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The Absence of Tertiary Interactions in a Self-Assembled DNA Crystal Structure

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Abstract

DNA is a highly effective molecule for controlling nanometer scale structure. The convenience of using DNA lies in the programmability of Watson-Crick base-paired secondary interactions, useful both to design branched molecular motifs, and to connect them through sticky-ended cohesion. Recently, the tensegrity triangle motif has been used to self-assemble 3D crystals whose structures have been determined; sticky ends were reported to be the only intermolecular cohesive elements in those crystals [Zheng J, Birktoft, JJ, Chen Y, Wang T, Sha R, Constantinou PE, Ginell SL, Mao C, Seeman NC. 2009. From Molecular to Macroscopic *via* the Rational Design of a Self-Assembled 3D DNA Crystal. *Nature* **461**:74–77]. A recent communication [Timsit Y, Varnai P. 2011. Cytosine, the double helix and DNA self-assembly. *J. Mol. Recognition* **24**:137–138] suggested that tertiary interactions between phosphates and cytosine N(4) groups are responsible for intermolecular cohesion in these crystals, in addition to the secondary and covalent interactions programmed into the motif. To resolve this issue, we report experiments challenging this contention. Gel electrophoresis demonstrates that the tensegrity triangle exists in conditions where cytosine-PO₄ tertiary interactions appear ineffective. Furthermore, we have crystallized a tensegrity triangle using a junction lacking the cytosine suggested for involvement in tertiary interactions. The unit cell is isomorphous with that of a tensegrity triangle crystal reported earlier. This structure has been solved by molecular replacement and refined. The data presented here leave no doubt that the tensegrity triangle crystal structures reported earlier depend only on base pairing and covalent interactions for their formation.

The control of structure on the finest possible scale and to the greatest possible extent is a central goal of modern structural science. DNA is arguably the most programmable molecule available for this purpose, because of the enormous amount of information content inherent in DNA base pairing secondary interactions [Seeman, 1982]. Timsit and Varnai [2011] have recently pointed out the potential importance of directing DNA intermolecular self-assembly in the lateral direction through tertiary interactions involving the N4 group of cytosine. While this is an important consideration for the control of DNA structure in three dimensions, its role is likely to be key only in those cases involving linear duplex DNA or flexible branched motifs (e.g., Holliday junctions) as the fundamental repeating motif. If one uses robust (inflexible) 2D and 3D DNA motifs, DNA structure can be controlled in self-assembled 2D arrays and in 3D crystals by using only sticky ended DNA contacts, as suggested originally in the early 1980s [Seeman, 1982]; the information content of sticky ends provides strong programmable control of intermolecular interactions. Examples of

robust motifs combined with sticky ends to self-assemble two-dimensional crystals include the double crossover (DX) [Li *et al.*, 1996; Winfree *et al.*, 1998], the triple crossover (TX) [LaBean *et al.*, 2000], the DX-triangle [Ding & Seeman, 2004], the 3D-DX motif [Zheng *et al.*, 2006], the DNA parallelogram [Mao *et al.*, 1999], the DX DNA parallelogram, the six-helix bundle, and the skewed TX triangle [Constantinou *et al.*, 2006]. Exclusive dependence on sticky ended intermolecular interactions enables programming of the crystallographic asymmetric unit to contain a designated number of motifs, ranging, to date, up to eight [Ding & Seeman, 2006]. In addition, aperiodic 2D arrays of DX molecules have been built successfully, using only sticky ends to direct their algorithmic self-assembly [Rothemund *et al.* 2004].

The tensegrity triangle [Liu *et al.*, 2004], which we have used to self-assemble high resolution 3D crystals [Zheng *et al.*, 2009], is the first example of a robust symmetric 3-space-spanning DNA motif. In our earlier communication, we reported nine different crystals, with triangle edge-lengths ranging from two to four double helical turns, whose unit cells were programmed using the sticky-ended interactions of tensegrity triangles [Zheng *et al.*, 2009]. The tensegrity triangle and the related 3D-DX motif, have been used to design sticky end-based crystals in both 2D [Zheng *et al.*, 2006; Liu *et al.*, 2004] and 3D [Zheng *et al.*, 2009], although the 3D crystals of the 3D-DX motif diffract only to poor resolution [Constantinou, 2005]. Recently, we reported another structure of the tensegrity triangle designed through its sticky ends to contain two molecules per asymmetric unit; this system was combined with covalently attached dye molecules to control the colors of the crystals [Wang *et al.*, 2010].

