Kimberly N. Rendek, Raimund Fromme, Ingo Grotjohann and Petra Fromme*

Department of Chemistry and Biochemistry,
Arizona State University, Tempe,
AZ 85287-1604, USA

Correspondence e-mail: pfromme@asu.edu

Received 16 October 2012
Accepted 31 December 2012

Crystallization of a self-assembled three-dimensional DNA nanostructure

The powerful and specific molecular-recognition system present in the base-pairing of DNA allows for the design of a plethora of nanostructures. In this work, the crystallization of a self-assembling three-dimensional B-DNA nanostructure is described. The DNA nanostructure consists of six single-stranded oligonucleotides that hybridize to form a three-dimensional tetrahedron of 80 kDa in molecular mass and 20 bp on each edge. Crystals of the tetrahedron have been successfully produced and characterized. These crystals may form the basis for an X-ray structure of the tetrahedron in the future. Nucleotide crystallography poses many challenges, leading to the fact that only 1352 X-ray structures of nucleic acids have been solved compared with more than 80 000 protein structures. In this work, the crystallization optimization for three-dimensional tetrahedra is also described, with the eventual goal of producing nanocrystals to overcome the radiation-damage obstacle by the use of free-electron laser technology in the future.

1. Introduction

Biomolecules such as DNA possess characteristics like sequence-specific base-pairing that allow their use as stable frameworks in nanotechnology (Feldkamp & Niemeyer, 2006). The physicochemical stability of DNA makes it a desirable candidate for the design of nanostructured materials as DNA is inherently nanoscalable. Double-stranded DNA (dsDNA) readily hybridizes in a highly reproducible process. Strands are separated at a 363 K peak temperature followed by a cooling step which facilitates hydrogen bonding between the complementary bases adenine–thymine and guanine–cytosine, and allows the dsDNA to relax into its characteristic helical B-DNA structure (Narayana & Weiss, 2009). Because of the reproducibility of experiments and well established protocols present in DNA technology, diverse three-dimensional nanostructures have previously been designed (Feldkamp & Niemeyer, 2006; Goodman *et al.*, 2005; Kato *et al.*, 2009), including a tetrahedron structure with 20 bp edges consisting of B-DNA (20T tetrahedron; Goodman *et al.*, 2005). Prior characterization of the 20T tetrahedron included an 8 Å resolution cryo-electron microscopy (cryo-EM) structure (Kato *et al.*, 2009) that provided the overall topography and verified the expected assembly of the structure. However, the resolution limit of cryo-EM prevents distinguishing between diastereomers and does not allow the identification of individual bases. The use of a DNA framework for hosting biomolecules was first proposed by Seeman (1982). The fairly large cavity size of the 20T (inner diameter of 70 Å) tetrahedron structure enables small proteins and designed functional molecules to be enclosed in the center of the construct and a report has been published on cytochrome *c* conjugation (Erben *et al.*, 2006). Further work includes the introduction of a two-dimensional scaffold of tetrahedra that is formed during the annealing step (Zheng *et al.*, 2009) and incorporation of the tetrahedron into antimony-doped tin oxide porous material (Simmons *et al.*, 2011).

A rationally designed three-dimensional network has many useful applications, especially as it has the ability to host macromolecules in a periodic array similar to a crystal lattice (Seeman, 1982). Addi-

© 2013 International Union of Crystallography
All rights reserved

tionally, encapsulating redox-active proteins or molecules inside the tetrahedron would allow the use of the system for electron-transfer reactions. However, to date no X-ray crystallographic structures of a single molecule inside a three-dimensional DNA nanostructure have been determined. The PDB reveals that of all the structures solved by X-ray diffraction, only 1.9% are nucleic acid structures and 4.6% are protein–nucleic acid complexes. Most of the published DNA structures solved by X-ray crystallography are DNA–protein complexes, short DNA sequences and tetraplexes (Narayana & Weiss, 2009; Phillips *et al.*, 1997; Lipscomb *et al.*, 1995). The pioneering work of Seeman and coworkers showed the first structure of a DNA lattice formed by annealing which is facilitated by ‘sticky ends’ (Goodman *et al.*, 2005), leading to a planar two-dimensional array of DNA triangles. Here, we report the first classical crystallization of a three-dimensional tetrahedron DNA nanostructure.

