Regulation of reversible conformational change, size switching, and immunomodulation of RNA nanocubes

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ABSTRACT

In biological systems, conformational changes and allosteric modulation play pivotal roles in regulating biological functions, such as the dynamic change of protein molecules, in response to binding or interacting with other factors such as pH, voltage, salt, light, or ligand. RNA can be manipulated and tuned with a level of simplicity that is characteristic of DNA or polymers, while displaying versatility in structure, diversity in function, and adaptability in a configuration similar to proteins. In the past, the work on the investigation of conformational change mainly focused on protein. The induced-fit and conformational capture in RNA have also been explored, such as in the study of riboswitches. Herein, we report the engineering of three-dimensional RNA nanocubes and demonstrated the operation and regulation for its configuration. We demonstrate the operation of reconfigurable RNA nanocubes whose shapes change precisely and reversibly in response to a specific trigger strand. The shape, size, and conformation can be regulated precisely and reversibly in response to the specific triggering signals. The shape and conformational conversion were observed by cryo-EM and gel electrophoresis, respectively. Harnessing the size, shape, conformation, and self-assembly capabilities of the RNA nanocube can provide a new potential use of this technology as nanocarriers for the treatment of various diseases.

Keywords: RNA nanotechnology; RNA nanostructures; RNA nanocube; reconfigurable switch; nanometer scale

INTRODUCTION

Conformational changes or molecular transformations are a common phenomenon in biological systems (Leulliot and Varani 2001; Pitici et al. 2002). The dynamics of conformational changes and molecular transformations have led to the development of many dynamic models. Protein molecules are usually flexible and dynamic. Their shape changes in response to binding or interaction with other factors. The transition between different states usually occurs reversibly within a very short time. To achieve a delicate control similar to proteins at the nanoscale level, development of a platform that can succeed in safe regulation of conformational changes and reversible switching is imperative. RNA provides such a platform.

RNA nanotechnology is an emerging research field that focuses on utilizing RNA as a unique and programmable biopolymer to auto-assemble nanoscale objects with sophisticated structures and rich functionalities (Jasinski et al. 2017). Similar to DNA, most of the RNA–RNA interactions are based on Watson–Crick base-pairings, and these base-pairings are largely predictable. However, in contrast to DNA, RNA–RNA interactions also involve many noncanonical base-pairings, and RNA can form complicated secondary structures such as single-stranded loops, bulges, hairpins, pseudoknots, and junctions (Tinoco and Bustamante 1999; Svoboda and Di Cara 2006; Lee et al. 2013). In addition, RNA is one of the most important
bimolecules in a cell and plays a critical role in normal physiology as well as in various diseases.

Previous research has shown that a variety of 2D and 3D RNA nanostructures, such as triangles (Khisamutdinov et al. 2014), squares (Khisamutdinov et al. 2014), pentagons (Khisamutdinov et al. 2014), tetrahedrons (Li et al. 2016), and prisms (Khisamutdinov et al. 2016), can be successfully constructed by utilizing naturally occurring RNA structures. Additionally, nanomechanical DNA/RNA devices that change conformations in response to various biomolecular stimuli have been introduced, such as DNA walkers (Sherman and Seeman 2004), nanotweezers (Ghimire et al. 2020), nanoboxes (Kuzuya and Komiyama 2009), cages (Raniolo et al. 2018), and even nanocubes (Afonin et al. 2010, 2011, 2014a; Severcan et al. 2010; Halman et al. 2017). These RNA nanostructures can also be easily functionalized through the incorporation of functional groups such as aptamers, siRNAs, and ribozymes (Yi et al. 2009; Shu et al. 2013; Cui et al. 2015; Pi et al. 2017). These functionalized RNA nanostructures have shown potential for many applications in biomedical and material sciences such as drug delivery, disease diagnosis, and tissue engineering. Hence, dynamic nanotechnology seeks to develop reconfigurable and autonomous nanodevices that respond to environmental cues and system inputs. These dynamic elements are particularly important for applications in nanomedicine, nanorobotics, and information processing.

In the present study, we report the design and construction of three-dimensional RNA nanocubes based on the stable pRNA 3WJ (Haque et al. 2018). By introducing hairpins into the nanocube, reconfigurable shape-switching from “open” to “closed” states was achieved with the help of hairpin or anti-hairpin RNA strands, respectively. The kissing loop (KL) interaction between two adjacent RNA hairpins ensures the “closed” state of the nanocube (Yingling and Shapiro 2007; Grabow et al. 2011; Afonin et al. 2014b; Rackley et al. 2018). Induced by anti-hairpin strands, the kissing loop interaction was disrupted, thus facilitating the shape switching by unfurling to an “open” state. The assembled RNA nanocubes have defined 3D structures as revealed by cryo-electron microscopy (cryo-EM). Furthermore, conformational change was confirmed by gel electrophoresis. Thus, this study uses the stable pRNA 3WJ as the building block unit in successful bottom-up auto-assembly of molecularly defined RNA nanocubes.

