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Micrometer-sized network structure of novel DNA-lipid conjugates induced by heat stimulation

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We have developed a novel lipid-bearing DNA that forms hairpin modules, including single RNA monomer; this can be used to create micrometer-sized structures from nanometer-sized building blocks during breakage at the RNA site. In the presence of divalent metal ions and heat stimulation, we observed transition of the self-assembly, which results in the formation of a three-dimensional network structure. To our knowledge, this is also the first report of heat-induced micrometer-sized molecular self-assembly of molecules that carry biological information.

1. Introduction

Current research into lipids and their self-assembly in the liquid phase addresses fundamental questions on self-assembly in soft matter physics, colloidal chemistry, and the biology of biomembranes. Due to their diverse potential applications, it is critical that the structure and intermolecular interactions of functional lipids are fully characterized.[1-3] Recent research into the self-assembly of lipids bearing DNA or RNA has drawn much attention, as it has elucidated the morphological transitions associated with their self-assembly.[4-6] Based on the functionalization of these self-assembling molecules, novel concepts of DNA and/or RNA molecules that encode biological or biophysical activities have been developed.

The constitution of nanometer-sized structures of DNA-modified molecules has already been reported. For example, the Gianneschi group assembled DNA-brush copolymers that underwent a morphological change from spherical to cylindrical micelles upon injection of the DNAzyme or a complementary DNA strand.[7] Kwak and Herrmann designed a block copolymer of linear DNA with poly(butadiene) that formed vesicles of about 80 nm in diameter. The novel behaviors of self-assembling structures of such amphiphilic oligonucleotides have also drawn attention.[8] In this regard, the Sleiman group demonstrated precise self-assembly of an amphiphilic polymer containing a hydrophobic polymer and a three-dimensional DNA nanostructure. Micelle formation and aggregation of the DNA polymer conjugate were precisely determined by the precise DNA cubic structure.[9] To date, however, there have been no reports on the induction of structural transition from the nanometer to the micrometer scale by the chemical conversion of informational materials. Heat stimulation can be used to effect such chemical conversion, and is a useful tool for comparing the structural transition energy and stability of the three-dimensional structure within the informational molecular assembly.

Here, we report the heat-induced constitution of micrometer-sized structures linked to informational molecules, and discuss the underlying molecular mechanisms. Among the informational molecules, DNA is generally more stable than RNA in aqueous solution, hence DNA is an attractive candidate molecule carrying information for heat-induced constitution of the micrometer-sized structures. However, the relatively rigid nature of the double helix secondary structure prevents it from adopting other morphologies. In order to link morphological changes and DNA information, we designed a DNA-RNA chimera with a stem-loop structure that was conjugated to lipid molecules. In previous reports,[10,11] binding of an RNA enzyme or a DNA enzyme to an RNA or a DNA oligo containing a single RNA monomer elicited a dramatic conformational change. Moreover, the structural stability of the stem-loop oligo DNA upon heat challenge is controlled by changing the length and sequence composition of both the stem and loop parts. Together, these data indicate that the structure of such chimeras will be sensitive to physical or chemical stimuli. Consistent with this, Mg^{2+} ions can accelerate the hydrolysis of RNA site under high temperature, and trigger aggregation of self-assembled particles in an aqueous phase.[12] In the current report, therefore, we focused on the structural changes of self-assembled and lipid-conjugated stem-loop oligo DNA-RNA chimeras (DNA-RNA chimera lipids) in the presence of Mg^{2+} and following heat stimulation (Fig. 1).

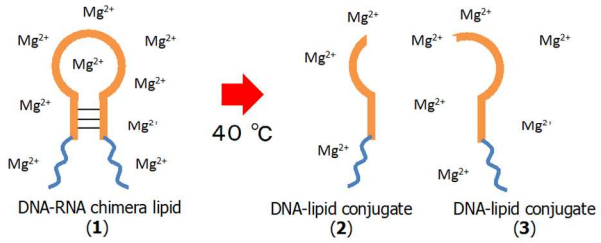

Fig. 1 Schematic illustration of the reaction of DNA-RNA chimera lipid **1** in the presence of Mg^{2+} and heat stimulation. Products **2** and **3** are capable of forming three-dimensional micrometer-sized structures.

