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Ultrastable Synergistic Tetravalent RNA Nanoparticles for Targeting to Cancers

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RAPID COMMUNICATION

Ultrastable synergistic tetravalent RNA nanoparticles for targeting to cancers

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Summary One of the advantages of nanotechnology is the feasibility to construct therapeutic particles carrying multiple therapeutics with defined structure and stoichiometry. The field of RNA nanotechnology is emerging. However, controlled assembly of stable RNA nanoparticles with multiple functionalities which retain their original role is challenging due to refolding after fusion. Herein, we report the construction of thermodynamically stable X-shaped RNA nanoparticles to carry four therapeutic RNA motifs by self-assembly of reengineered small RNA fragments. We proved that each arm of the four helices in the X-motif can harbor one siRNA, ribozyme, or aptamer without affecting the folding of the central pRNA-X core, and each daughter RNA molecule within the nanoparticle folds into their respective authentic structures and retains their biological and structural function independently. Gene silencing effects were progressively enhanced as the number of the siRNA in each pRNA-X nanoparticles gradually increased from one to two, three, and four. More importantly, systemic injection of ligand-containing nanoparticles into the tail-vein of mice revealed that the RNA nanoparticles remained intact and strongly bound to cancers without entering the liver, lung or any other organs or tissues, while remaining in cancer tissue for more than 8 h.

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Introduction

Living organisms possess wide assortments of elegant nanomachines, patterned arrays and highly structured macromolecules performing diverse biological functions. Macromolecules of DNA, RNA and proteins have intrinsically defined features at the nanometer scale and can serve as powerful building blocks for the bottom-up fabrication...
of biomimetic nanostructures and nanodevices [1,2]. RNA is a particularly attractive candidate for such applications [3—8], since it can be designed and manipulated with a level of simplicity characteristic of DNA, while possessing a versatile flexibility in structure and function similar to some properties of proteins [9]. Simple chemical modifications such as, 2′-Fluoro (2′F) can generate RNAs resistant to degradation without changing its folding into appropriate 3D structure, while retaining authentic biological and enzymatic functions [10,11].

There are many types of RNA molecules that could potentially be utilized for nanotechnology-based therapy such as small interfering RNAs [12—14], ribozymes [15—17], RNA aptamers [18,19], riboswitches [20,21], and miRNAs [22—24]. Although the methods for gene silencing with high efficacy and specificity have been achieved [22—24], the effective delivery of RNA to specific cells in vivo remains challenging. The development of a safe, efficient, specific and nonpathogenic nanodevice for the delivery of multiple therapeutic RNAs is in high demand. RNA nanotechnology holds great potential in this regard: (1) Homogeneous RNA nanoparticles can be manufactured with high reproducibility and known stoichiometry, thus avoiding unpredictable side effects or nonspecific toxicity associated with heterogeneous structures. (2) Using the bottom up approach, RNA nanoparticles can be assembled harboring multiple therapeutic, reporter and/or targeting payloads for synergetic effects [12]. (3) Cell type-specific gene targeting can be achieved via simultaneous delivery and detection modules which reduces off-target toxicity and lowers the concentration of the drug administered, thus reducing the side effects of the therapeutics. (4) RNA nanoparticle size typically ranges from 10—50 nm, an optimal size for a non-viral vector as they are large enough to be retained by the body yet small enough to pass through the cell membrane via the cell surface receptors mediated endocytosis. The advantage size has the potential to greatly improve the pharmacokinetics, pharmacodynamics, biodistribution, and toxicity profiles by avoiding non-specific cell penetration [25]. (5) Protein-free RNA nanoparticles with RNA aptamers as anti-receptors can yield superior specificity compared to protein anti-receptors while displaying lower antibody-inducing activity. This will provide an opportunity for repeated administration and treatment of chronic diseases. (6) RNA nanoparticles are treated as chemical drugs rather than biological entities, which might facilitate FDA approval.