RESULTS AND DISCUSSION

Design and self-assembly of the RNA cubes

A typical nanocube structure has eight vertexes, six square faces, and twelve edges. A 3D computational model of the RNA nanocube in Figure 1A was first generated using the software NanoEngineer (Allen et al. 2014). The resulting computational model exhibited authentic cubic conformation and the eight RNA 3WJs can be clearly observed in the model (Binzel et al. 2014). For designing the RNA cages with a nanocube shape, eight pRNA 3WJs (four of them are reverse pRNA 3WJs) are positioned into the vertexes and twelve double-stranded RNAs are positioned into the edges of the nanocube to link the individual pRNA 3WJs. A total of six RNA strands were designed to construct the RNA nanocube, with each RNA strand representing one square face of the RNA nanocube (Fig. 1B). For assembling the RNA nanocube, the designed six RNA strands were synthesized by in vitro transcription and then mixed in stoichiometric ratio and annealed in 1×TMS buffer in a one-pot manner. Stepwise assembly of the RNA cubic cages was observed by analysis in a 6% native PAGE, confirming the formation of the high molecular weight RNA nanocubes (Fig. 1B). Additionally, to determine whether the kissing loop design was successfully assembled on one of the square faces of the nanocube, different designs were constructed and run together in a native PAGE (Fig. 1C).

Imaging of the RNA nanocube cages by cryo-electron microscopy

To validate the formation of the RNA nanocube cages, nanocubes with a pair of hairpins on a single face of the cube were purified in “closed” and “open” conformation prior to acquiring the images, with only the “closed” conformation used for cryo-electron microscopy (cryo-EM) images (Fig. 1D). Cryo-EM was performed to directly image the RNA particles (Fig. 2A). The 2D computer projections of the reconstructed RNA nanocube model showed a match to the 2D class averages of the raw images, suggesting the reconstructed 3D model will correctly represent the native structure of the RNA nanocube (Fig. 2B). Cryo-EM images showed very clear RNA nanocubes with the expected dimensions. The single particle 3D reconstruction data revealed that the RNA nanocube has a clear overall shape and conformation in agreement with the computational 3D model and the predicted size of 100 Å or 10 nm (Fig. 2C). The six faces of the nanocube cage were characterized in the reconstructed 3D model. Single particle image processing and 3D reconstruction of the RNA nanocubes were performed from a RAW image for accurate representation of the RNA nanocubes (Fig. 3A). These were boxed to locate the nanocubes. The boxed nanocubes with 5106 particles were used for 2D averages (Fig. 3B), and 3D reconstruction was performed for refinement to make the final 3D model of the RNA nanocube (Fig. 3C).

Conformational change of a metamorphic RNA nanocube using unmodified RNA hairpin strands

In order to confirm that the nanocube experienced a conformational change by using hairpins, nanocubes with
hairpins and without hairpins were designed on one of the faces. The principle behind the conformational change is to introduce the component that will open up the hairpins to extend the size of that side of the nanocube (Fig. 4A). Auto-assembly of the nanocube with and without hairpin confirmation was performed via 6% native PAGE gel. After verification, both types of nanocubes were compared before and after the addition of RNA anti-hairpin strands using a 6% native PAGE. The RNA nanocubes containing hairpins unfurled to their “open” conformation, while the nanocube without hairpins remained the same size (Fig. 5A). To validate whether the conformational change is specific to the anti-hairpin strands, another gel was run for nanocubes treated with anti-hairpin or scramble strands.

FIGURE 1. (A) 3D computation model using NanoEngineer. (B) Nanocube formation using six different RNA strands and confirmed formation using 6% PAGE gel. (C) RNA hairpin (Hp) and kissing-loop (KL) were designed on the faces of RNA Cube to address the concern of whether the RNA hairpin and kissing loops were formed as designed. (D) Purification of RNA Cube-Hp with one kissing loop (KL) and RNA Trapezoidal Prism. RNA Cube-Hp was used for cryo-EM imaging in Figure 3.

