmembrane-signaling dimer DAP12, a covalently linked NKG2C-DAP12 trimeric[38] and a transmembrane domain homodimer ζ-ζ of the T cell receptor complex[6].
- Desalted and lyophilized DNA oligonucleotides (Invitrogen)

EQUIPMENT

- Shaker incubator, 37 °C
- BioProducts 96-well PCR plate (Fisher Scientific, cat. no. 21-402-441)
- Aluminum sealing tape for 96-well plates (Fisher Scientific, cat. no. 11806)
- Disposable multichannel pipetter basins (Fisher Scientific, cat. no. 13-681-500)
- Gilder fine bar grids (Ted Pella, cat. no. G400)
- Qiagen-tip 10000 (Qiagen, cat. no. 10091)
- Teflon tube, fluorinated ethylenepropylene (FEP) (Thomas Scientific, cat. no. 9567K10)
- Shigemi NMR tube
- Centricon-100 concentrators
- Dissecting microscope
- Low-DNA-affinity Teflon tube
- Microscope with polarizer and rotating analyzer
- Thermal cycler (MJ Research)
- NMR spectrometer equipped with a triple resonance probe head
- Protein labels; in our hands, RDCs were recorded on protein labeled with ^{15}N, ^{13}C
- NMRPipe and nmrDraw[50] software for processing and analyzing NMR spectra
- PALES[51,39] program for fitting of the dipolar couplings to the known ubiquitin structure by singular-value decomposition. The goodness of fit was assessed by both Pearson correlation coefficient (r) and the quality factor (Q)[52]

REAGENT SETUP

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. ▲ CRITICAL It is highly recommended that all buffers used for chromatography applications be filtered.

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.

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.

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.

PROCEDURE

Nanomole-scale folding of the DNA nanotube monomers ● TIMING ~26 h

1| In a multichannel pipette basin, prepare a 37.8-ml master mix containing the following for each monomer: 120 nM scaffold p7308 (**Box 2**), 720 nM (average) each staple (**Box 1** and **Supplementary Table 1**), 20 mM $MgCl_2$, 1 mM EDTA and 5 mM Tris (pH 8.0). Stock concentrations of scaffold and staples will vary, but an example of such a master mix is provided in **Table 2**. This volume is intended for 240 folding reactions at 150 µl per reaction and includes a 5% excess to account for pipetting error.

! CAUTION In order to prevent any evaporation during the folding step, it is highly recommended to leave an empty 'border' of wells on each plate. These border wells will be filled with water, leaving 60 wells per plate for nanotube folding reactions.

▲ CRITICAL STEP Magnesium concentrations have been observed to have a drastic 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 $MgCl_2$.

▲ CRITICAL STEP 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.

2| After preparing the master mix, use a multichannel pipette to distribute 150-µl aliquots into 96-well plates (BioProducts 96-well PCR plates).

3| Fill 60 wells on each of four plates for a total of 240 reactions.

▲ CRITICAL STEP Make sure that there are no air bubbles trapped in the wells, as they could promote the formation of artifacts during folding. This can be done after making aliquots by gently pipetting the wells up and down.

4| Seal the plates with an aluminum sealing tape for 96-well plates.
! CAUTION Ensure that the plates are very well sealed in order to prevent any evaporation during the thermal annealing step.

5| 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, then decrease by 1 °C every 40 min to 20 °C. Use a heated lid to minimize evaporation.

Nanomole-scale purification of DNA nanotube monomer
● TIMING **3–4 h**
6| 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.
! CAUTION Be careful not to pipette the wells filled with water on the border of each plate.

7| Once pooled, bring each sample volume to 100 ml with Buffer QBT.
! CAUTION Be sure to mix the samples after the addition of QBT to ensure homogenous distribution of DNA.

8| Remove 50 µl of each of the two pools for an analytical agarose gel (**Box 3**).

9| *Column equilibration*. 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.
▲ CRITICAL STEP Allow the buffer to flow through completely.

