

# Preparation of Chemically Modified RNA Origami Nanostructures

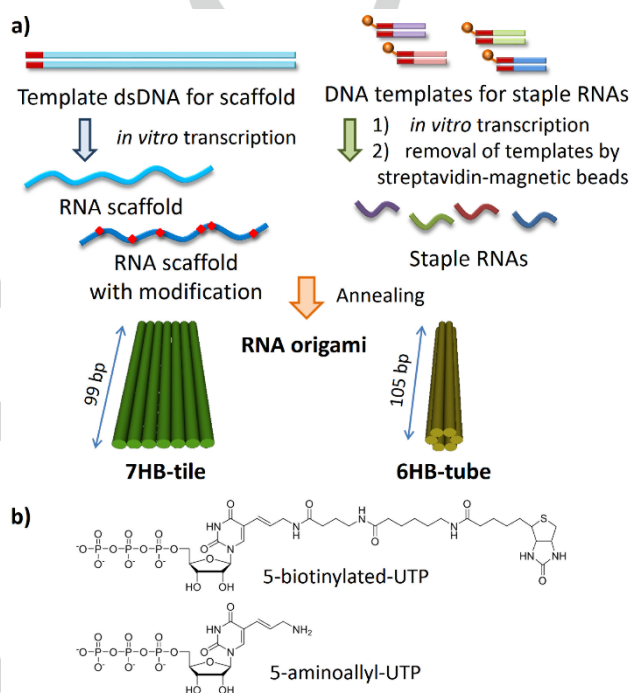
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**Abstract:** RNA molecules potentially have structural diversity and functionalities, and it should be required to organize the structures and functions in a designed fashion. We developed the method to design and prepare RNA nanostructures by employing DNA origami strategy. RNA transcripts were used as a single-stranded RNA scaffold and staple RNA strands for the formation of RNA nanostructures. After the annealing of the mixtures, we obtained 7-helix bundled RNA tile and 6-helix bundled RNA tube structures as pre-designed ones. These nanostructures were easily functionalized by introduction the chemical modification to the RNA transcripts. The DNA origami method can be extended and utilized to construct RNA nanostructures.

RNA nanotechnology is an emerging research field that has potential for applications in the imaging, diagnostics, and regulation of cell functions.<sup>[1–3]</sup> RNA molecules have structural diversity, which provides unique functions, such as catalysis and binding to specific molecules.<sup>[4]</sup> Functional RNAs have been explored by *in vitro* selection to create artificially ribozymes and aptamers. Riboswitches and short interfering RNAs (siRNAs) are now used to regulate gene expression.<sup>[5]</sup> For the construction of RNA nanostructures, small structural motifs have been used to build up 2D and 3D structures with functionalities.<sup>[6–9]</sup> Although RNA molecules have fascinating functions, the available structural motifs of short RNAs are limited; thus, it is still challenging to design RNA nanostructures artificially. To expand the variations of the RNA structures, we designed and constructed RNA nanostructures in a similar fashion to that used in DNA origami design.<sup>[10,11]</sup> Recently, RNA/DNA hybrid nanostructures were created to expand their structures, with synergistic effects.<sup>[12–14]</sup> Furthermore, RNA nanostructures designed using an “RNA origami” strategy should have potential to expand the variety of multidimensional structures for the design and construction of novel RNA nanostructures.

In this study, we designed and prepared RNA origami structures that were assembled from the single-stranded RNA

scaffold and staple RNA strands (Figure 1a). The RNA origami for a 7-helix bundled planar structure (7HB-tile) was designed with a geometry of 33 bp per three turns in the adjacent crossover points, which corresponds to a helical pitch with 11 bp/turn. The RNA duplex forms an A-form structure with an 11 bp/turn helical pitch, which means that a geometrical design with 11 bp/turn should be structurally stable. For the design of a 6-helix bundled tubular structure (6HB-tube), we used the geometry of the usual DNA origami design.<sup>[15]</sup>



**Figure 1.** Preparation of RNA origami. (a) The RNA scaffold was prepared by *in vitro* transcription using a template dsDNA containing the T7 promoter using T7 RNA polymerase. Staple RNA strands were prepared by *in vitro* transcription from biotinylated dsDNAs. Subsequently, a 7-helix bundled (7HB) tile and a 6-helix bundled (6HB) tube were assembled from purified RNA scaffold and staple RNA strands. (b) Modified uridine triphosphate (UTP), 5-biotinylated UTP and 5-aminoallyl UTP were used for the synthesis of modified RNA transcripts for the preparation of the chemically modified RNA origami nanostructures.