However, one of the challenges in this emerging field of RNA nanotechnology is the relative instability of the nanoparticles without covalent modifications or cross-linking, resulting in the dissociation at ultra low concentrations in vivo after systemic injection. This has seriously hindered the delivery efficiency and therapeutic applications of RNA nanoparticles.

The feasibility of RNA nanotechnology in disease therapy has been exemplified in the phi29 pRNA therapeutic system [11—15,17,26]. The DNA packaging motor of bacteriophage phi29 (Fig. 1A) is geared by a hexameric pRNA ring [27—29], which contains two functional domains [30,31]. The central domain of each pRNA subunit contains two interlocking loops, denoted as the right- and left-hand loops (Fig. 1B) that can be reengineered to form dimers or trimers via hand-in-hand interactions [32—36]. The helical DNA packaging domain is located at the 5′/3′ paired ends [31,37]. The two domains are connected by a three-way junction (3WJ) region [11]. We have recently demonstrated that the 3WJ region extracted from the pRNA can be assembled from three pieces of RNA oligos to construct trivalent RNA nanoparticles [11]. In this communication, we demonstrate that the centerfold domain of the pRNA could be engineered to form a X-shaped motif (Fig. 1C), which was thermodynamically stable, resistant to denaturation by 8M urea and remained intact at ultra-low concentrations. Incubation of four RNA oligos each carrying one of the four small RNA molecules, siRNA, receptor binding aptamer, or folate resulted in the formation of tetravalent RNA nanoparticles as potential therapeutic agents. We proved that each one of the four helices in the X-motif can serve as a sticky-end to link one siRNA or other therapeutic molecules without affecting the folding of the central pRNA-X core. Systemic injection of ligand-containing nanoparticles into the tail-vein
of mice revealed that the RNA nanoparticles remained intact and strongly bound to cancers without entering the liver, lung or any other vital organs or tissues.

**Results and discussion**

**Length and sequence requirements of the helical stem regions of the pRNA for the assembly of the X-motif**

The pRNA-X motif was constructed by (1) opening the right-hand loop of pRNA to insert 9 base pairs, thereby forming a double helical segment (Helix H2), and (2) extending the H3 helix by 4 base pairs (Fig. 1C). The length of the helices H1, H3 and H4 were 8 base pairs, respectively, while H2 was 9 base pairs long. Although 6 base pairs are sufficient for the assembly of the junction domain, 8 base pairs are necessary to keep the junction domain stable under strongly denaturing conditions [11].

**Thermodynamically stable properties displayed by the pRNA X-motif**

The pRNA-X motif was assembled by mixing four RNA oligos, denoted a, b, c, and d in stoichiometric ratio at room temperature. The affinity and efficiency of assembly was investigated by both gel shift assays and melting experiments conducted under the physiological buffer TMS in the presence of 5 mM magnesium and 100 mM sodium chloride at pH 7.6. The gel-shift assays demonstrate that if one or two strands are omitted (lanes 1—6), they have a faster migration rate compared to the pRNA-X-core (lane 7) (Fig. 2A, top). The core remained stable in 8 M urea (Fig. 2A, bottom), thereby demonstrating its stable properties. Melting experiments displayed a very smooth, high-slope temperature dependent melting curve indicating that the four oligos of the pRNA-X core ($T_m$ of 62.7 ± 3.2°C) have a higher affinity to interact favorably compared to any of the individual component strands (Fig. 2B). The robust attributes of the 3WJ core have already been demonstrated using competition assays at different temperatures and in presence of 0—8 M urea [11].

