Physicochemically Tunable Poly-Functionalized RNA Square Architecture with Fluorogenic and Ribozymatic Properties

Daniel Jasinski, Emil F Khisamutdinov, Yuri L Lyubchenko, and Peixuan Guo

*ACS Nano, Just Accepted Manuscript* • DOI: 10.1021/nn502160s • Publication Date (Web): 27 Jun 2014

Downloaded from [http://pubs.acs.org](http://pubs.acs.org) on July 15, 2014

**Just Accepted**

“Just Accepted” manuscripts have been peer-reviewed and accepted for publication. They are posted online prior to technical editing, formatting for publication and author proofing. The American Chemical Society provides “Just Accepted” as a free service to the research community to expedite the dissemination of scientific material as soon as possible after acceptance. “Just Accepted” manuscripts appear in full in PDF format accompanied by an HTML abstract. “Just Accepted” manuscripts have been fully peer reviewed, but should not be considered the official version of record. They are accessible to all readers and citable by the Digital Object Identifier (DOI®). “Just Accepted” is an optional service offered to authors. Therefore, the “Just Accepted” Web site may not include all articles that will be published in the journal. After a manuscript is technically edited and formatted, it will be removed from the “Just Accepted” Web site and published as an ASAP article. Note that technical editing may introduce minor changes to the manuscript text and/or graphics which could affect content, and all legal disclaimers and ethical guidelines that apply to the journal pertain. ACS cannot be held responsible for errors or consequences arising from the use of information contained in these “Just Accepted” manuscripts.
Physicochemically Tunable Poly-Functionalized RNA Square Architecture with Fluorogenic and Ribozymatic Properties

Daniel Jasinski,¹ Emil F. Khisamutdinov,¹ Yuri L. Lyubchenko,² and Peixuan Guo¹*

¹Department of Pharmaceutical Sciences, College of Pharmacy, Nanobiotechnology Center, and Markey Cancer Center, University of Kentucky, Lexington, KY 40536
²Department of Pharmaceutical Sciences, University of Nebraska Medical Center, Omaha, NE 68198

Keywords: pRNA-3WJ motif, Nanotechnology, Nanobiotechnology, RNA nanotechnology, Biomimetics

*Address correspondence to:
Peixuan Guo
Department of Pharmaceutical Sciences
College of Pharmacy
University of Kentucky
BioPharm Complex, Rm. 565
789 S. Limestone
Lexington, KY 40536, USA
Phone: (859) 218-0128
Email: peixuan.guo@uky.edu
ABSTRACT

Recent advances in RNA nanotechnology allow the rational design of various of nanoarchitectures. Previous methods utilized conserved angles from natural RNA motifs to form geometries with specific sizes. However, the feasibility of producing RNA architecture with variable sizes using native motifs featuring fixed sizes and angles is limited. It would be advantageous to display RNA nanoparticles of diverse shape and size derived from a given primary sequence. Here, we report an approach to construct RNA nanoparticles with tunable size and stability. Multifunctional RNA squares with a $90^\circ$ angle were constructed by tuning the $60^\circ$ angle of the three way junction (3WJ) motif from the packaging RNA (pRNA) of the phi29 bacteriophage DNA packaging motor. The physicochemical properties and size of the RNA square were also easily tuned by modulating the "core" strand and adjusting the length of the sides of the square via predictable design. Squares of 5, 10, and 20 nanometers were constructed, each showing diverse thermodynamic and chemical stabilities. Four "arms" extending from the corners of the square were used to incorporate siRNA, ribozyme, and fluorogenic RNA motifs. Unique intra-molecular contact using the pre-existing intricacy of the 3WJ avoids relatively weaker intermolecular interactions via kissing-loops or sticky ends. Utilizing the 3WJ motif, we have employed a modular design technique to construct variable size RNA squares with controllable properties and functionalities for diverse and versatile applications with engineering, pharmaceutical, and medical potential. This technique for simple design to finely tune physicochemical properties adds a new angle to RNA nanotechnology.
DNA, RNA, and proteins from living systems produce a variety of highly ordered nano-scale structures to perform diverse functions.\textsuperscript{1-5} The intricacies of these natural biomaterials have inspired their applications as building blocks to fabricate sophisticated nanodevices.\textsuperscript{6-17} In nanotechnology, RNA can be manipulated to assemble nanostructures with simple approaches as in DNA nanotechnology, while displaying versatility in structure and function similar to proteins. Thus, RNA has the advantage of two worlds, which garners tremendous interest in the directed design of a variety of RNA nanoarchitectures.\textsuperscript{18} Natural RNA molecules form a wide variety of complex structures by hierarchical folding that generates different motifs or modular units.\textsuperscript{19} These modular units can be “manually” extracted and used to fabricate artificial self-assembling RNA complexes of diverse 1D, 2D, and 3D nanoarchitectures with novel and unique functional features.\textsuperscript{2,16,20,21} Many RNA motifs exhibit phenomenal flexibility\textsuperscript{22-24} and the angles needed to form many nanoassemblies are encoded in the primary sequence of the structural motifs. The structure of a complex RNA architecture can be engineered by programming the information of a 3D structural motif into the nanoparticle’s primary sequences.\textsuperscript{17,18} However, the flexibility of RNA motifs has seldom been used to construct diverse shaped nanoparticles utilizing the same core structure.