First, an RNA transcript for the scaffold was obtained from a double-stranded DNA (dsDNA) template by *in vitro* transcription using T7 RNA polymerase. In the preparation of staple RNA strands, dsDNA templates were obtained from a biotinylated T7 primer and template single-stranded DNAs that were specific for the staple strands, using DNA polymerase (Klenow fragment). Subsequently, the staple RNA strands were obtained from biotinylated dsDNA templates using T7 RNA polymerase. Biotinylated dsDNAs were removed by streptavidin–magnetic beads and the solutions were subjected to ethanol precipitation or gel filtration for the purification of the RNA staple strands.

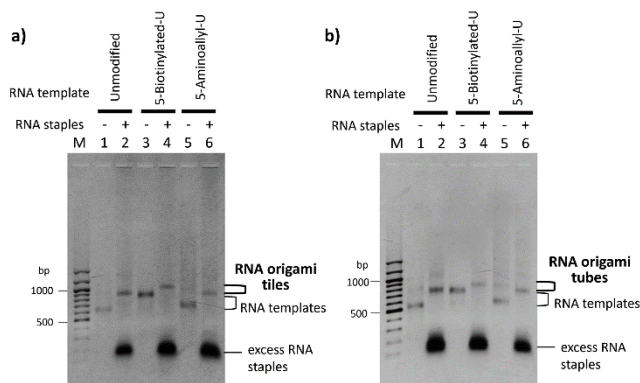
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This work was supported by Core Research for Evolutional Science and Technology (CREST) of JST and JSPS KAKENHI (Grant Numbers 24225005, 24310097, 24104002, 26620133). Financial supports from The Mitsubishi Foundation and The Asahi Glass Foundation to ME are also acknowledged.

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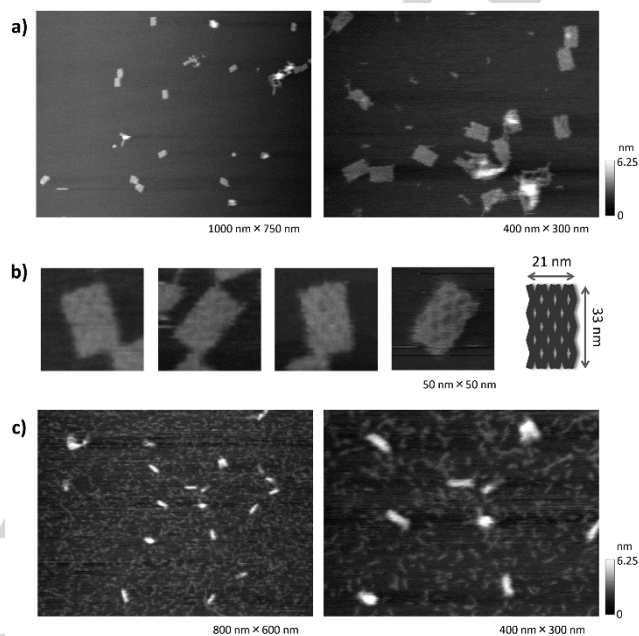
**Figure 2.** Complex formation of RNA origami structures. (a) Polyacrylamide gel electrophoretic (4%) analysis of 7HB-tile assemblies with modified UTPs. (b) PAGE analysis of 6HB-tube assemblies with modified UTPs.

