Lipid bilayer-assisted dynamic self-assembly of hexagonal DNA origami blocks into monolayer crystalline structures with designed geometries

Highlights
- Lipid bilayer-assisted self-assembly of 3D DNA origami blocks was achieved
- Time-lapse AFM imaging of the self-assembly processes was performed
- Different assembly patterns were achieved from a single DNA origami design
Lipid bilayer-assisted dynamic self-assembly of hexagonal DNA origami blocks into monolayer crystalline structures with designed geometries

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SUMMARY
The DNA origami technique is used to construct custom-shaped nanostructures that can be used as components of two-dimensional crystalline structures with user-defined structural patterns. Here, we designed an Mg2+-responsive hexagonal 3D DNA origami block with self-shape-complementary ruggedness on the sides. Hexagonal DNA origami blocks were electrostatically adsorbed onto a fluidic lipid bilayer membrane surface to ensure lateral diffusion. A subsequent increase in the Mg2+ concentration in the surrounding environment induced the self-assembly of the origami blocks into lattices with prescribed geometries based on a self-complementary shape fit. High-speed atomic force microscopy (HS-AFM) images revealed dynamic events involved in the self-assembly process, including edge reorganization, defect splitting, diffusion, and filling, which provide a glimpse into how the lattice structures are self-improved.

INTRODUCTION
Molecular self-assembly has attracted considerable attention as a method for the construction of novel supramolecular architectures. The scaffolded DNA origami method has enabled the one-pot preparation of almost arbitrarily shaped two-dimensional (2D) (Rothemund, 2006) and three-dimensional (3D) DNA nanostructures (Andersen et al., 2009; Dietz et al., 2009; Douglas et al., 2009a; Han et al., 2011; Kuzuya and Komiyama, 2009), which can be further used as components of higher-order architectures that self-assemble based on intermolecular interactions between DNA ends, such as sticky-ended cohesion (Liu et al., 2011; Rajendran et al., 2011; Tikhomirov et al., 2017; Zhang et al., 2018; Zhao et al., 2011) and blunt-ended stacking (Wagenbauer et al., 2017; Woo and Rothemund, 2011).

Among the various assembly approaches, mica-surface-assisted self-assembly (mica-SAS) is a promising method to obtain two-dimensionally ordered arrays of 2D DNA origamis (Aghebat Rafat et al., 2014; Kielar et al., 2018; Ramakrishnan et al., 2016; Woo and Rothemund, 2014; Xin et al., 2021). One key to the success of mica-SAS is the setting of appropriate adsorption conditions (i.e., not too weak and not too strong) to ensure the surface mobility of the component DNA origami on the mica substrate. In general, DNA origami structures are prepared in a buffer solution containing 10–20 mM Mg2+ in which they are strongly adsorbed onto mica surfaces and do not exhibit two-dimensional diffusion. Therefore, for the mica-SAS of DNA origami crystals, 200–700 mM NaCl was added to weaken the electrostatic interaction between DNA and the mica surface (Aghebat Rafat et al., 2014; Woo and Rothemund, 2014).

This strategy has been well established for the construction of monolayered crystalline structures from 2D DNA origami, but cannot be simply applied to those from 3D DNA origami, especially those that require high Mg2+ concentrations to connect with each other. Representative examples of such structures include the 3D DNA origami blocks with shape-complementary recession-protrusion patterns proposed by Gerling et al. (2015). These structures can be multimerized based on shape-fitting upon increasing the Mg2+ concentration, to neutralize the electrostatic repulsion between the shape-complementary interfaces. However, this type of assembly mechanism is unsuitable for conventional mica-SAS as increasing Mg2+ also strengthens the adsorption of DNA origami blocks onto the mica surface, making them immobile. Increasing the Na+ concentration is also unfeasible in this case due to its inhibitory effect on the multivalent cation-induced DNA-DNA interactions (Hibino et al., 2006).
To reconcile these conflicts, we employed the lipid bilayer-surface-assisted self-assembly (LB-SAS) (Avakyan et al., 2017; Kempter et al., 2019; Kocabey et al., 2015; Suzuki et al., 2015) to assemble Mg$^{2+}$-responsive 3D DNA origami blocks into monolayered crystalline structures. Artificial lipid bilayers, especially glass- or mica-supported lipid bilayers (SLBs), provide a flat but dynamic surface with the desired physicochemical properties (such as fluidity and surface charge) by tuning their lipid compositions, which enables us to realize conditions to ensure the lateral mobility of assembly components. In this study, we designed a 3D DNA origami block whose sides exhibited self-shape-complementary ruggedness. The electrostatic adsorption of DNA origami blocks onto a fluidic lipid bilayer membrane allows their diffusion on the surface. The subsequent increase in Mg$^{2+}$ promotes their self-assembly into a lattice with a designed geometry through homomultimerization based on a self-complementary shape-fit. The utilization of block-shaped components with prescribed thickness expands the constructible structures, whose thickness has been limited to ~2 nm in the conventional assemblies of sheet-like 2D DNA origami structures, via a surface-assisted approach. Our study provides a general approach for the construction of custom DNA origami lattices with the desired assembly patterns and nanoporous structures that will serve not only as a nano-micro scaffold for molecular patterning but also as an array of nanocompartmentalized spaces.