**Self-assembly of stable RNA nanoparticles harboring small RNA molecules linked to the X-motif**

Extension of the phi29 pRNA at the 3′-end does not affect the folding of pRNA global structure [11,34]. Accordingly, the sequences of the four RNA oligos a, b, c, and d were placed at the 3′-end of the pRNA monomer, Ab′. Mixing of the four resulting pRNA chimeras at equimolar concentrations led to the assembly of X-shaped branched nanoparticles harboring one pRNA at each branch. AFM images confirmed the formation of larger RNA complexes with four-branches (Fig. 3A and B), which were consistent with gel shift assays comparing monomer (Ab′), dimer (Aa′), 3WJ-3pRNA [11] and pRNA-X—4pRNA (Fig. 3C). The dimer (Aa′) formed via handin-hand interactions of interlocking loops is only stable up to 2 M urea, while the 3WJ—3pRNA and the pRNA-X—4pRNA
are both stable in presence of 8 M urea. The pRNA-X—4pRNA nanoparticles can also be co-transcribed and self-assembled in one step during transcription with high yield.

As a candidate therapeutic RNA nanoparticle, the pRNA-X constructs with four branches harboring multi-module functionalities have to remain intact after systemic delivery, where it will exist at ultra low concentrations due to dilution by circulating blood. To assay the dissociation, [α-32P] labeled pRNA-X nanoparticles were serially diluted to extremely low concentrations (100 nM to 1 pM). The concentration for dissociation was below the detection limit of the [32P]-labeling technology; at 25 pM in TMS buffer, the lowest detectable concentration, the pRNA-X nanoparticles showed no signs of dissociation (Fig. 3D).

Construction of a variety of therapeutic RNA nanoparticles using the X-motif as scaffold

Tetravalent RNA nanoparticles were constructed using the pRNA-X motif as a scaffold by incorporating four functional modules: MG (malachite green dye, triphenylmethane) aptamer, luciferase siRNA (siLuci), survivin siRNA (siSurv) and folate (FA) (Fig. 4A), denoted [pRNA-X/MG/FA/siLuci/siSurv] or corresponding scramble siRNA control. The presence of the functional moieties did not interfere with the formation of the pRNA-X core and the tetravalent complex assembled with high affinity (Fig. 4B). The purified constructs (lane 9, Fig. 4B) were stable in absence of magnesium and remained intact under strongly denaturing conditions, even after the incorporation of functionalities. In the next sections, we evaluated whether the incorporated RNA moieties in the pRNA-X nanoparticles retain their original folding and functionalities.

Assessment of MG fluorescence

MG binding aptamer [38] was used as model system for structure and function verification. Free MG is not fluorescent by itself, but emits fluorescent light after binding to the aptamer. Fused MG-binding aptamer retained its capacity to bind MG, as revealed by its fluorescence emission (Fig. 4C). The fluorescence is comparable to optimized positive controls and therefore confirming that the MG aptamer assembled from two strands of the pRNA-X after incorporation into the RNA nanoparticles.
Figure 4  Construction of multi-module RNA nanoparticles harboring MG (malachite green) aptamer, folate, luciferase siRNA, and survivin siRNA. (A) Schematic and sequences of the tetravalent pRNA-X constructs. (B) Step-wise assembly of RNA nanoparticles using the pRNA-X as scaffold with functionalities assayed by 8% denaturing urea PAGE. In the table, ‘+’ indicates the presence of the RNA strands in samples of the corresponding lanes. (C) Functional assay of the MG aptamer incorporated in pRNA-X nanoparticles. MG fluorescence was measured using excitation wavelengths 475 and 615-nm. (D) Target gene knock-down effects of survivin siRNA showed by RT-PCR (GADPH is the endogenous control) on mRNA level and Western Blot assay (β-actin bands served as loading control) on protein level. (E) Dual-luciferase assay for target gene knock-down of luciferase gene. The relative firefly luciferase activity reflects the level of luciferase gene expression and is obtained by normalizing firefly luciferase activity using the internal control renilla luciferase activity. Error bars represent s.d. (N = 3).
Targeted gene silencing assay in cancer cell model
Two pRNA-X nanoparticles were constructed for assaying the gene silencing effects harboring: (1) folate and survivin siRNA \([pRNA-X/MG/FA/siLuci/si/Surv]\); (2) folate and survivin siRNA scramble control \([pRNA-X/MG/FA/siLuci/si/Surv Scram]\). After 48-h transfection, both reverse transcription-PCR (RT-PCR) assayed on mRNA level and western blot assayed on protein expression confirmed reduced survivin gene expression level of \([pRNA-X/MG/FA/siLuci/si/Surv]\) nanoparticles compared to the scramble control (Fig. 4D). The mechanism of siRNA release from the pRNA nanoparticles is by Dicer processing, as established previously [14].