2. Materials and Methods

Materials.

All DNA-lipid conjugates, RNA-lipid conjugates, and the DNA-RNA chimera lipids were synthesized and purified by HPLC and the assignment was performed by mass spectrometry (Sigma-Aldrich). As shown in Fig. 2, DNA-RNA chimera lipid **1** had the following sequence; 5′-CCGCTATrAGGAAGAGACGG-3′ with both 5′ and 3′ terminals modified by amide bonds that are linked with oleic acid. rA indicates the RNA monomer. DNA-lipid conjugates **2** and **3** had the following sequences; 5′-CCGCTATrA- 3′ with the 5′ terminus modified by amide bonds linked to oleic acid, and 5′-GGAAGAGACGG-3′ with the 3′ terminus modified by amide bonds linking to oleic acid, respectively. Using the same sequence of **1**, a DNA-RNA chimera, **1**', and a DNA-RNA chimera lipid with shorter acyl chains (lauric acid) **1**" were also synthesized. Lipid conjugate **4** had the following sequence; 5′-CCGCTATAGGAAGAGACGG-3′ with both 5′ and 3′ terminals modified by amide bonds that are linked with oleic acid. RNA-lipid conjugate **5** had the sequence 5′-rCrCrGrCrTrArTrArGrGrArArGrArGrArCrGrG-3′ with both 5′ and 3′ terminals modified by amide bonds that are linked with oleic acid.

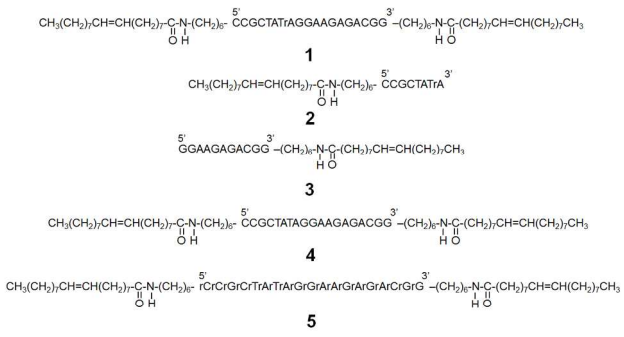

Fig. 2 Chemical structures of DNA-RNA chimera lipid **1**, DNA-lipid conjugates **2**, **3**, **4**, and RNA-lipid conjugate **5**.

Phase contrast microscopy observation.

Real-time observation of the transition of self-assembly of DNA-RNA chimera lipid **1** was performed under a phase contrast microscope (IX71, Olympus, Japan) equipped with a CCD camera (DP-72, Olympus, Japan). For heat stimulation of the buffered solution, we used commercially available pocket warmers ("HOKARON mini", Lotte, Japan). The four pocket warmers were shaken well and fixed around the specimen on the microscope stage by a packed tape (Fig. 3). The temperature of the specimen was kept in the range 39.7–40.2 °C over 6 hrs.

For image analysis of the area for the degree of agglomeration of the micrometer-sized structures, binarization processing of the brightness was performed on the microscopy images to clarify the edge information by using an image analysis software, ImageJ (NIH), and we calculated the area of