2. Materials and methods

2.1. Design of tetrahedron structures

For each construct, single-stranded DNA (ssDNA) strands were designed to anneal to form dsDNA that formed three-dimensional tetrahedra. The strands for the 20T tetrahedron were designed as described in the work of Goodman *et al.* (2005) forming a tetrahedral structure with 20 bp edges connected by adenines in each corner, and also a 20 × 30T tetrahedron with three 20 bp and three 30 bp edges (Goodman *et al.*, 2005) and a 30T tetrahedron consisting of 30 bp edges. A schematic indicating the overall dimensions for each construct is shown in Supplementary Figs. S1 and S2.¹

2.2. Oligonucleotide purification

The four single-stranded DNA oligonucleotides for all three constructs (Goodman *et al.*, 2005) were purified as described in the Supplementary Material. The oligonucleotides were quantified by their absorbance at 260 nm.

2.3. Annealing and purification of 20T tetrahedron

The annealing and purification scheme of the 20T tetrahedron is shown in Fig. 1. Information on the other constructs as well as experimental details can be found in the Supplementary Material. Size-exclusion chromatography provided a route for selectively isolating the individual tetrahedrons from traces of aggregation products and low-molecular-weight DNA fragments. Concentration during the annealing step was explored and the optimal concentration for annealing was 50 nM of each strand of DNA. The results are shown in Supplementary Fig. S4.

2.4. Tetrahedron characterization

Prior to crystallization experiments, native gel electrophoresis and dynamic light scattering (DLS) were used to analyze the homogeneity of the 20T tetrahedron. The corresponding methods and results are shown in Supplementary Fig. S5. A highly concentrated sample was achieved with less than $3.2 \times 10^{-6}\%$ aggregation.

2.5. Crystallization

Immediately following annealing, purification and characterization, crystallization experiments were conducted. For the initial screens, tetrahedron concentrations ranging from 1.5 to 4 mg ml^{−1}

were used. DLS experiments showed that some aggregates formed for samples with concentrations higher than 3 mg ml^{−1}. Therefore, 2.5 mg ml^{−1} was the concentration used for the optimization experiments for the crystallization. Sparse-matrix screens (Sigma, St Louis, Missouri, USA) were used to determine the initial crystallization conditions for the tetrahedron. Crystal optimization was performed by systematic variation of the temperature, PEG 1000 concentration and buffers and by pH variation, as well as the use of additives such as argininamide which stabilize the DNA structure. A 96-well-plate format with automatic imaging (Rigaku, The Woodlands, Texas, USA) was used for the large-scale screening. After several rounds of optimization, fine screening was performed in 24-well-plate format (Qiagen, Valencia, California, USA) in the range 29–34% PEG 1000, 10–25 mM L-argininamide and 50 mM sodium cacodylate pH 6.0. Crystals were grown in an incubator at 296 K and formed in approximately 2–3 d. In addition, heterogeneous nucleation experiments were performed and the best results were obtained using human hair as the nucleation agent.

2.6. Crystal cooling and X-ray diffraction

Fresh tetrahedron crystals were incubated in cryoprotectants before being cooled in liquid nitrogen. A large range of cryoprotectants was tested, including screening solutions from the NeXtal Cryos Suite (Qiagen, Valencia, California, USA) containing various cryoprotectants including 2-methyl-1,3-propanediol (MPD),

Figure 1
Schematic representation of 20T tetrahedron purification.

¹Supplementary material is available from the IUCr electronic archives (Reference: PU5389).

2-propanol, glycerol and various PEGs and different buffers including Tris, HEPES, PIPES and MES. However, the tetrahedron crystals dissolved or cracked in all of those cryoprotectant screen solutions. Therefore, an optimized cryoprotectant was developed by variation of the screen solution in which the crystals grew. After optimization, the best cryoconditions were established as 40% PEG 1000, 60 mM sodium cacodylate pH 6.0, 18 mM L-argininamide. Crystals were incubated for approximately 10 min before being flash-cooled in liquid nitrogen. X-ray diffraction experiments were performed on beamline 8.2.2 at the Advanced Light Source at Lawrence Berkeley National Laboratory and on beamline ID-19 at the Advanced Photon Source at Argonne National Laboratory.