RESULTS AND DISCUSSIONS

Design of a hexagonal DNA origami block

As the self-assembly component, we designed a hexagonal 3D DNA origami using caDNA2 (Figure 1A, see also Figure S1) (Douglas et al., 2009b). The sides of the hexagonal 3D origami (3D-Hx) exhibited shape-complementary ruggedness (Video S1), which imposes self-complementary shape-fitting to allow assembly into a homomultimeric two-dimensional lattice (Gerling et al., 2015). The 3D-Hx was prepared by annealing a mixture of 8064 nt scaffold DNA (p8064) and staple strands in folding buffer (5 mM Tris-HCl [pH 8.0], 15 mM MgCl$_2$, and 5 mM EDTA), and then purified using a glycerol gradient (Lin et al., 2013) (STAR Methods, Figure S2). Atomic force microscopy (AFM) imaging (Figure 1B) revealed the monodispersed nature of the purified 3D DNA origami blocks, with a size of 53 ± 4 nm (mean ± S.D., N = 85), which is in good agreement with the expected size.

2D self-assembly on the mica-supported lipid bilayer membrane

The purified 3D-Hx was self-assembled into honeycomb lattices on a mica-supported 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) bilayer. In this experiment, 3D-Hx was first adsorbed onto the DOPC bilayer in folding buffer (5 mM Tris-HCl [pH 8.0], 15 mM MgCl$_2$, and 5 mM EDTA), and then purified using a glycerol gradient (Lin et al., 2013) (STAR Methods, Figure S2). Atomic force microscopy (AFM) imaging (Figure 1B) revealed the monodispersed nature of the purified 3D DNA origami blocks, with a size of 53 ± 4 nm (mean ± S.D., N = 85), which is in good agreement with the expected size.

Dynamic events involved in lattice formation

Dynamic events, such as boundary reorganization, defect diffusion, and defect filling, occurring on an SLB were successfully monitored using time-lapse AFM imaging. Figure 3A shows the representative
Sequential images obtained after increasing the [Mg$^{2+}$] to 50 mM (Video S2). The dynamic association and dissociation of 3D-Hx monomers and oligomers frequently occur around the edges of the lattices (See also another example in Video S3). It should be noted that the area occupied by 3D-Hxs in the image area gradually increased with fluctuations in its value (Figure S4), indicating that diffusing 3D-Hxs entered and exited the area, some of which were incorporated into the already existing lattices. In particular, the main lattice island in the scanning area (Figures 3A, 0 s) increased with the incorporation of 3D-Hxs. Intriguingly, 3D-Hx monomers and oligomers dissociated from adjacent lattices were also incorporated into this lattice.