10| *Column loading*. Apply 100 ml of each monomer to the appropriate column and allow to flow through completely.
▲ CRITICAL STEP Collect all flow through to ensure the recovery of material.
? TROUBLESHOOTING

11| *Column washing*. After the nanotube pools have completely flowed through the column, wash the column six times with 100 ml of buffer QC.
! CAUTION To improve the wash step, allow each wash to flow through entirely before applying subsequent washes. Save the washes.
? TROUBLESHOOTING

12| *Nanotubes elution*. Elute each monomer from the column with 100 ml of buffer QF. At this stage, the DNA solution should be homogenous and clear.
? TROUBLESHOOTING

13| Remove 50 µl of each of the two eluted samples for an analytical agarose gel (**Box 3**).

14| Add $MgCl_2$ to a final concentration of 25 mM.
▲ CRITICAL STEP Magnesium stabilizes the DNA nanotubes after folding. Some precipitate may appear but does not interfere with subsequent steps.
■ PAUSE POINT The sample can be stored at 4 °C for at least 2 d.

Heterodimerization of DNA nanotube monomers ● TIMING **~2 h**
15| The DNA nanotubes to be used for NMR alignment experiments are heterodimers of a rear monomer and a front monomer. 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.
! CAUTION Be sure to mix by swirling after combining the two monomers.
▲ CRITICAL STEP 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.
? TROUBLESHOOTING

TABLE 2 | Nanomole-scale folding of DNA-nanotube monomer.

Rear or front monomer folding	1× reaction (µl)	252× reactions (ml)
Folding buffer, 20×	7.5	1.890
Scaffold p7308 (540 nM)	33.3	8.392
Staple oligos mix (3.6 µM each)	30.0	7.560
$MgCl_2$, 1 M	1.5	0.378
DH_2O	77.7	19.580
Total	150	37.800

Example mix reagents and their concentrations for nanomole-scale folding of DNA-nanotube monomers.

Box 3 | Agarose gel electrophoresis adapted for DNA origami nanostructures ● TIMING 1–2 h

Agarose gel electrophoresis currently provides the most effective method available for high-resolution analysis and separation of well-folded DNA nanostructures. The divalent cation magnesium is a cofactor in DNA-based molecular self-assembly reactions. When separating folded DNA origami nanostructures with agarose gel electrophoresis, additions of 11 mM $MgCl_2$ in both the gel and running buffer are required. This promotes a tighter folding of the nanostructures and ensures that minimal changes in the structure conformation takes place during the separation.

Additional materials

- Gel box (12 × 14 cm), OWL Easycast B2 apparatus (Thermo Scientific)

PROCEDURE

1. For a large 1.5% (wt/vol) agarose gel with a total volume of 120 ml of gel (12 × 14 cm OWL Easycast B2 apparatus), measure 1.8 g of agarose in a 600-ml beaker.
2. Add 0.5× TBE on a balance, to yield a total mass of 120 g.
3. Add additional dH_2O to a 150-g total mass to account for evaporation while the agarose melts.
4. Microwave on high for 2 min, swirl briefly, and then microwave an additional minute.

! CAUTION Wear well-insulated gloves while handling boiling agarose solutions.

5. Gently swirl in an ice-water bath until steam no longer rises from the beaker.

▲ CRITICAL STEP After cooling, add 1 ml of 1.32 M $MgCl_2$ for a final $MgCl_2$ concentration of 11 mM.

6. Add 6 µl of 10 mg ml^{-1} ethidium bromide for a final concentration of 0.5 µg ml^{-1}.

! CAUTION It is highly recommended to add ethidium bromide once the solution is below roughly 40 °C to minimize the toxic vapor.

7. Gently swirl until ethidium bromide is no longer visible.
8. Pour the gel into casting tray and insert a comb.
9. Once the gel is solid, fill the gel box with 0.5× TBE containing 11 mM $MgCl_2$.
10. Remove the comb and load the sample.
11. Set to 60 V and run for 2–3 h before imaging.

16| Warm the mixture by incubation in a 37 °C water bath for 15 min.

▲ CRITICAL STEP The heterodimerization is performed at 37 °C to improve the kinetics of the reaction.

? TROUBLESHOOTING

17| Incubate the mixture in a 37 °C room for an additional 1 h 45 min for a total of 2 h at 37 °C.

? TROUBLESHOOTING

■ PAUSE POINT 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.

18| Remove 50 µl of the mixture for an analytical agarose gel (**Box 3**).

19| The purity and purification efficiency can be checked using agarose-gel electrophoresis (**Box 3**). We recommend using 1.5% (wt/vol) agarose gels with this nanostructure.