Targeted gene silencing of luciferase
Dual-luciferase reporter system was used to quantitatively measure the gene silencing effects of the pRNA-X constructs harboring the siRNA targeting firefly luciferase gene [39] (Fig. 4A). The relative luciferase activity was
Figure 6  Construction of tetravalent pRNA-X nanoparticles harboring multiple siRNA for enhanced gene silencing effects. (A) Sequences and notations of siRNA used in tetravalent constructs. Blue: siLuci-1 and 1ʹ; red: siLuci-2 and 2ʹ; green: siLuci-3 and 3ʹ; orange: siLuci-4 and 4ʹ; black: control siRNA [42]. (B) and (C) Quantification of Luciferase gene expression: Effects of increasing number of different Luciferase siRNAs (siLuci-1, 2, 3 and 4) (A); and four identical siRNA constructs (siLuci-1, 2, 3 or 4) incorporated in the pRNA-X motif (B). RLU, relative luciferase units; siLuci-1, 2, 3 and 4 represent reversed siRNA sequences for siLuci-1, 2, 3 and 4, respectively. Error bars represent s.d. (N = 3).

used to reflect the expression level of firefly luciferase gene by normalizing the firefly luciferase activity with the internal control, renilla luciferase activity. The results indicated that [pRNA-X/MG/FA/siLuci/si/Surv] nanoparticles displayed ∼70% decrease in firefly luciferase gene expression (Fig. 4E).

Figure 7  Comparison of gene silencing effects for a single vs. multiple siRNAs incorporated in the pRNA-X motif. (A—D) Four identical siRNA (siLuci-1, A; siLuci-2, B; siLuci-3, C; siLuci-4, D) compared with a single siRNA harbored at each pRNA-X motif arm; RLU: relative luciferase units; siLuci-1, 2, 3 and 4 represent reversed siRNA sequences for siLuci-1, 2, 3 and 4, respectively. Error bars represent s.d. (N = 3).
Cell binding and entry of pRNA-X nanoparticles
Folate was incorporated in the pRNA-X nanoparticles to
serve as a cancer cell delivery agent via folate receptor-
mediated endocytosis [11,14,40]. Fluorescent pRNA-X
nanoparticles with folate conjugated into one of the
branches of the pRNA-X complex were tested for cell
binding efficiency. pRNA-X harboring FA and Cy3 labels
[Cy3—pRNA-X/MG/FA/siLuci/si/Surv] served as the test
sample, while the negative control harbored NH2 and Cy3
labels [Cy3—pRNA-X/MG/NH2/siLuci/si/Surv]. Flow cytom-
etry (Fig. 5A) and confocal imaging indicated a strong
binding of the RNA nanoparticles and efficient entry
into the targeted cells, as demonstrated by the excel-

tent co-localization and overlap of the fluorescent pRNA-X
nanoparticles (red) and cytoplasma (green) (Fig. 5B).

Gene silencing effects were progressively
enhanced as the number of siRNA in each pRNA-X
nanoparticles increased gradually from one, two
three to four
Tetravalent pRNA-X complexes were constructed harboring
multiple luciferase siRNAs to assay for enhanced gene silenc-
ing effects. Dual-luciferase reporter system was used to
quantitatively measure the gene silencing effects. For all
the constructs, the total concentration of RNA was kept con-
stant at 1.25 nM. The target sites on the luciferase gene for
the four siRNAs (Fig. 6A) were located at 153—173, 196—216,
498—518, and 846—869 positions, as published in the lit-
erature [41,42]. The incorporation of four identical siRNA
sequences compromised the assembly of the X-motif due to
self-folding of the complementary sequences of the respec-
tive siRNAs. To facilitate the assembly, the siRNA sequences
were reversed (denoted with a prime, such as siLuci-1) at
alternate helical branch locations. The reversed sequences
had no impact on the functionality of the siRNA.