Previous work in this lab has detailed the construction of RNA nanoparticles utilizing the pRNA from bacteriophage phi29 DNA packaging motor. Dimers, trimers, tetramers, and hexamers have been constructed utilizing hand-in-hand and foot-to-foot interactions as well as arm extension technologies which implement kissing-loop, sticky-end, and complementary interactions.\textsuperscript{1,2,20,21,23-30} Additionally, the 3WJ and X-way motifs from the phi29 motor pRNA, themselves, have proven successful as therapeutic delivery systems.\textsuperscript{2,20,21,26,31} Other RNA nanoparticle-based delivery systems have shown potential as therapeutic carriers.\textsuperscript{15,32-39}

Size and shape play an important role in the delivery efficacy, biodistribution and circulation time of nanoparticles.\textsuperscript{40} Control over these properties can be of paramount importance for in vivo applications. However, in many cases fine tuning of size, shape, and physicochemical properties of
nanoparticles is difficult to achieve. Having a library of diverse size nanoparticles with tunable properties is advantageous as pharmacokinetic mechanisms of drug delivery, distribution, and clearance are hard to predict. These facts are the motivation behind the development of a simple method to tune the size and thermodynamic and chemical properties of RNA nanoparticles.

The complexity of RNA’s tertiary structure complicates de novo design of such nanostructures; however, by utilizing a pre-defined 3D motif as the design template many complications can be avoided. This approach utilizes a modular design technique, sometimes referred to as RNA biomimetics. It involves the use of pre-defined structural motifs taken from biology as building blocks to construct RNA nanostructures of defined shape and size for a variety of applications including nanomedicine. The use of pre-defined RNA motifs has shown to be a successful method towards the rational design of RNA nanoarchitectures. Naturally, the first step in applying the pRNA 3WJ in modular design was to construct triangles based on the native 60° angle of the pRNA 3WJ motif(Figure 1A), ideal for the construction of an equilateral triangle. Utilizing this native angle triangular nanoparticles were constructed using the modular design technique. To create more diversity in size and shape, square shaped geometry was the next goal. By stretching the 3WJ, triangle, square, and pentagon can be formed. The next question to be addressed is whether we can tune the size of one polygon by using the same 3WJ while altering the length of each side of the polygon.

In this report, we show the construction of multiple variants of square-shaped RNA architectures based on a modular design technique using the same core structure, the pRNA 3WJ. By simply increasing or decreasing the length of the RNA duplex between each corner of the square, nanoparticles of 5, 10, and 20 nanometers (nm) were constructed with diverse thermal stabilities. The thermodynamic stability of certain RNA tertiary motifs and the strong Watson-Crick base pairing in RNA folding and certain predictable non-canonical base pairing and base stacking ensure predictability in structure that make it possible to increase or decrease size by simply adding or removing nucleotides, thereby regulating the length of RNA duplex in the RNA nanoparticle directly effecting size. Further control over nanoparticle stability was demonstrated by modulating the oligonucleotide used as the
“core” strand during construction of the square nanoparticle. Exploiting the different stabilities of RNA, DNA, and 2’-Fluorine modified RNA it is possible to add another dimension of regulation over the physicochemical properties of RNA nanoparticles. Moreover, due to the unique construction technique used, we show that it is possible to completely avoid intermolecular interactions such as kissing loop and sticky-end interactions during construction of the nanoparticles. The potential of using the resulting RNA square assembly for biomedical and imaging applications is evidenced by the incorporation of therapeutic RNA, ribozyme, and fluorogenic aptamers. As shown here, the assembly properties and tunability of RNA nanoparticles can be easily exploited to determine the best combination of size and stability for a wide array of applications.

RESULTS AND DISCUSSION

RNA Square Design

Square-shaped RNA nanoarchitectures were designed in silico utilizing planar geometry of the 3WJ structural motif. Swiss PDB viewer (www.spdbv.vital-it.ch) was used to align four 3WJ building blocks (PDB ID: 4KZ2)23 in a planar configuration utilizing the internal ∠AOB ∼60° angle at the four corners of the square (Figure 1A). A-type duplex RNA was then inserted to connect helix one (H1) with helix two (H2) (Figure 1A) of an addressable 3WJ element. The resulting design featured a square with four "arms" extruding outwards from each of the four vertexes of the square, facilitating the incorporation of additional RNA sequences, motifs, or functionalities attached to these arms. The final square contains five RNA strands, whereby four short strands (A, B, C, D) are complimentary to one long “core” strand E (Figure 1B, Supplemental Figure 1). This results in a nanoparticle containing a 3WJ at each vertex and A-type duplex RNA along each edge. Extension of both the core and external strands by increasing the number of complementary nucleotides was exploited to obtain RNA squares with variable but predictable sizes (Figure 2A). By such means, small, medium, and large square
architecture were designed using Swiss PDB Viewer and the sequences for all five strands were optimized to avoid non-specific interactions using the mfold program.47