FIGURE 2. (A) Cryo-EM images of RNA nanocube cages. (B) Cryo-EM RNA nanocube shape and dimension confirmation. (C) Cryo-EM comparison with 3D reconstruction match 2D class averages.
Minor broadening of the gel band was observed after the addition of scramble strands because RNA and DNA interaction and hybridization depends on stringency and sequence homogeneity. However, the results show that the conformational change is due to the addition of a specific type of component, namely, anti-hairpin strands (Fig. 5B). Furthermore, a modification on the RNA hairpin strands used to induce the conformational change could influence the formation of the “open” or “closed” state of the RNA nanocube.

**Nanocube conformational change using 2’F modified RNA hairpins and size control of nanocubes**

To validate that a modification on the RNA hairpins does not influence the hairpin’s effect on the nanocube, a 2’F modification to the hairpin was used for inducing the conformational change. The RNA nanocube was able to morph into the trapezoidal prism using the unmodified RNA anti-hairpin strands, while the 2’F modification to the hairpin was still able to change the trapezoidal prism back to a cube (Fig. 6A). The shape modification can be harnessed and controlled in a stepwise fashion. To demonstrate this, the nanocube, in its “closed” state with intact hairpins, gradually morphs to a trapezoidal prism and unfurls fully to an “open” state.

**FIGURE 3.** (A) Cryo-EM images containing auto-assembled RNA nanocubes at <20 nm for single particle image processing. (B) RNA nanocube boxed with 5106 particles were used for 2D class averages. (C) 4371 particles were used for final refinement of projections to generate final 3D map using a 16 Å Gauss low-pass filter.

**FIGURE 4.** (A) RNA nanocube cages with hairpin in with “close” and “open” conformation. (B) RNA nanocube formation compared with hairpin-containing nanocube confirmation. This contains the stepwise addition of each strand (S1–S6) to confirm proper connectivity.
state, making a cuboid as anti-hairpin strands are added and reversed back to a cube in the same order, as hairpin strands are added (Fig. 6B). The degree to which the capacity of regulation can be harnessed is further displayed by the capability of changing the shape in a stepwise manner and the ability to form intermediates of the “closed” and “open” states. As shown by Figure 6B, the stability of the nanocube increases as it unfurls to the “open” state. The stability of the “open” state is most likely due to the additional strain of the hairpins in the “closed” state. The unfurling of the hairpins causes a relatively large amount of energy to be released, thus increasing the stability of the nanocube in its “open” conformation (Bercy and Bockelmann 2015).

Additionally, the nanocube can be design-controlled and could be used to increase nanoparticle size on demand through the addition of hairpins on each of the faces on the nanocube, making a cube-6 KL (Fig. 7A). Nanocubes without a strand kissing loop (KL) and with KL were designed to compare migration in gel electrophoresis. The gel shows that if the kissing loop interaction was not present, the cube would migrate differently in the gel.

FIGURE 5. (A) RNA nanocube cages with hairpin changing from “close” to “open” confirmation using anti-hairpin. Nanocube without hairpins does not change shape. (B) RNA trapezoid formation with the addition of anti-hairpin strands. The nanocube did not reverse back to a trapezoid with the addition of scramble strands.

FIGURE 6. (A) RNA nanocube cages with hairpin transitioning from “closed” to “open” and back to “closed” conformational change using RNA anti-hairpin and hairpin strands, respectively. (B) RNA nanocube with dual hairpins conformation change to “open” state with the addition of anti-hairpin strands in stepwise fashion. After RNA nanocube reaches full “open” state to make a cuboid, the nanoparticle can be reversed to a nanocube shape using hairpin strands.
This especially becomes evident as more KLS are added. Furthermore, the migrations of the nanocube of interacting kissing loops versus noninteracting loops are different (Fig. 7B). Therefore, the KL containing nanocubes could be used in the same way to make an “open” conformation and increase the size of the nanocube using modified or unmodified RNA strands.

Conformational change of a metamorphic RNA nanocube using DNA hairpin strands

Using RNA hairpins and anti-hairpins opened up the possibility of harnessing the power of these controllable RNA nanocubes. To improve the efficiency of the conformational change, the RNA hairpins were made in the DNA variety. After administrating the DNA hairpins and anti-hairpins, the same morphing of the nanocube to a trapezoidal prism and the reverse was observed, respectively (Fig. 8). In contrast to the RNA hairpins/anti-hairpins, the DNA hairpin/anti-hairpins versions form the nanocube more efficiently when switching between “open” and “closed” states compared with RNA based hairpins (Fig. 9). This was possible while still harnessing the capability to control the size and conformational change of the nanocubes. The increased stability the DNA variety of conformational change inducing strands afforded a stable reversible conformational change from a “closed” cube-6 KL form to an “open” large cube form (Fig. 10).