Silencing effects with increasing number of different
luciferase siRNA
As the number of different luciferase siRNAs were gradu-
ally increased in the pRNA-X motif, progressive increase
in silencing effects were observed as follows, 25% (for
1 siRNA; 3 scramble siRNA), 57% (for 2 siRNA; 2 scramble
siRNA), 72% (for 3 siRNA; 1 scramble siRNA), and 81% (for 4
siRNA) (Fig. 6B).

Silencing effects of four identical luciferase siRNAs
Significant silencing effects were observed in presence of
four identical siRNAs fused to the pRNA-X motif, com-
pared to a single siRNA as follows, 74% (for four siLuci-1),
90% (for four siLuci-2), 80% (for four siLuci-3), and
72% (for four siLuci-4) (Fig. 6C). For comparison, we
constructed the X-motif harboring a single siRNA (either
siLuci-1 or 2 or 3 or 4) at helical locations H1, H2, H3
or H4, respectively (Fig. 7A—D). The silencing effects of
the four different siRNAs increased following the trend,
siLuci-2 > siLuci-1 ≈ siLuci-4 > siLuci-3. The functionality
of the siRNA was comparable at each of the arms of the X-
motif. The data demonstrated that greatly enhanced effects
were observed in presence of four identical siRNAs compared
to a single siRNA (Fig. 7A—D).
Tetravalent RNA nanoparticles for targeting to cancers

Silencing effects of pRNA-X harboring four luciferase siRNAs versus single standard siRNA

Firefly luciferase reporter was chosen as knockdown target for in vitro pRNA-X treatment. Two siRNA sequences (siLuci-1 and 2) were selected and tested in HT29 colon cancer cells with stable luciferase expression. To demonstrate that the pRNA-X system provides a superior delivery vehicle for siRNA, a direct comparison of the in vitro knockdown efficacy compared to standard siRNA (siLuciferase-1 and 2) was carried out (Fig. 8A and B). A 1% concentration of pRNA-X nanoparticles with four siRNA modules can achieve the same silencing effects (~60–70% decrease compared to scramble controls) as the siRNA by itself. Absence of nonspecific luciferase inhibition was confirmed by sulforhodamine B assay (Fig. 8C).

In vivo targeting of RNA nanoparticles to cancer xenograft by systemic injection

To confirm the chemical and thermodynamic stability of the X-shaped RNA nanoparticles for specific cancer targeting in vivo, RNA nanoparticles were constructed with one of the four RNA fragments carrying the folate to serve as a ligand for binding to the cancer cells, and another RNA fragment carrying the fluorescent dye Alexa-647. The nanoparticles were systemically injected (i.e., tail vein injections) into athymic nude mice bearing KB cells (folate receptor positive) xenografts in the subcutaneous flanks. Whole body imaging (at time points, 1-h, 4-h and 8-h) were carried out after intravenous administration of FA—pRNA-X—Alexa647 nanoparticles. The pRNA-X nanoparticles strongly bound to the tumor xenografts within 4-h (Fig. 9). The animals were sacrificed at 8-h time point and organ imaging revealed that fluorescence was localized specifically in the tumor and was not detected in other organs of the body, indicating that the particles are not trapped in the liver, lungs, kidneys or other tissues or organs after systemic injection (Fig. 9B). The results were confirmed to be reproducible by three independent labs with five trials. Together, these findings suggest a very selective targeting of the pRNA nanoparticles to tumors and not to normal tissues which would make this delivery system highly efficacious for future clinical applications.