**Structural Characterization**

Based on models, the size of the resulting square structures should be 5.1 nm, 10.2 nm and 20.1 nm along each edge. The size increase results from extension of the duplex RNA connecting the 3WJs at each corner. Based on nearest-neighbor thermodynamic parameters, larger size squares are expected to exhibit increased thermodynamic stability. As none of the 3WJ angles have a right angle according to its crystal structure (Figure 1A) the 3WJ motif must be stretched from ~60° to ~90° to accommodate square geometry. As a result, efficiency of self-assembly might be decreased. Consequently, our first experiments were aimed at characterizing the physical properties of our square construct designs.

Native PAGE was first used to test the assembly and analyze assembly efficiency. Nanoparticle assembly was performed via “one-pot” self-assembly procedure by thermal denaturation of all RNA strands in TMS buffer (400 mM Tris, 10 mM Mg, 100 mM NaCl) and gradual cooling over one hour. Using ImageJ software to analyze the intensity of gel bands. The yield of correctly folded 10 and 20 nm squares was estimated to be more than 90% and a slight decrease in folding efficiency observed for the 5 nm square (Figure 1C). Additionally, the hypothesis that each strand was involved in the assembly and that strand substitution was not occurring was tested. For this assay, each strand A through E was fluorescently labeled. Five different squares, each with one of five strands labeled, were then assembled and analyzed by native PAGE. The results demonstrate that each strand contributes to the assembly and therefore the overall stability of the square (Supplemental Figure 3).

While native PAGE experiments suggest the formation of compact, homogeneous, and distinct RNA nanoparticles, atomic force microscopy (AFM) was performed to demonstrate the formation of the square shaped RNA nanoarchitecture (Figure 2B). Resulting AFM images strongly support the formation of the medium and large square shaped RNA architecture. Image resolution of RNA nanostructures is in many cases limited by convolution due to tip size. Due to the resolution limits of the
AFM images shown in this report, the small square only appears as dots with no square shape obviously visible. The results are expected as the size of the small square model is 5.2 nm, a size too small to be clearly resolved. A small percentage of squares appear as diamond shape straying from square geometry. This can be attributed to the fact that AFM images were taken in air and not in solution, distorting the 3D shape when the nanoparticles are dried onto the mica surface. Alternatively, diamond shapes could be due to the slight flexibility of the 3WJ. Using the large square AFM images, particles were manually counted to determine efficiency of assembly by AFM. It was revealed that the large square formed with 70% efficiency.

When considering a nanoparticle for use as an in vivo therapeutic the size of the particle greatly affects the delivery efficacy. Too small a size and the particle will simply be excreted; too large a size and entry into the cell will be greatly hindered. Previous studies show that the optimal size for the cellular uptake of nanoparticles is 10-50 nm. Using dynamic light scattering (DLS), the average hydrodynamic diameters (D_h) of RNA square nanoparticles were discovered to be 4.0 ± 0.9 nm, 11.2 ± 1.3 nm, and 24.9 ± 0.5 nm for the small, medium, and large size squares, respectively (Figure 2C). These values are in close agreement with the predicted size of the model structures. At sizes of 4.0 nm, 11.2 nm, and 24.9 nm we have constructed variable size nanoparticles with potentially diverse physicochemical properties.

We have presented a simple method to adjust the size of RNA nanoparticles, however we aim to show that adjustment of size leads to diverse thermodynamic properties among square variants. To compare the thermodynamic stability of the squares, temperature gradient gel electrophoresis (TGGE) was used to determine the melting temperatures (T_M) of each square (T_M = 50% square formation). A temperature gradient perpendicular to electrical current was applied and increasing T_M corresponding to an increase in size was observed (Supplemental Figure 2). Due to the fact that each of the square constructs contains four 3WJ motifs, the increase in melting temperature can be attributed directly to an increase in the amount of base-pairing in the structures. This trend follows the nearest-neighbor predictions for increasing of thermodynamic stability.
Taken together, structural analysis by computer modeling, DLS, and AFM suggest the formation of compact, flat, square RNA nanoparticles. The native geometry, determined by crystallization, has H1 and H2 of the 3WJ in planar configuration. The computer model also results in planar geometry based on duplex alignment of connecting helices H1 and H2, strongly supporting the formation of planar square nanoparticles. Furthermore, the presence of square structures on AFM, and not triangular shapes, which would result from a 3D tetrahedron like structure, also supports flat, square geometry. As expected, AFM imaging demonstrates the size differences among the small, medium, and large squares.

