Fabrication of RNA 3D Nanoprisms for Loading and Protection of Small RNAs and Model Drugs

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In RNA nanotechnology, canonical Watson–Crick pairs combined with non-canonical base pair interactions create a large variety of RNA structural building blocks or motifs such as multi-way junctions, kissing loops, and sticky ends that have been utilized to create 2D RNA nanostructures. Combining diverse RNA connectivity into one nanostructure imparts complexity and allows for the addition of a third dimension in nanoparticle design. As a result, 3D RNA nanoarchitectures have been explored. 3D nanostructures are of particular interest as they hold tremendous potential for the encapsulation of small molecule drugs and therapeutic RNAs. Importantly, encapsulation may hold the distinct ability for controlled release of therapeutic modalities. Previously reported 3D DNA cages such as tetrahedrons, cubes, icosahedrons, and triangular prisms have been used as nanocontainers to hold proteins and gold nanoparticles and organic molecules. Although construction of 3D RNA nanocages has been previously reported, their potential to encapsulate small reporter molecules, drugs, and proteins is yet to be realized.

RNA aptamers are important candidates for therapeutic and diagnostic applications as they display high affinity and specificity for their target molecules. Binding of malachite green (MG), a triphenylmethane dye, to its respective RNA aptamer, the malachite green aptamer (MGA), causes a drastic increase in MG fluorescence. This fluorophore/RNA complex has been widely utilized as a marker in the construction of functional RNA nanoparticles. However, the application of MGA in vivo is limited due to RNA specific exo- and endonucleases inside cells. To prevent this, ribose ring modification at 2′-C/U in RNA is a common strategy to enhance RNA’s nuclease resistance. However, due to either structural hindrance or redistribution of the nearest neighbor landscape, 2′ ribose sugar or other chemical modifications can hamper the activities of aptamers selected with native RNA libraries. For example, 2′-fluorine (2′-F) modification to the MGA eliminates binding of the fluorophore, resulting in no fluorescence increase. Protection of RNA aptamers would be beneficial as their lifetimes could be significantly increased, thereby increasing their utility in vivo. One possibility to extend their lifetimes is encapsulation inside nuclease-resistant nanocages.

One advancement utilized for constructing RNA architectures was the finding of an unusually thermostable three-way junction (3WJ) derived from the pRNA of bacteriophage phi29 DNA packaging motor. The 3WJ can be used as a vehicle to integrate multiple functional RNA modules into one nanoparticle while retaining folding and function in vivo. Utilizing the crystal structure of the 3WJ, various RNA nanoparticles including triangles, squares, and pentagons were constructed. Upon systemic injection in mice, the RNA nanoparticles bind to xenograft and metastatic cells specifically and strongly with little or no accumulation in the liver or other healthy organs. Herein, we describe a strategy to fabricate 3D RNA nanocages resembling triangular prisms based on pRNA-3WJ. Importantly, this 3D nanoparticle was used to demonstrate encapsulation of a small fluorescent dye, MG, while increasing its fluorescence half-life in an RNA unfriendly environment. The modularity of the triangular nanoprism design also allowed single strand oligonucleotide–drug conjugates to be positioned precisely inside the inner cavity of the RNA complex. The functional properties of the RNA nanocage to simultaneously carry five folate ligands for cell targeting as well as six DNA CpG motifs for cell immune response were further evaluated in vitro. This work paves the way toward the application of RNA-based nanocaging as a delivery system for small reporter or therapeutic molecules.

The triangular nanoprism was designed employing the structural information of pRNA-3WJ following a bottom-up approach. As such, two helices of three adjacent 3WJs were connected with duplex RNA to form flat triangular nanoparticles, a nanoparticle construction method devised previously. To extend 2D triangle particles into a 3D RNA
complex, two triangular nanoparticles (T1 and T2) containing 21 nucleotide (nt) single-stranded (ss) complementary linker (L) regions on each vertex were tethered (Figure 1A, and Figure S1, Supporting Information). Each ssL region was designed to interact specifically with its compliment strands L1-L1', L2-L2', and L3-L3' forming RNA–RNA duplex of 21 bp. To provide structural flexibility and allow the linkers to bend to a 90° angle necessary for triangular prism complex, poly uracils (poly-Us) were embedded between the ssL and triangle vertices. This resulted in the formation of a “face-to-face” dimer with a total of eight RNA strands. The overall geometry was manually computed using SwissPDB Viewer software, the structure resembling triangular prism geometry with the pRNA-3WJ motif at the corners. The 3D RNA model displays a size of \( \approx 11 \) nm, measured from one vertex to an adjacent vertex, and a size of \( \approx 10 \) nm measured from one vertex to its nearest edge, as measured by the modeling software (Figure 1B). The largest diameter of the inner cavity of the prism was measured to be \( \approx 7 \) nm while the height was \( \approx 6 \) nm. Based on these dimensions, we assume that the central cavity of the prism could accommodate a spherical object of \( \approx 6 \) nm in diameter.