Conclusions

We demonstrated that the central domain of bacteriophage phi29 motor pRNA can be engineered into a stable X-motif to carry four functional modules in the absence of metal ions. The resulting nanoparticles were thermodynamically stable, and resistant to dissociation under strongly denaturing conditions or at ultra-low concentrations. Incubation of four RNA oligos representing therapeutic functional motifs resulted in the self-assembly of tetravalent RNA nanoparticles as potential therapeutic agents. Progressive enhancement of gene silencing effects were observed when the number of siRNA in each pRNA-X nanoparticles increased from one to two, three and four. The results demonstrated that a wide range of therapeutic RNA molecules targeting cancer and viral infected cells can potentially be fused to the multivalent pRNA-X motif to achieve enhanced silencing effects.

Experimental procedures

In vitro synthesis and purification of pRNA

The pRNA were synthesized by enzymatic methods as described previously [31]. RNA oligos were synthesized chemically by IDT (Iowa). RNAs were purified by 8 M urea 8% PAGE. The corresponding bands were excised under UV shadow and eluted from the gel over 4 h at 37°C in the elution buffer (0.5 M NH₄OAc, 0.1 mM EDTA, 0.1% SDS, and 0.5 mM MgCl₂) followed by ethanol precipitation overnight at −20°C (2.5 volume of 100% ethanol and 1/10 volume of 3 M NaOAc). The precipitate was pelleted by centrifugation (16500 × g, 30 min), washed with 70% ethanol, and dried by speed vacuum. Finally, the RNA dried pellet was rehydrated in 0.05% DEPC treated water and stored at −20°C.

Construction of multi-module RNA nanoparticles

The sequences for each of the RNA strands a, b, c, and d were added to the 3'-end of each 117-nt pRNA-Ab' and synthesized using two primers: 3'-end primer encoding the a, b,
c, and d, respectively, and a common 5′-end primer and the Ab′ template. The template was then used in transcription mixture and the pRNA was then purified in 8% urea PAGE gel in TBM buffer, as described previously [11].

The sequences for the luciferase siRNA, survivin siRNA, malachite green (MG) binding aptamer and folate labeled RNA were rationally designed with the sequences of the strands a, b, c, and d, respectively (Fig. 4). Multi-module nanoparticles pRNA-X/MG aptamer/Folate/luciferase siRNA and survivin siRNA, denoted [pRNA-X/MG/FA/siLuci/siSurv] or scramble control, denoted [pRNA-X/MG/FA/siLuci/siSurv Scram] were assembled from five individual fragments including a 26-nt folate labeled RNA (Trilink) or folate-DNA strand (synthesized in house). The individual RNA strands (fragments 1, 2, 3 and 4, Fig. 4A) were transcribed from DNA template amplified by PCR. Fluorescent dyes were labeled on one RNA strand by using the Label IT™ siRNA Tracker Intracellular Localization Kit, Cy3TM (Mirus Bio LLC). The five RNA strands were mixed after purification in TMS buffer at equal molar ratio and then heated up to 80°C for 5 min, followed by slow cooling to 4°C. The assembled nanoparticles were then purified from 8% native PAGE gel.

**Dilution assay to test dissociation at extremely low concentrations**

The stability of the tetravalent pRNA-X-4pRNA nanoparticles were evaluated by radiolabel assays. Purified [32P] pRNA-X-4pRNA complexes were serially diluted from 100 nM to 1 pM in TMS buffer, and then loaded onto 8% native PAGE gel for autoradiograph.

**Melting experiments**

The melting experiments were conducted by monitoring the fluorescence of the pRNA-X component strands using the LightCycler® 480 Real-Time PCR System (Roche). 1× SYBR Green I dye (Invitrogen) (emission 465—510 nm), which binds double-stranded nucleic acids, but not single-stranded ones was used for all the experiments. The respective RNA oligonucleotides were mixed at room temperature in physiological TMS buffer. The pRNA-X core strands were slowly cooled from 95°C to 20°C at the ramping rate of 0.11°C/s. Data was analyzed by the LightCycler® 480 Software using the first derivative of the melting profile. The Tm value represents the mean and standard deviation from 4 independent experiments.