Immunomodulation in macrophage cells by CpG DNA escorted RNA cubes

The synthetic CpG oligodeoxynucleotides (ODNs) are a type of DNA molecule that could induce a potent immune response both in vitro and in vivo (Ruan et al. 2014). Herein, based on the hypothesis that 3D display of CpG DNA by nanoparticles will create a synergistic effect and enhance the immune response, suggesting the potential of using the nanocube as carrier of the immunostimulatory strands. According to the literature, cubes that have had immunomodulation (Hong et al. 2018, 2019). However, an important point that would cause the difference in immunomodulation between this nanocube and previous cubes would be the shape, size, and sequence dependency (Guo et al. 2017). In the present study, the ELISA assay showed that CpG DNA delivered by the RNA nanocube induced an enhanced immune response, and the induced immune response is strongly dependent on the number of immunostimulatory molecules per RNA nanocube (Fig. 11). The more CpG DNA molecules incorporated into the RNA nanocube, the stronger immune responses observed. Additionally, the RNA nanocube without CpG DNA induced a negligible immune response. These results suggested that the newly designed RNA nanocube has the potential to be used as a novel vehicle for immune-active CpG DNA delivery because the immune response is dependent on the number of CpG DNA strands and not the nanocube.

MATERIALS AND METHODS

The design, synthesis, and self-assembly of RNA nanocubes

The NanoEngineer program (Allen et al. 2014) was used to facilitate the design of the RNA cages. Six RNA strands were designed for assembling the RNA nanocube. Each RNA strand represents one face of the RNA nanocube. The nucleotide sequence of the individual RNA strand was designed based on the pRNA 3WJ sequence and a perfectly base-paired double-stranded RNA sequence. RNA strands were synthesized by in vitro T7 transcription. Sequences of strands used to assemble the nanocube and a brief procedure are found in the Supplemental Information.
The RNA cubes were self-assembled in a one-pot manner by mixing the corresponding RNA strands in equimolar concentrations (0.3 µM) in 1× Tris buffer, heating them to 95°C, and slowly cooling them down to room temperature. The RNA cubes harboring CpG DNA were self-assembled in a one-pot manner by mixing the corresponding RNA and DNA strands in equimolar concentrations (5 µM) in 1× annealing buffer (10 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, pH = 8.0); then, they were heated to 95°C and slowly cooled down to room temperature. The stepwise self-assembly of the RNA nanocubes was verified by polyacrylamide gel electrophoresis (PAGE). The gels were stained with ethidium bromide (EB) and imaged by Typhoon FLA 7000 (GE Healthcare). For cryo-EM imaging, the assembled RNA cubes were further purified by 6% native PAGE.

CpG DNA decorated RNA nanocube design and immunomodulation assay

The constructed RNA nanocube to deliver CpG DNA by incorporating four CpG DNA oligos to each RNA nanocube. The CpG DNA oligo was added to the RNA cube via the branch extension approach, using the complementary sequence attached to the RNA sequence extending from the cube (Khisamutdinov

FIGURE 8. (A) A nanocube, in its “closed” state with intact hairpins, gradually morphs to a trapezoid and unfurls fully to an “open” state, making a cuboid as DNA anti-hairpin strands are added and reversed back to a cube in the same order, as hairpin strands are added. (B) This is similar to the RNA version of hairpin and anti-hairpins but the DNA variety has better efficiency for the nanocube conformational change.

FIGURE 9. A nanocube, in its “closed” state with intact hairpins, gradually morphs to a trapezoid as DNA anti-hairpin strands are added and reversed back to a cube as hairpin strands are added. The DNA version of the hairpin and anti-hairpin strands was more efficient at returning the trapezoidal prism back to a cube.
et al. 2014; Guo et al. 2017). RNA cubes harboring CpG DNA were then incubated with macrophage-like RAW 264.7 cells. The cell culture supernatant was collected to determine the TNF-alpha and IL-6 cytokine levels, correlated with inflammatory response, using Mouse Enzyme-Linked Immunosorbent Assay (ELISA) MAX Deluxe sets (BioLegend, Inc.), following protocols provided by the manufacturer. The method for immunomodulation cell study was carried out following the method described in previous publications (Khisamutdinov et al. 2014; Yin et al. 2019).

Arugose gel analysis
Annealed RNA structures were directly loaded on 1.5% agarose gel (if not otherwise indicated) and allowed to migrate for 3 h at 4°C (running buffer: 1× TBE, 10 mM MgCl2; running voltage: 80 V). The gel was stained with SybrGold (Life Technologies) and visualized with Typhoon Trio scanner (GE Healthcare Life Sciences).