Assembly efficiency of the RNA strands into the desired conformation was first assayed by 6% native polyacrylamide gel electrophoresis (PAGE). Upon addition of each of eight component strands, a step-wise decrease in electrophoretic migration can be clearly seen, indicating association of each complimentary ssRNA oligomer into the complex RNA structure (Figure 2A). The distinct band in lane 7 indicates the formation of a stable RNA complex containing all eight RNA strands. Integration of the intensity of gel bands results in an estimated assembly yield of 43.0 ± 3.1%. A large fraction of RNA aggregates are localized in the top of lane 7. Presumably, this is due to non-specific interactions between the 21 nt RNA single-stranded sticky ends. Interestingly, the RNA complex with no D and d' stands has a much higher assembly efficiency with estimated yield of 90% (Figure 2A lane 6).

Dynamic light scattering (DLS) was utilized to measure the apparent dimensions of the purified RNA prism. As measured by DLS, the hydrodynamic diameter of the RNA complex is 11.5 ± 1.6 nm, consistent with the 3D model structure of the nanoprism (Figure 2B). Atomic force microscopy (AFM) imaging of the pure RNA complex revealed that the particles do in fact display prismatic-like architecture (Figure 2C). AFM is 2D observation of the 3D RNA prism and cannot be used to conclusively indicate the formation of the triangular prism geometry, nevertheless, the particles do resemble the designed triangular prism in 2D projections.

To address the concern whether the folding of eight RNA stands resulted in 3D geometry, the purified RNA complex was visualized by single-particle cryo-electron microscopy (cryo-EM) (Figure 2D,E). Cryo-electron microscopy is a useful method to show the true 3D nature of the RNA nanoparticles, as AFM analysis causes distortion of 3D nanoparticles imaged in 2D.[35] The 3D reconstruction of the RNA nanoparticles was achieved implementing single-particle reconstruction using the EMAN2 approach as described in the Experimental Section. Reconstruction of the 3D structure of the RNA complex was determined at a resolution of 2.5 nm (10 nm RNA prism) and 2.2 nm (5 nm RNA prism), which demonstrated that the RNA particles have average sizes of 10 and 5 nm, respectively, in agreement with DLS and AFM results. More importantly, the overall geometry is similar to the computer-modeled 3D RNA prism. It is important to note that the reconstruction of the RNA prism by cryo-EM clearly demonstrates the presence of an inner cavity. Collectively, the data from native PAGE, DLS, AFM, and cryo-EM clearly indicate the formation of closed and compact RNA nanoparticles.

Figure 1. Triangular nanoprisms design and 3D model structure. A) Fabrication of the triangle nanoprisms from two planar equilateral RNA triangles. The triangle prisms nanostructure forms by hybridization of the flexible 21 nt ssRNA “linkers” on each triangle. The overall prismatic construct contains the preserved geometry of the pRNA 3WJ structural building block. B) Computer model structure of the triangular nanoprisms demonstrating its average dimension in 3D space.
The MGA is a commonly used tool in biochemistry for structure–function verification. This is attributed to its unique properties as free MG dye in solution displays little to no fluorescent signal, yet when bound to MGA the photoemission increases more than 2000-fold. However, fluorescence can only occur if the RNA MGA folds into its authentic 3D conformation. Previous attempts to achieve fluorescence using 2′-F modified RNA bases resulted in little to no fluorescence. Therefore, for RNA MGA to remain a useful tool within a cellular environment, it must be placed into a container that will protect the RNA from degradation. This is a common problem among many small RNAs and small molecule drugs with therapeutic utility. Using 2′-F modified RNA to construct the prism’s frame (2′-fluoro uridine (2′-F-U) and 2′-fluoro cytosine (2′-F-C)), which are known to display resistance to a degrading environment, we hypothesize that the RNA MGA will be protected when positioned inside the 2′-F modified nanocage. As a proof-of-principle, we employed the MGA RNA sequence by embedding it within the “D” and “d′” strands at their 3′ ends. The schematic encapsulation design is summarized in Figure 3A. Throughout this paper, we refer to the stable RNA complex formed by six 2′-F modified RNA strands without D or d′ strands as a “frame.” It was interesting to find that this complex is highly stable with apparent melting temperature (T_M) of 74 °C, with the assembly efficiency being Mg²⁺ dependent (Figure S2, Supporting Information).