To examine the RNA origami tile, we prepared the 7HB-tile structure (Figure 1). The RNA template was mixed with a staple RNA strand mixture (40 equivalents excess) in a solution containing 20 mM Tris buffer (pH 7.6), 10 mM MgCl<sub>2</sub> and 1 mM EDTA. The mixture was annealed from 75 °C to 20 °C at a rate of  $-0.5$  °C/min. The assembled structures were purified by gel filtration, and characterized by native polyacrylamide gel electrophoresis and atomic force microscopy (AFM). In the native polyacrylamide gel electrophoresis (PAGE) analysis, the RNA assembly showed a clear band (Figure 2a). The AFM images (Figures 3a and S1a) showed that RNA tile assemblies formed rectangular structures. The formation of the 7HB-tile structure was observed as a correct rectangular shape with dimensions of  $32.5 \times 21.2$  nm (Figure 3b). The yield obtained from the AFM images was 58% ( $N = 439$ ). The rise per base pair for the A-form RNA duplex was  $0.28$  nm,<sup>[16]</sup> thus, the observed length of the axial direction was 33 nm, which was 12% longer than expected length (28 nm). The difference in the sizes in the axial direction may be attributed to the expansion of the A-form structure in the constrained and packed nanostructure.

Next, a 6HB tubular structure was prepared from the RNA scaffold and staple RNA strands using the same annealing conditions (Figure 1c). The assembled structures were observed by AFM and analysed by native PAGE. In the native PAGE analysis, the assembled tube showed a clear band (Figure 2b). The AFM images showed that the assemblies formed rod-like structures that were 41.2 nm in length and 5.7 nm in height, in which the height was twice that of the planar tiles on the 3-aminopropyl-triethoxysilane-passivated mica surface (Figures 3c and S2a). The yield of tube formation determined after gel filtration was 44% ( $N = 81$ ). Therefore, the structural stress originating from the 10.5 bp/turn helical pitch in the RNA duplex seemed to be relatively small for the formation of the RNA tubular structure.

To compare the RNA origami structures with the RNA/DNA hybrid origami structures, RNA/DNA origami structures were prepared using the purified single-stranded RNA template and the DNA staple strands. RNA/DNA hybrid origami structures were correctly formed under the same conditions used for RNA origami formation (Figures S3a and S4a). The sizes of the

RNA/DNA hybrid tiles were compared with those of the RNA origami tiles (Table 1). In the case of the planar tiles, the axial lengths of the RNA/DNA tiles (33.4 nm) were similar to those of the RNA origami tiles (Table 1). Both the RNA tile and the RNA/DNA hybrid tiles formed similar structures.



**Figure 3.** AFM images of RNA origami structures. (a) AFM images of 7HB-tile assemblies. (b) Detailed images of the 7HB RNA origami tile. (c) AFM images of the 6HB RNA origami tubular structure.

**Table 1.** Observed dimensions of the 7HB-tile and 6HB-tube RNA origami structures and RNA/DNA hybrid structures.<sup>[a]</sup>