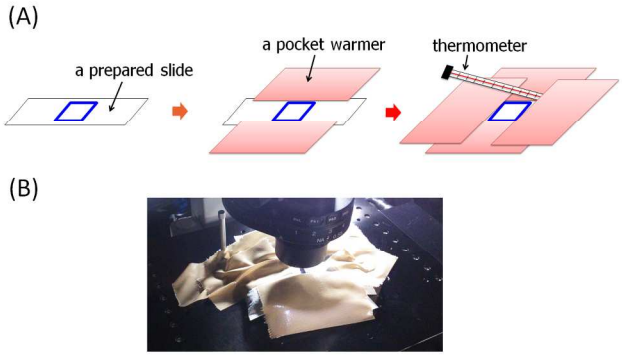

Fig. 3 Schematic illustration of the protocol for observing the specimen during heat stimulation. Mg^{2+}-containing buffered solution of DNA-RNA chimera lipid **1** was placed in the slide and the four pocket warmers were placed around the specimen (A). An actual photograph is shown in (B)

the micrometer-sized structures in pixel×pixel unit. The area unit was converged from pixel×pixel to μm^2 by using a graduated glass-scale plate (Olympus, Japan).

Transmission electron microscopy observation.

To clarify the three dimensional structure in detail, we negatively stained samples by absorbing them onto carbon-coated copper grids (400 mesh) and staining with 1% uranyl acetate solution for a few seconds. The samples were observed using a transmission electron microscope (JEM-1400Plus; JEOL Ltd., Tokyo, Japan) at an acceleration voltage of 80 kV. Digital images (2048 × 2048 pixels) were taken with a CCD camera (VELETA; Olympus Soft Imaging Solutions GmbH, Münster, Germany).

Atomic force microscopy observation.

Samples for atomic force microscopy observation were prepared as follows: DNA-RNA chimera lipid **1** (16 μM) was dissolved in a Mg^{2+}-containing buffered solution (HEPES, 50 mM, pH = 7.4, $MgCl_2$, 10 mM) and incubated for 20 minutes at 40°C, and then mounted on a fresh mica substrate at room temperature. The AFM imaging was performed using Nanowizard 3 (Bruker).

3. Results and Discussion

3.1. Micrometer-sized network self-assembly of DNA-lipid conjugates

DNA-RNA chimera lipid **1** was designed by the application of the DNA folding prediction. The mfold Web Server 1 employing the nearest-neighbor model enables prediction of the melting temperature T_m of DNA duplexes for the DNA sequence of **1**.[13,14] With $[Na^+]$ = 0 M, $[Mg^{2+}]$ = 0.01 M, the T_m of DNA sequence **1** was calculated to be 39.2°C.

DNA-RNA chimera lipid **1** was dissolved in HEPES buffer (50 mM, pH = 7.4) including $MgCl_2$ (10 mM) and the solution was observed under the phase contrast microscope (see ESI Movie S1). Fig. 4A shows images of the emergence of a