Using SwissPDB Viewer, we first modeled the complex structure of the RNA nanocage with encapsulated MGA by positioning the 3D structure of the MGA inside the inner cavity formed by the nanoprism. This allowed us to estimate the distance constraint in base pairs required to mount the RNA MGA (extracted from PDB ID: 1F1T) into the inner cavity of the prism. The complexation of the RNA MGA within the nanocage was evaluated on native PAGE (Figure S3, Supporting Information). The apparent gel shift of the band localized in lane 4 (2′-F-RNA frame with MGA) compared to lane 1 (2′-F-RNA frame only) shows complexation of the MGA sequences with the frame structure detected by total RNA stain with...
ethidium bromide (EB). Staining of the same gel with MG dye resulted in strong fluorescence emission from the distinct band on lane 4, indicating correct folding of the RNA MGA (Figure S3, Supporting Information). We performed additional control experiments based on migration properties of the closed (compact) and open (relaxed) 3D RNA complexes as it is generally accepted that more compact RNA structures migrate faster in comparison to relaxed RNA conformations. To fabricate the open 3D prism conformation, the interaction of one complementary sticky end linking two RNA triangles together was intentionally disrupted through sequence design (Table S1, Supporting Information). Comparing migration of the open RNA prism to the closed version revealed slight differences in migration distance (Figure S3, Supporting Information: lanes 4 and 5; Figure S4, Supporting Information). This indicates that the prism is closed and that the MGA was located, presumably, within the inner cavity of the nanocage. Data obtained from the mobility shift assay alone do not provide a concrete conclusion whether the RNA MGA formed inside of the closed cage; additional data supporting our assumption come from RNase T1 cleavage experiments as described below.

Encapsulation of an RNA module inside the negatively charged RNA nanoprisms is challenging due to the repulsion forces caused by the negatively charged phosphodiester backbone of both the inner cavity of the RNA cage and the RNA MGA. Development of a method to overcome this challenge would be exceptionally beneficial as the activity of functional RNA modules could be retained by protecting them from enzymatic degradation in vivo. To further confirm that the RNA MGA was localized inside the cavity of the nanocage, we treated the RNA complex with endonuclease RNase T1, which specifically cleaves unpaired guanosine (Gs) nucleotides. While ssGs are absent from the 2′-F-RNA prismoidal construct, there are several ssGs present in the RNA MGA itself that are essential for MG binding. Thus, we hypothesize that RNase T1 will cleave the exposed ssGs of the MGA sequence resulting in
loss of fluorescence. However, if the cage were to restrict RNase T1 access to the MG aptamer within the nanoprism, fluorescence emission would be retained or decay at a slower rate.

According to the modeled 3D structure of the prism, the estimated average dimensions of the inner cavity of the prism could accommodate a spherical object of about 6 nm. The average size of the RNase T1 structure was measured (PDB ID: 1YGW) to be 3.7 nm in diameter\textsuperscript{43} (Figure S5, Supporting Information) allowing RNase T1 to easily access the inner cavity and quench the fluorescence of the MGA. To address this obstacle, a nanoprism having a smaller cavity size of 3 nm was designed by decreasing the length of the RNA prism helical regions by one half (Table S1, Supporting Information). The schematic design of the experiment is summarized in Figure 3B. The smaller prism is expected to insulate the MGA from RNase T1 cleavage, thus extending fluorescence activity.