Concentration of DNA nanotubes and formation of DNA nanotube liquid crystals ● TIMING 4–5 h

20| Add 0.25 volumes of 20% (wt/vol) PEG8000 to the heterodimerized nanotubes.

21| Mix gently and incubate at room temperature for 15 min.

22| Spin down the nanotubes for 30 min at 15,000*g* and 4 °C.

23| Carefully decant the supernatant into another bottle.

▲ CRITICAL STEP Save the supernatant in case the nanotube pellet becomes dislodged from the bottle.

24| Spin the pellet once more for only 1 min at 15,000*g* and 4 °C to collect additional supernatant.

25| Carefully remove all remaining supernatant with a pipette.

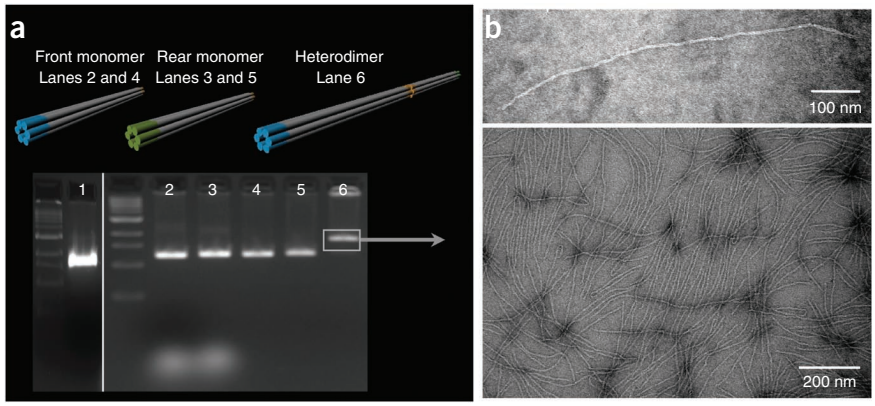

Figure 4 | Folding, purification and characterization of DNA six-helix bundle. (**a**) Cylinder models of 400-nm-long six-helix bundle monomers, 800-nm-long six-helix bundle heterodimer (not to scale) and gel analysis of the six-helix bundles after folding and purification, 1-kb ladder. Lane 1, p7308 scaffold; lanes 2 and 3, front and rear monomers folded before purification; lanes 4 and 5, front and rear monomers after purification; lane 6, heterodimer. (**b**) Both images show negative-stain transmission electron micrograph of a purified sample of six-helix bundle heterodimer.

26| To the nanotube pellet, add sufficient 0.5× folding buffer to achieve a concentration of 3 mg ml^{-1}, assuming 80% recovery from the Qiagen-tip ion-exchange columns. For the volumes described in this protocol, this will be ~6 ml.

▲ **CRITICAL STEP** Do not disturb the pellet initially. Simply add the buffer to the tube, and allow the buffer to diffuse into the pellet. 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.

27| Once the pellet has dissolved, mix the nanotube sample gently and transfer to a 50 ml conical tube.

28| Estimate the concentration of the nanotubes.

■ **PAUSE POINT** The nanotubes can be stored in 0.5× folding buffer at 4 °C for at least 6 d until one is ready to proceed with the concentration step.

29| Concentrate the nanotubes to ~30 mg ml^{-1} using Centricon-100 concentrator units. Prerinse the Centricon-100 concentrator units by adding 2 ml of water. Spin at 2,000*g* and 15 °C for 5 min to achieve concentration.

30| Remove excess water by inverting tubes and spinning at 900*g* for 2 min.

31| Weigh the Centricon-100 concentrator units, and then apply DNA-nanotube samples and record the mass of the concentrator unit with the DNA nanotubes.

32| Spin the nanotubes in 15-min increments at 1,500*g* and 15 °C. Estimate the concentration by periodically recording the mass of the concentrators with the DNA nanotubes. The beginning concentration of the DNA (3 mg ml^{-1}) is 10× lower than the desired concentration (30 mg ml^{-1}); therefore, a 10× decrease in the mass of the sample gives a good approximation of the desired concentration.

▲ **CRITICAL STEP** To prevent damage to the nanotube structure it is recommended that all spins be at speeds less than 2,000*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.