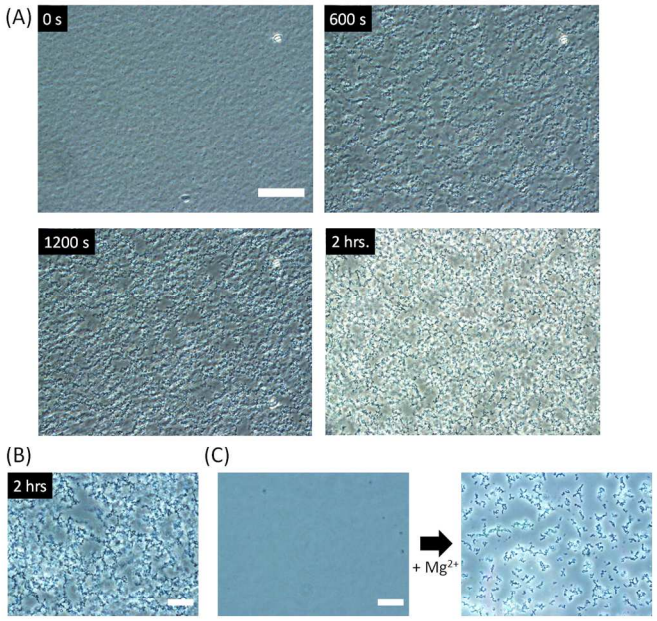

Fig.4 Microscopy images of the micrometer-sized structure induced by heat stimulation or Mg^{2+}. (A) Time-course change of phase contrast microscopy images of the emergence of the structure of DNA-RNA chimera lipid **1** in the Mg^{2+}-containing buffer at 40°C. Bar = 50 μm. (B) Phase contrast microscopy image of the DNA-RNA chimera lipid **1** aggregates in the Mg^{2+}-containing buffer after heat stimulation. Bar = 20 μm. (C) The phase contrast microscopy image of the granules of 1:1 mixture of DNA-lipid conjugate **2** and **3** structure in the buffer without Mg^{2+} at room temperature before $MgCl_2$ injection and that of the micrometer-sized structure after $MgCl_2$ injection. Bar = 20 μm.

three-dimensional network structure over time after heat stimulation at 40°C. The granular structures appeared in the specimen within the first 600 seconds of heat stimulation. Granular structures began to gather and grow in network manner at 1200 seconds; this process continued until 2700 seconds. High magnification images of the gathered granular structures (Fig. 4B) confirmed the network-like manner of the aggregates. When we applied heat stimulation to the Mg^{2+}-containing buffered solution of DNA-RNA chimera lipid **1** in an incubation chamber of 60°C and 95°C, a three-dimensional network structure developed (see ESI, Fig. S1). As a reference experiment, a solution composed of a 1:1 mixture of DNA-lipid conjugates **2** and **3** was also prepared with the buffer without Mg^{2+}, which did not afford granules. Buffer containing $MgCl_2$ (final concentration, 10 mM) was added to this solution and it was observed under the same conditions (in all cases, the final concentration of DNA was adjusted to 32 μM) at room temperature. This revealed that formation and aggregation of granules occurred within 1 minute of $MgCl_2$ injection into the chamber (Fig. 4C), which is similar to the situation observed with DNA-RNA chimera lipid **1**. These micrometer-sized network structures were stable in the chamber for more than two weeks. On the other hand, Tris-borate-EDTA injection (including 2 mM EDTA as the chelator of Mg^{2+}) resulted in the complete dissolution of heat-induced DNA-RNA chimera lipid **1** and of DNA-lipid conjugates **2** and **3** at room temperature (see ESI, Fig. S2).

Taking these phase contrast microscopy images into account, DNA-RNA chimera lipid **1** was converted to DNA-lipid conjugates **2** and **3**; this lead to the emergence of micrometer-sized structures from the nanometer-sized self-