Cryo-electron microscopy of the RNA cubes
For cryo-electron microscopy, 2 µL of the RNA nanocube cage sample (0.45 mg/mL) was applied onto a glow-discharged 200-mesh R1.2/1.3 Quantifoil grid. The grid was blotted and rapidly frozen in liquid ethane using a Vitrobot IV (FEI). Micrographs were recorded with a direct detection device (DDD) (DE-20 4k×5k camera, Direct Electron, LP) in movie mode at 25 raw frames per second at 40,000× microscope magnification (corresponding to a calibrated sampling of 1.64 Å per pixel) and a dose rate of ~20 electrons per second per Å² with a total exposure time of 3 sec. A total of 149 images of RNA cubes were collected with a defocus range of 2–4 µm.

Single particle image processing and 3D reconstruction of the RNA cubes
The image processing software package EMAN2 was used for the micrograph evaluation, particle picking, CTF correction, 2D reference-free class averaging, initial model building and 3D refinement of the cryo-EM data. A total of 5106 particles were boxed to generate the 2D class averages for building the initial model. Finally, 4371 particles were used for final refinement, applying the octahedron symmetry. The resolution for the final map was estimated by the 0.143 criterion of the FSC curve without any mask. A 16 Å Gauss low-pass filter was applied to the final 3D map displayed in the Chimera software package.

Cycling between open and closed states
For the two-state nanocube, to cycle between the “open” and “closed” states of a given edge, the appropriate hairpin or anti-hairpin strand, respectively, was added to the solution and incubated for 10 min, at room temperature. A slight excess of each

FIGURE 10. The process of opening and closing the nanocube using kissing loops is reversible after the addition of DNA hairpin/anti-hairpin strands. This makes the nanocube a configurable platform.

FIGURE 11. Four DNA CpG strands were incorporated into 2’F Cube nanoparticles. Six strands of the RNA Cube (left) and four strands of the RNA Cube (right) are used as control groups. (A) A stepwise increase in the amount of DNA CpG strands onto the nanocube shows a nonlinear increase in TNF-α activity, correlated with inflammation. (B) The synergistic effect is observed in IL-6 activity when adding DNA CpG strands to the nanocube from one to four in an increscent fashion. Statistics were calculated by unpaired t-test presented as mean ± SD, (***P < 0.001, (****) P < 0.0001.
subsequent addition of hairpin/anti-hairpin strand was added to compensate for any errors in stoichiometry. The four-state polyhedron was operated as above, except that incubation at 37°C for 30 min was used to increase the opening and closing rates for the second loop.

Statistical analysis
The results were presented as mean ± standard deviation. Statistical differences were evaluated using unpaired Student’s t-test, and P < 0.05 was considered significant.

Conclusion
In this study, we reported the successful bottom-up auto-assembly of molecular defined RNA nanocubes using the stable pRNA 3WJ as the building block. The constructed RNA cubes have defined 3D structures as revealed by cryo-EM. By incorporating hairpins with kissing loop interactions, we developed RNA nanocubes which can undergo complex structural rearrangement using an “open → close → open” strategy. The initial structures were self-assembled by thermal annealing and subsequently transformed through multiple strand displacement and hybridization steps. We showed that unfastening the hairpin interactions in the dynamic portions of the nanostructure led to the transformation process of opening the nanocube. In addition, we demonstrated that transformation between the structurally metamorphic RNA nano-objects was fully reversible and controllable. The success of our method proves that many simultaneous hairpin strand displacement events can be used to achieve an efficient structural reconfiguration. The reconfigurable nanocube and trapezoidal structures may also be used to control reversible switching of RNA nanoparticles for protein encapsulation and release. Additionally, this highly tamable nanoparticle could not only be used to encapsulate other large molecules that would be unstable otherwise but also be used to deliver a treatment that will only be activated using the hairpin strands that are present only in diseased states. Additionally, these nanocubes have the potential to be used in the field of immunotherapy by integrating immunostimulants onto the nanocube structures. Therefore, these nanoparticles have the potential for a variety of applications.

SUPPLEMENTAL MATERIAL
Supplemental material is available for this article.

COMPETING INTEREST STATEMENT
P.G. is the consultant of Oxford Nanopore Technologies, the co-founder of Shenzhen P&Z Bio-medical Co. Ltd., as well as co-founder of ExonanRNA, LLC and its subsidiary Weina Biomedical LLC in Foshan. The content is solely the responsibility of the authors and does not necessarily represent the official views of NIH.

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Author contributions: P.G. oversaw and led the entire project. P.J.B.C. participated in manuscript preparation and analysis of certain data from other coauthors. C.X. participated in manuscript preparation. S.G. performed the characterization of the nanoparticles used in this study. K.Z. and W.C. performed the cryo-EM experiments.

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