**Flow cytometry analysis of folate mediated cell binding**

Human cervical cancer Hela cells [American Type Culture Collection (ATCC)] were maintained in folate-free RPMI-1640 medium (Gibco), then trypsinized and rinsed with PBS (137 mM NaCl, 2.7 mM KCl, 100 mM Na2HPO4, 2 mM KH2PO4, pH 7.4). 200 nM Cy3 labeled pRNA-X complexes harboring folate [Cy3-pRNA-X/MG/FA/siLuci/siSurv], and folate-free control [Cy3-pRNA-X/MG/NH2/siLuci/siSurv] were each incubated with 2× 10⁵ KB cells at 37°C for 1 h. After washing with PBS, the cells were resuspended in PBS buffer. Flow cytometry (Beckman Coulter) was used to observe the cell binding efficacy of the Cy3-pRNA-X nanoparticles.

**Confocal microscopy**

Hela cells were grown on glass coverslides in folate free medium overnight. Cy3 labeled pRNA-X complexes harboring folate [Cy3-pRNA-X/MG/FA/siLuci/siSurv], and folate-free control [Cy3-pRNA-X/MG/NH2/siLuci/siSurv] were each incubated with the cells at 37°C for 2 h. After washing with PBS, the cells were fixed by 4% paraformaldehyde and stained by Alexa Fluor® 488 phalloidin (Invitrogen) for cytoskeleton and TO-PRO®-3 iodide (642/661) (Invitrogen) for nucleus. The cells were then assayed for binding and cell entry by Zeiss LSM 510 laser scanning confocal microscope.

**Malachite green (MG) aptamer fluorescence assay**

The pRNA-X tetravalent RNA nanoparticles [pRNA-X/MG/FA/siLuci/siSurv] harboring MG binding aptamer (100 nM) [38] was mixed with MG (2 μM) in binding buffer containing 100 mM KCl, 5 mM MgCl2, and 10 mM HEPES (pH 7.4) and incubated at room temperature for 30 min (Fig. 4C). The fluorescence was measured using a fluorospectrometer (Horiba Jobin Yvon; SPEX Fluolog-3), excited at 475 nm (scanning from 540 to 800 nm for emission) and 615 nm (scanning from 625 to 800 nm for emission).

**Assay for the silencing of genes in cancer cell model**

Two pRNA-X nanoparticles were constructed for assaying the gene silencing effects harboring: (1) folate and survivin siRNA [pRNA-X/MG/FA/siLuci/siSurv]; (2) folate and survivin siRNA scramble control [pRNA-X/MG/FA/siLuci/siSurv Scram].

Hela cells were transfected with 25 nM of the individual pRNA-X complexes using Lipofectamine 2000 (Invitrogen). After 48-h treatment, cells were collected and target gene silencing effects were assessed by both RT-PCR and Western blot assays.

Cells were processed for total RNA using illustra RNAspin Mini kits (GE Healthcare, Buckinghamshire, UK). The first complementary DNA strand was synthesized on mRNA template. The template was then used in transcription mixture and the cDNA was then purified in 8% urea PAGE gel in TBM buffer, as described previously [11].

**Primers for human GAPDH [43] and survivin [44] are:**

GAPDH left: 5′-ACGGATTTGGCTCATTTGGGCG-3′; GAPDH right: 5′-CTCCTGAAATGTTGATGAA-3′;

**References:**


[38] Lee et al. 2017.


Survivin left: 5′-GATGGGTGCCCCAGCGTGG-3′; Survivin right: 5′-GCTCCGAGCAGGCTCAAA-3′.

Cells were rinsed and harvested in lysis buffer. Protein concentrations were determined and equal amounts of proteins were loaded onto a 15% PAGE. Membranes were blocked, incubated with primary antibody to survivin and β-actin (R&D Systems, Minneapolis, MN), and conjugated to a secondary antibody (Sigma—Aldrich, St. Louis, MO). Membranes were then blotted by ECL kit (Millipore, Billerica, MA) and exposed to film.