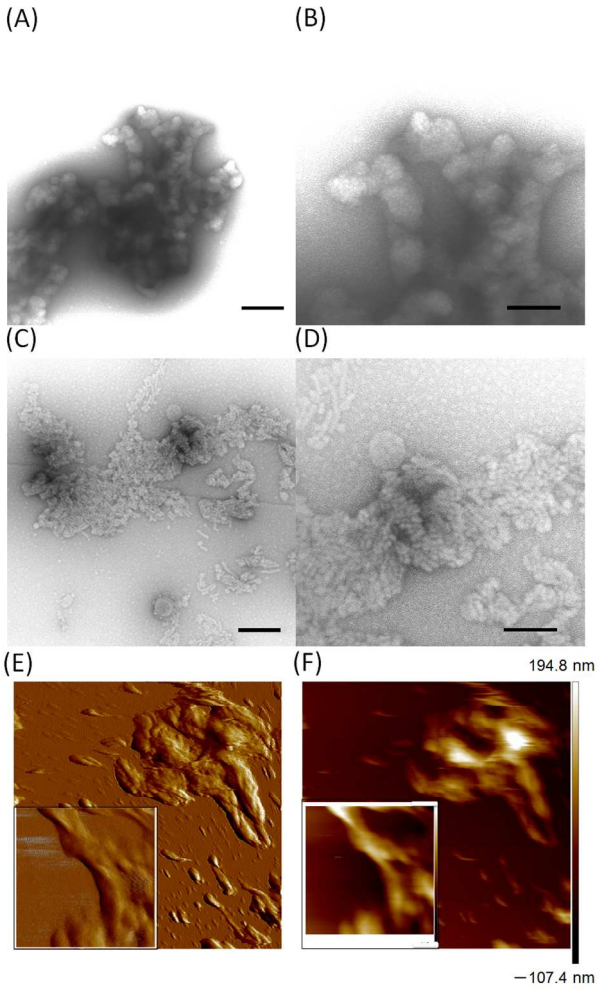

Fig. 5 Nanometer-size focused images of DNA-lipid conjugate self-assembly. (A)(B) DNA-RNA chimera lipid **1** incubated at 40 °C for 15 minutes in the Mg^{2+}-containing buffer by transmission electron microscopy. (C)(D) 1:1 mixture of DNA-lipid conjugate **2** and **3** incubated at 40 °C for 15 minutes in the Mg^{2+}-containing buffer by transmission electron microscopy. (E) The deflection images and (F) the raw images of DNA-RNA chimera lipid 1 incubated at 40 °C for 15 minutes in the Mg^{2+}-containing buffer by atomic force microscopy. Bar = 200 nm (A)(C). Bar = 100 nm (B)(D). (E)(F) 5.4 × 5.4 μm, caption 1.1 × 1.1 μm.

assembly during breakage at the RNA site. On the other hand, we confirmed that heat stimulation did not induce three-dimensional micrometer-sized structures in either DNA-RNA chimera **1'** or a DNA-RNA chimera lipid bearing lauryl chains (compound **1"**) (data not shown). Hence, we infer that the transition is due to the conformation change of amphiphilic molecules that mediate the changes in intermolecular hydrophobic interactions. Specifically, microscopic observation revealed that the transition was induced by the combined effects of heat stimulation and the presence of magnesium ions. Magnesium ions played two roles in the current experiments. First, they could induce breakage at the RNA site under not only high temperature but also room temperature (see ESI, Fig. S3).[15,16] Second, they were required to nucleate the three-dimensional micrometer-sized structures. We found that such structures were not observed after the heat stimulation in their

absence (see ESI, Fig. S4). Therefore, while both heat stimulation and magnesium ions induced the breakage at the RNA site, the formation of micrometer-sized structures required the magnesium ions. We imply that the DNA-lipid conjugates **2** and **3** the molecular weights of which are smaller than that of **1** slightly became hydrophilic and the intensity of the molecular motions of some of **2** and **3** eventually overcame the intermolecular hydrophobic interaction, resulting in bridging the granules in presence of Mg^{2+}.

Next, we focused on the roles that the sequence of the DNA-RNA chimera lipid **1** played in the structural transformation. The informational materials of DNA or RNA are inevitable to a logic for life phenomena including origins of life.[17,18] It has been suggested that studying the emergence of micrometer-sized granular networks from a mixture of more 'simple' informational materials may provide insights into the development of first primitive microorganisms.[19] We suggest that this study should extend to molecules such as DNA-lipid conjugates. As the 1:1 mixture of **2** and **3** formed three-dimensional micrometer-sized structures following addition of Mg^{2+}, we examined Mg^{2+}-containing HEPES buffer solutions of oligo-A lipid conjugate (bearing 8 and 11 monomers), oligo-T lipid conjugate (bearing 8 and 11 monomers), and a 1:1 mixture of them in the same condition at room temperature, respectively (see ESI, Fig. S5). Phase contrast microscopy revealed that the induction of micrometer-sized structures was influenced not only by the length, but also by the actual DNA base sequences.