33| When the 10× decrease in sample mass is achieved, recover DNA by inverting tubes into collection vials and spinning 3 min, 1,000*g*, 20 °C. The final total volume will typically be between 1 and 1.5 ml. Concentrated to 30 mg ml^{-1}, 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 (Step 34). If the sample is not birefringent, spin the nanotubes in 15-min increments at 1,500*g* and 15 °C until the sample appears viscous.

34| 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 **Figure 4**.

■ **PAUSE POINT** DNA nanotubes are very stable and can be stored at 4 °C for at least 12 months.

Measuring residual D_2O quadrupole coupling in the presence of DNA nanotubes ● **TIMING ~1 h**

35| Add D_2O to 250 µl of the DNA nanotube liquid crystal to a final concentration of 10% (vol/vol). Mix slowly by pipetting.

36| Use a low-DNA-affinity Teflon tube to transfer 250 µl of the nanotube sample with 10% D_2O into a Shigemi NMR tube.
▲ **CRITICAL STEP** 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.

37| Spin down the NMR sample at 500*g* and 15 °C for 2 min and add the Shigemi plunger.
▲ **CRITICAL STEP** At 30 mg ml^{-1}, 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.

38| Record 1D NMR spectrum at ^2H frequency.

39| Process 1D NMR spectra and measure D_2O splittings.

Preparation of NMR protein samples with DNA nanotubes ● **TIMING ~2 h**
40| Prerinse a Centricon-100 concentrator unit as in Steps 29–31.

41| 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^{-1}. Weigh the Centricon-100 concentrator unit with the DNA sample.

42| Exchange the DNA nanotubes into the desired protein buffer by diluting the nanotubes twofold with the protein buffer.

43| Mix the twofold-diluted sample slowly by pipetting up and down. Spin the nanotubes in 5 min increments at 1,500*g*, 15 °C.

44| Between each spin, mix the sample slowly by pipetting up and down. Stop the concentration when the columns reach roughly the starting weight.

45| Repeat Steps 42–44 three times to achieve sufficient exchange.

46| 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 1,500*g* and 15 °C.
▲ **CRITICAL STEP** 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 slowly up and down.

47| Recover the NMR sample from the Centricon concentrator unit.
▲ **CRITICAL STEP** 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 1,000*g* and 20 °C.
■ **PAUSE POINT** Store at 4 °C or temperature appropriate for protein of interest.

Long-term stability of DNA nanotubes with positively charged proteins
48| Much like other negatively charged alignment systems such as the Pf1 filamentous phage, high pI proteins can bind nonspecifically to DNA nanotubes. However, we have developed various approaches that can increase compatibility with protein-micelle complexes that carry significant positive charge. The following approaches can be routinely used to record accurate RDC constraints for NMR applications. Use option A for weak alignment at higher ionic strength (**Fig. 5a**), option B for weak alignment with magnesium chloride (**Fig. 5b**), or option C for weak alignment in the presence of small DNA-binding molecules (**Fig. 5c**). In option B, positively charged magnesium ions reduce the effective charge of the nanotubes by binding with greater electrostatic affinity to the negatively charged phosphate groups of the DNA (**Fig. 5b**). Of the four small molecules tested (ethidium bromide, 9-aminoacridine, DAPI and Hoechst 33258) that specifically bind DNA and shield the charge, we found the best performance with Hoechst 33258, a DNA groove binder, and this is used in option C.
? TROUBLESHOOTING
(A) Weak alignment at higher ionic strength
(i) Perform a small-scale screen of increasing NaCl concentrations for compatibility with the relevant protein/detergent complex. In each well of a 24-well plate, mix 1 µl each of purified protein and DNA nanotubes in a common buffer with specific NaCl concentrations (50, 100, 150, 200 and 300 mM NaCl). Incubate for 24–48 h at 18 °C.