The assembly of the small prism is highly efficient, and RNA MGA is functional according to native PAGE analysis (Figure 3C, lanes 1 and 2). Next, we carried out time-dependent MG fluorescence decay in the presence of RNase T1 to test the activity of the MGA embedded inside the large and small nanoprism. Figure 3D shows the fluorescence decay profiles of the large (black squares) and small (red circles) nanoprisms. The calculated lifetimes ($\tau$) were found to be 445.5 and 781.4 seconds (s) for the large and small nanoprisms, respectively. The small prism displayed a half-life almost double that of the large prism, suggesting that RNase T1 has limited access to MGA encapsulated inside of the small nanoprism. Complete protection against RNase T1 has not been observed presumably due to the dynamic nature of the interacting linker arms of two triangles. This is also supported by AFM images where significant fractions of prisms are in an open state, which allows RNase T1 to digest the MGA. Notably, fluorescence emission of the small prism was still observed after 1 h of incubation with RNase T1 while fluorescence in the large prism was negligible (Figure 3E). Additional control experiments were performed using a variety of other RNA control complexes containing RNA MGA. The calculated values of $\tau$ are summarized in Table 1. Mean lifetime comparison of the small RNA prism to the controls demonstrates that no control construct has greater $\tau$ than the small prism. Thus, the RNase T1 cleavage assay indicates the successful encapsulation of MGA encapsulated in both the small and large nanocages. More importantly, we have shown that by tuning the size of the inner cavity, fluorescence half-life can be significantly increased. This is synonymous with increasing the release time of a drug that can be potentially used in vivo system.

Taking advantage of the programmability of the D and d‘ prism strands, we next demonstrated the encapsulation of single-stranded oligodeoxynucleotides (ssODN) containing functional moieties such as fluorescence reporters and drugs. To this end, one long ssRNA was designed to connect D and d’ strands through a 22 nt linker. The resulting RNA sequence will be referred as a “core” RNA strand. While the 5’- and 3’-ends of the core RNA strand interact with the triangular edges of the prism, its single-stranded middle region is designed to localize inside the nanocage. The portion of the core strand inside the prism was utilized for encapsulation of either ssDNA or ssRNA carrying different functional groups via base pair hybridizations.

\begin{table}
\centering
\begin{tabular}{|c|c|}
\hline
Nanoparticles type and structure & Lifetime ($\tau$) [s] \\
\hline
Control 1 & 393.2 ± 39.3 \\
Control 2 & 352.5 ± 47.8 \\
Small prism & 781.4 ± 43.0 \\
Triangle prism & 445.5 ± 50.0 \\
\hline
\end{tabular}
\caption{Fluorescence lifetime of different RNA construct.}
\end{table}
shown previously. This is advantageous as the capacity of the nanocontainer can be controlled, enabling encapsulation of diverse size biomolecules or inorganic nanoparticles as well as increasing the payload of a single nanocontainer for increased detection sensitivity and increased efficacy in the treatment of disease. Moreover, from a medical point of view, size alternation of the nanoprism imparts differences in toxicity, cellular binding and internalization, renal clearance, pharmacokinetics and pharmacodynamics profiles. As such, the prismatic nanocontainer provides a unique system to further exploit these factors.

The principle behind chemical drug encapsulation relies on the conjugation of a drug at the 3′/5′-end or at both ends of DNA/RNA oligonucleotides, followed by hybridization of this conjugate inside the cavity of the RNA nanoprism. To demonstrate this in practice, short DNA sequences (22 nt) were labeled at the 5′-end with different chemicals including folic acid (FA), cyclodextrin, taxol, Cy5 fluorophore, and biotin. To verify the formation of the RNA nanoparticle with the conjugated DNA sequence, prism strands and corresponding DNA oligonucleotides were assembled in one-pot by mixing at equimolar concentrations, followed by native PAGE analysis (Figure S6B, Supporting Information). Formation of distinct bands corresponding to the frame structure and frame containing the core RNA sequence are shown in Figure S6 (Supporting Information), lanes 1 and 2, respectively. The additional lanes correspond to loaded nanocage with the 22 nt DNA containing a variety of small molecules. Based on the slower rate and almost identical distance of all five loaded cargoes as compared to the bands in lanes 1 and 2, we can conclude that the conjugated ssDNA were successfully hybridized inside the nanocage structure. Additionally, co-migration of Cy5 and EB signal of Cy5 loaded nanocage indicates correct assembly.

To demonstrate incorporation and function of the DNA–biotin conjugate within the RNA 3D cage, biotin–streptavidin binding was utilized (Figure S6C, Supporting Information). To separately visualize the biotin-nanoprism and the streptavidin, the DNA–biotin conjugate was tagged with Cy5 and the streptavidin with Cy3. The ensuing gel shift assay demonstrated complexation between the encapsulated biotin and streptavidin as the complex migrates much slower than the biotin-nanoprism or streptavidin alone (Figure S6C, Supporting Information, lane 1 vs 2 and 5). Colocalization of EB (RNA), Cy3 (streptavidin), and Cy5 (DNA–biotin) signals in the distinct band in lane 1 reveals the functional properties of the encapsulated molecule (Figure S6C, Supporting Information). Although PAGE results cannot directly confirm whether the functionalities were located inside the nanocage, results based on computer modeling and fluorescence functional assays are a strong indication of the encapsulation mechanism. Utilizing the programmability of RNA D and d′ strands as well as modularity of the ssDNA–drug conjugates we were able to demonstrate feasibility of the encapsulation approach, in addition to RNA MGA aptamer encapsulation, for caging of small molecules and proteins.