Previously, RNA triangles have been constructed using the same pRNA-3WJ. After analysis of the square nanoparticle, it was determined that the square and triangle display diverse properties in thermodynamic stability, size, shape, and stoichiometry: functions possible per nanoparticle. Although not explored in depth in this study, it is possible that utilizing these particles in different applications, where size and shape play an important role, could be beneficial.

**Physicochemical Properties are Tunable**

Native RNA structures are inherently sensitive to degradation by cellular nucleases, potentially limiting the application of RNA nanoparticles in clinical applications. Modifications to RNAs, such as 2'-Fluorine modification (2'F) on the ribose sugar of the RNA backbone, have been shown to reduce their sensitivity to cellular nucleases. These same 2'F modifications have also been shown to increase the thermodynamic stability of RNA nanoparticles. DNA has also proven to be more resistant to the degradation caused by cellular nucleases. In order to be useful as an *in vivo* therapeutic, modifications must be made to the native square nanoparticles to gain resistance to RNase while retaining the same structure. Previous studies demonstrating different chemical and thermodynamic stabilities of the pRNA-3WJ constructed of RNA, DNA, and 2'F-RNA were the driving force behind the replacement of the core strand of the square nanoparticle. Due to these diverse stabilities, it was predicted that replacement of the core strand with different oligonucleotides would result in a nanoparticle with diverse physicochemical properties.
To impart tunable chemical and thermodynamic stabilities onto the 10 nm square nanoparticle, the internal core strand E was replaced with both 2′F-C/U modified RNA and with single stranded DNA. Analysis by native PAGE demonstrated that all of the nanosquares co-migrated indicating formation of the same geometry (Figure 3A). Moreover, all nanoparticles assembled with comparable yields of ~90% as determined by integration of the intensity of gel bands (Figure 3A).

A degradation assay in fetal bovine serum (FBS) was performed on each 10 nm hybrid square assembly.² It was observed that substitution of 2′F modified core strand for unmodified RNA greatly enhances the stability of the nanoparticles in FBS (Figure 3B). After 14 hours, ~92% of square nanoparticles with 2′F modified core strand remained, as compared to ~20% for square nanoparticles with RNA and DNA core strands (Figure 3B, Supplemental Figure 4). While different hybrid duplexes exhibit different stabilities or susceptibilities to different RNases,⁵⁴,⁵⁵ serum comparison mimics more closely an in vivo environment and allows the direct comparison of each hybrid square. Overall, resistance to nuclease degradation was enhanced by replacing the core strand with 2′F modified RNA.

To further examine the effect the core strand has on the stability of the 10 nm square nanoparticle TGGE analysis was performed to determine the Tₘ of each hybrid. A temperature gradient, perpendicular to electrical current, was applied from 40°C-80°C. The Tₘ’s for the 2′F-RNA, RNA, and DNA hybrid square nanoparticles were calculated to be 68.4 ± 0.1 °C, 57.7 ± 0.2 °C, and 51.8 ± 0.1 °C, respectively (Figure 3C, Supplemental Figure 5). Melting temperatures were determined at 0.2 mM MgCl₂ simulating physiological conditions in which the cytoplasmic magnesium ion concentration is generally lower than 1 mM. At 10 mM magnesium concentrations no melting below 80 °C was observed (Supplemental Figure 5D, E, and F). Tertiary structure greatly affects the melting temperature, and therefore thermodynamic stability, of an RNA structure. The duplex cannot be solely responsible for the trend in melting temperature from 2′F-RNA hybrid to DNA-RNA hybrid since a large portion of each structure is 3WJ. It is well known that complicated tertiary structures are more stable in RNA than DNA resulting in a less stable a DNA-RNA hybrid 3WJ. Interestingly, the melting temperatures correlate with the FBS resistance assay indicating that the 2′F-RNA hybrid square was
the most stable, followed by the native RNA square, and the DNA hybrid architecture was the least
stable.

In clinical applications, considering pharmacokinetics, biodistribution, and toxicity, the most
stable nanoparticles will not always be the most effective treatment.\textsuperscript{41-43} Our results show that it is
possible to tune the stability characteristics of the RNA square nanoparticles by substituting the core
strand and varying the ratio of 2’F nucleotides. This simple modification produces physicochemically
diverse structures that can be easily tuned based on application.

**Functionalizing the Square Scaffold**

In order for the square architecture to be useful as a potential therapeutic in nanomedical
applications, functional molecules (RNAi, aptamers, ribozymes) must be successfully fused into the
core structure while it retains native folding and properties. Thus, the question of whether or not the
RNA scaffold can harbor different RNA functional moieties such as siRNA, ribozymes, and RNA
fluorogenic aptamers was addressed. More importantly, if the functional molecules retain their activity
when incorporated into the square scaffold.