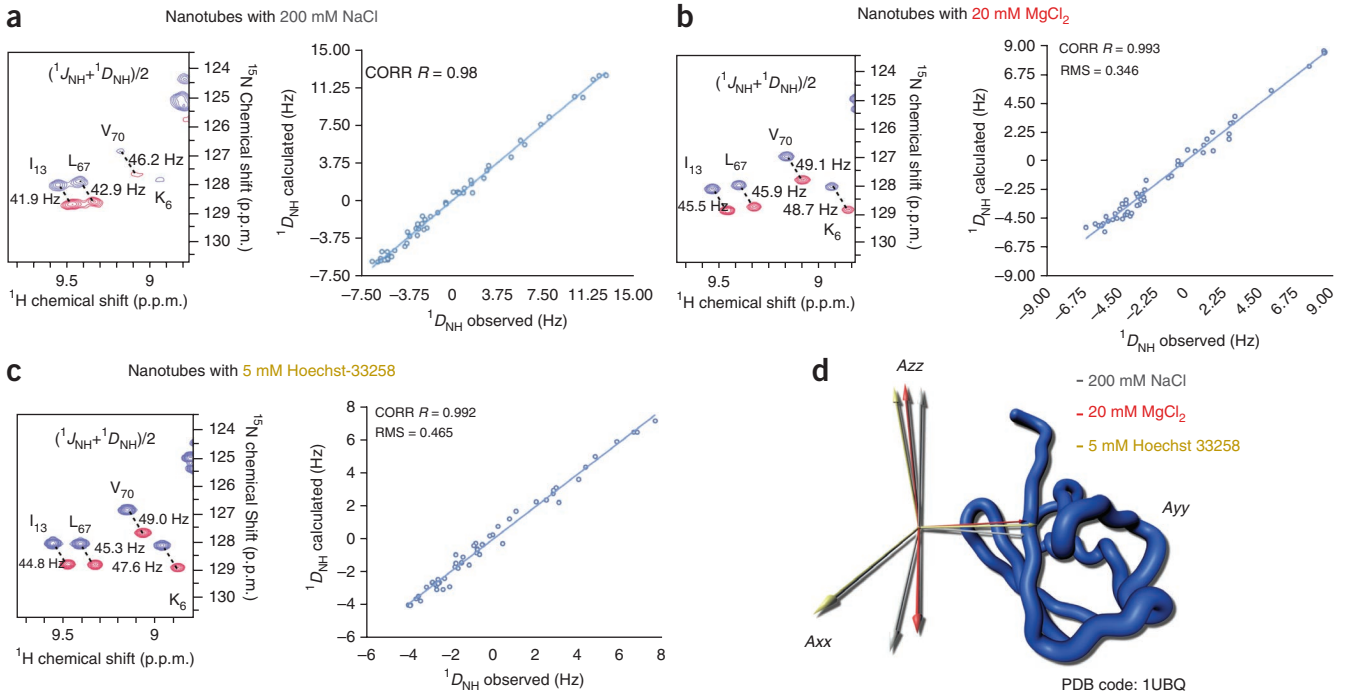

Figure 5 | Residual dipolar couplings measurement in the presence of small molecule. $^{1}J_{NH}/2$ splittings for the anisotropic samples. Superposition of the ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) (blue peaks) and transverse-relaxation-optimized spectroscopy (TROSY; red peaks) spectra recorded at 25 °C at 1H frequency of 600 MHz. The sample condition is 500 µM uniformly ^{15}N-labeled ubiquitin dissolved in 20 mg ml^{-1} DNA nanotubes with different buffer conditions. (**a**) With 200 mM NaCl: 20 mM Tris, 200 mM NaCl, pH 7.5. (**b**) With 20 mM magnesium: 20 mM Tris, 50 mM NaCl, pH 7, 20 mM $MgCl_2$. (**c**) With 5 mM Hoechst 33258: 20 mM Tris, 50 mM NaCl, pH 7, 5 mM Hoechst 33258. The ^{15}N-^{1}HN splittings (in Hz) are marked together with the sequential assignment. Correlation between measured ^{1}H-^{15}N RDCs of ubiquitin in 20 mg ml^{-1} DNA nanotubes and couplings calculated on the basis of X-ray crystal structure of ubiquitin are shown for each buffer condition. Linear regression applied to the data gives a correlation coefficient of 0.98, 0.993 and 0.992, respectively. (**d**) Structure of ubiquitin (PDB code: 1UBQ) shown with the orientation of the principal components of the alignment tensors in gray (200 mM NaCl), in red (20 mM $MgCl_2$) and in yellow (5 mM Hoechst 33258).

(ii) By using a microscope at ×100 magnification, evaluate the amount of precipitate formed for each NaCl condition. The lowest NaCl concentration in which precipitates no longer form is optimal.