Dissociation and re-association of the components occur not only at the edges of the lattices but also at the defects in the assembled lattice, resulting in defect diffusion. In the example shown in Figure 3B (see also Video S4), a defect equivalent of triangularly arranged 3 hexagonal blocks was observed in the initial frame. This defect split into a single defect and a twin defect, the latter of which moved from the left to the right of the image area, while the single defect remained at the same position. Figure 3C depicts another dynamic event, in which a large defect was reduced by incorporating 3D-Hx monomers. In the initial frame of this example (Figure 3C, see also Video S5), a large defect equivalent to seven hexagonal blocks was observed. The first monomer, which remained unadsorbed on the SLB surface, jumped into this defect, attempting to make a connection with the upper edge of the defect, but soon moved to the lower edge to make a tentative connection, indicating that the connection at the two sides was not sufficiently strong for the incorporation of the monomer into the already assembled lattice at this Mg$^{2+}$ concentration (50 mM). After the

**Figure 2. Lipid bilayer-assisted self-assembly of 3D-Hx into a honeycomb lattice**

(A) Schematic of the experimental procedure. 3D-Hx is deposited onto the mica-supported lipid bilayer membrane (mica-SLB). The connection among 3D-Hx blocks are then induced by increasing Mg$^{2+}$ concentration of the surrounding environment (buffer solution).

(B) AFM image of the honeycomb lattice assembled on the mica-SLB. Scale bars: 200 nm.

(C) Cross-sectional profile along the line a-b in (B). Errors represent standard deviations.
arrival of the second monomer, the first monomer again moved to the upper side and was incorporated into the lattice with the second monomer. Both the first and second monomers made connections on three of the six sides in the last frame. These observations provide a glimpse into how defects arising in the assembly process become smaller.
Self-assembly into depleted lattice and its surface modification

One of the unique features of this system is that we can inactivate the designated sides by disabling blunt-ended interactions through the addition of polyT tails at the ends of the protruding parts of the ruggedness. This simple customizability enabled us to realize different assembly modes of 3D-Hx. Here, ruggedness at the three symmetrical sides of the 3D-Hx was inactivated by staple extension with polyT tails (Figure 4A S5). Because the adjacent sides of our 3D-Hx origami showed upside-down patterns of ruggedness (Video S1, Figure S1), self-assembly of this 3-symmetric-side-inactivated hexagonal block (3D-Hx-135) resulted in a “depleted” honeycomb lattice in which face-down and face-up blocks are alternately laid out (Figure 4B). AFM images of the assembled lattices revealed structural features in which pores of two different sizes were arranged (Figure 4C and S6). To address the facing orientation of the 3D-Hx-135 blocks in the depleted honeycomb lattice, we prepared a functionalized block, with one of the faces having three biotin moieties, and performed post-self-assembly modification (Figure 4D). The depleted lattice was first prepared on the bi layer from one-sided biotinylated blocks. After lattice formation was confirmed, streptavidin was loaded onto the same sample. With this post-assembly treatment, only the blocks facing upwards could bind streptavidin. Figure 4E and 4F shows a representative AFM image after the post-assembly modification, where every other block in the lattice was decorated, demonstrating facing-up (modified) blocks and facing-down (unmodified) blocks alternately arranged in the depleted lattice, as expected.

Conclusion

Herein, we designed an Mg^{2+}-responsive hexagonal 3D DNA origami block with self-complementary features and demonstrated its ability to form LB-SAS in lattices with prescribed structural patterns. The primary advantage of the self-shape-complementary design employed here is that it can inactivate connections at arbitrary sides by simply extending the polyT tails from blunt ends. This enables the construction of lattices with different patterns from a single 3D DNA origami block design. To satisfy the conditions that allow both the surface diffusion of the origami blocks and their Mg^{2+}-induced assembly, we employed an artificial lipid bilayer membrane as a soft and flat surface to serve as an alternative to the naked mica surface. The lipid bilayer-adsorbed origami blocks retained their surface mobility and self-assembled into lattices with the desired geometries, in a process involving edge reorganization, defect splitting, diffusion, and filling, in response to the changes in Mg^{2+} concentration in the surrounding environment.

Figure 4. Lipid bilayer-assisted self-assembly of 3D-Hx into a depleted honeycomb lattice

(A) Schematic of the variation of the 3D-Hx whose ruggedness at three symmetric sides are inactivated. Active ruggedness and inactivated ruggedness are colored with orange and light orange, respectively. For convenience, the obverse and reverse of the 3D-Hx are indicated by different colors.

(B) Schematic illustration of the depleted honeycomb lattice.

(C) AFM image of the depleted honeycomb lattice assembled on the mica-SLB. Scale bars: 200 nm.

(D) Schematic of the experimental procedure of the post-assembly modification with streptavidin molecules.