3. Results and discussion

3.1. Tetrahedron designs

A schematic view of the tetrahedron construct investigated in this study is shown in Fig. 1, which is named 20T (20 base pairs on each edge). Fig. 1 also shows a schematic illustrating the tetrahedron purification scheme. The characterization results of other constructs explored in this work are shown in the Supplementary Material.

3.2. Crystallization of the 20T tetrahedron

Our tetrahedron crystallization experiments involved classical crystallization techniques in which the solubility of the drop in a vapor-diffusion experiment was reduced using precipitants. In the

initial crystallization screening, sparse-matrix screens were used (Sigma, St Louis, Missouri, USA). Initial screening indicated crystallites in the following conditions: (i) 50 mM sodium cacodylate pH 6.0, 20 mM argininamide, 1 mM barium chloride, 2 mM spermidine hydrochloride, 30% PEG 400; (ii) 50 mM HEPES pH 7.0, 1 mM cobalt hexamine chloride, 35% PEG 400; (iii) 50 mM sodium cacodylate pH 6.5, 20 mM lithium chloride, 5 mM cobalt(II) chloride, 35% PEG 600; (iv) 50 mM HEPES pH 7.0, 20 mM ammonium chloride, 5 mM MgCl₂, 35% PEG 600; (v) 1 mM cobalt hexamine chloride, 2 mM putrescin hydrochloride, 35% PEG 600 and (vi) 50 mM sodium cacodylate pH 6.0, 1 mM cadmium chloride, 30% PEG 1000.

Comparing the conditions, we noticed that all crystallization conditions that produced initial hits had a pH between 6.0 and 7.0. Most of the crystals were very small and showed visible defects, such as being hollow or clustered. Crystals were analyzed for their diffraction at synchrotron sources and the resolution was around 10 Å using PEG 1000 as the precipitant. We screened multiple additives including argininamide, which gave the initial hit to improve crystal quality. Most of these additives contained the positively charged amine groups that may aid in stabilizing the structure of the DNA *via* electrostatic interactions, minimizing charge repulsion of the phosphate backbone. An additive screen using argininamide has been proposed by Sauter *et al.* (1999) and has been successfully used for crystallization and structure determination of a DNA aptamer by Robertson *et al.* (2000). The work of Robertson and coworkers also showed the tight structural interactions between the DNA and argininamide. After several rounds of optimization, optimized conditions were established in 29–34% PEG 1000, 10–25 mM L-argininamide, 50 mM sodium cacodylate pH 6.0 using the hanging-drop vapor-diffusion technique. Crystal sizes in the sitting-drop setup ranged from approximately 50–100 µm in conditions with higher PEG 1000 concentrations to 100–500 µm at the largest with lower PEG 1000 concentrations. Under optimized conditions, tetrahedron crystals

Figure 2

Crystal size comparison of the largest DNA tetrahedron crystals observed (a), and crystals obtained through heterogeneous nucleation techniques (b).

Figure 3

Single X-ray diffraction pattern of 20T crystals to a resolution limit of approximately 3.4 Å.

Table 1
Crystallographic data analysis of the 20T tetrahedron.

Space group	<i>C</i> 2
Unit-cell parameters (Å, °)	<i>a</i> = 105, <i>b</i> = 171, <i>c</i> = 60, $\alpha = 90, \beta = 92.7, \gamma = 90$
Solvent content (%)	50
Wavelength (Å)	1
Photon flux (s ⁻¹)	10 ¹²
Beam dimensions (µm)	250 × 200
Exposure time (s)	3
Detector size (pixels)	3072 × 3072
Resolution (Å)	3.4

grew in approximately 2–3 d at 296 K. The crystal shape was consistently rectangular, colorless and transparent, but even after optimization many crystals showed visible defects. Fig. 2(*a*) shows a large crystal grown in 50 mM sodium cacodylate pH 6.0, 10 mM L-argininamide, 29% PEG 1000. The large crystal was 400 µm long and 40 µm thick. Fig. 2(*b*) shows crystals grown in an experiment under similar conditions with the use of human hair as a seed. These crystals were grown in 50 mM sodium cacodylate pH 6.0, 15 mM argininamide, 32% PEG 1000. Most of the crystals grew on the surface of the hair and their average size was 50 µm in length and 10 µm in diameter. They also displayed a more solid and stable appearance during crystal handling, mounting and freezing. These smaller crystals

showed fewer defects than the larger crystals. Crystals of different sizes were cooled as described in §2.