One potential advantage of RNA 3D nanocontainers is the ability to safely deliver and release encapsulated drugs in a fine-tunable fashion. Development of controlled release is extremely challenging yet highly desirable in RNA nanotechnology. To release the encapsulated drug(s), one approach is to use the help of naturally occurring nucleases to break down RNA 3D nanoscaffold. It was our hypothesis that changing the percentage of 2′-F nucleotides in the nanoprism scaffold would result in diverse degradation profiles. To change the amount of 2′-F nucleotides in the nanoprism, the ratio of 2′-F cytidine triphosphate (CTP) and uridine triphosphate (UTP) nucleotides to unmodified cytidine triphosphate uridine triphosphate were adjusted during in vitro transcription reactions to 4:0, 3:1, 2:2, 1:3, and 0:4, respectively. After transcription and purification, five different nanoprism were assembled with 2′-F-C/U modifications of =100%, 75%, 50%, 25%, and 0%. The degradation profiles of each nanoprism were observed over the course of 1 h in 2% fetal bovine serum (FBS) solution at a final concentration of 200 × 10⁻⁹ m and analyzed on 3% agarose gel (Figure 4A).

Figure 4. Tuning degradation profile of nanoprism in fetal bovine serum. A) Gel images of nanoprism with different percentages of 2′-F modified pyrimidines used during assembly. Nanoprism of 200 × 10⁻⁹ m concentration were incubated in 2% FBS solution and analyzed by 3% agarose gel. B) Prism relative gel band intensity plotted versus time. The intensity of each band was compared to the zero time point for each nanoprism.
intensity at each time point was compared to initial gel band intensity for each individual nanoprism (Figure 4B). As shown by the time course plot, decreasing the percentage of 2′-F nucleotides increases the digestion rate of the nanoprism scaffold. This approach shows feasibility of controlled drug release by degradation of the partially 2′-F modified nanocontainer, which can be fine-tuned to the scale of minutes.

The ability of the RNA nanocage to successfully bind and deliver drugs or reporter molecules into cancer cells would tremendously benefit the advancement of nanomedicine. To investigate whether the RNA nanocage can serve as a container to deliver small molecules, the 2′-F RNA frame structure was decorated with five copies of DNA oligonucleotide–folate conjugates and one copy of DNA–Cy5, as shown in Figure 5A (assembly shown in Figure S7, Supporting Information). Folate-containing nanocontainers are known to have a strong affinity to cancer cells of epithelial origin as these types of cancer cells overexpress folate-binding protein receptors.[46] After incubation of the folate-functionalized RNA nanoprism with KB cells, strong binding of the RNA complex with the cells was observed (Figure 5A) in comparison to control complex having the same RNA 3D nanostructure but lacking folate molecules. This result demonstrates the applicability of the 3D RNA system for detection and delivery of the reporter molecules.

CpG ODN is a short synthetic DNA sequence containing unmethylated C and G nucleotides that has recently gained attention in immunology as it has shown to be useful as a vaccine adjuvant or immunotherapy reagent.[37,47,48] It was of particular importance to further investigate whether the unique 3D conformation of the RNA triangular nanoprism can be used to enhance the immunomodulation effect in vitro. Six CpG oligonucleotides were incorporated onto the 2′-F-RNA frame construct (Figure S8, Supporting Information). The immunostimulatory efficacy of the nanoprism was evaluated by measuring the release of cytokines TNF-α after addition to mouse macrophage-like RAW264.7 cells (Figure 5B), as previously described.[49,50] The results demonstrate that increasing the number of CpG induced a stronger immune response, observed by measuring the level of the induction of TNF-α. The results suggest that the cytokine released by the CpG prism complex significantly increases the immunostimulatory activity compared to CpG alone (Figure 5B). Interestingly, the same observation was obtained by previous studies using RNA polygonal nanoconstructs,[17] as with more CpG per nanoparticle a higher level of host immune response can be triggered. These data combined with cell binding studies demonstrate that the 3D RNA nanoprism can be readily utilized in cell studies to effectively carry functional moieties. This paves the way to further in vivo applications.