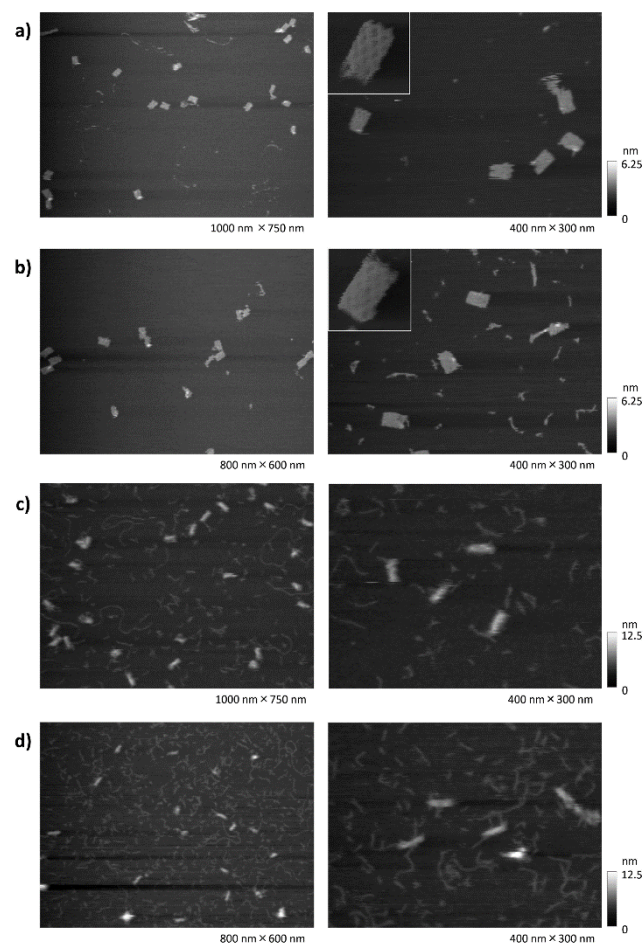
| Structure                | Dimension length x width or height (nm)          |
|--------------------------|--|
| RNA origami 7HB tile     | $32.5 \pm 1.9 \times 21.1 \pm 0.9$               |
| RNA origami 6HB tube     | $41.2 \pm 2.8 \times 5.7 \pm 0.4$ <sup>[b]</sup> |
| RNA/DNA origami 7HB tile | $33.4 \pm 1.5 \times 22.5 \pm 1.5$               |
| RNA/DNA origami 6HB tube | $39.0 \pm 1.1 \times 4.0 \pm 0.1$ <sup>[b]</sup> |

<sup>[a]</sup> Mean  $\pm$  S.D. <sup>[b]</sup> Height of the structure.

To expand the chemical modification of origami structures, the modified RNA templates were used for RNA origami formation. Native UTP was fully replaced by 5-biotinylated UTP and 5-aminoallyl UTP for the preparation of chemically modified RNA scaffolds.<sup>[13]</sup> The RNA scaffolds containing modified uracil and staple strands were annealed using the conditions described above.

In the native PAGE analysis, the modified RNA assemblies showed clear bands (Figure 2a). Both bands were shifted

compared with that of the unmodified RNA origami tile. The hydrophobicity of biotin and the cationic property of the aminoallyl group in the modified RNA scaffolds changed their migration in the polyacrylamide gel. In the case of the 5-biotinylated uracil modification, the hydrophobicity of biotin led to a slower migration compared with the unmodified RNA origami tile. In the case of the 5-aminoallyl uracil modification, the positive charge of the primary amine neutralized the negative charge of phosphates, resulting in a slightly slower migration. These results showed that modification at the 5-position of uracil changes the physical properties of the assembled RNA origami structures. These assembled structures were observed by AFM (Figures 4 and S3). The AFM images showed that RNA origami tiles were also formed using RNA scaffolds that contained modified uracil (Figure 4). The observed dimensions of the structures that included 5-biotinylated uracil and 5-aminoallyl uracil were  $32.8 \times 22.2$  nm and  $33.8 \times 21.6$  nm, respectively (Table 2). The yields of origami tile formation using the RNA templates containing 5-biotinylated uracil and 5-aminoallyl uracil were 53% ( $N = 158$ ) and 52% ( $N = 104$ ), respectively. The observed sizes were similar to those of the unmodified RNA



origami (Table 2).

**Figure 4.** AFM images of 7HB-tile and 6HB-tube RNA origami using an RNA template in which uracil was replaced fully by 5-biotinylated uracil and 5-aminoallyl uracil. (a) 7HB-tile assemblies prepared using an RNA template with 5-biotinylated uracil. (b) 7HB-tile assemblies prepared using an RNA template with 5-aminoallyl uracil. (c) AFM images of 6HB-tube assemblies

prepared using an RNA template with 5-biotinylated uracil. (D) 6HB-tube assemblies prepared using an RNA template with 5-aminoallyl uracil. Inset image size:  $50 \times 50$  nm.