3.2. Self-assembly of DNA-lipid conjugates observed at the nanometer scale

We next investigated the details of the self-assembly product that was derived from **1** after heat stimulation. Transmission electron microscopy (TEM) was used to reveal the nanometer scale structures of the DNA-lipid conjugates. Fig. 5 (panels A and B) shows the product obtained by incubating a 32 μM DNA-RNA chimera lipid **1** at 40°C for 15 minutes. Nanometer-sized 3D complex structures and dimples with dimensions of 200–500 nm were observed. The structures resembled the granule aggregation that is shown in Fig. 4A. Fig. 5 (panels C and D) shows the 1:1 mixture of DNA-lipid conjugates **2** and **3** (16 μM final concentration of each) after incubation at 40°C for 15 minutes. Segmented and aggregated structures with a width of 10–20 nm and length of 30–50 nm were observed. This morphology was again similar to that shown in Fig. 4C. Note that, although the shape of the nanometer-sized self-assembly found in the specimen of the hydrolyzed products of **1** is different from that in the 1:1 mixture of DNA-lipid conjugates **2** and **3**, the manner of aggregation was similar. However, these nanometer-scale self-assemblies and their structures were more complicated than those observed with DNA-lipid conjugate **3** alone (data not shown). These differences observed in nanometer scale plausibly comes from the self-assembly process. While the 1:1 mixture of DNA-lipid conjugates **2** and **3** or sole **3** aggregated from the randomly dispersed state as the initial stage, the self-assembly derived from **1** after heat stimulation involved the reorganization of the DNA-lipid conjugates **2** and **3** in the preformed granules of **1**. The structure of DNA-RNA chimera lipid **1** at the nanometer scale after heat stimulation was also confirmed by AFM. Structures with a height of 300 nm on a mica surface were consistent with similar structures found in the TEM images (Figs. 5E and F).

3.3. Agglomeration degree evaluation of the micrometer-sized structures

As a reference experiment, we examined whether DNA-lipid conjugate **4** and RNA-lipid conjugate **5** (Fig. 2) produced micrometer-sized structures after heat stimulation. Fig. 6A shows that this was indeed the case for DNA-RNA chimera lipid **1** following a stimulus of 40°C for 20 minutes. Fig. 6 (panels B and C) shows Mg^{2+}-containing buffer solutions of DNA-lipid conjugate **4** and RNA-lipid conjugate **5** treated under the same conditions. No micrometer-sized structures were found in the "all DNA"-lipid conjugate **4** after heat stimulation. On the other hand, micrometer-sized structures derived from "all RNA"-lipid conjugate **5** in Mg^{2+}-containing buffer solution were observed, although they were not fully networked. We thus analyzed the degree of agglomeration of the micrometer-sized structures by measuring their area (Fig. 6D) instead of other optical evaluation methods such as turbidity, because the amount of sample was limiting. We then investigated why DNA-RNA chimera lipid **1** gave a more extensive network structure than those of DNA-lipid conjugate **4** and RNA-lipid conjugate **5**. We serially diluted DNA-RNA chimera lipid **1** in Mg^{2+}-containing HEPES buffer and observed the structures formed in the Mg^{2+}-containing buffer after heat