(iii) Exchange 180 µl of the DNA nanotube liquid crystal at 25 mg ml^{-1} into the relevant protein buffer containing the optimal concentration of NaCl (see Steps 42–44 for nanotube buffer exchange).

(iv) If the purified protein is not in a buffer containing the optimal concentration of NaCl, add an appropriate amount of a concentrated NaCl stock to achieve the optimal concentration. Once the DNA nanotubes are in the appropriate buffer, the NMR samples are prepared by mixing the protein and the DNA (1:1) and then re-concentrating down to the appropriate volume and protein concentration as described previously[50,52].

(v) Measure the isotropic and anisotropic splittings in the unaligned and aligned protein samples using the pulse sequences appropriate to the protein as described previously[42,43].

(B) Weak alignment with magnesium chloride

(i) Exchange 180 µl of the DNA nanotube liquid crystal at 25 mg ml^{-1} into the relevant protein buffer containing 20 mM of $MgCl_2$ (see Steps 42–44 for nanotube buffer exchange).

(ii) If the purified protein is not in a buffer containing 20 mM $MgCl_2$, add an appropriate amount of a concentrated $MgCl_2$ stock to achieve the 20 mM. Once the DNA nanotubes are in the appropriate buffer, the NMR samples are prepared by mixing the protein and the DNA (1:1) by volume and then re-concentrating to the appropriate volume and protein concentration as described above (Steps 46 and 47).

(iii) Measure the isotropic and anisotropic splittings in the unaligned versus aligned protein samples using the pulse sequences appropriate to the protein as described above (Steps 38 and 39).

(C) Weak alignment in presence of dicationic Hoechst 33258 molecule

(i) Exchange 180 µl of the DNA nanotube liquid crystal at 25 mg ml^{-1} into the relevant protein buffer containing 5 mM Hoechst 33258 (see steps 42–44 for nanotube buffer exchange). After the initial dilution of the nanotubes with the protein buffer containing 5 mM Hoechst 33258, incubate at room temperature for 15 min.

(ii) Once the DNA nanotubes are in the appropriate buffer, the NMR samples are prepared by mixing the protein and the DNA (1:1) by volume and then re-concentrating down to the appropriate volume and protein concentration as described above (Steps 46 and 47).
(iii) Measure the isotropic and anisotropic splittings in the unaligned versus aligned protein samples using the pulse sequences appropriate to the protein as described above (Steps 38 and 39).

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Solution
Nanomole-scale purification of DNA-nanotube monomer			
10–12	Low purification yield	Column was overloaded	Check the sample volume and yield against the capacity of the Qiagen-tip 10000. The maximum DNA-binding capacities of the Qiagen-tip 10000s are at least 10 mg. Inappropriate salt or pH conditions in buffers may lower this capacity dramatically. Ensure that any buffers prepared in the laboratory were prepared according to the instructions provided for each purification step. Above pH 7, the column shows reduced affinity for DNA nanotubes
Heterodimerization of DNA nanotube monomers			
15–17	Low dimerization yield	After the purification step, ensure that each monomer is produced in a 1:1 stoichiometric ratio	Equimolar amounts of each monomer have to be mixed to form 100% of heterodimer. If one of the monomers is formed in excess, its amount should be reduced to a stoichiometric quantity before mixing
Weak alignment at higher ionic strength			
48	Long-term stability of DNA nanotubes with positively charged proteins	Highly positively charged proteins may have an inherent tendency to stick to the negatively charged DNA nanotubes	Salt concentrations above 100 mM are sufficient in many of these cases for enabling the acquisition of a reasonable spectrum. However, the tumbling of the proteins may slow down slightly, which in turn may compromise the resolution of the acquired RDCs. As an example, ubiquitin has several peaks of weak intensity while in a 20 mg ml^{-1} DNA-nanotube liquid crystal (see lysine residue 6 in **Fig. 5a**). Additionally, high ionic strength has an adverse effect on the sensitivity gains of NMR experiments in general. The use of NMR buffers made of ions with low mobility provides a way to improve the sensitivity of NMR experiments at the high-salt concentrations that may be necessary to prevent nonspecific binding to DNA nanotubes. This can be done by using salts of alkaline earth metals, such as magnesium, that have a high affinity for the DNA nanostructure, low conductivity and low ion mobility. This will result in substantial shortening of the pulse length and improvement of the charge compatibility between the protein and DNA (**Fig. 5a**)
Weak alignment with magnesium chloride			
48	Long-term stability of detergents with magnesium ions	Some detergents can be incompatible with magnesium ions	To accommodate such detergents, charge shielding can be done via positively charged small molecules that specifically bind to DNA