The proof-of-concept for a technique to construct 3D RNA nanocontainers to encapsulate small RNA molecules and a method for loading the nanocontainers with model drugs was demonstrated. The assembly of RNA triangular nanoprism utilizes pRNA-3WJ motifs positioned at the prism vertices, imparting stability into the system and allowing for future in
vivo delivery. Loading the container with model drugs (MG) was accomplished via diffusion of the model drug into the container with subsequent immobilization by RNA receptors (MGA) pre-encapsulated within the nanoprisms. The concept of RNA aptamer encapsulation is a promising platform for exploring new applications in pharmaceutical and materials science. The RNA-directed triangular prism brings together the functionality, biocompatibility, and self-assembly potential with fine control of geometry, positioning, and the responsive characteristic of RNA nanoparticles.

**Experimental Section**

**RNA Sequence Design and 3D Modeling:** RNA sequences were optimized using RNA 2D folding software Mfold (www.mfold.bioinfo.rpi.edu) prior to synthesis. Computer models of the RNA prism were assembled from two previously constructed RNA triangles.[21] The vertices of the triangles were assembled by overlapping helical domains of the triangular “arms” to position them at the correct angle and distance using the “autofit” tool in Swiss-POB Viewer (www.swis婆婆.nrl.ch) and manual adjustment. The detailed procedure for the triangle design can be found in previous reports.[22] Each triangle vertex contains a 21 nt ssRNA tethered to the 3’ end using a poly-uracil (poly-U) linker. The poly-U was inserted to provide a hinge allowing for the characteristic of RNA nanoparticles.

**RNA Synthesis, Purification, and Assembly:** Templates for RNA transcription were prepared by standard protocols described in detail elsewhere.[23] Briefly, PCR was used to prepare transcription templates using PCR reaction kit (Promega Corporation, GoTaq FlexiDNA Polymerase) for the amplification of DNA primers (IDT). Following purification of DNA templates on spin columns (QIAGEN), the RNA transcription were prepared by standard protocols described in detail elsewhere.[24] Detailed 2D structures and sequence of the nanoprisms as well as synthetic oligonucleotides containing conjugates are provided in, Table S1 (Supporting Information).

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**AFM Images:** For all samples, specially modified mica surfaces (APS mica) were used. The APS mica was obtained by incubation of freshly cleaved mica in 167 × 10⁻⁶ M 1-(3-aminopropyl)silatrane following previously reported protocol.[25] The RNA samples were diluted with 1× TMS buffer to a final concentration of 3–5 × 10⁻⁸ M. Then, 5–10 μL was immediately deposited on APS mica. After 2 min incubation on the surface, excess samples were washed with DEPC treated water and dried under a flow of argon gas. AFM images in air were acquired using MultiMode AFM NanoScope IV system (Veeco/Digital Instruments, Santa Barbara, CA) operating in tapping mode.

**Dynamic Light Scattering:** The hydrodynamic diameter of the RNA nanoparticles was determined at a concentration of 1 × 10⁻⁶ M in 50 μL TMS buffer using a Zetasizer nano-ZS (Malvern Instrument, Ltd.) at 25 °C.

**Prism MG Degradation Assay:** The function of the MGA within the triangular prism nanoparticles was assayed by mixing the RNA complex at a concentration of 0.1 × 10⁻⁶ M with 1 × 10⁻⁶ M MG dye in 1× TMS buffer. The solutions were allowed to equilibrate at room temperature for 10 min. The fluorescence emission spectrum (recorded from 630–750 nm) of the complex was measured using a fluorospectrophotometer (Horiba Jobin Yvon) with an excitation wavelength positioned at 615 nm. 1 μL of 500 U mL⁻¹ RNase T1 (Thermo Scientific) was added to 99 μL of the above RNA-MG complex directly in a fluorometer cuvette. Fluorescence at λₑᵥₑ = 650 nm was immediately recorded and monitored every 1 s for a total time of 3600 s. The time-resolved fluorescence lifetime measurements were performed by the software integrated within the fluorometer. The obtained data were fit using exponential decay function \( f(t) = l_0 \cdot e^{(-t/\tau)} \), where \( l_0 \) is the initial intensity (at time zero) and \( \tau \) is the mean lifetime defined as the time for the intensity to drop by 1/e or to ~37%. All fluorescence decay experiments were conducted in triplicate with indication of ± for mean standard error (SEM).

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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