(E) AFM images of the streptavidin-bound depleted honeycomb lattice. Scale bar: 100 nm.

(F) Surface plot of the streptavidin-bound depleted honeycomb lattice.
In our protocol for DNA origami preparation, four times the excess amount of staple strands are mixed against a scaffold DNA (p8064, 0.75 USD per pmol). Because each staple DNA costs 0.12 USD per base at a molar yield of 10 nmol and 756 bases of the scaffold were folded into a designed shape with staples, total cost of the origami per pmol is 1.1 USD (i.e., $1.8 \times 10^{-12}$ USD per origami). Given that the area occupied by a single hexagonal DNA block is about $1.5 \times 10^{-11} \text{cm}^2$ (1500 nm$^2$), the cost of the DNA required to assemble the honeycomb lattice is estimated to be 0.12 USD per cm$^2$, which is comparable to the cost required for the mica-assisted self-assembly of conventional 2D DNA origami lattices (Xin et al., 2021).

This cost can be lowered, owing to the development of methods for the mass production of DNA strands (Praetorius et al., 2017) and the further improvement of DNA synthesis technology.

Lipid membrane-interacting DNA nanostructures have recently attracted great attention in the fields of synthetic biology (Czogalla et al., 2016) and molecular robotics (Hagiya et al., 2014) for their potential to mimic the structures and functions of membrane proteins (Burns et al., 2013, 2016; Krishnan et al., 2016; Langecker et al., 2012; Thomsen et al., 2019), membrane-binding proteins (Czogalla et al., 2015; Franquelim et al., 2018; Grome et al., 2018; Khmelinskaia et al., 2018), and membrane-cytoskeleton networks (Kocabey et al., 2015; Kurokawa et al., 2017). We believe that our approach to assembling lattices with 3D features matches with this direction, not just for its application as a scaffold for the periodic placement of protein molecules and metal nanoparticles.

Other possible applications may result from the use of nanospaces compartmentalized by origami blocks. It has been implicated that reaction kinetics of various biological molecules in the nanoconfinement spaces differ from those in bulk solutions (Kuchler et al., 2016). DNA origami nanotechnology has enabled the construction of a designed 3D nanospace, in which a single to several molecules of interest are enclosed and applied to reveal the effects of nanoconfinement on enzymatic reactions (Grossi et al., 2017; Zhao et al., 2016) and the structural transitions of DNA (Jonchhe et al., 2018; Shrestha et al., 2017). The porous crystalline DNA origami structures can be regarded as arrays of such nanoconfinement spaces, which provide a tool for multiplexed single-molecule analysis (Sakamoto et al., 2020) that will enable the simultaneous monitoring of reactions occurring in individual nanopores.

**Limitations of the study**

One of the primary limitations of this study is that in our origami design and surface-assisted approach, self-assembly fundamentally occurs in 2D, although the assembly component itself is a 3D object. However, extending the assembling process into 2nd, 3rd, or more layers could be investigated in the future by designing interactions between the top and bottom faces of the origami block. Introducing specific interactions among multiple different components, which should lead to the surface-assisted self-assembly of lattices with predetermined shapes and sizes, will also be challenging.

Our 3D-Hx can be regarded as a modular structure because we can prevent an arbitrary side from connecting with others by disabling blunt-end stacking via staple extension with polyT. However, the current design has a symmetric appearance and thus does not allow us to define the combination of sides to be connected, as only one type of shape-complementary ruggedness is employed. This drawback should be addressed by designing specific pairs of complementary ruggedness patterns at specific pairs of sides. Such strategy could be applied not only to our hexagonal blocks but also to other 3D structures, such as cubes and cuboids. The implementation of photoresponsive moieties (Asanuma et al., 2007; Kamiya and Asanuma, 2014; Yoshimura and Fujimoto, 2008) to control the blunt-ended stacking (Gerling and Dietz, 2019; Willner et al., 2017) is also a fascinating direction, which would enable in situ switching of the assembly mode of the DNA origami component, thus leading to controlled reorganization of the lattice geometry from honeycomb to depleted, and vice versa.

**STAR+METHODS**

Detailed methods are provided in the online version of this paper and include the following:

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- Lipid bilayer-surface-assisted self-assembly of hexagonal 3D origami structures
- AFM observation

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104292.