Timsit and Varnai [2011] suggest that a cytosine at the branched junction of the tensegrity triangle plays a key role in the assembly of our complex, similar to the role it plays in a non-covalent triangle motif seen in a 1989 crystal structure [Timsit *et al.*, 1989]. By contrast, we reported that other than covalent bonds, the structure relies exclusively on secondary structural interactions (Watson-Crick nucleotide pairs), both within the intramolecular DNA triangle structure and in the intermolecular interactions [Zheng *et al.*, 2009]. Our earlier work extended structural DNA nanotechnology [Seeman, 1982] from 2D crystals to 3D crystals in our quest for programmable control of the structure of matter on the finest possible scale; consequently, it is of key importance to provide experimental evidence that resolves these differing interpretations, and we do so here.

Superficially, the tensegrity triangle resembles the 1989 triangle motif, in that it is 3-fold symmetric, with an over-and-under structure; however, tertiary interactions involving cytosine do not appear to play a role in its formation. It should be recognized that the 1989 triangle results from the association of three duplex DNA molecules (the fundamental motif in that work), whereas the tensegrity triangle is a single self-assembled robust motif that can form at low concentrations, and whose crystal-forming intermolecular associations are governed exclusively by sticky-ended interactions. It is incorrect to ascribe Figure 1c of Timsit & Varnai [2011] to our structure, because it shows a cluster of three double helices, rather than a tensegrity triangle. The cytosines shown in red in Figure 1c of Timsit & Varnai [2011] are in fact all parts of the same covalent strand in the tensegrity triangle, as illustrated in red in Figure 1 here. At the 4 Å resolution of our crystals, we find no evidence for the tertiary interactions discussed by Timsit & Varnai [2011]. The closest contact involving N4 of cytidine to a phosphate oxygen in our structure is between two successive residues on the same covalent (red) strand in Figure 1, a cytosine analogous to one in the 1989 structure, and a phosphate adjacent to it; this distance is 4.6 Å (± 0.8 Å), longer than acceptable for a hydrogen bond and not the type of intermolecular interaction they describe.

We were aware of Timsit & Varnai's contention about a year ago, because they kindly provided us with a preprint of a note that they had prepared. In the interval, we have performed experiments to gather evidence regarding this issue. In the first experiment, we have used PAGE to demonstrate that we really do have a construct containing 63 nucleotide pairs, and that it coheres at moderate and low concentrations of DNA. Figure 2 shows a non-denaturing gel that establishes this point. Lanes 2, 3 and 4 contain the sequence of the dodecamer DNA duplex used in the 1989 crystal structure where the tertiary interaction involving N(4) C - PO₄ was discovered [Timsit *et al.*, 1989]. Lanes 5, 6 and 7 contain the tensegrity triangle that we have reported, but lacking sticky ends, so as to avoid aggregation on the gel. The concentrations of DNA are 12, 21 and 30 μ M in lanes 2, 3 and 4, respectively, whereas they are 4, 7 and 10 μ M in lanes 5, 6 and 7. μ This factor of three allows the duplex concentration to be equal across equivalent lanes. It is evident from this gel that the tensegrity triangle is not merely a crossover product of three DNA duplexes, but is a single intact entity at the concentrations employed. It is equally evident from the gel that the duplex has remained a duplex without trimerizing under these conditions. Thus, the tensegrity triangle does not use the tertiary N(4) C - PO₄ interaction to form, because we have shown here that the tertiary interaction does not occur under the conditions tested. We refer the reader to Figure 3 of Liu *et al.* [2004] for AFM images of 2D tensegrity triangle arrays constructed at low concentrations.