As such, four functional RNA groups, RNA spinach (SPIN) aptamer,\textsuperscript{56} RNA malachite green
(MG) aptamer,\textsuperscript{57,58} hepatitis B virus ribozyme,\textsuperscript{59} and siRNA targeting luciferase gene\textsuperscript{60} were
incorporated at each corner of the 10 nm square nanoparticle (Figure 4A, Supplemental Figure 6). The
multi-functional square showed more than 90% efficiency as analyzed by integration of gel (Figure 4B).
Additional AFM imaging was performed to demonstrate the retention of square geometry with the fused
functionalities (Figure 4C).

The MG and spinach aptamers are synthetic RNA aptamer sequences, previously obtained
though SELEX (Systematic Evolution of Ligands by Exponential Enrichment),\textsuperscript{61} that bind the
triphenylmethane and 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI) dyes
respectively.\textsuperscript{2,21,58,62,63} Free fluorophores in solution do not exhibit fluorescence and emit light only upon
binding to their correctly folded RNA aptamer structures.\textsuperscript{64,65} To assay if folding was retained when
incorporated into the multi-functional square, fluorometer measurements were taken for the
triphenylmethane and DFHBI chemicals alone and in the presence of the functional RNA square (Figure 5). The multi-functional square was simultaneously incubated with both fluorophores and fluorescence measurements were then taken. Both aptamers retained their folding and bind their respective fluorophore as evidenced by an increase in triphenylmethane and DFHBI emission upon binding. As a positive control, a 3WJ with the MG aptamer and the SPINACH aptamer were used, both of which have been previously shown to exhibit increased fluorescence signal when incubated with their respective fluorophores.\textsuperscript{2,63} Furthermore, we aimed to show the simultaneous dual-mode fluorescent properties of the functional square architecture. The PAGE gel was stained for both MG and spinach aptamer dyes. Individual fluorescence of each aptamer was detected when incorporated into the same nanoparticle (Supplemental Figure 7).

The hepatitis B virus (HBV) ribozyme is a hammerhead ribozyme that cleaves hepatitis B genomic RNA.\textsuperscript{66} Ribozymes show potential as therapeutic agents; however they traditionally have shown low efficiency when tested \textit{in vivo}. This can be attributed to the misfolding of ribozymes in an intracellular environment and their susceptibility to exonucleases.\textsuperscript{67} One method to protect the ribozymes and prevent their mis-folding is to harbor them in some larger, higher-order structure, such as tRNA. However, ribozymes must maintain their native folding, to retain functionality, when fused to RNA or incorporated into nanoparticles.\textsuperscript{68} Upon incubation of the multi-functional square with HBV genomic RNA, cleavage of the RNA genome occurs. Figure 6A demonstrates that the HBV ribozyme retains its catalytic activity when fused with the RNA square, as the cleaved products are clearly seen on the gel and the bands are comparable with a positive pRNA-HBV control construct.\textsuperscript{20}

To test the ability of the RNA square to carry siRNA for gene silencing effects, an \textit{in vitro} assay was performed with a dual-luciferase reporter system.\textsuperscript{62} An siRNA anti-sense strand, which targets the firefly luciferase gene, was hybridized with its complimentary sense strand used as a sticky end fused onto the multi-functional square (Figure 4A). A decrease in luminescence indicates a decrease in reporter gene protein production, designating a positive result. In comparison to the positive control, a 3WJ nanoparticle with fused luciferase siRNA,\textsuperscript{62} the multi-functional square harboring luciferase siRNA
caused a similar decrease in luminescence indicating similar gene knockdown activity (Figure 6B)
Overall, the results indicated that the RNA square nanoparticles carrying luciferase siRNA displayed
~40% gene knockdown activity.

In order for DICER to recognize and process double stranded (ds) RNA, the 3’-overhang must
be accessible and not trapped inside the inner cavity of the square nanoparticle. This possible
obstruction proves to be no problem. This newly constructed square makes it simple to incorporate
other diverse functional moieties into one of the four "arms" of the square. This can be done by simply
fusing siRNA into one of the four external scaffold strands.

The results demonstrate that diverse functionalities retain their activity when incorporated into
the square scaffold, making the RNA square an addressable and programmable poly-functional
nanoscaffold for potential delivery of RNA therapeutics for application in medicine. Due to the unique
five-strand construction of the square architecture functionalities such as RNA aptamers and siRNA for
gene therapy can be added by simply encoding for functionalities in the primary sequence of one strand
in the square.

CONCLUSION

Here we report the design and construction of poly-functional RNA square architectures utilizing
the 3WJ motif of bacteriophage phi29. The conserved 60°angle from the 3WJ was expanded to 90° and
formation of square geometry was achieved. The length of the connecting helix was increased or
decreased to generate three distinct sizes of nanoparticles. These three sizes of nanoparticles
demonstrate diverse thermodynamic stabilities. By modulating the material used for the core strand we
were able to tune the physicochemical properties of the square scaffold. The four arms extending from
the four corners of the square were used to incorporate RNA functionalities, each of which was
demonstrated to fold independently and correctly while retaining their authentic functionalities. The
square nanoscaffold shows great promise for biomedical and imaging applications due to its tunable
thermodynamic and biochemical stabilities. The technique shown here for simple design to finely tune
physicochemical properties adds a new angle to exploit RNA nanoparticles in a clinical setting.
MATERIALS AND METHODS

RNA synthesis, purification and labeling.