3.3. X-ray data analysis

X-ray diffraction experiments were performed on beamline 8.2.2 at the Advanced Light Source at Lawrence Berkeley National Laboratory and on beamline ID-19 at the Advanced Photon Source at Argonne National Laboratory (for details of data collection, see Supplementary Material). A partial data set of 200 images was collected from a large crystal of 200 µm in length and 50 µm in diameter. The crystal diffracted to 3.4 Å resolution. The crystal was shifted several times during data collection to reduce the effects of X-ray damage. The diffraction pattern is shown in Fig. 3. However, large anisotropy was observed in the crystal diffraction pattern, as shown in Fig. 3. While the crystal diffracts very well in one orientation, the data set is anisotropic, where peaks are smeared out to lines in some orientations. At the beginning of the data collection (Fig. 4*a*) spots are visible which represent a pattern more characteristic of diffraction-quality crystals, whereas in Fig. 4(*f*) the spots not only diminish but also become very broad and faint. The small crystals showed significantly lower anisotropy but were very sensitive to X-ray exposure and fewer than 20 images could be collected before

Figure 4
X-ray diffraction from a single 20T tetrahedron crystal over time.

Figure 5

20T tetrahedron crystals grown in varying PEG concentrations with the goal of growing micro/nanocrystals. (a) Crystals grown in 50 mM sodium cacodylate pH 6.0, 10 mM L-argininamide, 30% PEG 1000, (b) crystals grown in 50 mM sodium cacodylate pH 6.0, 10 mM L-argininamide, 31% PEG 1000, (c) crystals grown in 50 mM sodium cacodylate pH 6.0, 10 mM L-argininamide, 32% PEG 1000. The scale bars are 200 μ m in length.

the peaks diminished. The results of preliminary data evaluation are shown in Table 1.

4. Discussion

The X-ray data analysis of the tetrahedron crystals shows a common dilemma of difficult-to-crystallize biomolecules. Large crystals show long-range disorder leading to anisotropic resolution. Small crystals are better ordered; however, they are more prone to X-ray damage and therefore data collection is difficult, even at microfocused beamlines. In X-ray crystallography, cooling and X-ray structure analysis of the crystals under cryogenic conditions (77–100 K) reduce the secondary radiation damage to the sample. Cooling leads to a significant reduction in X-ray damage in biomolecular crystals (for more details, see the review by Garman & Owen, 2006), but even under cryogenic conditions 85% of the X-rays are absorbed leading to X-ray damage while only 5% are scattered. The X-ray dose limit for biomolecular crystals has been calculated to be 2×10^7 Gy for the diffracting power of cryocooled protein crystals (Owen *et al.*, 2006). Recently, femtosecond crystallography has shown a path to overcome the X-ray damage limitation of small crystals. In femtosecond crystallography, X-ray data are collected from a liquid stream of nanocrystals smaller than 1 μ m at room temperature using free-electron lasers. Each X-ray pulse is so short that X-ray diffraction is observed before the crystal is destroyed, thereby overcoming the radiation-damage problem in X-ray crystallography (Gaffney & Chapman, 2007; Chapman *et al.*, 2011). Our group and collaborators have recently pioneered this method and established a method for the growth of protein nanocrystals. We have applied these methods to try and grow nanocrystals of the tetrahedron. The initial results are depicted in Fig. 5, showing the development towards the production of small crystals of the tetrahedron. The crystal size visibly decreased at higher PEG concentrations, leading to showers of small crystals of 0.5–2 μ m in size in Fig. 5(c). A beamline proposal for DNA nanocrystallography has been submitted to LCLS (Linac Coherent Light Source, SLAC National Accelerator Laboratory) to investigate the diffraction quality of the microcrystals.