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AUTHOR CONTRIBUTIONS
The manuscript was written largely by Y. S., with sections contributed by I. K. Y. S. and I. K. designed the hexagonal DNA origami block. Y. S. performed AFM experiments. Y. S., E. M., and K. W. performed biochemical experiments and analysis. All the authors approved the final manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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STAR METHODS

KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Chemicals, peptides, and recombinant proteins | NIPPON GENE, Japan | 310-90361 |
| Magnesium chloride | NIPPON GENE, Japan | 312-90614 |
| Tris-HCl (pH 8.0) | NIPPON GENE, Japan | 318-91041 |
| 5×TBE | NIPPON GENE, Japan | 318-90041 |
| Ethylenediaminetetraacetic acid (EDTA), pH 8.0 | Thermo Fisher Scientific, USA | 15575-038 |
| SYBR™ Gold Nucleic Acid Gel Stain | Thermo Fisher Scientific, USA | S11494 |
| Glycerol | Wako, Japan | 072-00626 |
| Agarose S | Wako, Japan | 312-01113 |
| Streptavidin | Wako, Japan | 198-17861 |
| 1,2-Dioleoyl-sn-glycer-3-phosphocholine (DOPC) | Avanti Polar Lipids, USA | 850375C-25mg |
| Oligonucleotides | | |
| Unmodified ssDNA strands, see Tables S1–S3 | Eurofins Japan, Japan | https://www.eurofins.co.jp |
| Biotin-labeled DNA strands, see Table S4 | Eurofins Japan, Japan | https://www.eurofins.co.jp |
| Software and algorithms | | |
| caDNAno2 | Douglas et al. 2009b | https://cadnano.org |
| ImageJ | Schneider et al. 2012 | https://imagej.nih.gov/ij/ |
| Other | | |
| Scaffold ssDNA (p8064) | tilibit nanosystems, Germany | https://www.tilibit.com |
| 100 kDa Amicon Ultra filters | Merck, Germany | UFC510096 |
| himac 2.2PA TUBE | Koki Holdings, Japan | S300536A |
| Micro ultracentrifuge | Hitachi, Japan | himac CS150GXII |
| Swing rotor | Hitachi, Japan | S55S-2017 |
| Gel imager | BIO-RAD | ChemiDoc™ MP Imaging System (170-8280) |
| Mica | Furuuchi chemical, Japan | Clear mica, Φ3.0 mm |
| High-speed atomic force microscopy | Olympus, Japan | BIXAM |
| Cantilever | Nanoworld, Switzerland | USC-F0.8-k0.1-T12 |

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources should be directed to and will be fulfilled by the corresponding author: Yuki Suzuki (yuki.suzuki.e6@tohoku.ac.jp).

Materials availability
This study did not generate any new reagents.

Data and code availability
- Data reported in this paper will be shared by the lead contact upon request.
- This study does not report any original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.
**METHOD DETAILS**

**Preparation of DNA origami structures**

The 8064 nt scaffold single-stranded DNA (p8064) was purchased from tilibit Nanosystems (Garching, Germany). All staple strands were purchased from Eurofins Genomics (Tokyo, Japan). DNA origami structures were designed using caDNAno2 software (Douglas et al., 2009b) (Figure S1). DNA origami structures were folded by mixing 10 nM scaffold ssDNA (p8064) with 40 nM staple strands in 20 μL of folding buffer containing 5 mM Tris-HCl (pH 8.0), 15 mM MgCl₂, and 1 mM EDTA. The mixture was incubated at 65°C for 15 min, then annealed by reducing the temperature from 60°C to 45°C at a rate of 1.0°C/h.