RNA oligomers were prepared by *in vitro* T7 transcription from dsDNA containing the T7 promoter prepared by polymerase chain reaction (PCR) as previously described. DsDNA was prepared with chemically synthesized single-stranded (ss) DNA oligomers purchased from Integrated DNA Technologies (IDT).

Cy5 whole chain labeling was carried out on the RNA oligomers following the Mirus FITC Label IT Nucleic Acid whole chain labeling kit. Briefly, diethylpyrocarbonate (DEPC) treated water was mixed with Mirus 10X Labeling Buffer, Mirus Label IT Tracker Reagent (Cy5), and the selected RNA oligomer, and incubated for one hour at 37°C. One tenth volume 3 M sodium acetate was added and ethanol precipitation was used to remove excess dye. The samples were then vacuum dried and re-suspended in DEPC treated water.

For TGGE analysis of core strand hybrid squares, the Perkin Elmer 5'-[γ-32P]-ATP end labeling kit was used to label one of the short external strands. The RNA square nanoparticles and hybrids were then assembled with the labeled strand and TGGE experiments ran, as described below. Total RNA staining by ethidium bromide staining was used for TGGE compariso of the different sized square constructs.

Assembly of RNA Nanoparticles

To construct the square the three component strands of the 3WJ, a3WJ, b3WJ, and c3WJ (Figure 1A), were deconstructed and b3WJ was connected to c3WJ while a3WJ was interconnected fusing H1 and H2 with a "connecting" helix. Equimolar amounts of RNA oligomers were mixed to a final concentration of 1 μM in 1X TMS with a magnesium ion concentration of 10 mM. The mixture was heated to 95°C for 5 minutes and then slow cooled from 80°C to 4°C at a rate of 2°C/minute on the Eppendorf Mastercycle Thermocycler. Confirmation of the assembly products were then run on native PAGE (described below).
**Native PAGE and TGGE analysis**

All native PAGE experiments were performed on 7% (29:1) polyacrylamide gels (PAGE) and run at 100 V in a 4°C cold room for 120 minutes. Cy5 labeled RNAs were then scanned on the Typhoon FLA 7000 and total RNAs were stained with ethidium bromide (EB).

Temperature gradient gel electrophoresis (TGGE) analysis was performed on 7% native PAGE in a buffer containing 50 mM TRIS pH = 8.0, 100 mM NaCl, and 0.2 mM MgCl$_2$. A gradient temperature of 40-75°C was applied perpendicular to electrical current, and experiment was run for 1 h at 20 W. A total RNA concentration of 80 nM was used in TGGE analysis of the core strand hybrid constructs and 100 nM RNA concentration was used for TGGE comparison of the different sized squares.

Apparent $T_M$ values corresponded to the temperature at which half of the square fractions were dissociated, and apparent $T_M$ values for multiple RNA strands were calculated, as described previously (Supplemental Figure 5).$^{20}$

Quantification analysis was performed using *ImageJ* software.$^{69}$ Equal-sized boxes were drawn around the lanes corresponding to the square complexes and corresponding quantified values for each hybrid square were divided by the sum of the values presented in the corresponding lane. All plots were generated using *OriginPro 8* software.

**AFM Imaging**

RNA polygons were imaged with MultiMode AFM NanoScope IV system (Veeco), as per previously reported methods.$^{70}$ Briefly, the RNA samples were diluted with 1X TMS buffer to a final concentration of 3-5 nM. Then, the droplet of samples (5-10 µL) was immediately deposited on APS mica. After 2 min incubation on the specifically modified APS mica surface,$^{71,72}$ 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.
**FBS resistance assay**

The square nanoparticles with RNA, 2'F RNA, and DNA core strands were incubated in 2% FBS solution at 37°C, and time points were taken from 0 minutes to 14 hours. After removal from 37°C incubation after their respective time points, the samples were frozen on dry ice to prevent any further nuclease degradation. The samples were then analyzed on 7% Native PAGE as described previously. ImageJ software was used to integrate the intensities of the assembled square nanoparticles in the gel. Integration areas for each time point were compared to the integration area for the 0 minute time point to construct an RNAse degradation comparison between the RNA, DNA, and 2'F-RNA hybrids (Figure 4B). All plots were generated using OriginPro 8 Software.