● TIMING

Steps 1–4, nanomole-scale folding of the DNA nanotube monomers: ~26 h
Steps 6–14, nanomole-scale purification of DNA nanotube monomer: 3–4 h

Steps 15–19, heterodimerization of DNA nanotube monomers: ~2 h
Steps 20–34, concentration of DNA nanotubes and formation of DNA nanotube liquid crystals: 4–5 h
Steps 35–39, measuring residual D_2O quadrupole coupling in the presence of DNA nanotubes: ~1 h
Steps 40–47, preparation of NMR protein samples with DNA nanotubes: ~2 h
Step 48, long-term stability of DNA nanotubes: variable
Box 1, hydrating and pooling staple strands: ~2 h
Box 2, nanomole-scale production of M13 bacteriophage ssDNA scaffold: 2 d
Box 3, agarose gel electrophoresis: 1–2 h

ANTICIPATED RESULTS

Production of the DNA scaffold

Lane 1 in **Figure 4a** shows typical results obtained from the p7308 scaffold purification. With the protocol described, usually between 20 and 25 nmol of p7308 scaffold can be obtained per 3.6 liters of culture with a purity greater than 95%.

Heterodimerization of DNA nanotube monomers

Figure 4a shows typical results obtained from six-helix bundle after folding, purification and dimerization. With the protocol described, 25–40 mg of DNA nanotube can be obtained with a purity greater than 95%. This purification protocol has been consistently successful in generating nanotubes of sufficient quality and quantity for subsequent NMR experiments. The scale of the preparation can be easily scaled by modifying master mix quantities and Qiagen-tip column capacities.

Measuring residual D_2O quadrupole coupling in the presence of DNA nanotubes

A DNA nanotube liquid crystal with 10% (vol/vol) D_2O yields stable residual quadrupole coupling for the ^2H lock signal (**Fig. 2b**). Splitting of ~6 Hz is routinely observed for large-scale preparations at ~25 mg ml^{-1}, although greater splitting can be achieved at higher nanotube concentrations (**Fig. 4c**).

Weak alignment at higher ionic strength

The optimal NaCl concentrations will vary from protein to protein, depending on the extent of the positive charge. For ubiquitin, which is highly positively charged, the optimal NaCl concentration is 200 mM.

Weak alignment with magnesium chloride

The critical DNA concentration required to promote anisotropic protein behavior is unchanged in the presence of 20 mM $MgCl_2$, which leads to a much lower dissipation of RF power and therefore a higher signal-to-noise ratio relative to NaCl. To achieve the same signal-to-noise ratio requires an acquisition time twice as long in 200 mM NaCl compared with 20 mM $MgCl_2$ (**Fig. 5b**). Additionally, the alignment tensor induced under 200 mM NaCl is slightly different than that under 20 mM $MgCl_2$ (**Fig. 5d** and **Table 1**). Although this approach provides a general solution for the spectral optimization of many other proteins that suffer from interfacial line broadening caused by nonspecific binding to the DNA nanotubes, some detergents can be incompatible with magnesium ions. To accommodate such detergents, refer to the following section, in which we describe charge shielding via positively charged small molecules that specifically bind to DNA.

Weak alignment in the presence of dicationic Hoechst 33258

The critical DNA concentration required to promote anisotropic protein behavior is the same in the presence of 5 mM Hoechst 33258, and the nanotubes show similar deuterium splitting. As with Mg^{2+} charge shielding, Hoechst 33258 shielding substantially reduces observed line broadening in ubiquitin spectra, but more substantially alters the alignment tensors compared with the sample shielded with 200 mM NaCl (**Fig. 5c**,**d** and **Table 1**).

Modification of the charged surface of the DNA nanotube is not only a useful means of making positively charged proteins more compatible with the system, but also an effective approach to achieving multiple alignment tensors with the same alignment medium.

Note: Supplementary information is available in the online version of the paper.

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