**Assay for the silencing of genes in cancer cell model**

Dual-luciferase assays were used to test the potential of the pRNA-X complex in escorting siRNA delivered into cells. For dual-luciferase assays, Hela cells were seeded in 24-well plates. Gene silencing assays were performed by co-transfecting [pRNA-X/MG/FA/siLuci/si/Surv] with both plasmid pGL3 and pRL-TK (Promega, Madison, WI) coding for firefly and renilla luciferase, respectively. The latter served as an internal control to normalize the luciferase data (Dual-luciferase Reporter Assay System; Promega). Cells were washed once with PBS and lysed with passive lysis buffer. The plates were shaken for 15 min at room temperature. 20 μL of lysate were added to 100 μL of luciferase assay reagent (LAR II) and firefly luciferase activity was measured. Upon addition of 100 μL of Stop & Glo Reagent, control measurements of renilla luciferase activity were then obtained. The data was then normalized with respect to the renilla activity for determining the average ratio of firefly to renilla activity over several trials.

HT29 colon cancer cells, that express GFP and firefly luciferase (HT29 GFP-Luc), were plated at 10,000 cells/well in a 96-well black plate (Corning Life Sciences; Tewksbury, MA) overnight and then transfected with control siRNA (NCT), siLuc#1, and siLuc#2 using Lipofectamine (Invitrogen). The siRNAs and pRNA-X nanoparticles were transfected into HT29 GFP-Luc cells at 100 nM and 1 nM each, correspondingly. About 100 μL of Stop & Glo Reagent, control measurements of renilla luciferase activity were then obtained. The data was then normalized with respect to the renilla activity over several trials.

Animal trial: **in vivo targeting of tumor xenograft by systemic injection of pRNA-X nanoparticles**

Male athymic nude (6–8 weeks old) mice were obtained from Taconic (Hudson, NY) and housed in clean, pathogen-free rooms in an environment with controlled temperature (27 °C), humidity, and a 12 h light/dark cycle. All animal experiments were approved by the Institutional Animal Care and Use Committee at University of Kentucky and conducted in accordance with guidelines issued by the National Institutes of Health for the care of laboratory animals. Mice were fed a folate-free diet (Harlan Laboratories; Indianapolis, IN) for a total of 2 weeks before the experiment and injected with KB cells (3 × 10^6 cells per mouse in 100 μL PBS) subcutaneously. When the tumors grew to about 500 mm^3, the mice were anesthetized using isoflurane gas (% in oxygen at 0.6 L/min flow rate) and injected intravenously through the tail vein with a single dose of 3 μg of pRNA per gram of body weight of 2′-F U/C modified Folate-Alexa647-labeled pRNA-X nanoparticle in 300 μL of PBS. Whole-body imaging (Ex-Max 650 nm/Em-Max 668 nm) was carried out at 1-h, 4-h and 8-h after pRNA administration on IVIS Spectrum station (Caliper Life Sciences; Hopkinton, MA). Composite images obtained were comprised of black and white digital photos with an overlay of images reflecting fluorescent activity. The density map, measured as photons/second/cm^2/steradian (p/s/cm^2/sr), were created using the Living Image 3.1 (Caliper Life Sciences; Hopkinton, MA) software and represented as a color gradient centered at the maximal spot. Following CO2 asphyxiation at 8-h after pRNA administration, the tumors, liver, heart, lung and kidney of the mice were dissected and individually imaged.

**Authors contributions**

P.G. conceived, designed and led the project; F.H., D.S., and Y.S., designed and conducted the experiments; L.S. obtained the AFM images. P.G., F.H., D.S., and Y.S. analyzed the data and co-wrote the manuscript. R.P and B.M.E carried out the xenograft and animal imaging.
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References


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