**Spinach and Malachite Green Aptamer Testing**

Native 7% PAGE was used to detect the fluorescence emission of fluorogenic nanoparticles. The gel was stained simultaneously in a mixture of 5 µM MG and 5 µM DFHBI and scanned for the MG-apt fluorescence λ_{exc} centered at 635 nm, and for SPINACH-APT fluorescence λ_{exc} centered at 473 nm. After recording images at different excitation wavelengths the total RNA stain in EB solution was performed to detect all RNAs in the gel.

Fluorescence emission in solution was measured, as previously reported. Briefly, assembled square nanoparticles (0.1 µM) harboring MG and SPINACH aptamers in TMS buffer were mixed with MG (2 µM) or with DFHBI (2 µM) (Lucerna, Inc, http://www.lucernatechnologies.com) and incubated at room temperature for 30 min (Fig 3e and 3f). Fluorescence was measured using a fluorospectrometer (Horiba Jobin Yvon), excited at 450 nm (565–750 nm scanning for emission) for SPINACH and 615 nm (625–750 nm scanning for emission) for MG dyes.

**Dual Luciferase Assay**

The dual luciferase assay was carried out to assay the efficiency of knockdown achieved by the luciferase siRNA harbored in the square nanoparticle. Experiments were carried out as previously described. Briefly, for square experiments carried out here, controls of cell only, plasmid only, and Lipofectamine 2000(Lipo) only were used. Cell only contains luminescence data of cells only, with no
transfection reagent or plasmid DNA. Lipo only samples contained just Lipofectamine 2000 transfection reagent. The plasmid only samples contained DNA plasmid only, which were transfected into the cell utilizing the transfection reagent Lipofectamine 2000. For RNA controls, the 3WJ with luciferase siRNA was used, which has previously been shown to cause knockdown.\textsuperscript{62} Square scaffold and a square utilizing scrambled siRNA sequence were utilized as negative controls.

**HBV Ribozyme Activity Assay**

HBV ribozyme activity assay was carried out as previously detailed. \[\text{ref: Bacterial Virus Phi29 PRNA as a Hammerhead Ribozyme Escort To Destroy Hepatitis B Virus}\] Briefly, HBV RNA substrate was 5'-Cy5 labeled using the Mirus Cy5 labeling kit. The labeled substrate was incubated with square nanoparticle conjugated with HBV ribozyme for 60 minutes at 37\(^\circ\) in buffer containing 20 mM MgCl\(_2\), 20 mM NaCl, and 50 mM Tris-HCl (pH 7.5). pRNA conjugated to HBV ribozyme served as a positive control and pRNA with a disabled HBV ribozyme was used as a negative control. The cleaved fragments were analyzed on the Typhoon Fluorescent Imaging system.

**Acknowledgements**

We thank Luda Shlyakhtenko for AFM imaging at the Nanoimaging Core Facility supported by the NIH SIG program and the UNMC Program of ENRI to Y.L. The work was supported by NIH grants U01-CA151648 and R01-EB003730, and funding to Peixuan Guo’s Endowed Chair in Nanobiotechnology position from the William Fairish Endowment Fund. The content is solely the responsibility of the authors and does not necessarily represent the official views of NIH. We would like to acknowledge the core facilities of the Markey Cancer Center at the University of Kentucky.

**Competing financial interests**

P.G. is a co-founder of RNA Nano, LLC, and Biomotor and RNA Nanotechnology Development Corp., Ltd.
Supporting Information Available. RNA sequences for all squares, additional assembly experiments, gel images for FBS and melting temperature assays, and secondary structures of the three squares and multi-functional square. These materials are available free of charge via the internet at

http://pubs.acs.org.
Reference List


Figure Legends

Figure 1. Design and assembly of RNA square. (A) Crystal structure of pRNA-3WJ showing the ~60° angle ∠AOB and the three component strands of the pRNA-3WJ, a3WJ (blue), b3WJ (purple), and c3WJ (green). (B) Three-dimensional structure of square shaped nanoparticle modeled on Swiss PDB viewer, based on pRNA-3WJ. (C) Native PAGE assembly gel with ethidium bromide total RNA staining showing assembly of each square structure: Lane 1 is 5 nm square, Lane 2 is 10 nm square, Lane 3 is 20 nm square.

Figure 2. Physical Properties of Square. (A) Schematic of small, medium, and large squares drawn to scale to each other for size comparison. Four external strands (blue, purple, red, orange) are complimentary to the core internal strand (green). (B) AFM imaging of small, medium, and large RNA squares. Zoomed pictures of individual squares located on the right. On the far right is a mixture of squares, each square is indicated by arrows. (C) Dynamic light scattering (DLS) to determine the size of square nanoparticles. Far right panel shows comparison of all three square constructs.

Figure 3. Comparison of RNA, 2’F-RNA, and DNA core strand hybrids. (A) Structure and native PAGE assembly of square nanoparticle hybrids. Blue strands represent RNA, red strands represent 2’F-RNA, black strands represent DNA. Lane 1 through 4 show the stepwise assembly from monomer to tetramer. (B) Nanoparticle stability in serum was compared between the RNA core strand, 2’F-RNA core strand, and DNA core strand to test the effect of the core strand on nanoparticle stability in FBS. (C) TGGE melting temperature profiles of square RNA nanoparticle along with 2’F and DNA core strand hybrids.