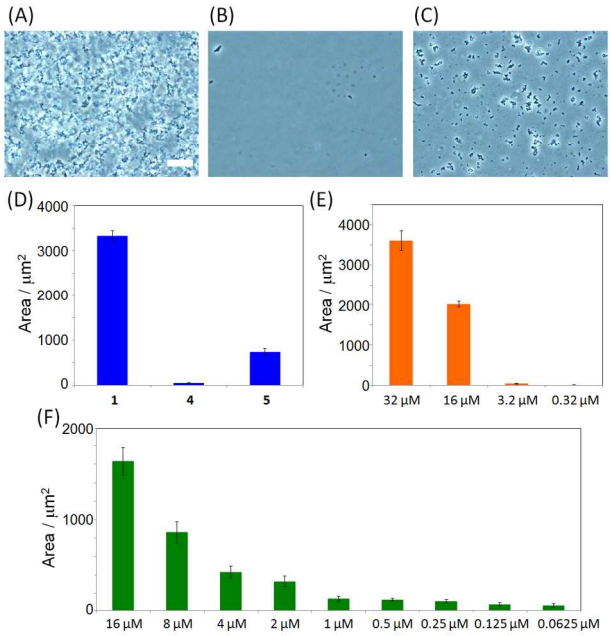

Fig. 6 Image analysis of the area for the degree of agglomeration of the micrometer-sized structures. The microscopy images of the micrometer-sized structures of DNA-RNA chimera lipid **1** (A), DNA-lipid conjugate **4** (B) and RNA-lipid conjugate **5** (C) incubated at 40 °C for 20 minutes in the Mg^{2+}-containing buffer are shown. Bar = 20 μm. (D) Diagram of the area (average and standard deviation) filling with structures of each specimen (**1**; N = 19, **4**: N = 19, and **5**; N = 23). (E) Diagram of the area of filling with structures of DNA-RNA chimera lipid **1** incubated at 40 °C for 15 minutes in the Mg^{2+}-containing buffer with different concentration (32 μM; N = 11, 16 μM; N = 17, 3.2 μM; N = 17, and 0.32 μM; N = 18) (F) Diagram of the area of filling with structures of dilution series of the specimen of 32 μM DNA-RNA chimera lipid **1** incubated at 40 °C for 15 minutes (16 μM; N = 13, 8 μM; N = 10, 4 μM; N = 17, 2 μM; N = 18, 1 μM; N = 13, 0.5 μM; N = 15, 0.25 μM; N = 15, 0.125 μM; N = 14, 0.0625 μM; N = 12).

stimulation in order to evaluate degrees of agglomeration using the area measurement. No structure (the area of structure filling; 44 ± 18 μm^2) was observed when less than 3.2 μM of DNA-RNA chimera lipid **1** was used (Fig. 6E). For comparison, we evaluated a dilution series of the micrometer-sized structures that were derived from heat treatment of 32 μM of same DNA-RNA chimera lipid **1** (Fig. 6F). This revealed that structures with an area of 331 ± 113 μm^2 were present when 2 μM of DNA-RNA chimera lipid **1** was used. Image analysis (Figs. 6E and F) indicates that there is a threshold of the concentration of DNA-RNA chimera lipid **1** which is required in order to generate a three-dimensional micrometer-sized structure.

The aggregation of DNA-RNA chimera lipid **1** was characterized as follows: (i) formation of the three-dimensional network structure triggered by an increase in temperature in the presence of Mg^{2+}, (ii) a structure composed of the hydrolyzed products of DNA-lipid conjugates, which had specific DNA sequences, and (iii) an irreversible nature (unless Mg^{2+} was removed). These characteristics are different from the aggregation behaviors of conventional nonionic surfactants (the temperature of the aggregation is known as the cloud point)[20] or of DNA-binding gel networks (the aggregation is induced by a reduction in temperature).[21] As the growth of the network resembled a cluster-cluster aggregation (see ESI Movie S1), we infer that the three-dimensional network structures are formed due to percolation once a certain threshold has been reached.[22,23]

4. Conclusions

We conclude that the DNA-RNA chimera described here undergoes dramatic structural reorganization upon heat stimulation. This transition was likely caused by the hydrolysis of the DNA-RNA chimera lipid to produce two DNA-lipid conjugate molecules that subsequently underwent conformational change. The flexible nature of these novel compounds, and the ability to equip them with functional groups, provides an effective resource for self-assembling and bioactive nanostructures.

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Notes and references

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Electronic Supplementary Information (ESI) available: Phase contrast micrographs and a movie of the Mg^{2+}-containing buffer solutions of the series of DNA lipid conjugates. See DOI: 10.1039/b000000x/

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