**Purification of DNA origami by density gradient centrifugation**

DNA origami structures were purified using a glycerol gradient (Lin et al., 2013). To prepare a linear glycerol gradient (5–45%, v/v), nine layers (200 μL per layer) of glycerol solution in 1×TE-Mg buffer (5 mM Tris-HCl [pH 8.0], 15 mM MgCl₂, and 1 mM EDTA) were carefully added into a 2.2 mL ultracentrifuge tube with 5% concentration decrement per layer, starting with a 45% glycerol solution at the bottom. The tube was incubated overnight at 25°C to prepare a glycerol gradient. A solution of DNA origami nanostructures (200 μL) containing 5% glycerol was then loaded on top of the glycerol gradient. The tube was then centrifuged at 50,000 rpm (214,288g) for 1.5 h at 4°C using an ultracentrifuge (himac CS150GXII, Hitachi, Tokyo, Japan) equipped with a swing-rotor (SS55S-2017, Hitachi, Tokyo, Japan). After centrifugation, 15 fractions (133 μL each) were collected sequentially from the top to bottom of the tube. Aliquots of each fraction were subjected to agarose gel electrophoresis (Figure S2). The fractions exhibiting the target bands were mixed and concentrated using Amicon Ultra-0.5 mL centrifugal filters (MWCO 100 kDa) (Merck KGaA, Darmstadt, Germany). The glycerol-containing buffer was also replaced with glycerol-free buffer during ultracentrifugation.

**Agarose gel electrophoresis**

The samples were loaded for electrophoresis on a 1.0% or 1.5% agarose gel containing 5 mM MgCl₂ in a 0.5× TBE (Tris-borate-EDTA) buffer solution (pH 8.0) at 50 V and 4°C. The gels were then imaged with ChemiDOC MP (Bio-Rad Laboratories, Inc., CA, USA) using SYBR Gold nucleic acid gel stain (Thermo Fisher Scientific, MA, USA) as the staining dye.

**Preparation of mica-supported lipid bilayers**

Mica-supported lipid bilayers (mica-SLBs) were prepared by employing a vesicle fusion method (Mingeot-Leclercq et al., 2008), as previously described (Suzuki et al., 2015; Uchihashi et al., 2012), with some modifications. Lipid dry films were prepared by adding 20 μL of 10 mg/mL 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Avanti Polar Lipids, AL, USA) solution dissolved in chloroform in a round-glass tube, followed by overnight drying under vacuum with argon gas. Ultrapure water (400 μL) was then added to the dried lipid film to obtain a lipid concentration of 0.5 mg/mL. The glass tubes were vortexed and sonicated to generate small unilamellar vesicles (SUVs). Mica-SLBs were formed by depositing 2 μL of the SUV solution, followed by the addition of 1 μL of buffer containing 20 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, and 1 mM EDTA onto freshly cleaved mica disks, with a diameter of 3.0 mm (Furuuchi Chemical, Tokyo, Japan). To prevent drying of the bilayers, the samples were incubated at room temperature (25°C) in a sealed container containing a piece of Kimwipe moistened with Milli-Q water. After 30 min of adsorption on mica, the sample was rinsed with buffer to remove the unadsorbed SUVs. The procedure, from deposition to rinsing, was repeated twice to completely coat the mica surface with a bilayer.

**Lipid bilayer-surface-assisted self-assembly of hexagonal 3D origami structures**

A drop (3 μL) of hexagonal 3D DNA origami (3D-Hx) nanostructures in folding buffer (10 nM) was deposited onto the preformed SLB. After 5 min of incubation in the sealed container described above, the sample was soaked in 120 μL of buffer containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 15 mM MgCl₂ without surface rinsing, and then further incubated for 30 min at room temperature. Surface-assisted self-assembly was then induced by adding an appropriate volume of 1 M MgCl₂ to the buffer, to a final concentration of 50 mM. After an additional 60 min of incubation, the sample was subjected to atomic force microscopy (AFM) imaging in the same buffer. For time-lapse imaging of the dynamic events during self-assembly, this additional incubation was omitted, and imaging was started immediately after the addition of MgCl₂.
AFM observation

High-speed AFM (HS-AFM) imaging was performed using tip scan high-speed AFM (BIXAM, Olympus, Tokyo, Japan), which was improved based on a previously developed prototype AFM (Suzuki et al., 2013; Yoshida et al., 2018). Small cantilevers (9 μm long, 2 μm wide, and 100 nm thick) with a spring constant of 0.1 N/m (USC-F0.8-k0.1-T12; Nanoworld, Neuchâtel, Switzerland) were applied. The images were collected at a scan rate of 0.5 frames per second (fps). The image sequences were analyzed using AFM scanning software (Olympus) and ImageJ software (http://ImageJ.nih.gov/ij/) (Schneider et al., 2012).