Figure 4. Design and assembly of multifunctional square. (A) Design of square structure with Spinach (SPIN) aptamer, malachite green (MG) aptamer, hepatitis B virus (HBV) ribozyme, and Luciferase siRNA incorporated into the square. (B) Native PAGE assembly of native square nanoparticle and multifunctional square nanoparticle. (C) AFM images of multi-functional square nanoparticle with visible aptamers on the corners of the nanoparticles.

Figure 5. Fluorescence assays for multi-functional square. (A) Spinach aptamer assay. Increased fluorescence is shown upon incubation with DFHBI dye. (B) Malachite green aptamer assay. Increased fluorescence is shown upon incubation with malachite green dye.

Figure 6. Ribozyme and siRNA Activity. (A) Catalytic activity of the HBV ribozyme. Cleaved products are boxed. pRNA-HBV-Rbz was used as a control. (B) Luciferase siRNA activity. Gene knockdown was measured by a decrease in luminescence. Cell only, Lipofectamine(Lipo) only and plasmid only were used as controls. Plasmid only control will exhibit the highest luminescence as the cells are only transfected with plasmid and no RNA. For a positive control pRNA-3WJ-siRNA showing knockdown was used. As negative control, naked square (no luciferase siRNA) and a scrambled siRNA sequence fused onto square were used. The square tested contained all four functional moieties. RLU is relative luminescence units.
TOC Graphic
Figure 1. Design and assembly of RNA square. (A) Crystal structure of pRNA-3WJ showing the $\sim 60^\circ$ angle $\angle AOB$ and the three component strands of the pRNA-3WJ, a3WJ (blue), b3WJ (purple), and c3WJ (green). (B) Three-dimensional structure of square shaped nanoparticle modeled on Swiss PDB viewer, based on pRNA-3WJ. (C) Native PAGE assembly gel with ethidium bromide total RNA staining showing assembly of each square structure: Lane 1 is 5 nm square, Lane 2 is 10 nm square, Lane 3 is 20 nm square.

127x44mm (300 x 300 DPI)
Figure 2. Physical Properties of Square. (A) Schematic of small, medium, and large squares drawn to scale to each other for size comparison. Four external strands (blue, purple, red, orange) are complimentary to the core internal strand (green). (B) AFM imaging of small, medium, and large RNA squares. Zoomed pictures of individual squares located on the right. On the far right is a mixture of squares, each square is indicated by arrows. (C) Dynamic light scattering (DLS) to determine the size of square nanoparticles. Far right panel shows comparison of all three square constructs.
177x101mm (300 x 300 DPI)
Figure 3. Comparison of RNA, 2’F-RNA, and DNA core strand hybrids. (A) Structure and native PAGE assembly of square nanoparticle hybrids. Blue strands represent RNA, red strands represent 2’F-RNA, black strands represent DNA. Lane 1 through 4 show the stepwise assembly from monomer to tetramer. (B) Nanoparticle stability in serum was compared between the RNA core strand, 2’F-RNA core strand, and DNA core strand to test the effect of the core strand on nanoparticle stability in FBS. (C) TGGE melting temperature profiles of square RNA nanoparticle along with 2’F and DNA core strand hybrids.

82x175mm (300 x 300 DPI)
Figure 4. Design and assembly of multifunctional square. (A) Design of square structure with Spinach (SPIN) aptamer, malachite green (MG) aptamer, hepatitis B virus (HBV) ribozyme, and Luciferase siRNA incorporated into the square. (B) Native PAGE assembly of native square nanoparticle and multi-functional square nanoparticle. (C) AFM images of multi-functional square nanoparticle with visible aptamers on the corners of the nanoparticles.

124x43mm (300 x 300 DPI)
Figure 5. Fluorescence assays for multi-functional square. (A) Spinach aptamer assay. Increased fluorescence is shown upon incubation with DFHBI dye. (B) Malachite green aptamer assay. Increased fluorescence is shown upon incubation with malachite green dye.

82x37mm (300 x 300 DPI)
Figure 6. Ribozyme and siRNA Activity. (A) Catalytic activity of the HBV ribozyme. Cleaved products are boxed. pRNA-HBV-Rbz was used as a control. (B) Luciferase siRNA activity. Gene knockdown was measured by a decrease in luminescence. Cell only, Lipofectamine(Lipo) only and plasmid only were used as controls. Plasmid only control will exhibit the highest luminescence as the cells are only transfected with plasmid and no RNA. For a positive control pRNA-3WJ-siRNA showing knockdown was used. As negative control, naked square (no luciferase siRNA) and a scrambled siRNA sequence fused onto square were used. The square tested contained all four functional moieties. RLU is relative luminescence units.