5. Conclusion and outlook

In this paper, we have described the first crystallization of three-dimensional tetrahedral DNA nanostructures. Crystals of the 20T

tetrahedron have been grown and have been characterized by X-ray diffraction, with observed improvements in the resolution limits from an initial 10 to 3.4 Å. Crystals of different sizes were investigated with large anisotropy being observed for large crystals and X-ray damage limiting resolution and data-set completeness for small crystals, even at cryogenic temperatures. Current work is focused on further reduction of anisotropy of large crystals using seeding techniques, as well as probing the use of femtosecond crystallography (Gaffney & Chapman, 2007; Chapman *et al.*, 2011; Boutet *et al.*, 2012) for analysis of nanocrystals of DNA nanostructures. Femtosecond crystallography is based on the collection of diffraction data at room temperature from a stream of nanocrystals which are exposed to ultrafast femtosecond X-ray pulses of high intensity. The crystals are destroyed in each X-ray pulse, but diffraction is observed before the onset of any damage. Thereby one can overcome X-ray dose limits by the ‘diffract before destroy’ principle (Barty *et al.*, 2012). Solving the X-ray structure of the tetrahedron would prove to be a very important step in the X-ray crystallography of DNA nanostructures. A high-resolution structure would provide insight into the conformation and location of each nucleic acid. The structure of the tetrahedron would also provide valuable information that would enable us to facilitate the attachment of guest molecules inside the cage that can be used as synthetic active sites of proteins. We also hope that techniques could subsequently be used to incorporate redox-active proteins involved in photosynthetic electron transfer. This would enable the rational design of covalent linkages of peptides and proteins in the future, such as, for example, the encapsulation of a synthetic oxygen-evolving complex capable of splitting water into protons and electrons that would provide the necessary driving force to power a solar fuel cell.

We would like to thank Dr Hao Yan, Dr Yan Liu and Chad Simmons for helpful discussion. This material is based upon work supported as part of the Center for Bio-Inspired Solar Fuel Production, an Energy Frontier Research Center funded by the US Department of Energy, Office of Science, Office of Basic Energy Sciences under Award No. DE-SC0001016.

References

- Barty, A. *et al.* (2012). *Nature Photonics*, **6**, 35–40.
- Boutet, S. *et al.* (2012). *Science*, **337**, 362–364.

- Chapman, H. N. *et al.* (2011). *Nature (London)*, **470**, 73–77.
- Erben, C. M., Goodman, R. P. & Turberfield, A. J. (2006). *Angew. Chem. Int. Ed. Engl.* **45**, 7414–7417.
- Feldkamp, U. & Niemeyer, C. M. (2006). *Angew. Chem. Int. Ed. Engl.* **45**, 1856–1876.
- Gaffney, K. J. & Chapman, H. N. (2007). *Science*, **316**, 1444–1448.
- Garman, E. F. & Owen, R. L. (2006). *Acta Cryst.* **D62**, 32–47.
- Goodman, R. P., Schaap, A. T., Tardin, C. F., Erben, C. M., Berry, R. M., Schmidt, C. F. & Turberfield, A. J. (2005). *Science*, **310**, 1661–1665.
- Kato, T., Goodman, R. P., Erben, C. M., Turberfield, A. J. & Namba, K. (2009). *Nano Lett.* **9**, 2747–2750.
- Lipscomb, L. A., Peek, M. E., Morningstar, M. L., Verghis, S. M., Miller, E. M., Rich, A., Essigmann, J. M. & Williams, L. D. (1995). *Proc. Natl Acad. Sci. USA*, **92**, 719–723.
- Narayana, N. & Weiss, M. A. (2009). *J. Mol. Biol.* **385**, 469–490.
- Owen, R. L., Rudiño-Piñera, E. & Garman, E. F. (2006). *Proc. Natl Acad. Sci. USA*, **103**, 4912–4917.
- Phillips, K., Dauter, Z., Murchie, A. I., Lilley, D. M. & Luisi, B. (1997). *J. Mol. Biol.* **273**, 171–182.
- Robertson, S. A., Harada, K., Frankel, A. D. & Wemmer, D. E. (2000). *Biochemistry*, **39**, 946–954.
- Sauter, C., Ng, J. D., Lorber, B., Keith, G., Brion, P., Hosseini, M. W., Lehn, J.-M. & Giegé, R. (1999). *J. Cryst. Growth*, **196**, 365–376.
- Seeman, N. C. (1982). *J. Theor. Biol.* **99**, 237–247.
- Simmons, C. R., Schmitt, D., Wei, X., Han, D., Volosin, A. M., Ladd, D. M., Seo, D.-K., Liu, Y. & Yan, H. (2011). *ACS Nano*, **5**, 6060–6068.
- Zheng, J., Birktoft, J. J., Chen, Y., Wang, T., Sha, R., Constantinou, P. E., Ginell, S. L., Mao, C. & Seeman, N. C. (2009). *Nature (London)*, **461**, 74–77.