So as to exclude completely the possible role of the cytosine implicated by Timsit & Varnai [2011], we have flanked the branch point by a different junction. The junction used in the nine crystals reported previously (Figure 3a) contains the J1 sequence [Seeman & Kallenbach, 1983] at its crossover points. We have switched the junction-flanking sequence to that of another well-characterized junction (Figure 3b). Both triangles shown in Figure 3 contain three turns in each edge. The crossover preference of the second junction is well-known [Duckett *et al.*, 1988], and it contains a T, rather than a C, at the key site under discussion here. The electrophoretic mobilities of the two junctions are compared in Figure 3c. It is clear from the gel that the two junctions behave identically under conditions of electrophoresis. We have crystallized both the triangles shown in Figure 3. The triangle with the T diffracts to 6.7 Å resolution, not dissimilar to the other 3-turn triangles (including the one in Figure 3a) whose cell dimensions we have reported previously [Zheng *et al.*, 2009]: In space group R3, the cell dimensions of the earlier crystal were $a = 102.0$ Å and $\alpha = 112.7^\circ$, while those of this crystal are $a = 101.9$ Å and $\alpha = 112.7^\circ$. The structure has been solved by molecular replacement to a resolution of 6.7 Å, and has been refined to an R_{free} of .137 and R_w of 0.126. The mean deviations of bonds are 0.014 Å, and those of angles are 1.6°. The structure has been deposited at RCSB with the accession code of 3UBI. The crystal structure of this triangle is shown in stereographic projection in Figure 4. Except for the extra turn of DNA in each edge, it is evident that the structure is identical to that shown in Figure 1.

Thus, the covalent bonds present have established the structure of the tensegrity triangle motif in a fashion that appears to all existing evidence to be sequence independent. We have demonstrated by gel electrophoresis and by X-ray crystallography that tertiary interactions involving cytosine N(4) protons at the junctions are not responsible for the structures of the triangles. The structures of the triangles are established by the covalent bonds, and the crystal structure has been programmed by sticky-ended cohesion at the tips of the helices forming the edges. Timsit and Varnai's assertion [2011] that 'the strand exchange that occurs between the duplexes of Zheng *et al.*'s triangle has no influence on the architecture...' is inconsistent with the evidence presented here.

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Figure 1.

A stereoscopic image of the tensegrity triangle. The three unique strands are shown in individual colors. The blue strands are on the periphery of the triangle, the yellow strands form continuous helices and the red strand is a cyclic covalent strand containing a single nick distal to any crossover points.

Figure 2. Gels that Compare the Electrophoretic Behaviors of the Tensegrity Triangle and a Linear Duplex DNA Molecule that Forms a Similar Structure
 Lanes 2–4 contain the duplex at concentrations of 12, 21 and 30 μM , respectively. Lanes 5–7 contain the tensegrity triangle at concentrations of 4, 7 and 10 μM , respectively. Lanes 1 and 8 contain 10 bp markers. This is an 8% non-denaturing gel run at 4 $^{\circ}\text{C}$ in TAE buffer containing 100 mM Mg^{2+} .

Figure 3. The Sequences and Non-Denaturing PAGE of 3-Turn Triangles with Different Junction-Flanking Sequences

(a) The 3-turn/edge triangle with the junction reported in reference 13. (b) The 3-turn/edge triangle with a junction lacking the cytidine discussed by reference 2. The key nucleotide pairs are drawn in red. (c) A non-denaturing gel containing the two junctions in (a) and (b). Lane 1 contains 100 bp markers, and lane 2 contains 10 bp markers. Lane 3 contains the triangle in (a), and Lane 4 contains the triangle in (b). It is evident that the two triangles migrate similarly.

Figure 4. Stereographic Projection of the 3-Turn Triangle Lacking a C at the Crossover Site in the Crossover Strand

The color scheme is identical to that in Figure 1. It is clear that the triangle shown here and the one in Figure 1 are identical, except for the number of base pairs in the edges of the triangles.