In addition, tubular structures were obtained from the RNA scaffolds that contained 5-biotinylated uracil and 5-aminoallyl uracil. Their formation was confirmed by gel electrophoresis (Figure 2b), and their observed dimensions were 37.6 nm and 38.3 nm in length and 6.2 nm and 4.3 nm in height, respectively (Figures 4c,d and Table 2). The heights of the modified tubes, because of the modification, changed by 0.5 and  $-1.4$  nm for 5-aminoallyl uracil and 5-biotinylated uracil, respectively, compared with that of the unmodified tube (5.7 nm). The yields of origami tube formation from the RNA templates that contained 5-biotinylated uracil and 5-aminoallyl uracil were 46% ( $N = 125$ ) and 47% ( $N = 52$ ), respectively.

**Table 2.** Observed dimensions of modified 7HB-tile and 6HB-tube RNA origami structures with 5-biotinylated uracil and 5-aminoallyl or -containing RNA template.<sup>[a]</sup>

| Modification        | RNA origami 7HB-tile               | RNA origami 6HB-tube                    | RNA/DNA origami 7HB-tile           | RNA/DNA 6HB-tube                        |
|---------------------|------------------------------------|---|------------------------------------|---|
| 5-biotin-uracil     | $32.8 \pm 2.2 \times 22.2 \pm 1.1$ | $37.6 \pm 3.7 \times 6.2 \pm 0.4^{[b]}$ | $33.9 \pm 1.2 \times 23.5 \pm 1.7$ | $37.6 \pm 2.5 \times 3.8 \pm 0.4^{[b]}$ |
| 5-aminoallyl-uracil | $33.1 \pm 1.6 \times 21.6 \pm 0.8$ | $38.3 \pm 1.2 \times 4.3 \pm 0.1^{[b]}$ | $33.1 \pm 1.1 \times 22.5 \pm 1.1$ | $40.0 \pm 2.5 \times 3.7 \pm 0.1^{[b]}$ |

<sup>[a]</sup>Mean  $\pm$  S.D. Dimension, length  $\times$  width or height (nm). <sup>[b]</sup>Height of the structures.

The modified RNA origami structures were also compared with the RNA/DNA hybrid origami structures. The hybrid tiles formed good assemblies, as assessed by gel electrophoresis, whereas the hybrid tubes did not form well (Figures S3b and S5). In the case of the tube design, we used the 10.5 bp/turn helical pitch, which is not suitable for the formation of tubular structures based on an RNA/DNA hybrid. These results indicate that the RNA/DNA hybrid duplex is not as flexible as the RNA duplex.

In conclusion, we successfully prepared RNA origami structures using RNA scaffold and designed staple RNA strands which were simply prepared via *in vitro* transcription. Furthermore, the physical properties of the assembled structures were changed by modification of the RNA scaffolds. This method can be applied for the introduction of various functional molecules into RNA nanostructures via chemical modifications that express specific functions.

## Experimental Section

**Materials.** Single-stranded M13mp18 DNA and streptavidin magnetic beads were purchased from New England Biolabs (Ipswich, MA). The

staple strands and primers were purchased from Operon Biotechnology (Tokyo, Japan). Modified oligonucleotides were purchased from Nippon Bio Service (Saitama, Japan). PCR amplification was carried out using GoTaq Green Master Mix (Promega). 5-biotinylated UTP (Bio-16-UTP) and 5-(3-aminoallyl)-UTP were purchased from Ambion (Austin, TX). The gel-filtration column and the Sephacryl S-300 were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA) and GE Healthcare (Buckinghamshire, UK), respectively.

**Preparation of template dsDNA for RNA synthesis.** The PCR amplification of the template dsDNA was carried out from a plasmid-containing EGFP coding region (717 nt) using Go Taq (Promega) with two primers by following the manufacture's protocol, and the product was purified using a PCR purification kit (Qiagen).

**Preparation of RNA template and RNA staples.** A T7 promoter-containing template dsDNA was used for the transcription to prepare RNA template. RNA synthesis was performed using 10 nM template dsDNA, 40 mM Tris-HCl (pH 8.0), 5 mM DTT, 8 mM MgCl<sub>2</sub>, 2 mM spermidine, 0.5 mM NTP, and 0.25 μM T7 RNA polymerase (Takara) at 42 °C for 1.5 h. In the cases of the preparation of modified RNA templates, UTP was replaced to 5-aminoallyl UTP and 5-biotinylated UTP. The transcribed RNA was treated with DNAase (Takara) and purified using RNeasy Mini kit (Qiagen). The product was confirmed by gel electrophoresis.

**Preparation of RNA staple strands.** A biotine-attached primer of T7 promoter top strand was annealed with bottom strand containing complementary strand of the target RNA staple strand and T7 promoter bottom strand. The DNA strands were incubated with Klenow fragment with dNTP to prepare dsDNA. After the deactivation of Klenow fragment by heating at 65 °C for 20 min, the prepared dsDNA templates were incubated with T7 RNA polymerase using the same condition above. The reaction mixtures were mixed together and the biotinylated dsDNA templates were removed by streptavidin-magnetic beads twice. The RNA mixture was purified by gel filtration and used without further purification.

**Preparation of RNA origami.** The RNA origami was designed by a caDNA software<sup>[17]</sup> using the geometry of 11.00 bp/turn for tile and 10.50 helical pitch for tube. The sequences of the staple strands are listed in Table S1. For the tile formation, sample solution (40 μL) containing 0.02 μM RNA template, 1 μM staple RNA strands (40 eq), 20 mM Tris-HCl (pH 7.6), 1 mM EDTA, and 10 mM MgCl<sub>2</sub> was annealed from 75 °C to 15 °C at a rate of -1.0 °C/min. For the tube formation, sample solution (40 μL) containing 0.02 μM RNA template, 0.4 μM staple RNA strands (40 eq), 20 mM Tris-HCl (pH 7.6), 1 mM EDTA, and 10 mM MgCl<sub>2</sub> was annealed from 65 °C to 15 °C at a rate of -0.1 °C/min. These structures were purified using a gel filtration column (Sephacryl 300, GE Healthcare). The purified RNA origami was analysed with a 4% native polyacrylamide gel electrophoresis (4%) run at 4 °C in 1xTBE buffer with 5 mM MgCl<sub>2</sub>.

**Preparation of RNA origami with modified UTP.** For the preparation of modified RNA templates, *in vitro* transcription was performed with ATP, CTP, GTP, and 5-biotinylated UTP or 5-aminoallyl UTP. The purified modified RNA templates and staple strands were assembled as described above.

**AFM imaging.** AFM images were obtained using an AFM system (Nano Live Vision, RIBM, Tsukuba, Japan) with a silicon nitride cantilever (Olympus BL-AC10EGS). Samples (2 μL) were adsorbed onto an aqueous 3-aminopropyltriethoxysilane (APTES, 0.1%) passivated mica plate for 5 min at room temperature and then washed three times using the same buffer solution. Scanning was performed in the same buffer solution using a tapping mode.

## Acknowledgements

This work was supported by Core Research for Evolutional Science and Technology (CREST) of JST and JSPS KAKENHI (grant nos. 24310097, 24104002, 25253004, ). Financial supports from The Mitsubishi Foundation and The Asahi Glass Foundation to ME are also acknowledged.

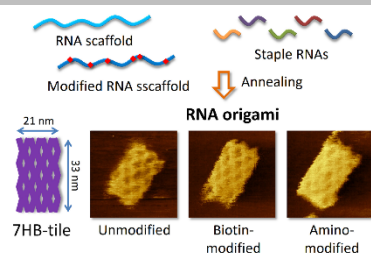
**Keywords:** RNA • nanostructure • transcription • chemical modification • AFM

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## Entry for the Table of Contents

## COMMUNICATION

RNA nanostructures were designed and prepared using the DNA origami strategy. Predesigned 7-helix bundled RNA tile and 6-helix bundled RNA tube nanostructures were obtained by annealing of a single-stranded RNA scaffold and staple RNA strands. These nanostructures were easily functionalized by introduction of the chemical modification to the RNA transcripts.



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